RADIOIMMUNOASSAY OF INSULIN AND GLUCAGON WITH STUDIES ON THE OBESE HYPERGLYCAEMIC SYNDROME IN MICE

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SUMMARY

Sensitive and precise radioimmunoassays for insulin and glucagon have been established. Although it was possible to employ similar precepts to the development of both hormone assays, the establishment of a reliable glucagon radioimmunoassay was complicated by the poor immunogenicity and instability of the peptide. Thus, unlike insulin antisera which were prepared by monthly injection of guinea pigs with crystalline insulin emulsified in adjuvant, the successful production of glucagon antisera was accomplished by immunisation of rabbits and guinea pigs with glucagon covalently linked to bovine plasma albumin. The conventional chloramine-T iodination with purification by gel chromatography was only suitable for the production of labelled insulin. Quality tracer for use in the glucagon radioimmunoassay was prepared by trace iodination, with subsequent purification of monoiodinated glucagon by anion exchange chromatography. Separation of free and antibody bound moieties by coated charcoal was applicable to both hormone assays, and a computerised data processing system, relying on logit-log transformation, was used to analyse all assay results.

The assays were employed to evaluate the regulation of endocrine pancreatic function and the role of insulin and glucagon in the pathogenesis of the obese hyperglycaemic syndrome in mice. In the homozygous (ob/ob) condition, mice of the Birmingham strain were characterised by numerous abnormalities of glucose homeostasis, several of which were detected in heterozygous (ob/+) mice. Obese mice exhibited pancreatic alpha cell dysfunction and hyperglucagonaemia. Investigation of this defect revealed a marked insensitivity of an insulin dependent glucose sensing mechanism that inhibited glucagon secretion. Although circulating glucagon was of minor importance in the maintenance of hyperinsulinaemia, lack of suppression of alpha cell function by glucose and insulin contributed significantly to both the insulin insensitivity and the hyperglycaemia of obese mice.

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GLOSSARY OF TERMS

Accuracy: the extent to which the mean of an infinite number of measurements of an antigen agrees with the exact amount of antigen which is present (Midgley et al., 1969a).

Affinity: the energy of binding of an antigen for a particular antibody (Hurn & Landon, 1971).

Antigen: a substance that will combine with a specific antibody (Hurn & Landon, 1971).

Avidity: the energy of binding of an antigen for a particular antibody (Hurn & Landon, 1971).

Hapten: a substance that is not usually immunogenic but which becomes so when complexed with another compound (Sönksen, 1974).

Immunogen: a substance that will provoke an immune response (Hurn & Landon, 1971).

Metameter: the transformed value of a dose or a response (Ekins, 1976).

Precision$^{\phi}$: the extent to which a given set of measurements of the same sample agrees with the mean (Midgley et al., 1969a).

As illustrated in figure 9 , the precision of measurement (Δp) of an unknown concentration of antigen (p) is related both to the precision of measurement of the response metameter (ΔR) and the slope of the response curve at the corresponding point.

Reproducibility: the extent to which an estimate is duplicated upon repeat measurement (Midgley et al., 1969a).

Sensitivity$^{\phi}$: the smallest amount of unlabelled antigen which can be distinguished from no antigen (Midgley et al., 1969a).

As illustrated in figure 9, the precision of measurement (Δ po) of zero concentration of antigen (po), is related both to the precision of measurement of the response metameter (Δ Ro) and the slope of the response curve at the corresponding point. Thus sensitivity represents a limiting case of the concept of precision in the particular instance where p, the measured amount, is equal to zero.

The term sensitivity as applied to an assay technique commonly refers to the ability of the system to measure small amounts of antigen with acceptable precision. Thus a technique is regarded as more sensitive if it enables smaller amounts to be measured with the same precision, or the same small amount with a greater precision.

Specificity: the extent of freedom from interference by substances other than the one intended to be measured (Midgley et al., 1969a).

Titre: the final dilution of the antiserum in the incubation mixture required to bind the appropriate amount of labelled antigen in the absence of unlabelled antigen (Hurn & Landon, 1971).

ϕ Precision and sensitivity have been defined by Yalow and Berson in terms of the slope of the dose response curve (Yalow & Berson, 1968, 1969a). These definitions are only valid in circumstances where the error in the measurement of the response metameter remains constant at any point on the curve.

Note: the shape of the dose response curve has been assumed to give a reasonable indication of both assay precision and assay sensitivity in many of the figures presented in this thesis. The validity of this approach can be evaluated by reference to figure 53 which demonstrates the relationship between the error in the measurement of the response metameter and the position on the response curve for 47 assays. Data from both insulin and glucagon assays have been combined.

NONSTANDARD ABBREVIATIONS ø

General

GLI	glucagon-like immunoreactivity, a heterogeneous group of peptides which react with N-terminal and/or C-terminal reactive glucagon antisera.
C-GLI	glucagon-like protein which reacts with C-terminal reactive glucagon antisera.
N-GLI	glucagon-like protein which reacts with N-terminal reactive glucagon antisera.
KIU	kallikrein inhibitor unit.
TRIS	tris-(hydroxymethyl)-aminomethane.
EDTA	ethylenediaminetetraacetic acid.

Basic radioimmunoassay

P	unlabelled antigen.
P *	radioactive, isotopically labelled antigen.
Q	antibody.
PQ	complex between the unlabelled antigen and the antibody.
P*Q	complex between the labelled antigen and the antibody.
B	bound antigen.
F	free antigen.
p	concentration of the unlabelled antigen.
p*	concentration of the labelled antigen.
q	concentration of antibody binding sites.
K	affinity constant.
k_1, k^1	association and dissociation rate constants.
ΔF^o	standard free energy change.
ΔH^o	enthalpy change.
ΔS^o	entropy change.
ΔR	precision of measurement of the response metameter.
Δp	precision of measurement at an arbitrary dose of unlabelled antigen.
Δpo	precision of measurement at zero dose of unlabelled antigen (sensitivity).
S	specific activity of the labelled antigen.
V	final volume of the incubation mixture.

Dose-response curve

B	bound antigen.
F	free antigen.
T	total labelled antigen.
N	nonspecific counts, counts recorded in the absence of antiserum.
B/F, R	ratio of bound-to-free antigen.
Ro	ratio of bound-to-free antigen at zero dose of unlabelled antigen.
B/T	ratio of bound-to-total antigen.
$(B/T)_o$	ratio of bound-to-total antigen at zero dose of unlabelled antigen
B/Bo, Y	antigen bound at arbitrary dose relative to that at zero dose of unlabelled antigen.
Var(Y)	variance of Y.
Y'	logit Y, Ln (Y(1-Y)), $o<y<1$.
$\bar{Y}'$	mean Y'.
Var(Y')	variance of Y'.
$\hat{Y}'$	value of Y' predicted by (previous) regression line.
$\hat{Y}$	value of Y predicted by (previous) regression line.
y'	working logit.
y	general response variable.
W	weight for a single point (X', Y').
ao, a,	weighting coefficients for equation 36.
X	concentration of the unlabelled antigen.
X'	$\log_{10}X$.
$\bar{X}'$	mean X'.
$\hat{X}'$	value of X' predicted by the regression line.
$\hat{X}$	value of X predicted by the regression line.
x	general dose variable.
m, c	constants of regression equation, slope, intercept.
Vp	pipetting error.
V1, V2	misclassification errors.

D	standard deviation of total counts.
t	student's t value.
n	number of points, observations.
df	degrees of freedom.

Ø A variety of abbreviations have been used in radioimmunoassay literature. The present nomenclature, based on that introduced in the mathematical treatises of Ekins and Rodbard, is now well established in the field of saturation analysis.

CONTENTS

CHAPTER 1

GENERAL PRINCIPLES AND PRACTICAL ASPECTS OF RADIOIMMUNOASSAY

INTRODUCTION

During the past twenty years, radioimmunoassay has made an explosive impact upon endocrinology and other areas of medical research which rely implicitly on the accurate measurement of biologically potent compounds (Yalow, 1974a; Antoniades, 1976; Parker, 1976). The technique has found general acceptance as the method of choice for the determination of a great variety of substances, ranging from hormones and non-hormonal peptides to drugs; enzymes; cyclic nucleotides; parasitic; microbial and viral agents (Glick & Rothenberg, 1973; Skelly et al., 1973; Jaffe & Behrman, 1974; Simmons & Ewing, 1974; Sönksen, 1974). The widespread and diverse application of the technique in the biomedical field is not only a result of the general practicability of the method, but is also due to its fundamentally inherent chemical specificity, and its exquisite sensitivity which permits precise quantitative estimations of minute concentrations of biologically active agents (Diczfalusy & Diczfalusy, 1969; Kirkham & Hunter, 1971; Odell & Daughaday, 1971; Parker, 1972). Indeed, much of our present knowledge of clinical and investigative medicine can be directly attributed to the development of a technique which offers a unique combination of characteristics unmatched by contemporary bioassay methods (Chayen et al., 1974; Fleisher & Schwartz, 1975; Antoniades, 1976; Loraine & Bell, 1976).

HISTORICAL PERSPECTIVE

The development of radioimmunoassay by Berson and Yalow during the late 1950's represents a milestone in the history of endocrinology (Yalow & Berson, 1971; Yalow, 1974a; Freeman & Blauxfox, 1975). The fundamental principle of the technique was first realised following the demonstration and characterisation of insulin-binding antibodies in the sera of insulin-dependent diabetics (Berson et al., 1956a, b). Investigations on the quantitative aspects of the insulin-antibody reaction (Berson & Yalow, 1957) and the species specificity of available antisera (Berson & Yalow, 1959,a,b,c)

were however required to translate the theoretical concepts of radioimmunoassay from the measurement of exogenous insulin in the plasma of rabbits (Berson & Yalow, 1958) to the determination of insulin in unextracted human plasma (Yalow & Berson, 1959). Following the initial description of radioimmunoassays for insulin (Grodsky & Forsham, 1960; Yalow & Berson, 1960 a, b), the technique was rapidly applied to other proteins and polypeptide hormones, including glucagon (Unger et al., 1959; Grodsky et al., 1961; Unger et al, 1961) and growth hormone (Utiger et al., 1961, 1962). The development of the chloramine - T iodination procedure for growth hormone (Hunter & Greenwood, 1962; Greenwood et al., 1963) provided a convenient and reproducible method for the preparation of many radioiodinated peptide hormones, and was fully exploited in the digitoxin radioimmunoassay (Oliver et al., 1966, 1968). The extension of the immunoassay principle to drugs and other non-antigenic compounds has widened the field of application of radioimmunoassay to embrace virtually all compounds of biological interest, and has accordingly revolutionised many of our methodological approaches to experimental medicine (Glick & Rothenberg, 1973). The impact of recent radioimmunoassay technology has been particularly apparent in the field of gastroenterology, where the development and application of new techniques has led to considerable advances in our understanding of both the structure and function of the intestine (Barrington & Dockray, 1976; Rayford et al., 1976; Pearse et al., 1977; Johnson, 1977). The application of radioimmunoassay in this field has not only provided, for the first time, reproducible methods for the determination of physiological concentrations of enteric hormones (Bloom, 1974, 1977), but has also been implicit in the development of techniques for the histochemical demonstration of individual cell types (Fujita, 1976) and purification of their secretory products (Murphy et al., 1971, 1973).

GENERAL PRINCIPLES

Radioimmunoassay is based on the principle of isotopic dilution (Hevesy & Hobbie, 1931) which depends on the competition between radioactively-labelled antigen and unlabelled antigen for the binding sites of specific antibodies (Berson & Yalow, 1957). Increasing amounts of unlabelled antigen in the test fluid cause proportionate decreases in the binding of labelled antigen to the antibodies (Yalow & Berson, 1959). Thus, the level of radioactivity associated with the antigen-antibody complex is inversely related to the concentration of unlabelled antigen in the original test fluid. The latter value is obtained by comparing the radioactivity bound with that produced by standard solutions containing known amounts of unlabelled antigen (figure 1).

In contrast to the requirements for validation of classical isotopic dilution techniques (Hevesy & Hobbie, 1931), radioimmunoassay neither requires uniform mixing of labelled and unlabelled antigens, nor identical behaviour of these two components in the immune system. The sole requirement in radioimmunoassay is that the standard and unknown antigens behave identically in competitively inhibiting the binding of the labelled species (Berson & Yalow, 1973). Accordingly, the control of non-specific interference, and the demonstration of immunochemical identity between standards and unknowns are two of the most important criteria upon which the establishment of a valid radioimmunoassay is based.

The fundamental reaction in radioimmunoassay is, by definition, immunochemical in nature. Since there is no guarantee that the immunological and biological determinants reside at the same site of the antigen molecule, the immunochemical reactivity of the antigen is not necessarily correlated with its biological activity (Berson & Yalow, 1964). Thus, fragments of the antigen molecule, precursor forms, and other structurally related antigens all represent potential sources of error in radioimmunological estimations (Heding, 1966; Yalow, 1974b).

The selection of 'antigen-specific' antisera and the use of suitable standard preparations are therefore of paramount importance in assuring the immunochemical and biological identity of the unknown.

BASIC KINETICS AND THERMODYNAMICS

Quantitative evaluation of the concentration of antibody-binding sites and the kinetics of the antigen-antibody reaction has been extensively studied by Berson and Yalow (1957, 1959d). Using radioiodinated beef insulin as tracer, and antisera from insulin resistant diabetics they demonstrated that the steady state and transient state kinetics of most immune sera were consistent with a model in which a univalent antigen reacts with two or more distinctly different orders of antibody-binding sites (Berson & Yalow, 1959d).

The exact nature of the antigen-antibody reaction in radioimmunoassay is complex, and will vary according to the heterogeneity of the antibody, and the physical, chemical and immunochemical properties of the labelled, standard and unknown antigen (Ekins et al, 1970). However, despite these intricate considerations, it is generally accepted that the simplest possible model, i.e. the reaction between homogeneous antigen and a single order of antibody binding sites, is a reasonable approximation to the antigen-antibody interaction in most radioimmunoassay systems (Ekins et al., 1968; Yalow & Berson, 1968).

The antigen and antibody react to form a non-precipitating complex which is in reversible equilibrium with the uncomplexed species (Berson & Yalow, 1957):

$$P + Q \underset{k_1^{-1}}{\overset{k_1}{\rightleftharpoons}} PQ \quad , \qquad (1)$$

$$P^* + Q \underset{k_2^{-1}}{\overset{k_2}{\rightleftharpoons}} P^*Q \quad , \qquad (2)$$

where P represents the unlabelled antigen; P* the labelled antigen; Q the antibody; PQ the complex of unlabelled antigen and antibody; P*Q the complex of labelled antigen and antibody; k_1 and k_2 the association rate constants; and k_1^1 and k_2^1 the dissociation rate constants.

At equilibrium, according to the law of mass action, we have

$$K = k_1/k_1^1 = [PQ] / [P][Q] , \qquad (3)$$

$$K^* = k_2/k_2^1 = [P^*Q] / [P^*][Q] . \qquad (4)$$

These equations define the affinity constants K and K* . By virtue of the assumption that the labelled and unlabelled antigens behave identically in the immune systeme, K* = K. The bound-to-free ratio for the antigen is therefore defined as

$$R = B/F = [P^*Q] / [P^*] . \qquad (5)$$

The total concentration of bound antigen is given by

$$B = [PQ] + [P^*Q] \qquad (6)$$

and the bound-to-total ratio by

$$B/T = B/B+F = R/(1 + R) = [P^*Q] /p^* = [PQ] /p , \qquad (7)$$

where

$$p = [P] + [PQ] , \qquad (8)$$

$$p^* = [P^*] + [P^*Q] . \qquad (9)$$

p* is the total concentration of labelled antigen, and p is the total concentration of unlabelled antigen. The total concentration of antibody is defined as

$$q = [Q] + [P^*Q] + [PQ] . \qquad (10)$$

The concentrations are usually expressed in moles per litre; accordingly the K values are expressed in litres per mole.

Combining equations 4, 5, 6 and 10 we obtain

$$R = K(q-B) \quad , \tag{11}$$

where

$$B = [PQ] + [P^*Q] = B/T(p^* + p). \tag{12}$$

Equation 11 indicates that the ratio of bound-to-free antigen (R) is a linear function of the concentration of bound antigen (B) and a plot of R vs B yields a straight line with a slope of -K, an abscissa intercept of q and an ordinate intercept of Kq (figure 2). This plot, introduced for the interactions between proteins and small molecules by Scratchard (1949) and applied to radioimmunoassay by Berson and Yalow (1959d), is of fundamental importance in determining the total concentration of binding sites and the affinity constant for the antiserum. Systems containing many different orders of antibody binding-sites demonstrate a curvilinear relationship (figure 3), which may or may not, over certain ranges, approximate to straight lines (Berson & Yalow, 1959d).

Combining equations 11 and 12 we obtain

$$R = K\left[q - B/T(p^* + p)\right] \quad . \tag{13}$$

It is apparent from this equation that the ability to detect a concentration of antigen, p, depends on the relationship between K and p.

Berson and Yalow (1973) have demonstrated that for the condition $q = p$ and $Ro = 1$, where Ro represents R at zero dose of unlabelled antigen,

$$K \geq 1/p \tag{14}$$

According to this relationship, the sensitivity that can be achieved with any given antiserum is dependent on the affinity constant of the predominating antibodies. Antisera suitable for radioimmunoassay in the

picomole range should have affinity constants of 10^{12} to 10^{10} litres per mole.

Changes in both the antibody affinity constant and concentration have been shown to markedly affect the shape and sensitivity of the standard curve (Berson & Yalow, 1973). Figure 4 illustrates the experimental curves derived from several antisera to insulin. Antisera with high affinity constants produce the most sensitive standard curves (Ekins et al., 1968; Yalow & Berson, 1968).

The kinetics of the antigen-antibody reaction can be defined in terms of the rate constants (see equations 3 and 4). With radioimmunoassays conducted at low concentrations of reactants, the reaction may require several days to reach equilibrium (figure 5).

The influence of temperature on the reaction between the antigen and antibody is illustrated in figure 6. In general the affinity constant for the reaction increases as the temperature is lowered, and providing that equilibrium is reached, antigen binding is maximal at $4^{\circ}C$ (Berson & Yalow, 1959d). At high temperatures the reaction proceeds more rapidly since the frequency of collisions between the reactants is increased. This property can be fully exploited when the affinity constant of the reaction does not display temperature dependence, and thus a high incubation temperature can be used for the rapid attainment of equilibrium without affecting the binding and thus the sensitivity potential of the antiserum (Keane et al., 1976).

The relationship between the affinity constant (K) and the temperature is defined by the equations:

$$\Delta F^{\circ} = -RT\mathrm{Ln}K \quad , \qquad (15)$$

$$\Delta F^{\circ} = \Delta H^{\circ} - T\,\Delta S^{\circ} \quad , \qquad (16)$$

where ΔF° represents the standard free energy change; R the gas constant (note that the same symbol, R, was used in a different context above); T the absolute temperature: LnK the natural logarithm of the affinity constant; and ΔH° and ΔS° the enthalpy and entropy changes.

These equations demonstrate that the temperature dependence of the affinity constant is governed by the relative influence of the enthalpy and entropy changes on the standard free energy change of the antigen antibody reaction. The magnitude of the enthalpy and entropy components is determined by the intricate nature of the binding process (Karush, 1962; Padlan, 1977). The entropy change is generally a reflection of the extent of hydrophobic bond formation, while the formation of more complex bonds of the Van der Waals type is associated with the enthalpy change of the reaction (Karush, 1962).

Antigen-antibody reactions have been classified according to the relative contributions made by the enthalpy and entropy components to the standard free energy change (Keane et al., 1976). Keane and colleagues were able to show that in reactions characterised by a small enthalpy and large entropy component, the affinity constant was not temperature sensitive. Antigen-antibody reactions which relied on the enthalpy change for their free energy of binding were however, shown to possess large but temperature dependent affinity constants.

Most radioimmunoassays allow the simultaneous competition between the labelled and unlabelled antigen for the limited number of antibody binding sites to reach equilibrium before stopping the reaction (Zettner, 1973):

$$P^{*}Q \rightleftharpoons P^{*} + Q + P \rightleftharpoons PQ \qquad (17)$$

The termination of the reaction prior to the attainment of equilibrium results in the production of a shallow standard curve with poor precision (Feldman & Rodbard, 1971; Rodbard et al., 1971). The sensitivity of the method, as defined by either Ekins or Yalow and Berson, is therefore greatly reduced.

In radioimmunoassays performed with delayed addition of labelled antigen, the antigen-antibody reaction is often terminated prior to reaching equilibrium (Zettner & Duly, 1974). This technique, first introduced by Hales and Randle (1963a) for the radioimmunoassay of insulin, often results in better sensitivity than can be achieved with the

more conventional equilibrium conditions (Rodbard et al., 1971). The effect is illustrated in figure 7, where dose-response curves obtained by the equilibrium and delayed addition methods are compared. The delayed addition curve is steeper, indicating greater sensitivity. The useful working range of this curve is however more limited than that of the equilibrium assay.

In the equilibrium method P* competes with P during their simultaneous incubation for the available binding sites of Q, while in the delayed addition method such competition is essentially eliminated. Whatever competition occurs is confined to the second incubation step. At zero dose of P, the amount of P*Q formed in both techniques will be identical, but the same finite dose of P will bring about a greater decrease of P*Q in delayed addition than in the equilibrium method, resulting in a steeper dose-response curve. If the precision of both assay forms is the same, increased sensitivity will be observed (Ekins, 1974a).

The limiting factor in the success of the delayed addition method is the extent of dissociation of the bound unlabelled antigen that occurs during the second incubation step (Rodbard et al., 1971; Zettner & Duly, 1974). This is dependent on the dissociation rate constant which is related by definition to the affinity constant of the antiserum (equations 1 & 3). Figure 8 illustrates the dose response curves obtained by equilibrium and delayed addition methods with one particular glucagon antiserum. The long incubation time relative to the dissociation velocity results in the establishment of quasi-equilibrium conditions and thus essentially superimposable curves.

The effect of duration of the second incubation in delayed addition radioimmunoassays has been extensively studied (Feldman & Rodbard, 1971; Rodbard et al., 1971). Using computer simulation of the binding reaction, Rodbard and colleagues were able to demonstrate that as time progressed during the second incubation, the fraction of labelled antigen bound for any arbitrary concentration of unlabelled antigen increased progressively

approaching the equilibrium value. Thus, providing that the precision remained unchanged, the termination of the reaction soon after the addition of the labelled antigen and prior to the attainment of a second equilibrium, would result in an apparent steepening of the dose response curve and an improvement in assay sensitivity. Unfortunately, as noted by Zettner and Duly (1974), this is usually only true in situations where the concentrations of reactants are high and relatively short incubation times are used. When the concentrations of the reactants are low, minor differences between the timing of individual tubes can cause significant differences in the dissociation of the complex PQ, allowing differing amounts of P* Q to form, and thereby adversely affecting both the precision and accuracy of the assay. For this reason, many delayed addition radioimmunoassays employing high affinity antisera utilise a prolonged second incubation, thereby accepting a less spectacular shift of the dose response curve but nevertheless achieving improved assay sensitivity (Albano et al., 1972; Utiger, 1974).

FUNDAMENTAL PRINCIPLES OF ASSAY DESIGN

The fundamental purpose of all radioimmunoassay techniques is the accurate measurement of an unknown concentration of the antigen in a biological fluid or extract. Accuracy, defined by Midgley and colleagues (1969) as the extent to which the mean of an infinite number of measurements of an antigen agrees with the exact amount of the antigen present, embraces the concepts of both precision and specificity, since, in order for the measurement to be accurate it must be both reproducible and unbiased.

Precision reflects the extent to which a given set of measurements of the same sample agrees with the mean (Midgley et al, 1969). As illustrated in figure 9, the precision of measurement ($\triangle$ p) of an unknown concentration of antigen (p) is related both to the precision of

of measurement of the response metameter (Δ R) and the slope of the response curve at the corresponding point:

$$\text{precision } (\Delta p) = \frac{\Delta Rp}{\text{slope (at Rp)}}$$

Accordingly, at zero concentration of antigen (po), the precision of measurement (Δ po) represents the detection limit or sensitivity of the assay (Ekins, 1974a):

$$\text{sensitivity } (\Delta po) = \frac{\Delta Ro}{\text{slope (at Ro)}}$$

Thus the ability of an assay system to distinguish a measured antigen concentration as being significantly different from zero is governed by the precision of the measurement of zero antigen (Midgley et al., 1969a; Ekins & Newman, 1970). Similarly, the ability of the system to distinguish between two concentrations on the response curve is dependent upon the precision of measurement of antigen concentration at that point (Ekins & Newman, 1970; Reeves & Calhoun, 1970). Since in general both the slope and the precision of measurement of the response metameter change at different points on the curve, the precision of antigen measurement will likewise vary as a function of the antigen concentration (Midgley et al., 1969a; Rodbard, 1974; Ekins, 1976).

As illustrated in figure 10, the precision of measurement of the response metameter (Δ R) is dependent upon the statistical sum of two independent errors: the experimental errors arising from pipetting and other sample manipulations, and the statistical counting errors inherent in radioactivity measurements. Thus for any unknown concentration of antigen (p), the precision of measurement (Δ p) depends on the relationship between three principle parameters: the slope of the response curve, and the two component sources of error incurred in the measurement of the response metameter (Δ R). These in turn each depend upon a number of

factors, some of which are within the direct control of the investigator, whilst others reflect his personal expertise or are characteristic of the reagents or techniques used and are therefore essentially fixed (Ekins, 1974a). The selection of optimal assay parameters makes it possible to maximise the precision of measurement of any hormone concentration and, in particular, to maximise the sensitivity of the assay (Ekins et al., 1970). The fundamental aim of assay design is the selection of an optimal assay system such that the precision in the measurement of a defined target concentration is maximal (Ekins & Newman, 1970).

The selection of assay design parameters for the optimisation of radioimmunoassays has been the subject of considerable controversy and misunderstanding (Ekins & Berson, 1969; Ekins, 1971; Yalow & Berson, 1971). The problem which has arisen from the conflicting concepts of sensitivity and precision within the radioimmunoassay field, has resulted in the development of greatly contrasting theoretical solutions by Yalow and Berson (Berson & Yalow, 1964; Yalow & Berson, 1968) and by Ekins and colleagues (Ekins et al., 1968; Ekins & Newman, 1969, 1970) to the common problem of assay design.

In contrast to the definitions described above, Yalow and Berson (1968, 1969a) have defined sensitivity as the slope of the dose response curve relating B/F or B/T to total antigen concentration:

$$\frac{d(B/F)}{dp} \quad \text{or} \quad \frac{d(B/T)}{dp} \quad ,$$

and precision as the slope of the response curve relating B/F or B/T to the fractional change in antigen concentration:

$$\frac{d(B/F)}{dp/p} \quad \text{or} \quad \frac{d(B/T)}{dp/p} \quad .$$

Although these definitions have been bitterly contested by Ekins on numerous occasions(Ekins, 1969, 1970b), careful inspection reveals that they differ only in as much as that the definitions proposed by Ekins and colleagues (Ekins et al., 1968; Ekins & Newman, 1970) include an error term. In practice, this means that the slope of the response curve cannot be taken as an indication of sensitivity or precision of hormone measurement except under special circumstances where the error in the measurement of the response metameter remains constant at any point on the curve.

The traditional approach to radioimmunoassay design as developed by Yalow and Berson (Berson & Yalow, 1964; Yalow & Berson, 1968, 1969a, 1970a), and adopted by most workers in the field, entails the preliminary search for high affinity antisera and the development of methods for the synthesis of high specific activity labelled antigen. In setting up an individual assay, the concentration of antibody binding sites is adjusted to yield 33 to 50% binding of total immunoreactive counts, in the absence of unlabelled antigen. If a very sensitive assay is required, a minimum workable amount, usually 6,000 counts per minute, of labelled antigen of the highest practical specific activity is added to each tube. Following the reaction between both the labelled and unlabelled antigens for the binding sites of the antibody, the free and antibody bound antigens are separated, and the radioactivity in either or both fractions is counted, usually to 10,000 counts to ensure high counting precision. The distribution of radioactivity is finally expressed in terms of one of a number of response metameters, e.g. B/F or B/T, and plotted as a function of the amount or concentration of the unlabelled antigen. The dose of unlabelled antigen producing a 10% fall in bound counts, and the midpoint of the assay, the dose which lowers the response metameter to one half of its initial value, are then evaluated by visual inspection of the response curve. These parameters give a rough guide to the sensitivity of the assay

and to the range of hormone concentrations measured with the greatest precision. The optimisation of assay parameters is then carried out on an empirical basis, such that the precision in the measurement of a defined target concentration is maximal. As noted previously (figure 7), the timing of addition of the labelled and unlabelled antigens can drastically alter the shape of the response curve. However, the most effective and common method used to change the sensitivity and midpoint of an assay is to vary the dose of labelled antigen, thereby reducing the concentration of antibody binding sites. This phenomenon is illustrated in figure 11, where a simple halving of the tracer dose results in an approximate four-fold increase in sensitivity. The working range of this assay is however greatly reduced.

The theoretical validity of this essentially empirical approach to assay design has been justified in numerous mathematical treatments by Yalow and Berson (Berson & Yalow, 1964; Yalow & Berson, 1968, 1969a). The analyses which are based on a number of simplifying assumptions start from the fundamental relationship between the ratio of bound-to-free antigen and the concentration of bound antigen (equation 11):

$$B/F = K(q-B) .$$

Assuming $P*Q \cong 0$, $p-B = F$. Substituting for F in equation 11 enables B to be solved in terms of p. By substituting this value for B in the right side of equation 11, B/F can be given as a function of p to provide the dose-response relationship. The resulting expression is simplified by expansion of the binomial series. Neglecting higher order terms beyond the first, the resulting expression is

$$B/F \cong Kq - \left(\frac{K^2qp}{Kq + Kp + 1}\right) . \qquad (18)$$

Differentiating this equation with respect to p and simplifying, we obtain

$$\frac{d.B/F}{d.p} \cong \frac{(B/F)^2}{q(Kq+1)} \quad . \qquad (19)$$

This equation determines sensitivity, defined as d(B/F)/dp. Sensitivity increases as q decreases. From equation 11, it is seen that $B/F \leq Kq$ and that $q \longrightarrow (B/F)/K$ as $B \longrightarrow 0$ (or $p \longrightarrow 0$). Substituting for this value of q in equation 19, we obtain the maximal sensitivity at any given B/F:

$$\frac{d.(B/F)}{d.p} \cong \frac{B/F}{B/F + 1} K \quad . \qquad (20)$$

It follows that this degree of sensitivity will be obtained when the concentration of labelled antigen is sufficiently low for B to be much less than q. At higher concentrations, when this condition is not fulfilled, B/F is smaller than Kq, and the sensitivity is less than that given by equation 20.

It is important to determine the B/F value at which the percentage bound changes maximally with hormone concentration. Since $(B/T)/(F/T) = (B/T)/(1-B/T)$, it follows that

$$\frac{d(B/T)}{d(B/T)/(F/T)} = (1-B/T)^2 \quad .$$

Since $(B/T)/(F/T) = B/F$, we obtain from equation 20

$$\frac{d(B/T)}{d(B/T)/(F/T)} \frac{d(B/F)}{dp} \cong \frac{d(B/T)}{dp} \cong -K(B/T)(1-B/T)^2 \quad . \qquad (21)$$

We find the maximum and minimum by differentiating with respect to B/T and setting the result to 0, thus

$$1 - 4(B/T) + 3(B/T)^2 = 0$$

The absolute value of $d(B/T)/dp$ is a maximum at $B/T = 0.33$ $(B/F = 0.5)$ and is a minimum at $B/T = 1$.

Thus the maximal sensitivity for any given antibody is attained when q is adjusted to yield $Kq = 0.5$ and the concentration of tracer is negligibly small so that $B/T \cong 0.33$ in the absence of unlabelled antigen, and that under these conditions

$$\frac{d(B/T)}{dp} = -\frac{4}{27} K \quad . \qquad (22)$$

The conditions for maximal precision are different than for maximal sensitivity,

$$\frac{d(B/F)}{dp/p} \cong -B/F \left(1 - \frac{B/F}{Kq}\right) \quad . \qquad (23)$$

Maximal precision is obtained not at trace concentrations of hormones, but at concentrations that result in virtual saturation of the antibody combining sites, that is, at $B/F \ll Kq$. Under such conditions $B \cong q$ obtains. Since by definition B/T equals B/p, then under the conditions described $B/T \cong q/p$ which, when logarithms are taken becomes

$$\mathrm{Ln}(B/T) \cong \mathrm{Ln}(q) - \mathrm{Ln}(p) \quad . \qquad (24)$$

Differentiating with respect to p and rearranging gives

$$\frac{d(B/T)}{dp/p} = -B/T \quad . \qquad (25)$$

In contrast to sensitivity, maximal precision is independent of the affinity constant (K). When $B/F \ll Kq$ precision is maximal when B/T approaches unity. Generally good precision is obtained when B/T is between 0.4 and 0.75.

An alternative approach to radioimmunoassay design has been developed by Ekins and colleagues (Ekins et al., 1968; Ekins & Newman, 1969, 1970). The mathematical calculations involved in the selection of optimal reagent

concentrations and incubation times are highly complex, generally necessitating the use of computer optimisation techniques. Such optimisation programmes have been developed by Ekins and colleagues (Ekins & Newman, 1970; Ekins et al., 1970), Rodbard (1971) and Wilson et al (1971), but as pointed out by Rodbard (1974) they must still be regarded as experimental since each makes numerous simplifying assumptions. An essential prerequisite of this approach to assay design is the accurate determination of the affinity constant of the predominating antibody binding sites. This necessitates the temporary adoption of methods employed in the classical approach to radioimmunoassay for the production of the first dose response curve. The energy of binding is estimated from the slope of the response curve expressed in terms of the Scratchard co-ordinates (Scratchard, 1949). Ekins and colleagues (Ekins et al., 1968; Ekins & Newman, 1969, 1970) have shown that both sensitivity and precision can be defined in terms of this fundamental assay parameter. The analyses which are based on the same basic assumptions as used by Yalow and Berson start, in contrast, from the quadratic equation obtained by combining equations 3 to 10:

$$R^2 + R(1 + Kp + Kp^* - Kq) - Kq = 0 \quad . \qquad (26)$$

This equation which is the key to all dose response relationships for the reaction between homogeneous antigen and a single order of antibody combining sites, and which has been used extensively in computer simulation studies of radioimmunoassay, is used to derive the slope of the response curve at any point:

$$\frac{dR}{dp} = -\frac{R}{qR + 1/KR} \quad . \qquad (27)$$

The slope increases as K increases and q decreases.

The precision of measurement of the response metameter is given by the equation:

$$\Delta R = R\sqrt{\frac{(1+\sqrt{R})^2(R+1)}{p*SVTR} + e^2}\ , \qquad (28)$$

Where S represents the specific activity of the tracer (in counts per minute, per unit weight); T represents the total time devoted to counting each free and bound pair; V represents the volume of incubation mixture that is subjected to separation and to radioactive measurements; and e represents the experimental errors. Counting errors depend in a predictable way on the total available counting time, and the concentration and specific activity of the labelled antigen. Experimental errors will depend on the technique used and the skill of the experimenter. They will also be a function of the concentrations of hormone and binding sites present in the assay system.

Combining equations 27 and 28, we may derive the precision of measurement of any concentration of the antigen. The following expression concerns zero concentration, the detection limit or sensitivity of the assay (Δpo):

$$\Delta po = \Delta Ro(dp/dR) = (qRo + 1/RoK)\sqrt{\frac{(1+\sqrt{Ro})^2(Ro+1)}{Rop*SVT} + e^2}\ . \qquad (29)$$

Thus to optimise precision we must choose values of q and p to minimise Δp. In general this can only be achieved using computer optimisation techniques. The optimisation of assay sensitivity which is equivalent to that of assay precision when p = o may however be derived algebraically. Assuming that the only error in the determination of R is the counting error, i.e. e = o, optimal sensitivity is achieved at:

$$p* = 4/K, \quad q = 3/K \quad \text{and} \quad R = 1.$$

A significant concentration of labelled antigen is required for optimal sensitivity under these conditions. The minimal detectable concentration of unlabelled antigen is given by

$$\Delta \text{ po min} = \frac{4\sqrt{2}}{\sqrt{KSVT}} \quad . \qquad (30)$$

This equation reveals the fundamental dependence of assay sensitivity on the affinity constant. The precision of target antigen concentrations greater than zero does not always display this relationship. Indeed, a situation may be reached where the precision of target antigen measurement is essentially independent of the affinity constant.

If the experimental error, e, is re-introduced into the analysis (assumed minimal when R = 1), the optimal concentration of binding sites is given by the quadratic equation:

$$e^2STV\,(q - 1/K)^2 + 2(q - 1/K) - 4/K = 0 \; . \qquad (31)$$

The optimal concentration of labelled antigen can be similarly derived. Using these equations as the basis for a computer optimisation study, Ekins and colleagues (1970) have confirmed that as the experimental errors decrease towards zero, Kq tends to 3 and Kp* to 4, regardless of the values of S, V, T and K. The analysis also demonstrated the importance of the magnitude of the experimental error in the determination of assay sensitivity. Accordingly, sensitivity can only be increased to a limited extent by reducing the counting error.

Despite the contrasting conclusions regarding optimisation of radioimmunoassay which have arisen as a result of fundamental differences in the mathematical approach to assay design adopted by Yalow and Berson (Berson & Yalow, 1964; Yalow & Berson, 1968, 1969a) and by Ekins and colleagues (Ekins et al., 1968; Ekins & Newman, 1969, 1970), the optimal concentrations of labelled antigen and antibody predicted by the two methods often correspond quite closely (Ekins, 1976). Indeed, using

computer simulation of the binding reaction, Feldman & Rodbard (1971) were able to demonstrate that the traditional ($p^* = o$, $q = 0.5/K$, $R = 0.5$) and computerised ($p^* = 4/K$, $q = 3/K$, $R = 1$) approach to optimisation of assay sensitivity yielded similar response curves. It must however be re-emphasised that both mathematical approaches are based on numerous simplifying assumptions:

1. the antigen is present in homogeneous form, consisting of only one chemical species;
2. the antibody is present in a single homogeneous chemical form:
3. both antigen and antibody are univalent;
4. no allosteric or co-operative effects are present:
5. the radioactively-labelled and unlabelled antigen have the same physical, chemical and immunochemical properties;
6. the antigen and antibody react until equilibrium is reached;
7. bound and free forms of the labelled antigen are separated perfectly without disturbing equilibrium;
8. the ratio of bound-to-free antigen or the ratio of bound-to-total antigen can be measured perfectly.

These conditions do not necessarily prevail in any assay system, particularly since every antiserum contains a heterogeneous mixture of antibodies (Parker, 1976). Consider the sensitive, precise radioimmunoassay of serum insulin relying on charcoal separation of bound and free hormone moieties reported by Albano, Ekins, Maritz and Turner (1972). The method is based on the non-simultaneous competition between human insulin (standard or unknown) and radioiodinated beef insulin for the antibody binding sites of guinea pig antiporcine insulin antiserum. Clearly these assay parameters alone invalidate assumptions 5 and 6. Since insulin antisera contain at least two orders of antibody binding sites (Berson & Yalow, 1959d; Kerp & Kasemir, 1976) which invariably

cross react with proinsulin (Steiner et al., 1968) assumptions 1 and 2 are also invalidated. The insulin antibody is divalent as is the insulin molecule under certain circumstances (Følling, 1976), this gives rise to co-operative and allosteric effects (Rodbard & Bertino, 1973) as indicated by the demonstration of the hook effect (Yalow, 1976). Thus assumptions 3 and 4 do not hold either. Finally, it is self-evident that in radioimmunoassay there exist numerous inherent unavoidable errors (Rodbard, 1971; Rodbard & Catt, 1972) which invalidate assumptions 7 and 8. However, despite all these anomalies, the assay which was developed according to the principles acclaimed by Ekins and colleagues (Ekins et al., 1968; Ekins & Newman, 1970) has a formal sensitivity of 1.8 pg/ml. This compares almost identically with the corresponding assay of Yalow and Berson (1970b) which can readily detect 1 pg/ml. Accordingly, it should be emphasised that in spite of the relatively unrealistic assumptions upon which the two theoretical models are based, they each provide a logical basis for the optimisation of assay design.

In practice, the traditional approach to radioimmunoassay design as developed by Yalow and Berson (Berson & Yalow, 1964; Yalow & Berson, 1968, 1969a) has gained widespread acceptance. The popularity of this approach is probably a reflection of its general practicability and does not therefore represent a genuine preference on theoretical grounds. The principle is simple and requires no previous knowledge of either the specific activity of the labelled antigen or the affinity constant of the antibody binding sites. Assays developed along traditional lines are often suboptimal, but nevertheless comparable with those developed using more sophisticated techniques (Ekins, 1974a). The theoretical approach to assay design developed by Ekins and colleagues (Ekins et al, 1968; Ekins & Newman, 1970) however, facilitates the optimisation of assay parameters to the determination of any target concentration of antigen. The method, which is based on a highly complex mathematical model of the

binding reaction can only be exploited by the use of numerical methods involving computer optimisation techniques (Ekins, 1974a, 1976). The affinity constant of the antibody binding sites features predominantly in the mathematical derivation of assay parameters and must therefore be considered of fundamental importance in the optimisation of assay design. Unfortunately, it is not always possible to obtain a reliable estimate of the affinity constant by Scratchard analysis of the binding reaction (Walker, 1977). The Scratchard relationship holds only for ideal systems, and curvilinear plots (figure 3) are frequently obtained due to the presence of multiple orders of antibody binding sites (Berson & Yalow, 1959d; Kerp & Kasemir, 1976). An effective affinity constant (Ekins, 1970a; Ekins, 1971; Albano et al., 1972) can however be accurately derived by computerised techniques (Wilson et al., 1971; Rodbard & Catt, 1972; Rodbard, 1973). More recently, Ekins has extended the mathematical model to permit optimisation of assay parameters in those instances where the afore mentioned assumptions do not apply (Ekins, 1974a, 1976). Although this development undoubtedly represents a major advancement, the intricate nature of the analysis has necessarily limited its general applicability to one or two specialist centres.

PREPARATION OF REAGENTS

The principle of radioimmunoassay is based on the competition between radioactively labelled antigen and unlabelled antigen (standard or unknown) for a limited number of binding sites of specific antibodies. The performance of radioimmunoassay procedures therefore depends on the induction of specific antibodies with a high affinity for the antigen, the synthesis of radiolabelled antigen and the production of a suitable standard or reference preparation of the antigen. A degree of impurity in each of these basic reagents is permissible providing that the immunochemical reaction is not affected. Accordingly, the presence of

antibodies other than those directed towards the specific antigen in the immune serum is unimportant so long as they do not react with either the labelled or native antigen (Berson & Yalow, 1973). Similarly, minor impurities in the tracer preparation can be permitted providing that they are neither labelled nor immunologically active in the assay system (Hunter, 1974). Finally, the presence of contaminants in the standard preparation does not necessarily invalidate its usage, providing that the impurities do not participate in the fundamental immunochemical reaction (Bangham & Cotes, 1974).

ANTIBODY

The sensitivity and specificity of radioimmunoassay depend predominantly on the affinity and structural configuration of the antibody binding sites (Kirkham & Hunter, 1971; Berson & Yalow, 1973). Consequently, the production of a high quality antiserum is an essential prerequisite to all physiological studies based on radioimmunoassay techniques. Despite the fundamental role of the antibody in determining the potential usefulness of the assay system, the production of antisera for radioimmunoassay is conducted on an empirical basis (Hurn & Landon, 1971; Hurn, 1972).

An initial difficulty in most radioimmunoassays is raising an antibody of high affinity and specificity for the antigen to be measured (Jaffe & Behrman, 1974). The successful production of useful antisera is dependent upon many factors, the most important of which concerns the availability and immunogenicity of the antigen (Bloom, 1974). The target antigen concentration is also an important consideration in the evaluation of the degree of success (Ekins, 1974a). Antisera suitable for immunoassay in the picomole to nanomole range must have affinity constants of approximately 10^{12} to 10^{10} litres per mole respectively (Yalow & Berson, 1970a). The immunogenicity of a substance depends on both its size and its chemical composition (Kabat & Bezer, 1958;

Sela, 1966). Although proteins and most large polypeptides are potent stimulators of antibody formation, other compounds including steroids and smaller peptides are not immunogenic in their native state and must be conjugated to larger carrier molecules before they will initiate an immune response (Abraham & Grover, 1971b; Parker, 1971; Skowsky & Fischer, 1972). A variety of chemical procedures are available for the covalent linkage of such substances to larger molecules (Kennedy et al., 1976; Parker, 1976). Based largely on the experience in raising antibodies to steroid hormones, cyclic nucleotides, and a variety of drugs, it is now evident that small poorly immunogenic peptide hormones such as glucagon can also be rendered immunologically potent by these coupling procedures (Abraham & Grover, 1971b; Bloom, 1974; Marks et al., 1974).

Numerous methods exist for the covalent coupling of substances to large carrier molecules (Kennedy et al., 1976; Parker, 1976). Small molecular weight antigens have been coupled to various proteins by reaction with diisocyanates, diimido esters, dihalonitrobenzenes, glutaraldehyde, bifunctional diazonium salts, alkyl chloroformates, isoxazolium salts and carbodiimides (Parker, 1971; Kennedy et al., 1976). The choice of conjugation procedure is influenced by the physical and chemical properties of the hapten (Parker, 1976). The most commonly employed method for hapten conjugation is the reaction with albumin using carbodiimide as the coupling agent (Goodfriend et al., 1964). The carbodiimide condensation reaction first applied to the problem of antibody production for immunoassay by Oliver and colleagues (1966, 1968) in connection with the digitoxin radioimmunoassay, involves the activation of carboxyl groups of the hapten so that they react with the amino groups of the protein, forming a CO-NH bond (Abraham & Grover, 1971a; Parker, 1971). Where the hapten does not contain appropriate functional groups, or where these groups must be left undisturbed for maximal antibody specificity, it is necessary to add new functional groups to the hapten

by organic synthesis. In the case of digitoxin, cyclic AMP and cyclic GMP, a free carboxyl group can be added by succinylation and the resulting derivative is conjugated to albumin using carbodiimide (Oliver et al., 1966, 1968; Steiner et al., 1969; Steiner, 1974). The desired level of substitution is controversial, however for most antigens a conjugate containing between 5 and 15 haptenic groups per molecule of protein provokes a good immunological response (Niswender & Midgley, 1970; Abraham & Grover, 1971a).

Most antisera for radioimmunoassay purposes have been raised in small laboratory animals. Although the amount of sera that can be obtained from these species is relatively small, the high working dilution at which a good antiserum is used renders even a restricted supply sufficient for most purposes (Ekins, 1976). Rabbits are usually the species of choice, they are easy to handle and 50 ml of blood can be taken from the marginal ear vein at frequent intervals. Guinea pigs are large enough to survive regular bleedings of 10-15 ml, however the blood must be obtained by cardiac puncture with its associated risks. Nonetheless they are cheaper to maintain than rabbits and need slightly less immunogen, enabling larger numbers to be used. There are very few instances where the species of animal used is of overriding importance in the successful production of antisera. The virtual necessity of using guinea pigs for the production of insulin antisera is a rare exception (Morse, 1959; Robinson & Wright, 1961). Indeed the fact that an immunogen may be related to, or even identical with, a substance naturally circulating in the immunised animal does not necessarily prevent the immune response (Hurn & London, 1971; Playfair et al, 1974). Antibodies have been produced against numerous native hormones including arginine vasopressin (Roth et al., 1966; Edwards, 1970), human growth hormone (Roth et al., 1964) and both porcine and bovine insulins (Renold et al., 1964; Lockwood & Prout, 1965). The interaction of antibodies with

endogenous hormone in these circumstances is poorly understood, since theoretically the animal should exhibit symptoms of hormonal deficiency with fully saturated antibodies. Under appropriate conditions however, antibody mediated resistance to insulin (Yalow & Berson, 1971), growth hormone (Parker & Daughaday, 1964) and glucagon (Grey et al., 1970) can be demonstrated. Whether circulating hormones bind the high affinity antibodies, reducing the sensitivity potential of an antiserum, is unknown.

The production of high affinity antisera can only be achieved with the use of an adjuvant. The most widely used adjuvant is that introduced by Freund (1951) which consists of a neutral detergent (Arlacel A), parafin oil, and in the complete form, dried heat killed mycobacteria. The hydrophilic and lipophilic groups of the detergent bind both the oil and the aqueous solution of the immunogen allowing the formation of a stable emulsion (Herbert, 1973). A single inoculation of an immunogen in a water-in-oil emulsion stimulates a high and long-lasting antibody response (Talmage & Dixon, 1953). The immunogen has been detected in the emulsion depot for as long as one year after inoculation and the adjuvant activity appears to be closely related to the slow release of immunogen for which emulsions of this type are ideally suited (Herbert, 1966, 1968). This slow release of immunogen over a period of weeks avoids its rapid degradation by circulating proteolytic enzymes and, in the case of certain hormones such as insulin, permits the use of potentially lethal doses (Heding, 1972). The use of complete adjuvant is especially valuable for the provocation of autoimmune phenomena (Freund & Lipton, 1955). Mycobacteria increase the inflammatory response in regional nodes, amplifying the immune response and helping stimulate the eventual formation of high affinity antibody (Hurn & Landon, 1971). A disadvantage of the complete adjuvant is the extensive granuloma that is inevitably formed and the frequent occurence of an abscess at the innoculation site (Herbert, 1973).

Antibody response is virtually independent of immunogen dosage over a wide range (Hurn & Landon, 1971). The minimum effective quantity varies from one substance to the next, but even poor immunogens can usually be employed at primary doses of 0.1 mg (Playfair et al., 1974). In practise however, larger quantities are used and most antigens are administered at doses of 0.2 to 2.0 mg, although the absolute range extends from 0.02 to 15.0 mg (Skelly et al., 1973). Although the immunogen should theoretically be as pure as possible, homogeneity is rarely necessary, and commercial grade preparations are frequently employed for immunisation (Berson & Yalow, 1973; Parker, 1976). As indicated previously, the presence of contaminating antibodies in the immune serum does not interfere in radioimmunoassay (Playfair et al, 1974), however if the immunogen is less than 10% pure, antigenic competition can lead to an unsatisfactory immune response (Parker, 1976). When the amount of immunogen available for immunisation is limited, small doses can sometimes be used with good results, particularly if the antigen is pure (Vaitukaitis et al., 1971). The role of immunogen dose in determining the quality of the antisera in these instances may be explained by the existence of two populations of antibody producing cells (Parker, 1971, 1976). At low immunogen dosage, cells with high affinity sites are stimulated to produce high affinity antibody. At high doses of immunogen, these cells are inhibited by excess immunogen, but other cells capable of producing low affinity antibody are stimulated (Paul et al., 1968; Parker & Vavra, 1969). In most instances however, antisera display marked heterogeneity of binding characteristics suggesting that the cells involved in the formation of both high and low affinity antibodies are being stimulated simultaneously (Eisen & Siskind, 1964; Kerp & Kasemir, 1976).

The immunisation procedure may be as important as the immunogen in determining the success in developing antibodies (Odell et al., 1971).

Immunisation of five or more mature adolescent animals is usually carried out with 0.2 to 2.0 mg antigen incorporated in 50 to 80% complete adjuvant in a final volume of 0.5 to 1.0 ml (Hurn & Landon, 1971; Berson & Yalow, 1973). The water-in-oil emulsion may be injected by various routes (Herbert, 1973), however it is usually convenient to employ either the subcutaneous or the intramuscular route (Hurn & Landon, 1971; Playfair et al., 1974). When only small amounts of the immunogen are available, the multiple intradermal injection procedure (Vaitukaitis et al., 1971) may be more successful. The method in which very small amounts of the immunogen (1-5 ug per injection site) are injected at 40 or more sites spread over the body surface of the recipient animal, produces a near maximal antibody response within a few weeks of initial immunisation (Vaitukaitis et al., 1971). Regardless of the route or dose of immunogen employed, the timing of booster injections is important in ensuring a maximal response (Odell et al., 1971). Antibody levels rise slowly after the primary immunisation, reaching a peak some six weeks later (Hurn & Landon, 1971). Booster doses, often reduced to 5 to 50% of the initial dose (Vaitukaitis et al., 1971) and given in incomplete adjuvant, are injected near the original sites at monthly (Hurn & Landon, 1971) or less regular intervals (Odell et al., 1971) depending on personal preference. Antibody levels rise to maximum about 10 to 14 days after each booster injection and animals are bled 7 to 14 days after the third and subsequent boosters (Playfair et al., 1974). The serum is separated and stored frozen undiluted or diluted 1:100 in assay buffer (Landon, 1971).

The screening and characterisation of resulting antisera entails the assessment of titre, sensitivity and specificity (Hurn & Landon, 1971). Although a thorough analysis of each antiserum may involve the study of its ability to neutralise hormonal activity (Midgley et al., 1971), its immunohistochemical (Midgley & Pierce, 1962) and immunodiffusion

(Midgley et al., 1961) characteristics, antisera for use in radio-immunoassay are primarily assessed by their ability to bind the labelled antigen (Berson et al., 1956b). In practice, titre of each antiserum is determined from the binding characteristics of a tracer dose of labelled antigen incubated for 1 to 2 days with serial dilutions of the antiserum (Odell et al., 1971). Figure 12 illustrates the antibody dilution curves of a single bleeding of a guinea pig immunised with porcine insulin. The curves which represent 40 and 100 pg amounts of tracer have 50% maximum binding titres of 1:320,000 and 1:680,000 respectively. The divergence between the two dilution curves gives an indication of the effective affinity constant of the antiserum (Hunter, 1971a, 1973). The production of high titre antisera to small molecules and hapten-protein conjugates is however more difficult (Parker, 1971; Skowsky & Fischer, 1972). Figure 13 shows the antibody dilution curves of sera obtained from rabbits immunised with a glucagon-bovine serum albumin conjugate prepared by carbodiimide condensation. Despite the use of a common immunisation programme, only a small proportion of the sera can be used at a working titre in excess of 1: 2,000. It should be emphasised however that the titre of antibody is of secondary importance in most radioimmunoassays. Thus, provided that the antibody concentration is sufficiently high to permit the performance of a large number of assays from a reasonable volume of antiserum, the usefulness of an antiserum for the determination of low concentrations of antigen is dependent upon the energy of the reaction between the antigen and the predominating order of antibody binding sites (Ekins et al., 1968; Yalow & Berson, 1968). Although the effective affinity constant can be directly estimated (Scratchard, 1949; Odell et al., 1969), the potential sensitivity of the antiserum is usually evaluated in terms of the slope of the dose response curve (Yalow & Berson, 1968; Berson & Yalow, 1973). Visual inspection of the response curve permits a direct comparison of the sensitivity and working range of

different antisera (figure 14). The specificity of an antiserum is assessed from response curves in which potentially crossreactive substances have been substituted for the native antigen (figure 15). The consequence of variable cross reactivity with structurally related molecules is that the estimated concentration will depend on both the relative contributions of the immunoreactive components and the ability of antisera to recognise these components.

LABELLED ANTIGEN

The sensitivity, precision and specificity of radioimmunoassay is directly related to the nature and properties of the radiolabelled antigen (Hunter, 1969 a, b). Thus, the appraisal of any given antiserum, in terms of titre, sensitivity or specificity, will depend on both the specific activity and the immunological identity of the labelled antigen employed (Hunter, 1973). Accordingly, the preparation of high quality tracer is a requisite of all immunoassay techniques (Hunter, 1971a, 1974).

One of the most important considerations in radioimmunoassay concerns the preparation of radiolabelled antigens of sufficient specific activity to fully exploit the sensitivity potential of each antiserum (Yalow & Berson, 1969b; Hunter, 1973). In many instances, lowering the dose of labelled antigen to a minimal working amount causes a dramatic increase in both the antibody titre and the precision in the low dose region of the response curve (figures 11 and 12). Consequently, the use of high specific activity labelled antigens can be recommended, since not only does it help to preserve stocks of valuable antisera and improve assay sensitivity, but it also permits the analysis of dilute plasma samples thereby minimising incubation damage of the tracer (Eisentraut et al., 1968; Hunter, 1969b; Heding, 1971).

The specific activity of the labelled antigen is predominantly determined by the radionuclide incorporated into the molecule. Steroids,

drugs and other non-peptide antigens are commonly labelled with tritium, however proteins and peptide hormones can be labelled to high specific activity using radioisotopes of iodine (Eshkol, 1969). Although the iodine isotope ^{131}I was originally employed for iodination (Unger et al., 1959; Yalow & Berson, 1959), because of its relatively short half life and more hazardous radiation characteristics, its use has been superceded in recent years by ^{125}I (Hunter, 1973). Thus, despite the fact that pure ^{131}I has a theoretical potential specific activity of 19.25 Ci/μmole, approximately seven times the specific activity of pure ^{125}I, the isotopic abundance of commercially available ^{131}I is frequently only 15% and rarely exceeds 30% (Yalow & Berson, 1968). In contrast, ^{125}I is available in higher isotopic abundance, approaching 100% (Freedlender, 1969). The greater isotopic abundance of ^{125}I, combined with its high counting efficiency (Helman & Ting, 1973; Bell, 1974) and the stability of the radiolabelled antigen (Catch, 1971), has resulted in ^{125}I emerging as the radionuclide of choice for use in radioimmunoassay systems (Ekins, 1976). Indeed, despite problems of synthesis, iodinated derivatives of small compounds such as drugs, cyclic nucleotides and steroids have become preferred as alternatives to the conventional tritiated tracers (Sönksen, 1974).

Radioiodine may be incorporated into proteins and peptides by iodination of tyrosine, and under certain conditions, histidine and sulphydryl residues of the molecule (Hughes, 1957). Numerous methods are available for the iodine labelling of proteins (Chervu & Murty, 1975); these rely on the use of iodine monochloride (McFarlane, 1958; Samols & Williams, 1961), on chloramine-T (Hunter & Greenwood, 1962; Greenwood et al., 1963; Butt, 1972), on electrolytic oxidation (Rosa et al., 1964) and more recently on enzymatic oxidation (Marchalonis, 1969; Morrison et al., 1971; Thorell & Johansson, 1971). The chloramine-T method introduced by Hunter & Greenwood (1962) has been employed for the small

scale iodination of numerous proteins and has in recent years been modified to permit iodination of non-tyrosine containing peptides (Bolton & Hunter, 1972, 1973) and haptens (Oliver et al., 1968; Steiner et al., 1969; Horgan & Riley, 1973; Maclouf et al., 1976; Gomez-Sanchez et al., 1977).

The chloramine-T method is now almost universally used for the preparation of iodinated proteins and peptides of high specific activity (Hunter, 1971a). In alkaline solution the sodium salt of the N-chloro-derivative of p-toluene sulphonamide, chloramine-T, slowly generates hypochlorous acid which by oxidation of iodide permits quantitative incorporation of cationic iodine into the tyrosine residues of the molecule (Hughes, 1957; Hunter, 1973). In practice the reaction between 5 μg protein (minimum 90% pure) and 1 mCi radioiodine is carried out in 0.04 ml sodium phosphate buffer, pH 7.5, using 50 μg chloramine-T. The reaction is terminated immediately by the rapid addition of 120 μg sodium metabisulphite and the final volume is made up to 1 ml by the addition of buffer containing 100 μg potassium iodide. The radioiodinated protein is separated from unreacted radioiodine and badly damaged products by gel chromatography on a column previously saturated with protein (Greenwood, 1971). The yield of the reaction, which has an optimum at pH 7.5, is strongly dependent on the concentration of the protein, and the relative amounts of radioiodine and chloramine-T (Hunter, 1973).

Although gel filtration has proved to be the most convenient method for the purification of a wide range of radioiodinated proteins, it does not permit the full exploitation of the quality of the tracer (Hunter, 1969a). Alternative techniques have frequently been employed to suit the differing properties of the proteins being iodinated and of their damaged products (Hunter, 1971a). The alternatives to purification by gel chromatography include: adsorption chromatography using powdered cellulose (Yalow & Berson, 1960a) or synthetic silicates (Ratcliffe &

Edwards, 1971), chromatoelectrophoresis on polyacrylamide (Hunter, 1967) or starch gel (Franchimont, 1969) and ion exchange chromatography (Franchimont, 1969; Düsterdieck & McElwee, 1971; Jørgensen & Larsen, 1972). These methods enable a more complete separation of chemically damaged tracer, and accordingly yield high quality radiolabelled proteins for use in radioimmunoassay (see Hunter, 1971a). Purification by ion exchange chromatography is particularly effective in this respect since it also removes unreacted protein from the preparation, thereby increasing the specific activity (Jørgensen & Larsen, 1972).

Although extensive purification can make a good tracer even better, it is eminently apparent that even the most exquisite purification procedure cannot counterbalance the consequences of a poorly designed iodination procedure. The percentage incorporation of iodine into protein can, in theory, be progressively increased until each tyrosine residue of the molecule is di-iodinated (Berson & Yalow, 1973). High levels of substitution, however, often lead to unacceptable chemical and immunological damage (figure 16), thereby restricting the potential usage of the preparation (Berson & Yalow, 1973). A well-designed iodination procedure is therefore characterised by the attainment of a maximum practical specific activity without reducing the immunological properties of the protein. The optimal level of substitution for each protein must be determined empirically, using the antiserum employed in the assay to monitor immunological potency (Freedlender & Cathou, 1971). Substitution to the extent of 5.9 atoms of iodine per molecule may be tolerated in some instances (Izzo et al., 1964), however, in the absence of direct evidence of antiserum indifference to high levels of substitution, an average of 0.5-1.0 atoms per molecule generally ensures full immunochemical identity (Berson & Yalow, 1973; Hunter, 1974). Indeed, the specific activity achieved by this level of substitution will often be adequate for practical purposes, in these instances there will also be minimal iodination damage (Hunter, 1974).

Following purification of the labelled peptide, the preparation must be assessed in terms of radiochemical purity, specific activity, immunoreactivity and sensitivity. The radiochemical purity and specific activity of the tracer may be estimated from the elution profile of radioactivity obtained during the purification procedure (see Hunter, 1973). Although the extent of chemical damage can be determined by wick chromatography, precipitation with tricarboxylic acid or adsorption by charcoal (Sönsken & Refetoff, 1971; Jørgensen & Larsen, 1972; Bansal et al., 1974), this is rarely beneficial and the first practical step is usually the construction of an antibody dilution curve (figure 12). An approximate indication of the limits of sensitivity may be obtained by running two curves with different amounts of tracer (Hunter, 1971a, 1973). The quality of the labelled hormone may be assessed from the percentage binding to excess antibody (Greenwood, 1971). With this information in hand, the next step is to accurately determine the specific activity (Chervu & Murty, 1975; Walker, 1977) and construct the first response curve. A high quality ^{125}I-labelled hormone may be used, if stored under the appropriate conditions (Hunter, 1971b), over a period of several months.

STANDARD

In radioimmunoassay, reference preparations are required for dose interpolation to determine the potencies of the unknown samples. Although the availability of suitable standard preparations of substances such as digitoxin, cyclic nucleotides and steroids is not restricted, since synthetic preparations are readily attainable in homogeneous chemical form, the provision of International standards for peptides represents a vexing problem of considerable practical and conceptual importance (see Bangham & Cotes, 1971, 1974; Bangham, 1976).

For proper validation of radioimmunoassay procedures, the standard and unknown antigen must behave identically in competitively inhibiting the binding of the labelled species (Berson & Yalow, 1973). Any difference between the constitution of the epitope will lead to non-identical affinity constants and disparate assay results (Ekins et al., 1970; Ross, 1971). This requirement poses a fundamental problem in the radioimmunoassay of peptide hormones where extensive purification of the standard preparation can lead to the introduction of configurational artifacts (Rudinger, 1971; Bangham & Cotes, 1974). In contrast to the subtle conformational changes introduced during manipulation and storage of the standard, is the marked inter-species variation in the primary structure of many peptide hormones. Accordingly, considerable variation in immunochemical cross reactivity may be observed with the same hormone from different species, and that the degree of variation will itself depend on the antiserum used (figure 17). Thus it is essential before using a hormone of one species as standard for the endogenous hormone of another species to test the comparative reactivities of the two hormones with the antiserum in question. If a suitable reference preparation is not available from the species whose endogenous hormone is to be measured it is not possible to assign absolute values to the concentration of endogenous hormone.

An additional problem in providing reference preparations for use in peptide hormone radioimmunoassay is contamination by heterogeneous species and other immunologically related peptides (Cotes, 1974). Thus, although the presence of impurities does not necessarily invalidate the potential usage of a standard preparation (Bangham & Cotes, 1971), the existence of immunologically active contaminants represents an important source of error in radioimmunoassay (see Sönksen, 1971; Soeldner et al., 1974).

Standards for use in radioimmunoassay are provided by international organisations, national research agencies, manufacturers and in some instances individual research workers. Material suitable for use as a reference preparation should be fully characterised in terms of stability, identity, homogeneity and biological activity (Bangham & Cotes, 1971, 1974).

RADIOIMMUNOASSAY

The design of immunoassay is dependent on both the kinetic and thermodynamic properties of the antiserum and the physico-chemical characteristics of the labelled and native antigen. Thus for each application of radioimmunoassay there exists an optimal set of assay conditions which when used in conjunction with the appropriate separation procedure results in the accurate measurement of antigen concentration (Ekins & Newman, 1970; Ekins, 1974a).

INCUBATION

As indicated earlier, the choice of optimal assay conditions is largely determined by the affinity constant of the predominating antibody binding sites and the target antigen concentration (Ekins & Newman, 1970; Yalow & Berson, 1970a). Consequently, many important assay parameters including the concentration of reagents, timing of addition of the labelled species, total incubation time and the temperature of incubation are governed by the apparent energy of the binding reaction (Ekins, 1974a; Keane et al., 1976). However, during incubation, additional factors inherent in the incubation medium or introduced in the unknown sample can markedly affect the antigen-antibody reaction, thereby reducing the full potential of the assay system (Heding, 1966a). These non-specific factors can influence the binding reaction directly, or indirectly by restricting the availability of one or more of the reactants.

The selection of appropriate incubation conditions and the control of non-specific interference are essential prerequisites to the development and optimisation of all radioimmunoassay techniques (Ekins, 1976). Ionic strength, pH and monovalent anion concentration of the buffer, together with the presence of trace quantities of detergents, pharmaceuticals and other substances in the incubation mixture, can directly influence the antigen antibody reaction (Morgan & Lazarow, 1963; Heding, 1966 a, b; Berson & Yalow, 1968; Balázsi & Stützel, 1972; Andersen et al., 1976). Other factors such as the choice of assay tubes and the protein content of the incubation medium influence the binding reaction by affecting the degradation of the antigen, the adherence of reactants to glassware and their tendency to concentrate in foam (Eisentraut et al., 1968; Ensinck et al., 1972; Heding, 1972; Kubasik et al., 1976).

Although non-specific effects introduced in the unknown sample can be largely eliminated by analysis of dilute or extracted plasma (Hunter, 1969b; Heding, 1971) or by running the standard curve with the appropriate volume of charcoaled plasma (Albano et al., 1972; Weir et al., 1973), the presence of unsuitable constituents in the incubation medium itself can give inaccurate results (Heding, 1966a). Accordingly, the selection of suitable assay components merits careful consideration when designing assay systems. In general, radioimmunoassay is conducted in phosphate, borate or veronal buffered saline containing an appropriate concentration of protein to reduce glassware adsorption. However, as illustrated for the glucagon assay (figure 18), the choice of diluent can markedly affect the shape of the standard curve and must therefore be determined empirically for each given antigen. The use of albumin preparations contaminated with peptidases (Rutner et al., 1974), immunoreactive components (Midgley, 1969; Kourides et al., 1976) or other potential interfering substances (Cohn et al., 1947) dramatically reduces assay sensitivity.

SEPARATION

The penultimate step in radioimmunoassay involves the separation of free and antibody bound antigen prior to determination of the radioactivity associated with either or both fractions. Although numerous methods exist for the physicochemical or immunological separation of the two components, the choice of technique and the care applied to its usage are almost as important as the antibody in determining the sensitivity and specificity of the assay system (Ekins, 1976).

Since the original application of paper chromatoelectrophoresis to the separation of free and protein bound insulin (Berson et al., 1956b), a wide variety of separation techniques have been specifically developed for use in radioimmunoassay (see Daughaday & Jacobs, 1971; Hunter & Ganguli, 1971; Skelly et al., 1973; Antoniades, 1976). These methods rely on:

1. differential migration of bound and free fractions using paper chromatoelectrophoresis (Yalow & Berson, 1960a), electrophoresis (Hunter & Greenwood, 1964), wick chromatography (Ørskov, 1967; Ørskov et al., 1968) and gel filtration (Haber et al., 1965);
2. chemical coupling of the antigen (Miles & Hales, 1968a, b; Beck & Hales, 1975), the first (Catt et al., 1966; Wide & Porath, 1966) or the second antibody (Den Hollander & Schuurs, 1971; Koninckx et al.,1976) to a finely divided solid phase support, or the physical adsorption of the first antibody to the inside surface of plastic tubes (Catt & Tregear, 1967), or its covalent linkage to a polymer coated iron oxide (Hersh & Yaverbaum, 1975; Nye et al., 1976; Nye and Landon, 1976);

3. adsorption of free antigen to solid adsorbants such as charcoal (Ekins & Sgherzi, 1965; Herbert et al., 1965), silicates (Rosselin et al, 1966), ion exchange resin (Meade & Klitgaard, 1962), or of the bound fraction to DEAE-cellulose (Frenkel et al., 1966);

4. precipitation of bound antigen by chemical or immunochemical methods:

   (a) chemical separation using ethanol (Odell et al., 1965; Heding, 1966b), sodium sulphite (Grodsky & Forsham, 1960), sodium sulphate (Grodsky et al., 1961), ammonium sulphate (Chard et al., 1971), polyethylene glycol (Desbuquois & Aurbach, 1971), dioxan (Thomas & Ferin, 1968), or trichloroacetic acid after enzyme proteolysis of the free antigen using glutathione-activated ficin (Mitchell & Byron, 1967);

   (b) immunochemical separation using pre-precipitation (Hales & Randle, 1963 a, b) or post-precipitation (Hales & Randle, 1962, 1963; Morgan & Lazarow, 1962, 1963; Utiger et al., 1962) with a second antibody.

Although each of these techniques carries potential advantages and disadvantages in terms of cost, convenience and susceptibility to non-specific interference, none has emerged as the single method of choice for radioimmunoassay (Ratcliffe, 1974). Thus each method must be judged on its own merits and particularly on its ability to achieve complete separation both in the absence and presence of potentially interfering substances (Ekins, 1976). Accordingly, the selection of a suitable separation procedure is based on consideration of the properties of the antigen and biological fluid to be assayed, the requirements of the assay in terms of sample capacity, sensitivity, specificity, and available expertise (Ratcliffe, 1974).

The most commonly employed separation techniques in radioimmunoassay include adsorption of free antigen by coated charcoal (Ekins & Sgherzi, 1965; Herbert et al., 1965), chemical precipitation of bound antigen by ethanol (Odell et al., 1965; Heding, 1966b) and immunological precipitation with second antibody (Utiger et al., 1962; Hales & Randle, 1963a; Morgan & Lazarow, 1963). Since each method offers a unique combination of contrasting characteristics, the selection of a suitable separation procedure for a new antigen often involves a preliminary study of these techniques (figure 19) followed by a detailed examination of the optimal conditions for the one chosen (Hunter & Ganguli, 1971). In general, charcoal separation is preferred since it is simple, cheap, reproducible and permits control of non-specific effects (Buchanan & McCarroll, 1971; Hunter & Ganguli, 1971). However, when the charcoal method fails due to stripping of low affinity antibodies (Ratcliffe, 1974), inappropriate sensitivity to incubation damage (Hunter & Ganguli, 1971), or other factors (Schopman & Hackeng, 1971), separation can usually be achieved using ethanol precipitation or the double antibody technique. Separation using ethanol provides a simple and cheap method to sequestrate the bound and free fractions in a number of assays (see Skelly et al., 1973; Antoniades, 1976). However, when unextracted plasma is analysed large and variable non-specific trapping can occur in the bulky precipitate (Buchanan & McCarroll, 1971; Hunter, 1973). Unlike the other methods double antibody techniques can be applied to essentially all radioimmunoassays and have therefore been employed with success by a great many investigators (Burr et al., 1969; Midgley et al., 1969b). In addition to added expense, the main disadvantages of this method are those of complexity, since a second immunological reaction is introduced which may be influenced by the concentration of serum proteins, the presence of anticoagulants as well as other less well-defined factors (Morgan et al., 1964 a, b; Midgley et al., 1969b). These non-specific

effects are eliminated in the precipitation method (Hales & Randle, 1963b). However, as illustrated in figure 19, the sensitivity is less than that achieved using more conventional techniques (Hunter, 1973; Malvano et al., 1974).

Regardless of the technique employed, the control of non-specific effects on the separation of free and antibody bound antigen represents a practical problem common to all radioimmunoassay techniques (Hunter & Ganguli, 1971). As indicated in the previous section, control of non-specific interference can be achieved by ensuring that the incubation milieux of both standard and unknown are as uniform as possible (Hunter, 1973). This can be achieved by the addition of 'antigen free' plasma to the standard tubes (Albano et al., 1972; Weir et al., 1973).

STANDARD CURVE

The final stage in the successful completion of radioimmunoassay concerns the plotting of the standard or response curve, relating the distribution of radioactivity observed in a set of standard incubation mixtures to the antigen concentration, followed by dose interpolation for the unknown samples. This analysis is usually performed by graphical representation of the binding reaction followed by visual interpolation. However, in recent years there has been a gradual trend towards automation (Powell & Stevens, 1973; Ertinghausen et al., 1975; Shorey, 1975; Green et al., 1976) and consequently numerous time saving methods for routine data processing have emerged (Rodbard et al., 1969; McHugh & Meinert, 1970; Rodbard, 1974; Rodbard & Frazier, 1975).

The dose response curve can be portrayed in a number of different co-ordinate systems (Midgley et al., 1969a; Bliss, 1970; Rodbard et al., 1970; Ekins, 1974b, 1976). Thus using either arithmetic or logarithmic scales for the dose, any one of the following response variables may be employed: B, bound counts; F, free counts; B/T, fraction bound; F/T fraction free; B/F, R, bound-to-free ratio; B/B0, Y, counts bound relative to counts bound for zero dose. In addition to these

hyperbolic and sigmoidal curves, various degrees of linearisation can be achieved with plots of the reciprocal of any of the previously mentioned response variables against arithmetic dose, or the special metameters log (T/B - T/Bo), log (F/B - Fo/Bo) and logit B/Bo against logarithmic dose.

Although many different co-ordinate systems have been employed in radioimmunoassay data processing, each having characteristic advantages and disadvantages, a number of particularly popular response metameters have emerged (Rodbard et al., 1968, Midgley et al., 1969a). Some of these are illustrated in figures 20 to 22, using the same data from one particular assay of insulin. The plot of B/F, B/Bo or B/T against arithmetic dose (figure 20) gives a hyperbolic curve. It is important to note that despite the fact that the B/F ratio originates from the theoretical derivation of the Scratchard equation (Scratchard, 1949; Berson & Yalow, 1958), it is often more convenient to employ B/Bo or B/T as the response variable. These metameters standardise the scale and avoid both the error arising from the use of the free fraction as denominator and the need to calculate the B/F ratio. The use of B/T is particularly compelling since it provides important information concerning the initial binding while at the same time offers greatest stability. Unlike the previous method, logarithmic transformation of the dose co-ordinate (figure 21) results in sigmoidal curves which can generally be approximated to straight lines following truncation. This representation is particularly applicable in situations where the potency estimates fall in the central region of the response curve. In contrast to the previous co-ordinate systems, the plot of Bo/B versus arithmetic dose, and log (T/B - T/Bo) or logit B/Bo against logarithmic dose (figure 22) will provide adequate linearisation of the dose response curve. Such methods are readily applicable to programmable desk-top electronic calculators and are consequently of immense value when the throughput of

assay samples is particularly great. Aside from their use in automated curve-fitting programmes, these expressions can clearly be used as a basis for the manual calculation of assay results.

The selection of a suitable co-ordinate system for the analysis of the binding data will depend on numerous factors including: the separation procedure; the fraction counted; the target antigen concentration; the available expertise and the personal preference of the investigator. However, in addition to enabling the accurate estimation of potency for the unknown samples, the method should provide important diagnostic information about the assay system (Rodbard et al., 1968, 1970; Woo and Cannon, 1976).

Figure 1 Schematic representation of a radioimmunoassay dose response curve (modified from Rodbard & Hutt, 1972)

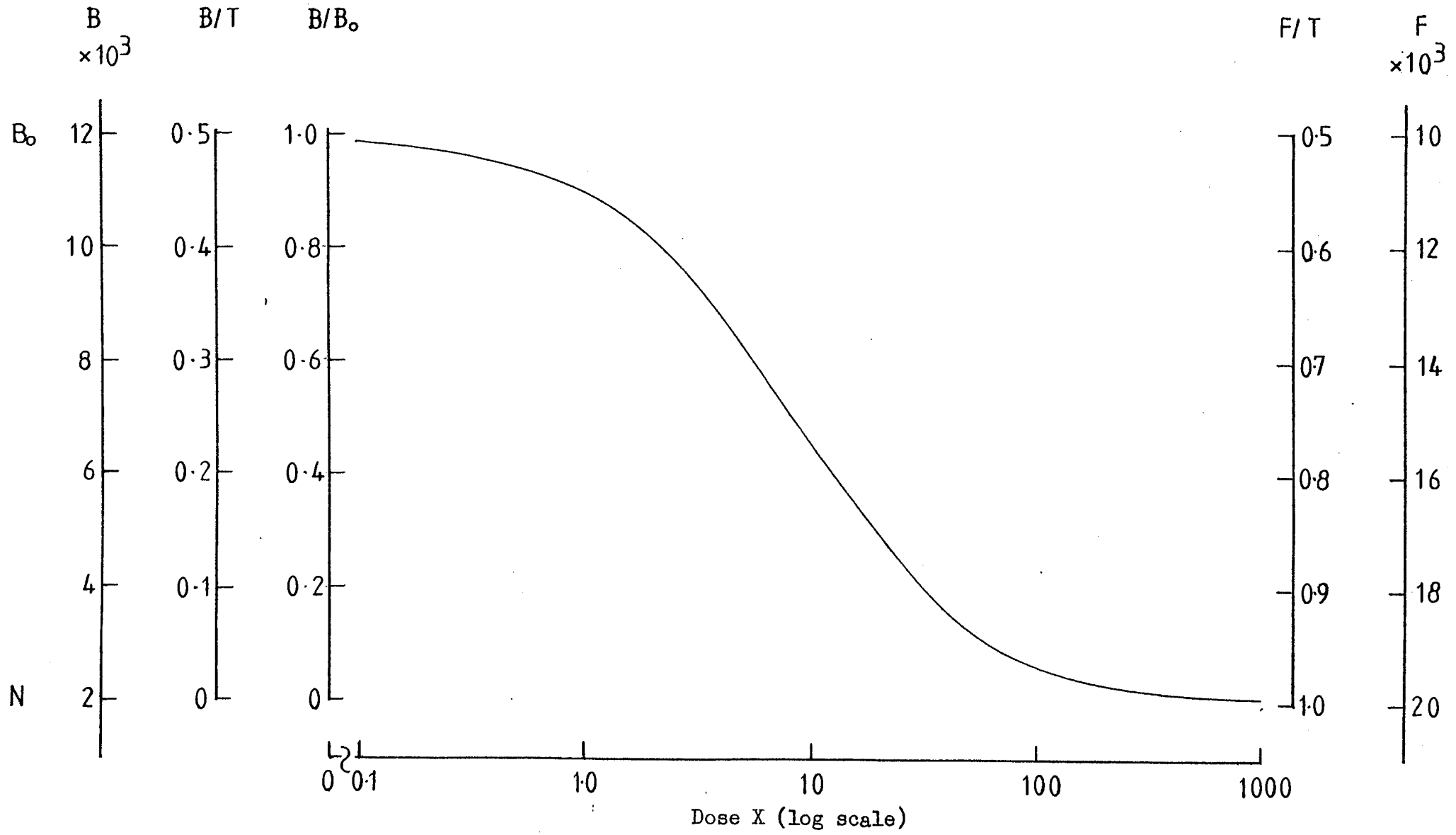
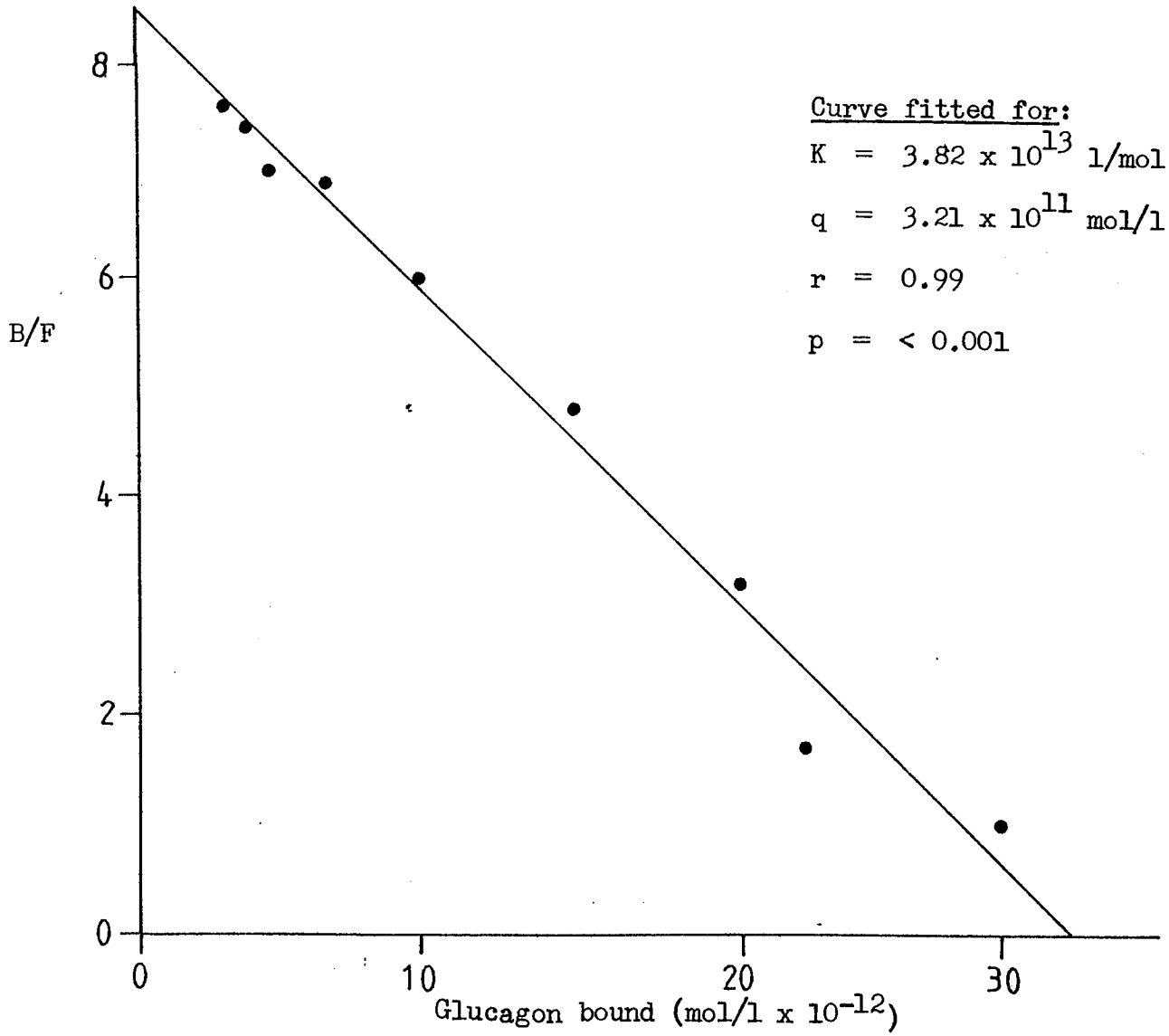
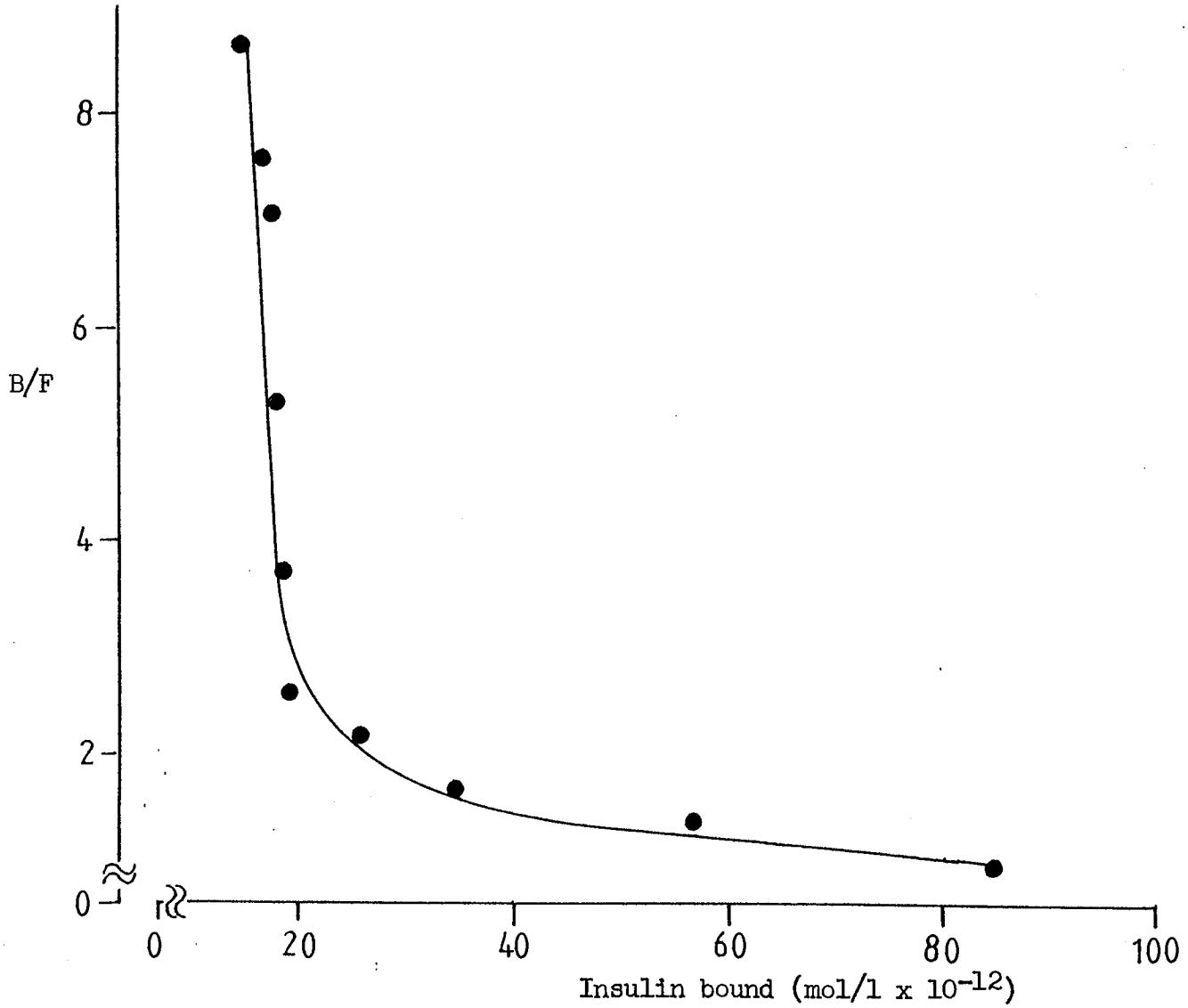


Figure 2 Scratchard plot for glucagon antiserum (ROG1/2). Curve fitted for univalent antigen reacting with a single order of antibody combining sites[ø].



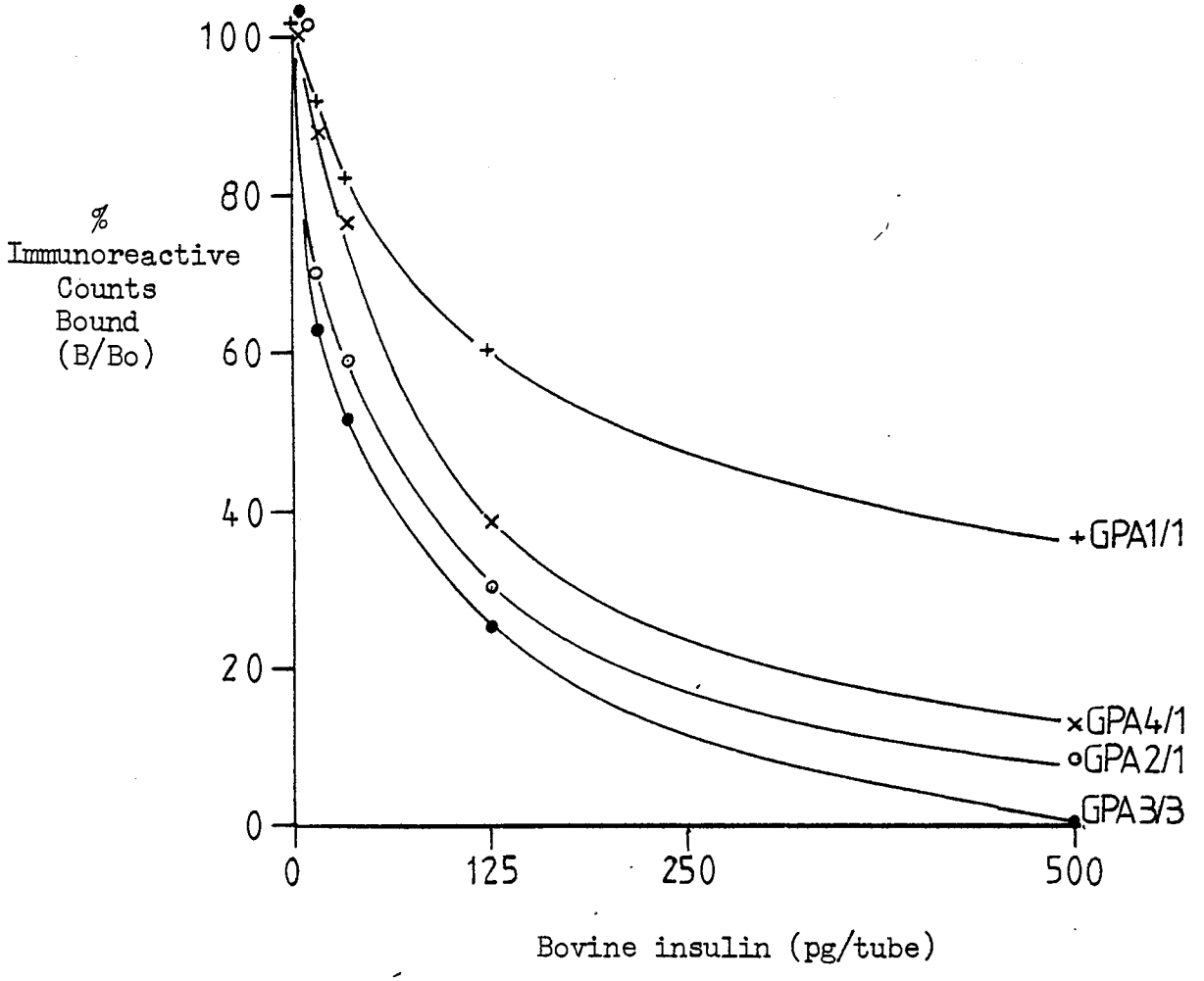
ø Experimental details are given in Chapter 3.

Figure 3 Scratchard plot for insulin antiserum (GPA2/3). System containing multiple orders of antibody combining sites$^{\phi}$.



ϕ Experimental details are given in Chapter 2.

Figure 4 Variation in the slopes of inhibition curves with four different guinea pig antibovine insulin antisera$^{\phi}$.



$^{\phi}$Full experimental details are given in chapter 2.

Figure 5 Effect of time on the ^{125}I-insulin-antibody reaction at 4°C.

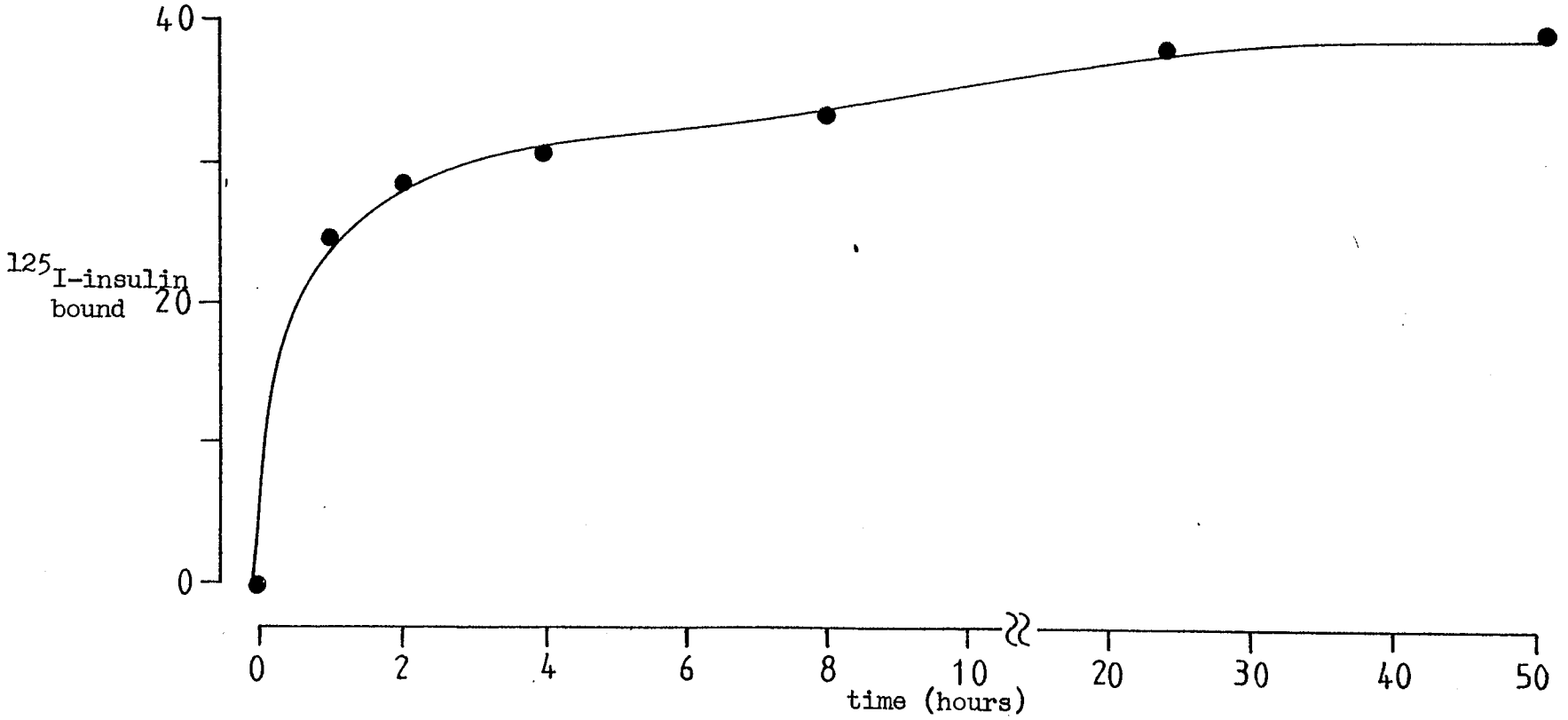


Figure 6 The influence of temperature on the ^{125}I-insulin antibody reaction.

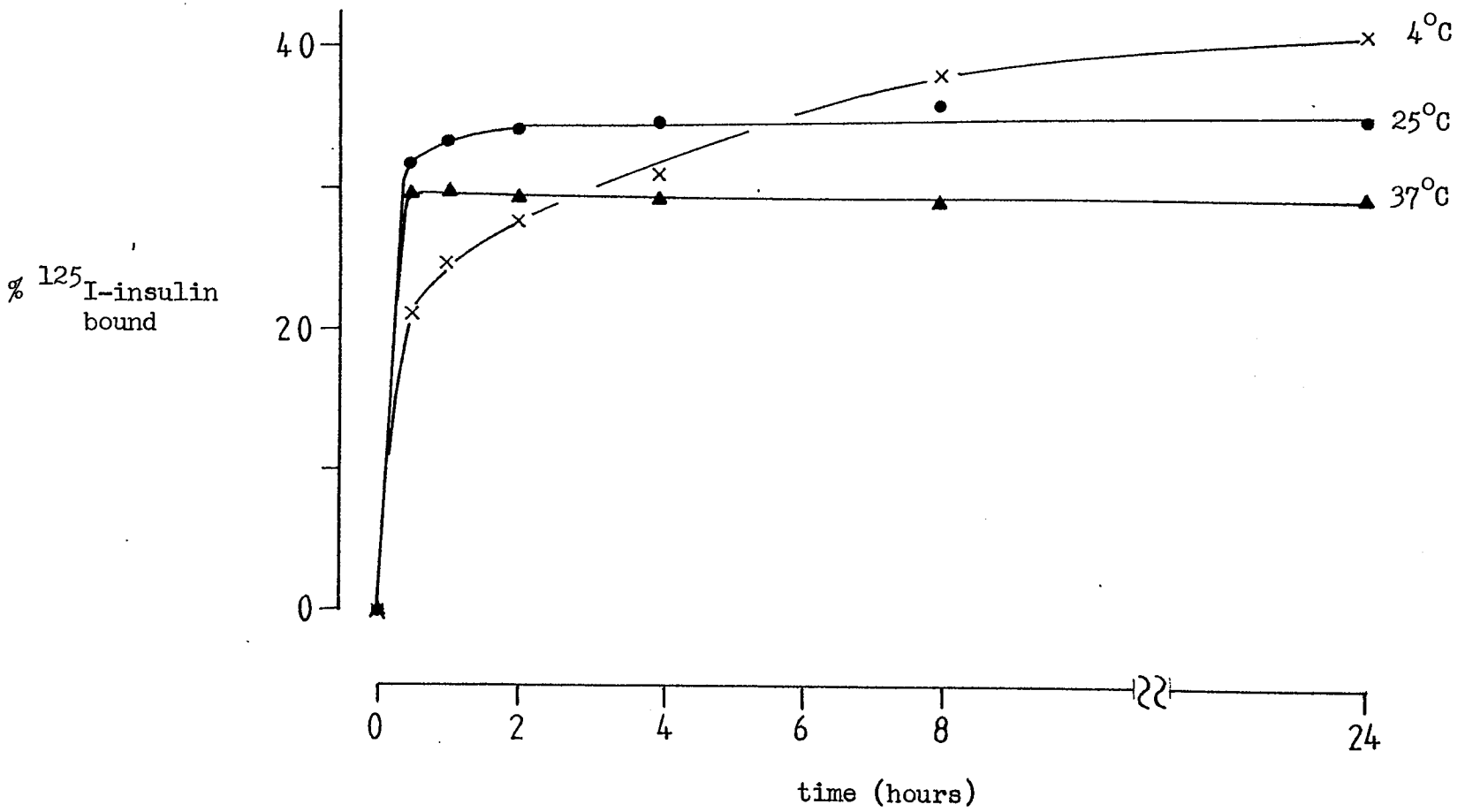
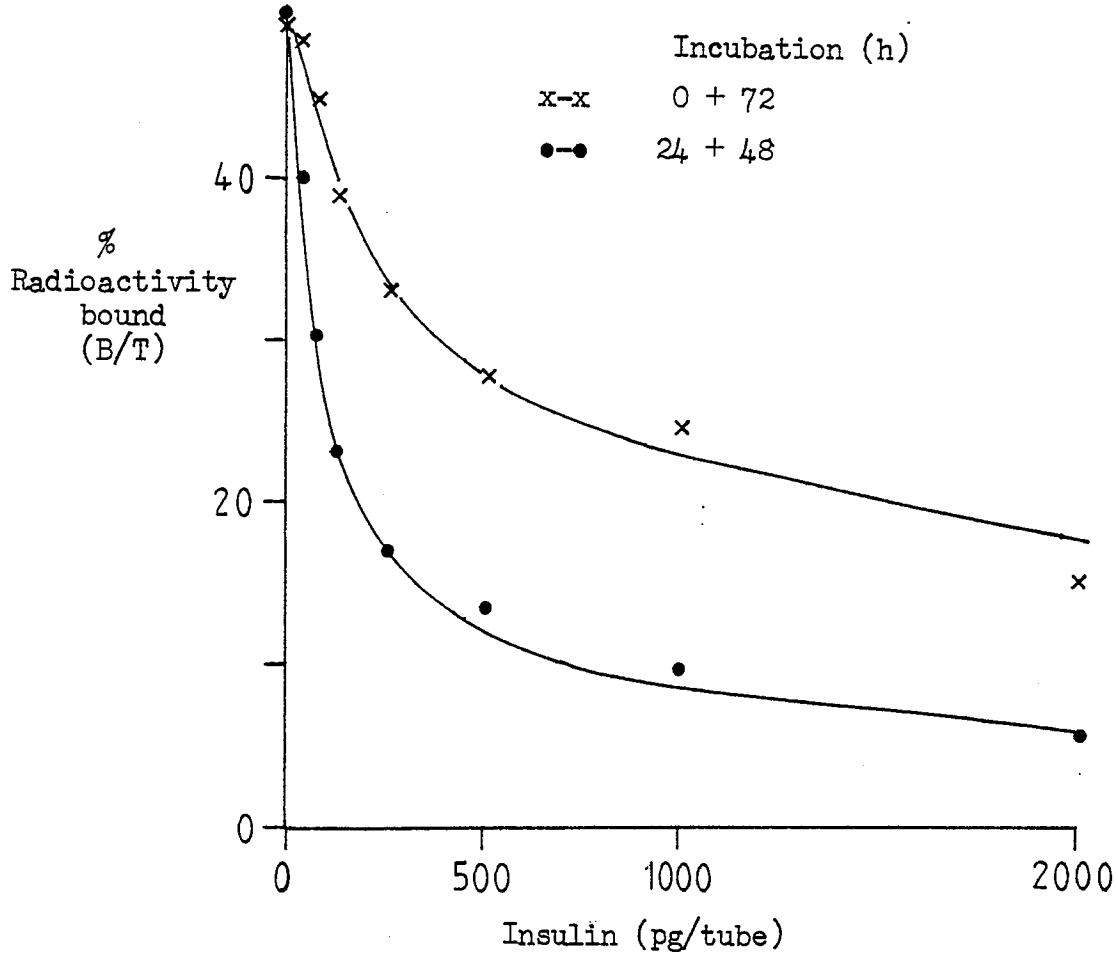
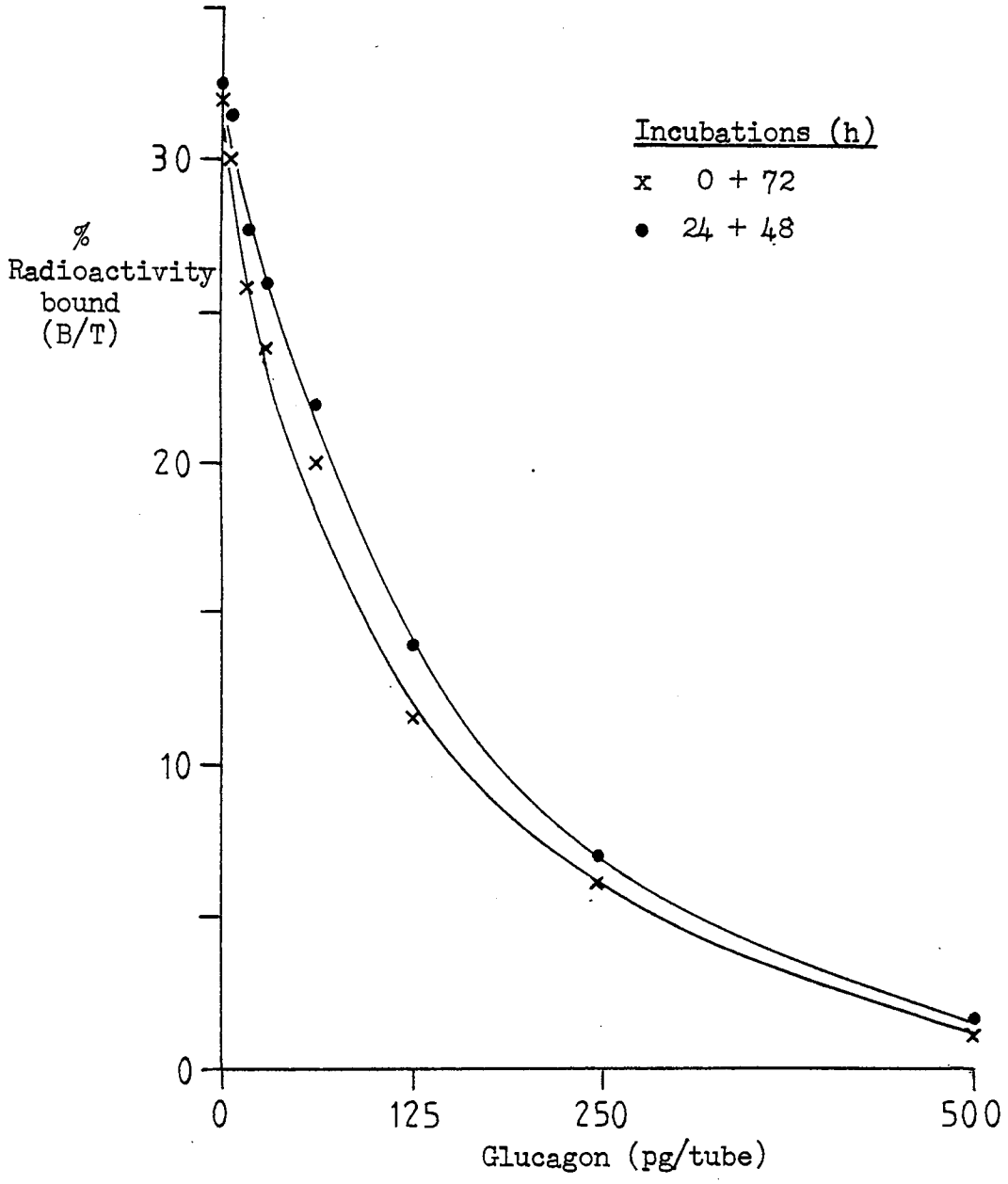


Figure 7 Effect of delayed addition of ^{125}I-insulin on the slope of the insulin dose response curve$^{\phi}$.



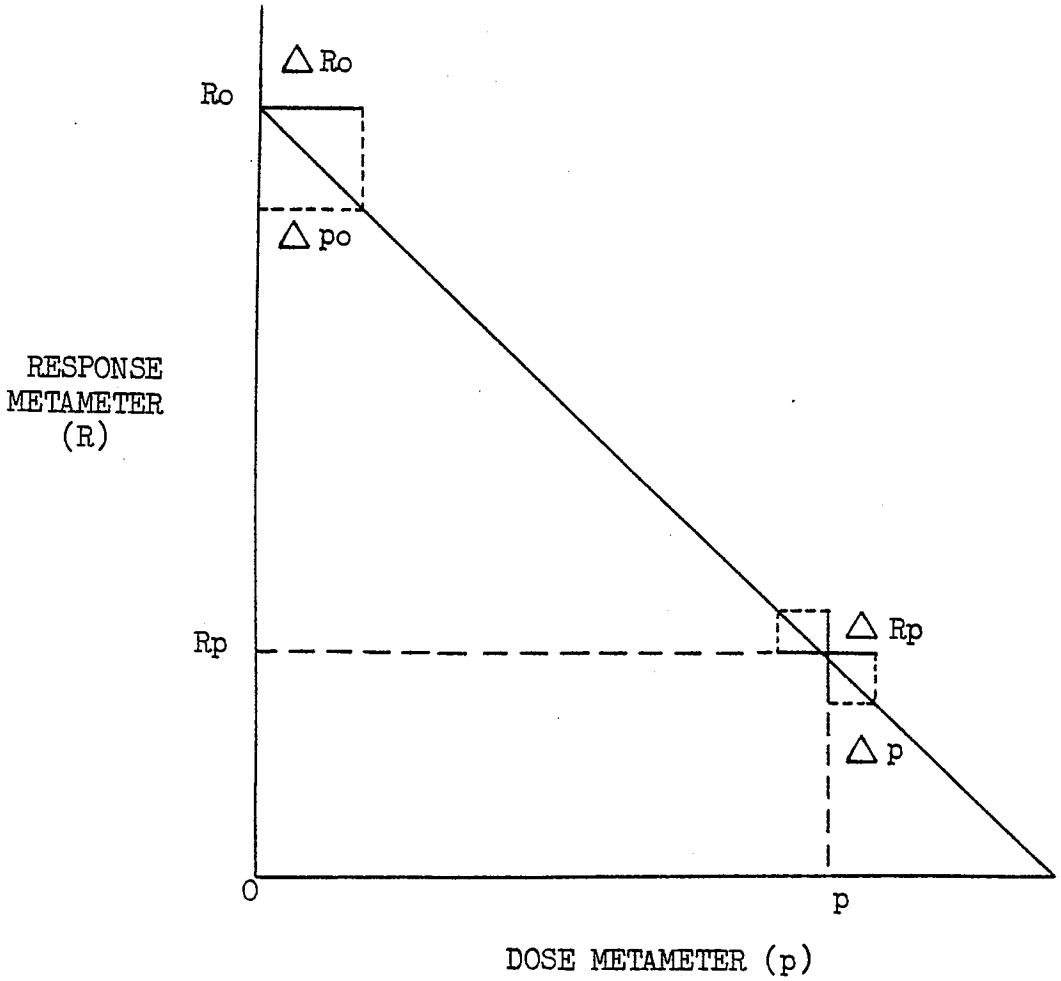
$^{\phi}$Experimental details are given in Chapter 2.

Figure 8 Effect of delayed addition of ^{125}I-glucagon on the shape of the glucagon dose response curve$^{\phi}$.



$^{\phi}$Glucagon antiserum YY118. Experimental details are given in Chapter 3.

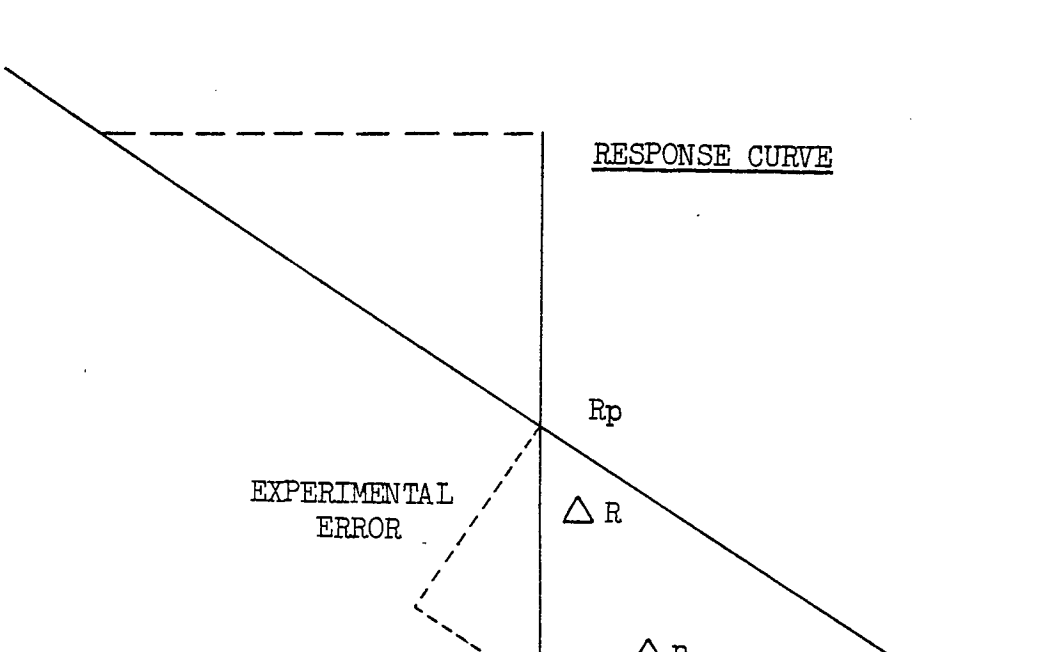
Figure 9 Definitions of precision ($\triangle$ R) and sensitivity ($\triangle$ Ro) as illustrated for the logit-log plot.



$$\text{Precision } (\triangle p) = \frac{\triangle Rp}{\text{slope at Rp}}$$

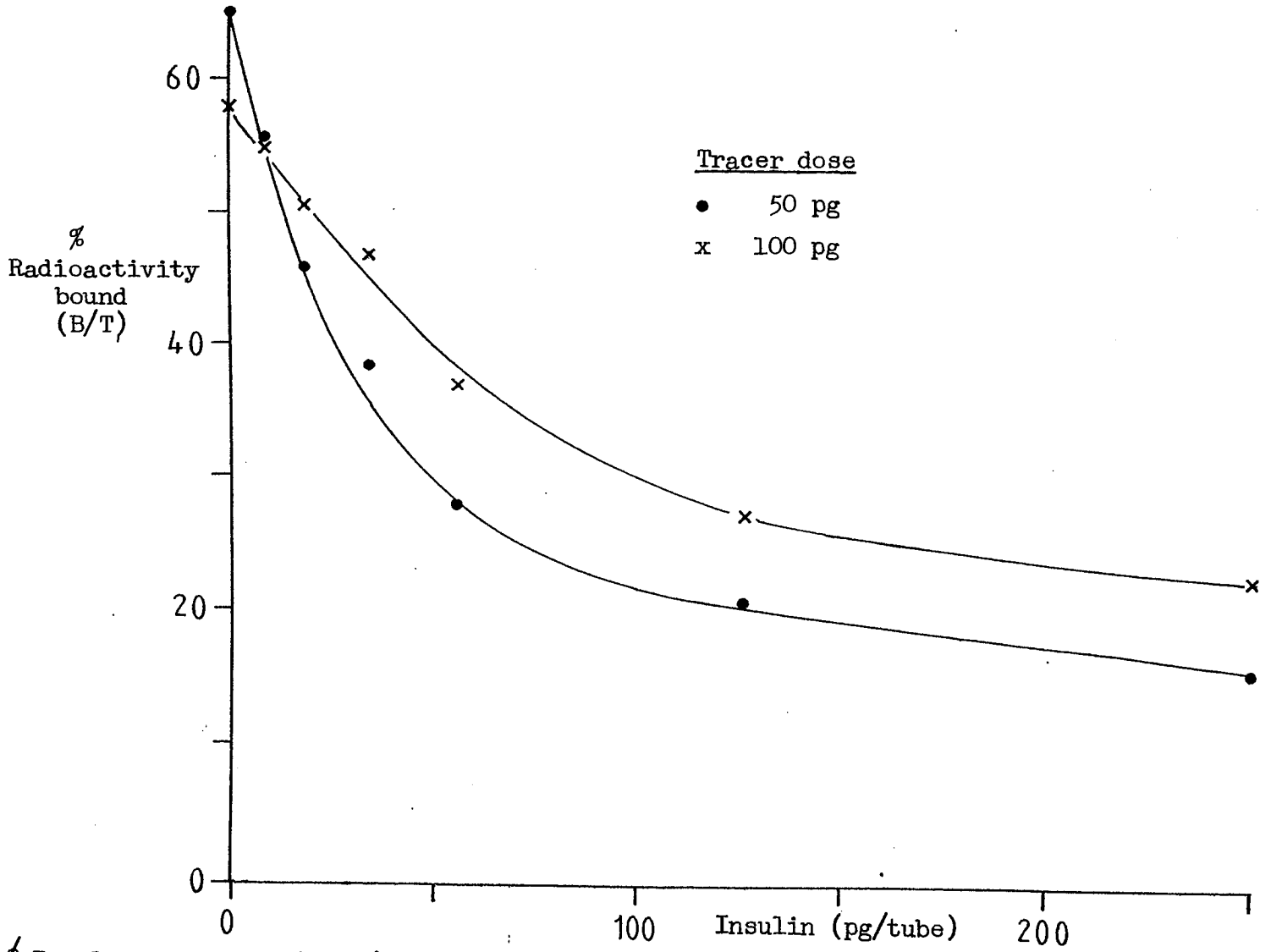
$$\text{Sensitivity } (\triangle po) = \frac{\triangle Ro}{\text{slope at Ro}}$$

Figure 10 Factors affecting the precision of measurement of the response metameter (R).



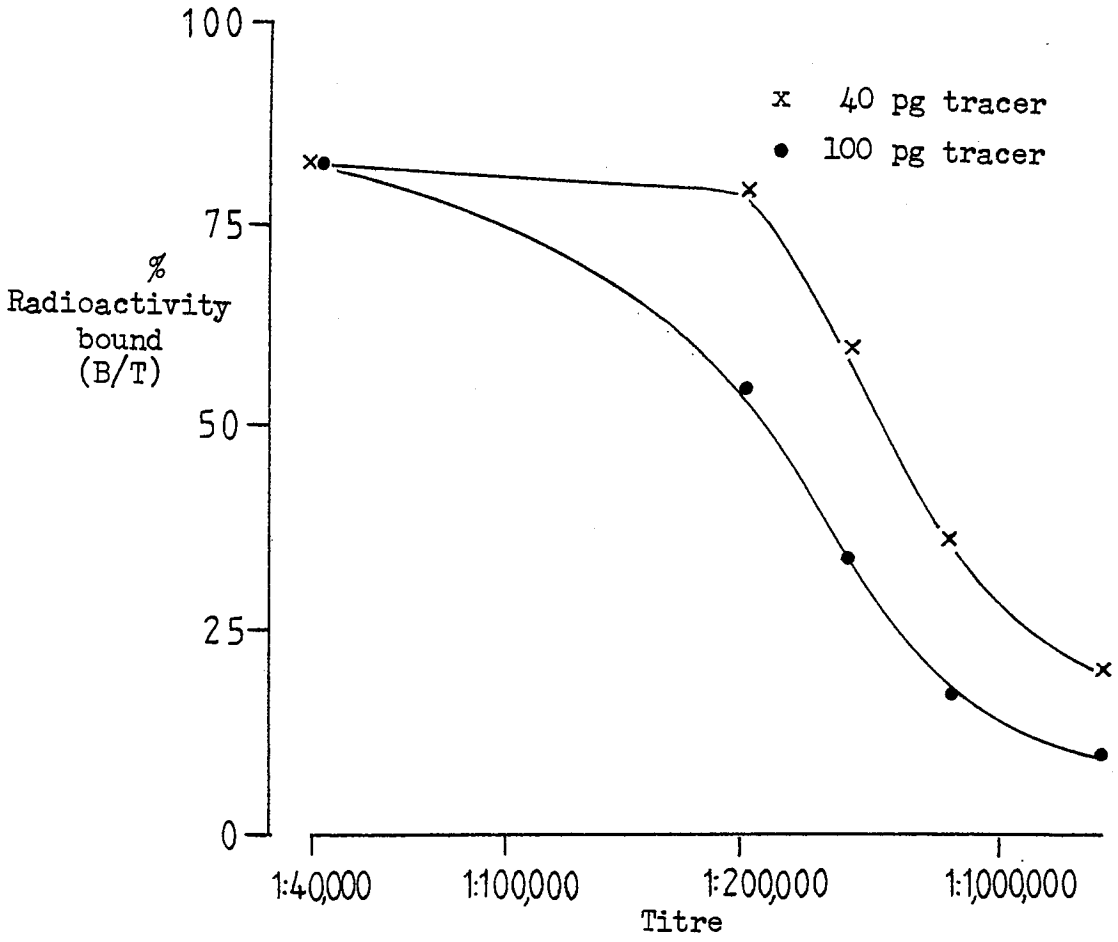
EXPERIMENTAL ERROR	pipetting and other manipulations misclassification of free and bound activities reagent impurities concentrations of reagents
COUNTING ERROR	specific activity of tracer counting time per sample volume of incubation mixture concentrations of reagents
SLOPE OF RESPONSE CURVE	energy of reaction concentration of reagents

Figure 11 Effect of tracer dose on the shape of the insulin dose response curve$^{\phi}$.



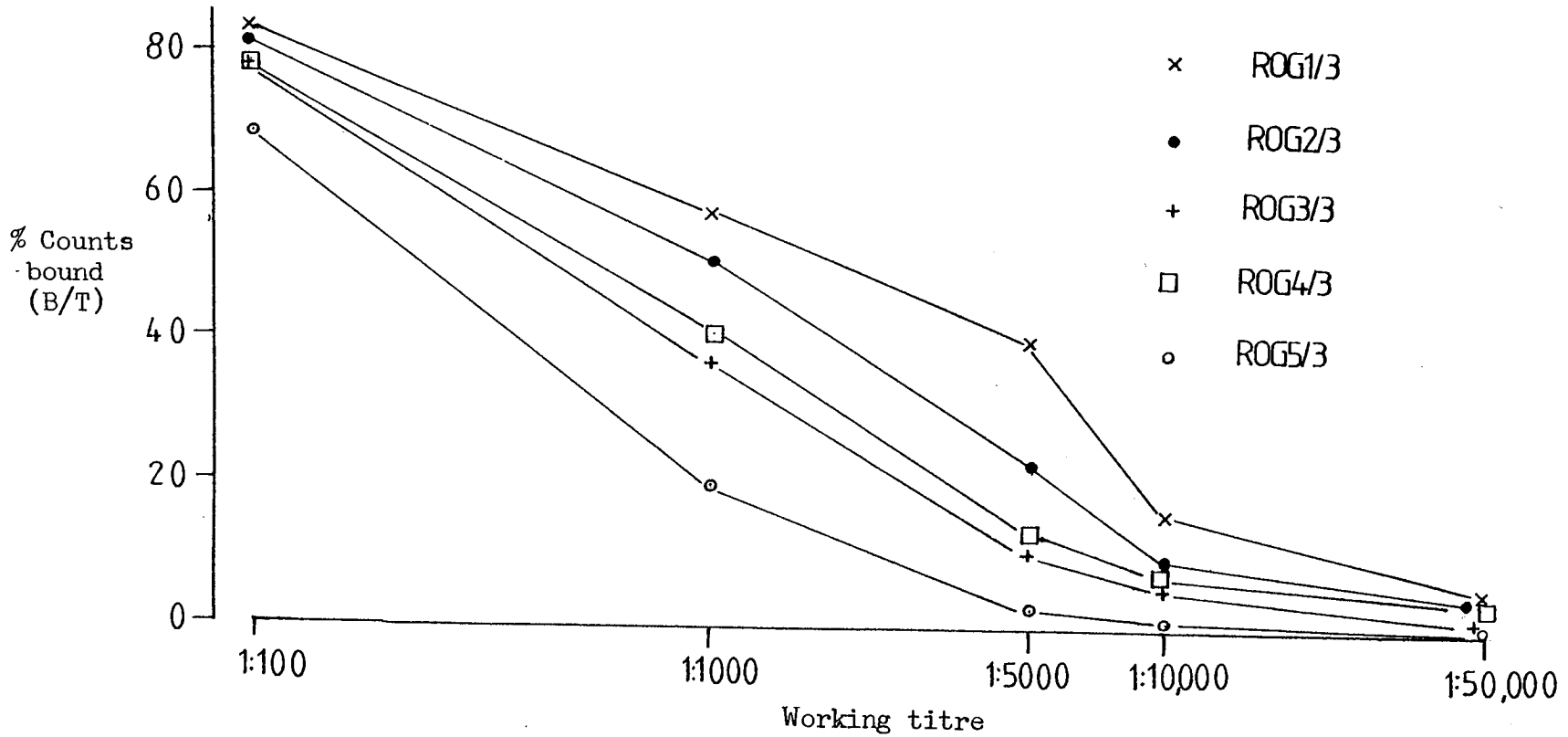
$^{\phi}$ Insulin antiserum (GPB1/3). Experimental details are given in Chapter 2.

Figure 12 Insulin antibody dilution curves prepared with 40 pg and 100 pg amounts of ^{125}I-insulin$^{\phi}$.



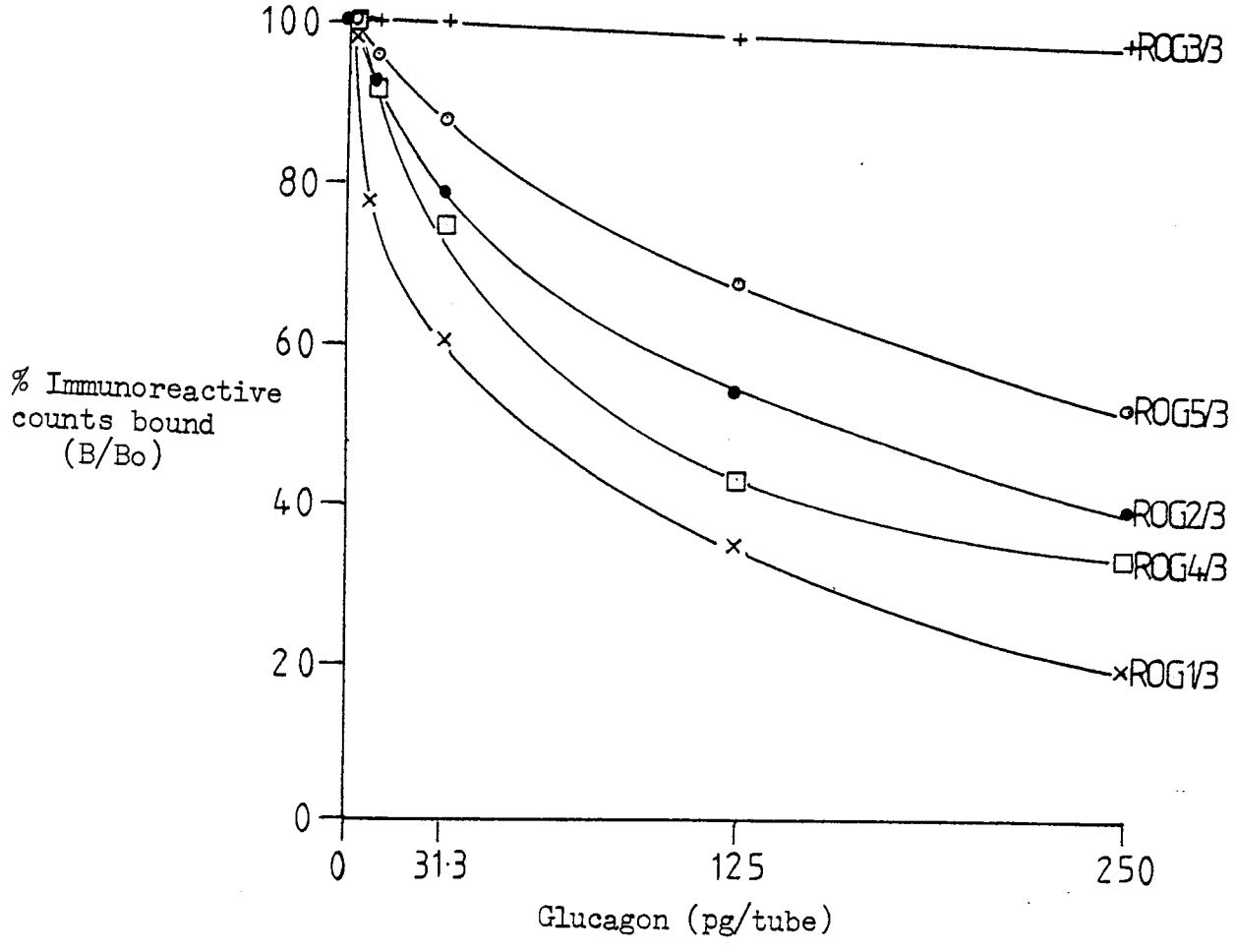
$^{\phi}$Insulin antiserum (GPB1/3). Experimental details are given in Chapter 2.

Figure 13 Antibody dilution curves of sera derived from rabbits immunised with a glucagon-bovine plasma albumin conjugate$^{\phi}$.



$^{\phi}$Full experimental details are given in Chapter 3.

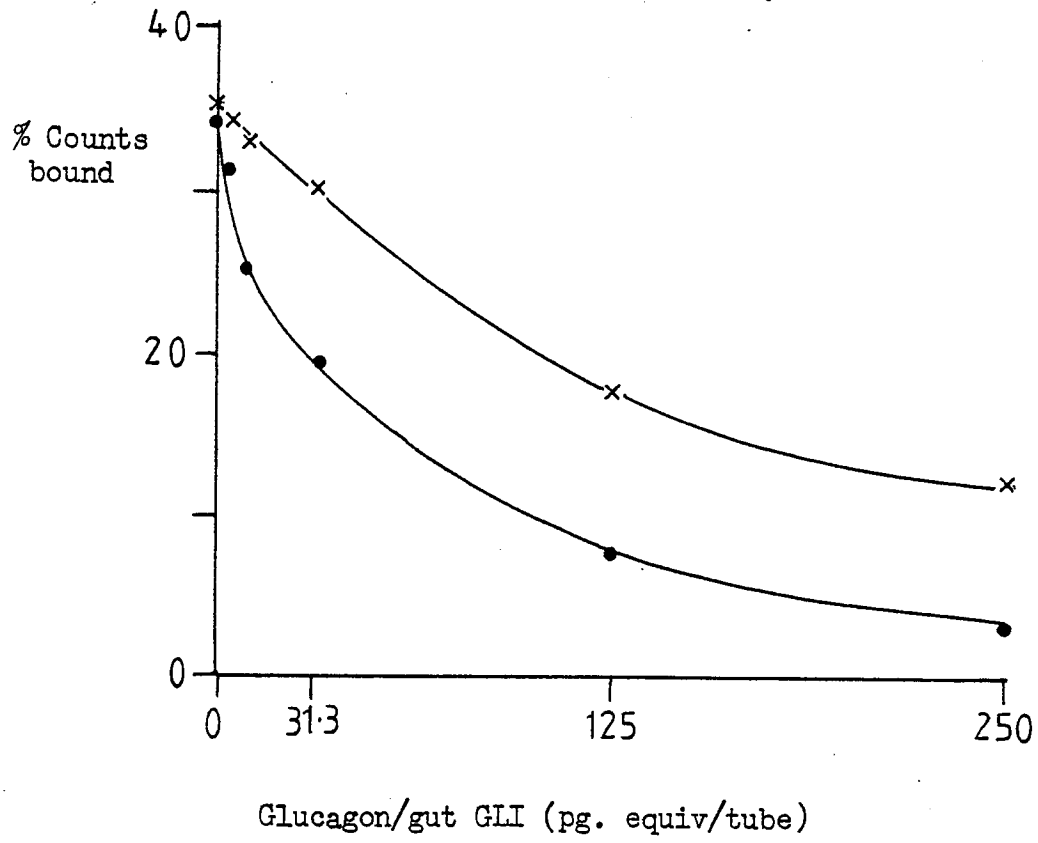
Figure 14 Variation in the slopes of the dose response curve with five different rabbit glucagon antisera[ø].



[ø]Initial binding (Bo/T): ROG1/3, 0.33; ROG2/3, 0.37; ROG3/3, 0.54; ROG4/3, 0.41; ROG5/3, 0.46.

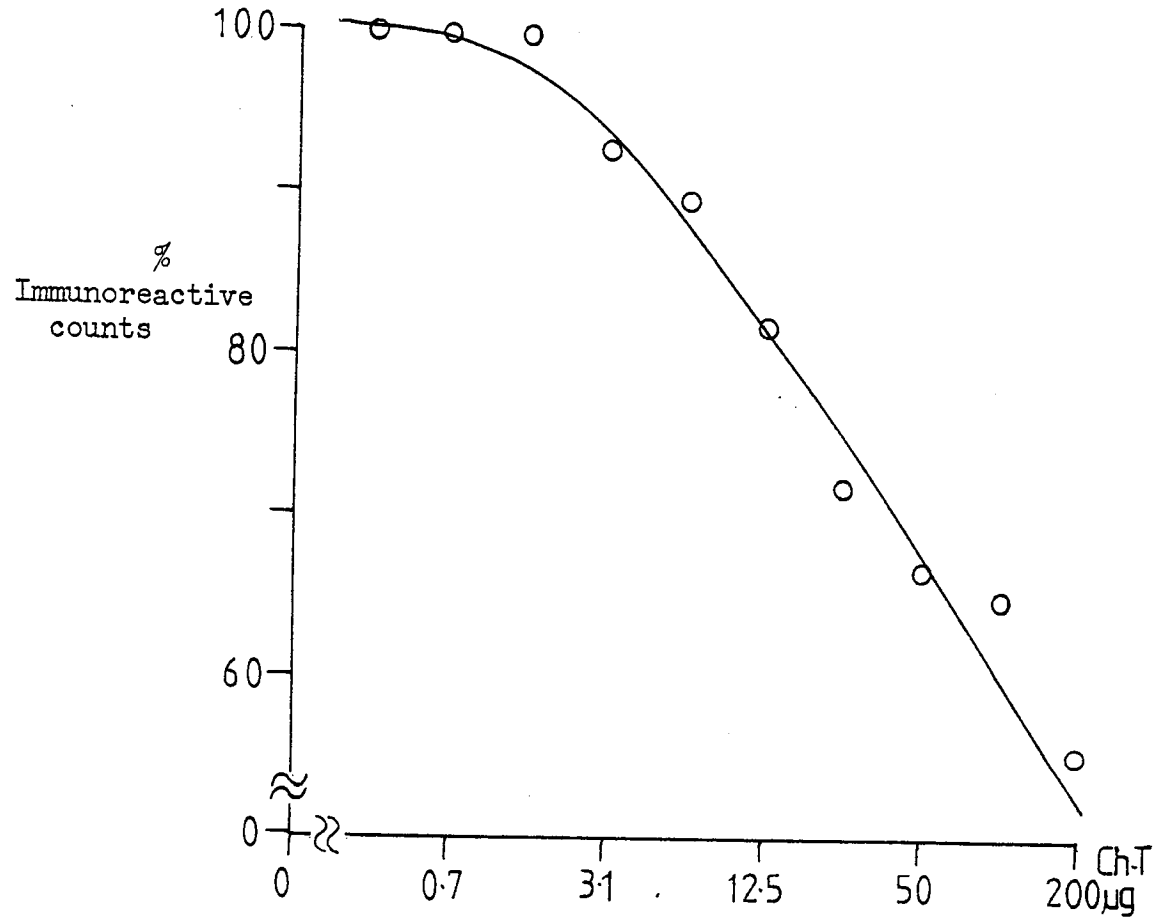
Full experimental details are given in Chapter 3.

Figure 15 Reaction of guinea pig glucagon antiserum (GPD4/3) with glucagon (●) and gut GLI (X) ϕ



ϕ Experimental details are given in Chapter 3.

Figure 16 Conventional iodination of glucagon: Relationship between the amount of Chloramine-T (Ch-T) and the incorporation of radioactivity into immunoreactive and trichloroacetic acid (TCA) precipitable protein. Full experimental details are given in Chapter 3.



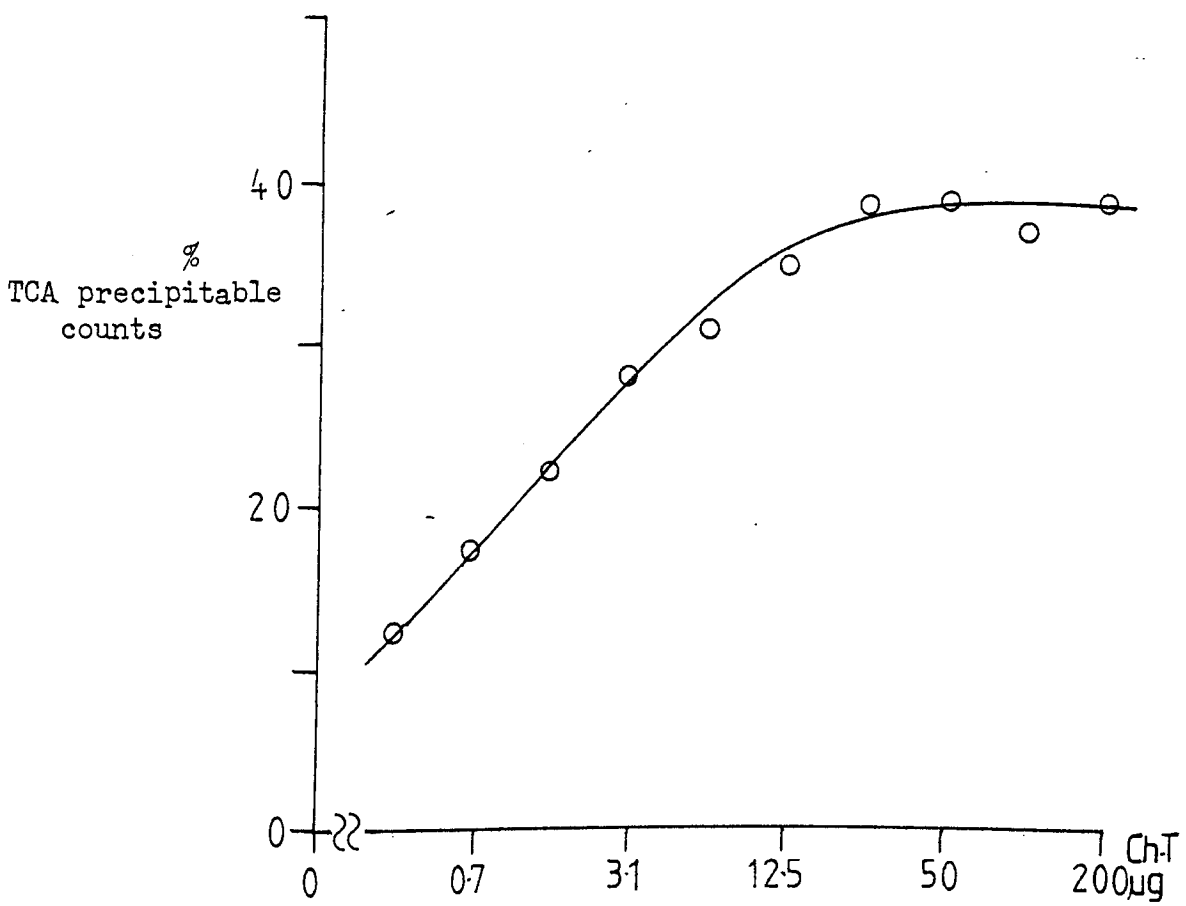
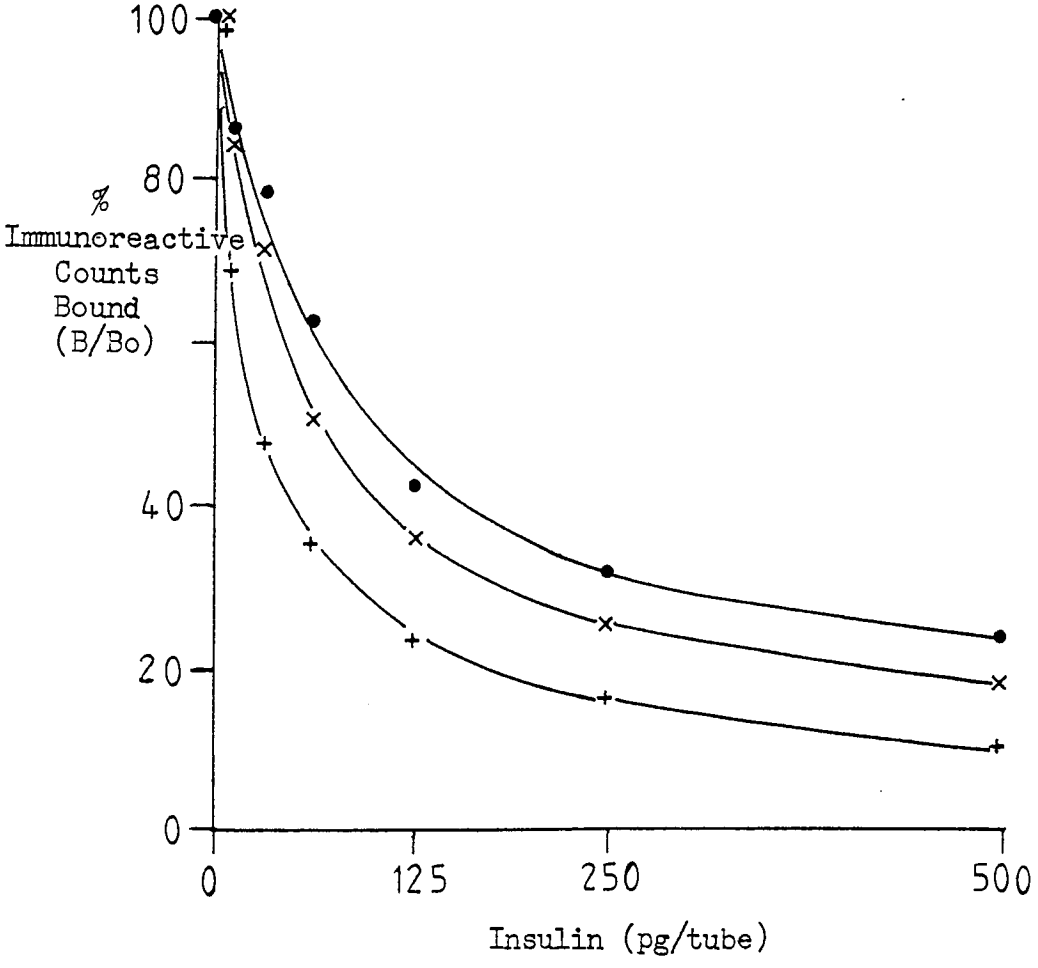
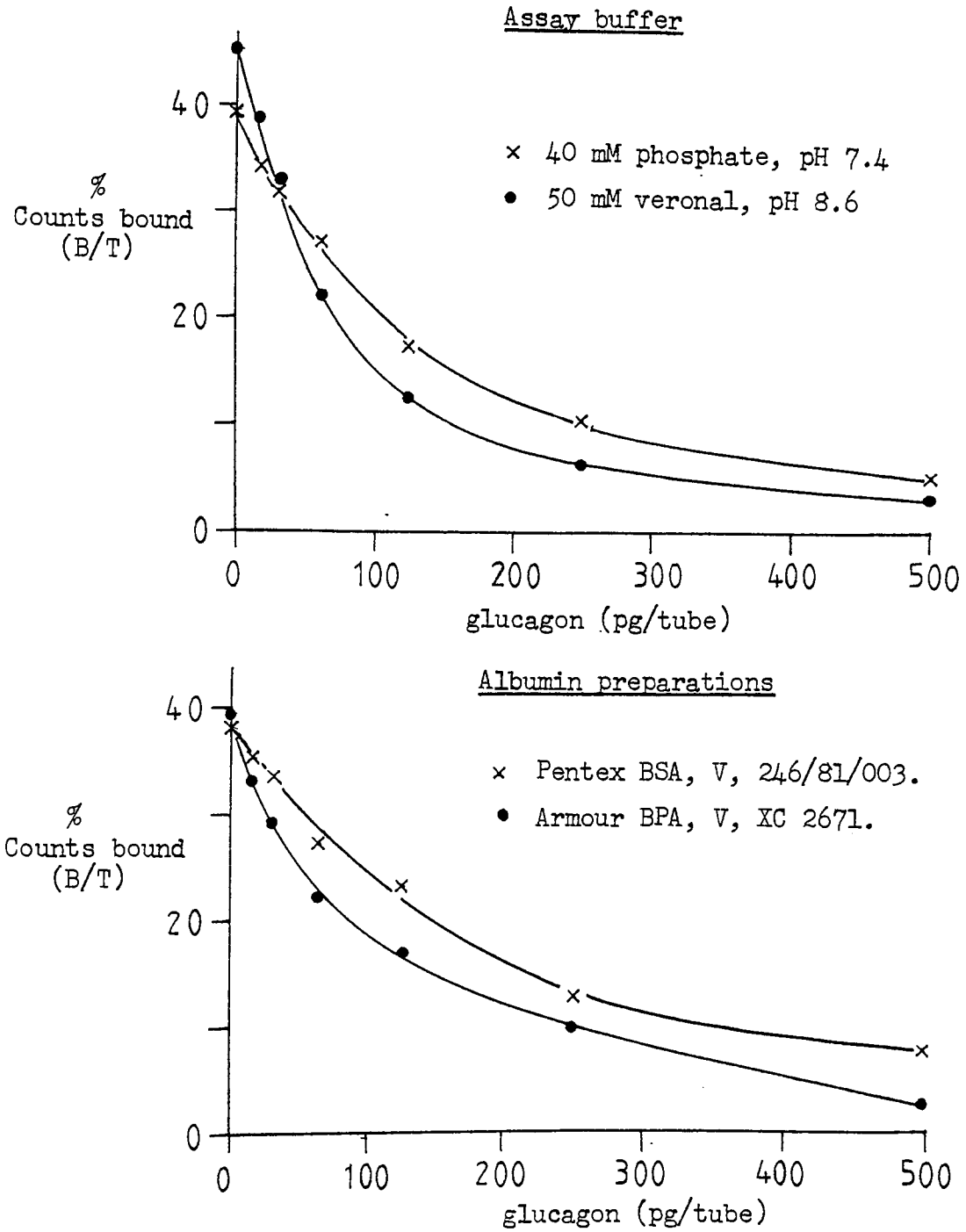


Figure 17 Reaction of guinea pig antiporcine insulin antiserum (GPB1/3) with standard preparations of porcine (+), human (X) and mouse (•) insulin.ϕ



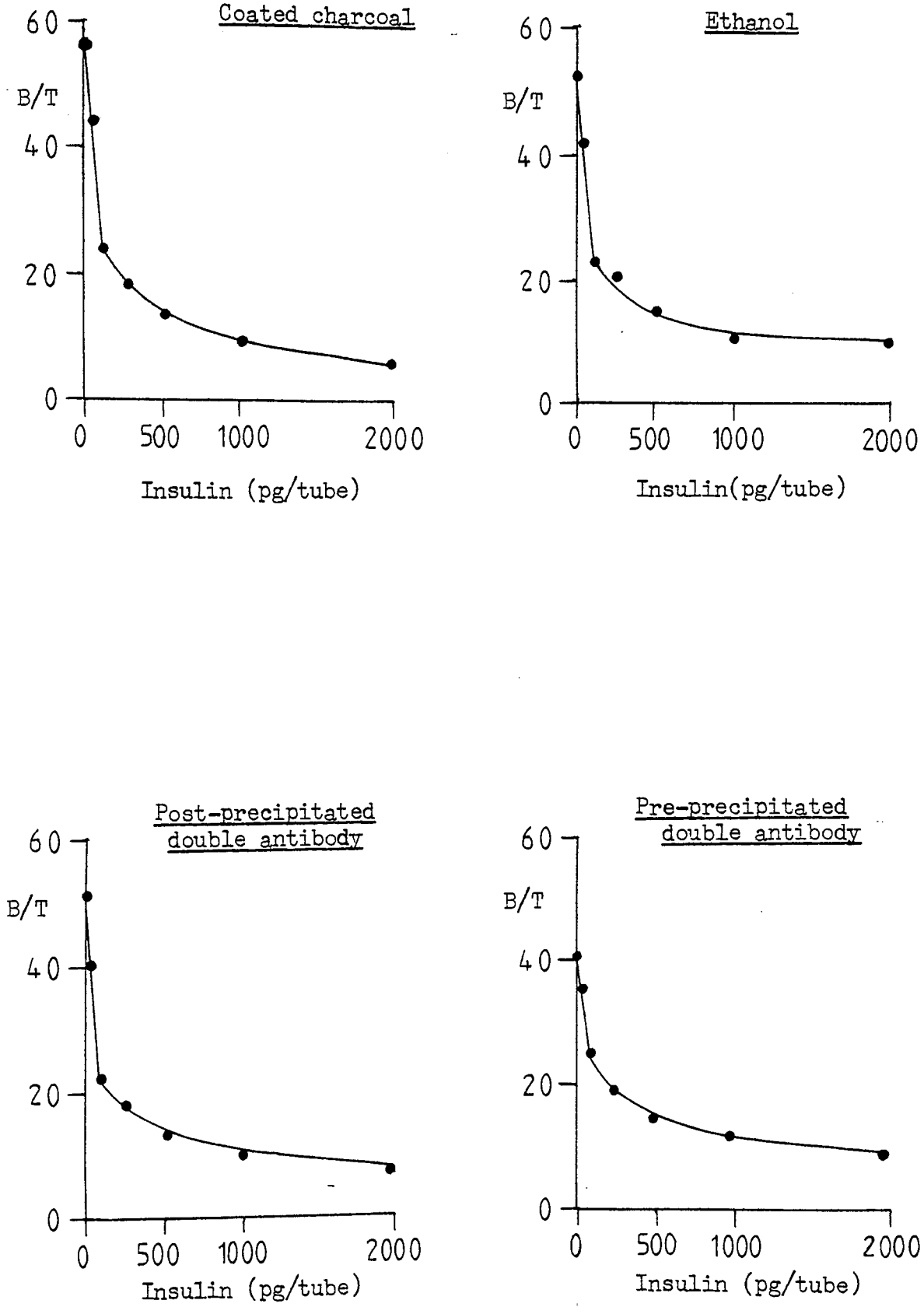
ϕExperimental details are given in Chapter 2.

Figure 18 Effect of assay diluent and different albumin preparations on the glucagon dose-response curve$^{\phi}$.



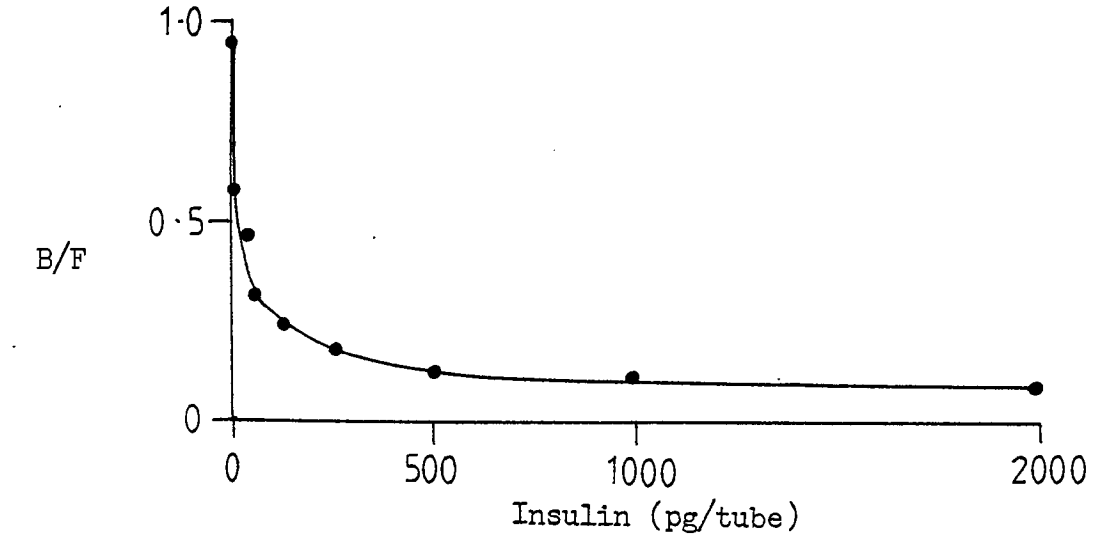
$^{\phi}$Full experimental details are given in Chapter 3.

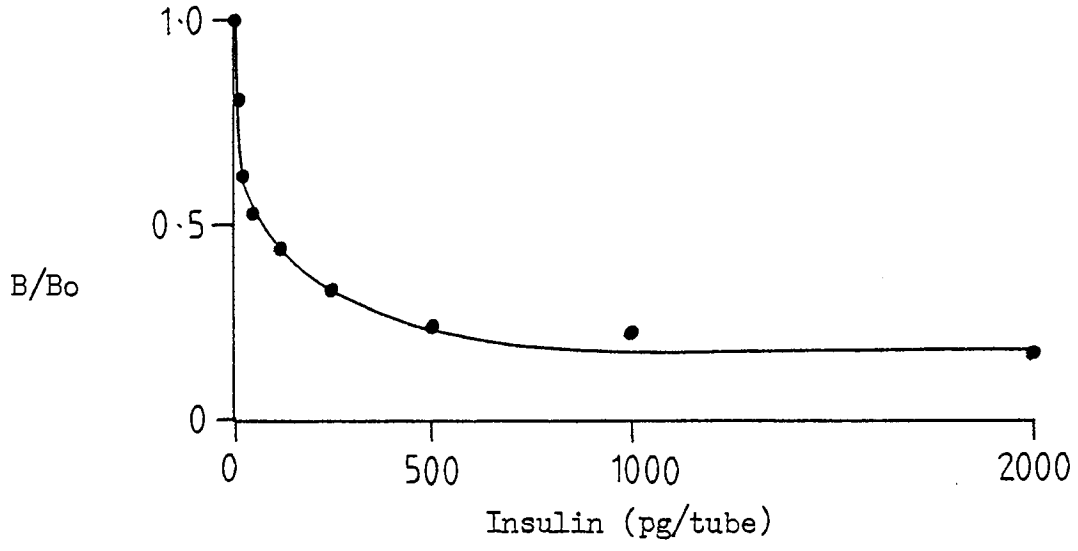
Figure 19 Comparison of separation methods commonly employed in the radioimmunoassay of insulin$^{\phi}$.



$^{\phi}$ Experimental details are given in Chapter 2.

Figure 20 Radioimmunoassay dose response curves.
I. B/F, B/Bo and B/T vs. arithmetic dose.





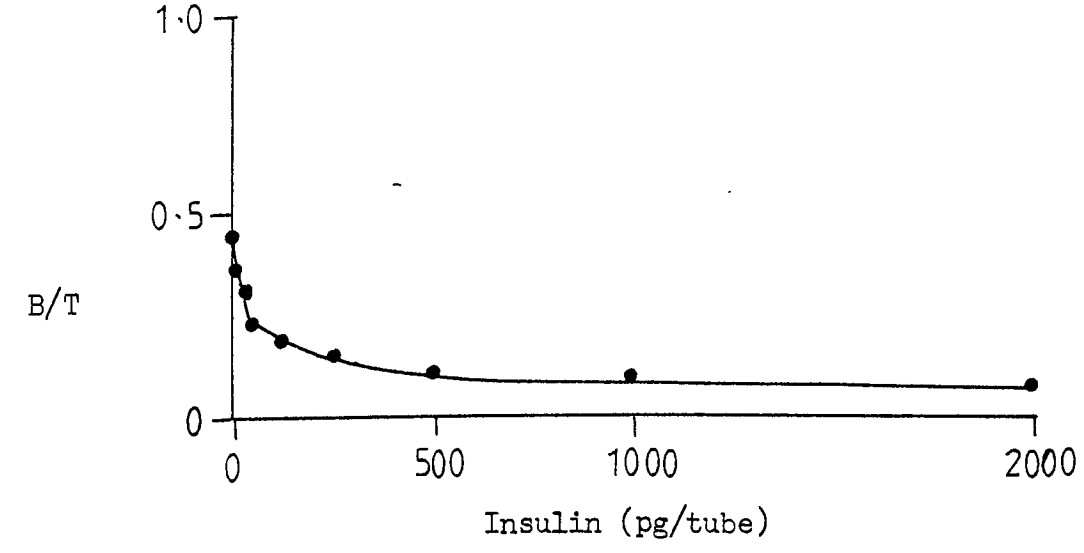
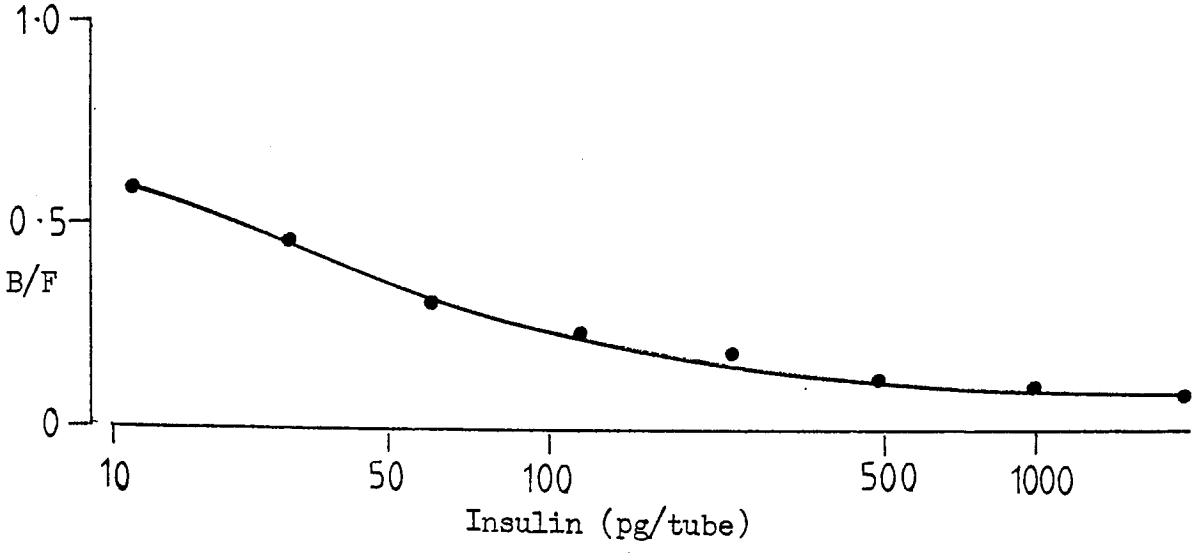
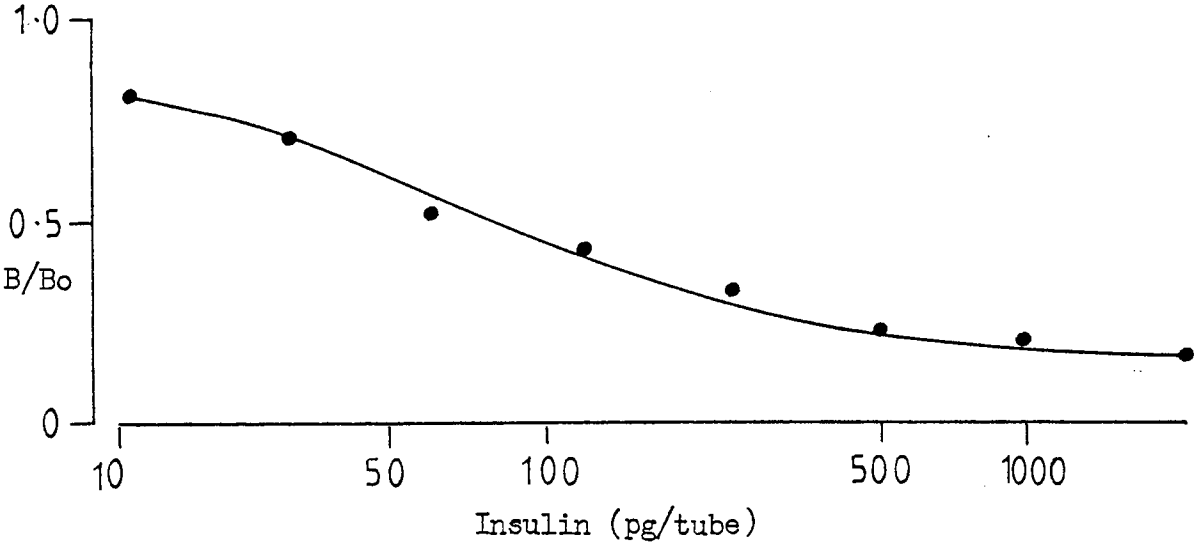


Figure 21 Radioimmunoassay dose response curves.
II. B/F, B/Bo and B/T vs. logarithmic dose





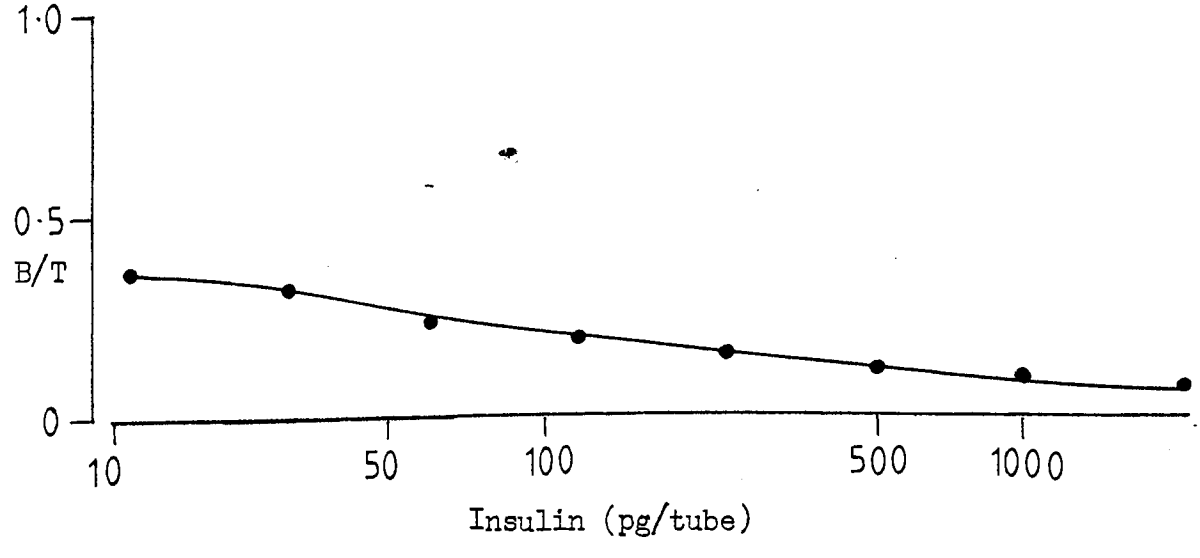
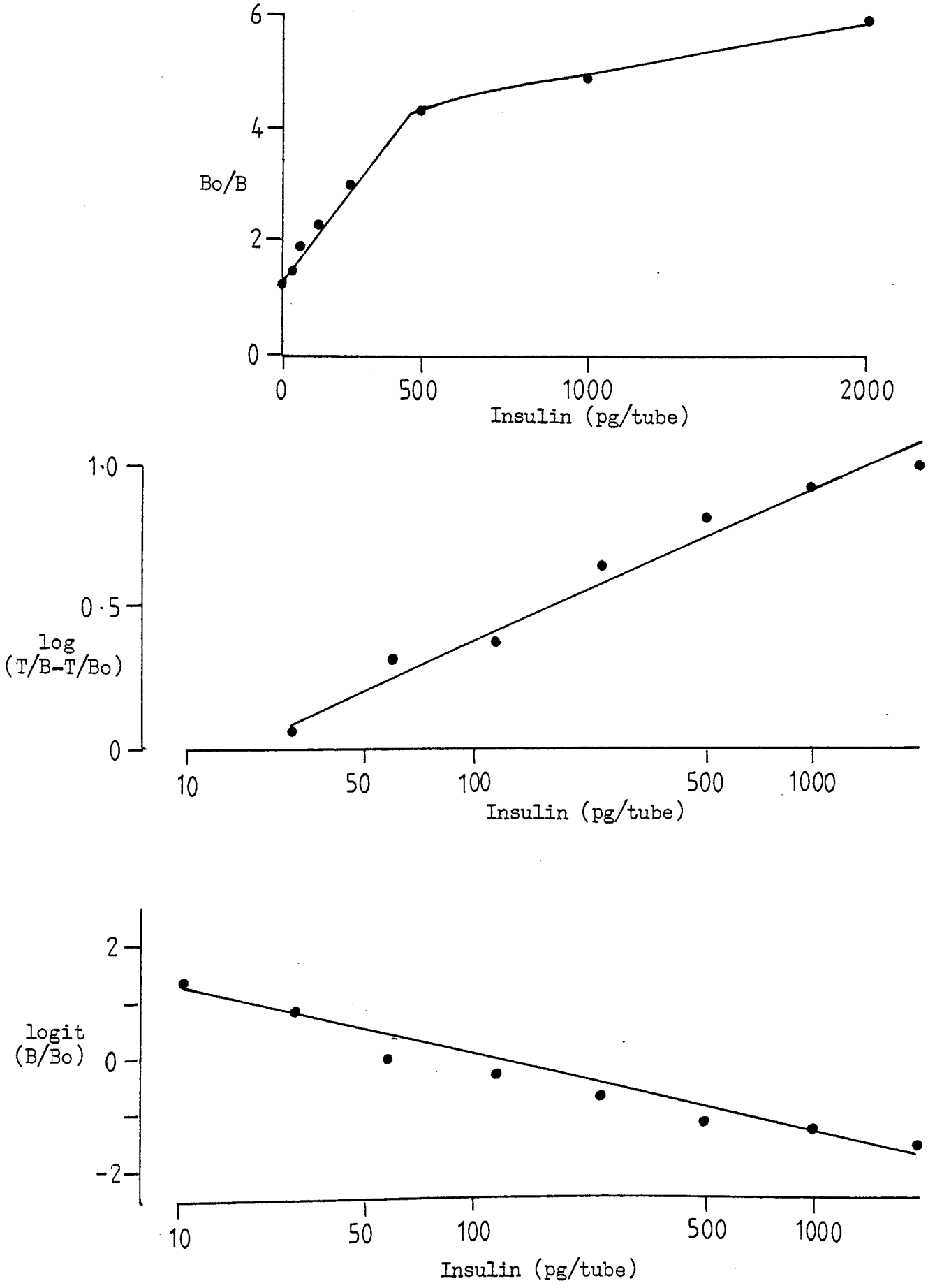


Figure 22 Radioimmunoassay dose response curves.
III. Reciprocal plots.



CHAPTER 2

RADIOIMMUNOASSAY OF INSULIN

GENERAL INTRODUCTION

A variety of procedures have been described for the quantitative estimation of insulin in biological fluids. Early attempts were based upon the biological activity of the hormone (Stewart, 1960; Vallance-Owen & Wright, 1960), but these have been largely supplanted in recent years by immunological methods of assay, in particular radioimmunoassay methods, which offer a unique combination of specificity, sensitivity, precision and practicability for the microdetermination of insulin in unfractionated mixtures (Berson & Yalow, 1964; Ashford et al., 1969; Ditschuneit & Faulhaber, 1975; Yalow, 1976).

The original bioassay procedures were performed *in vivo* and depended on the ability of the hormone to produce hypoglycaemia in rabbits (Banting et al., 1922), hypoglycaemic convulsions in mice (Young & Lewis, 1947) and to lower the blood sugar level of various surgically prepared alloxan diabetic rodents (Bornstein, 1950; Hagedorn, 1954; Beigelman & Antoniades, 1958; Baird & Bornstein, 1959). Additional *in vitro* techniques were developed which involved the measurement of glucose uptake and glycogen deposition by diaphragms (Randle, 1956; Manchester et al., 1959), epididymal fat pads (Beigelman, 1958; Martin et al., 1958) and isolated adipocytes (Gliemann, 1965) of rats. However, the sensitivity and specificity of these methods was not always adequate to enable the accurate determination of insulin in body fluids and the procedures were invariably expensive, complicated to perform and lacking in reproducibility (Randle, 1957; Randle & Taylor, 1960; Yalow & Berson, 1960c). Consequently, soon after its development (Berson & Yalow, 1957; Yalow & Berson, 1959) radioimmunoassay became the method of choice for the routine estimation of insulin, and most of our current knowledge of the mechanisms involved in the regulation of insulin secretion in normal and pathogenic states has been obtained using radioimmunoassay methodology (Steiner & Freinkel, 1972; Hasselblatt & Bruchhausen, 1975; Bajaj, 1977).

The introduction of radioimmunoassay by Yalow and Berson (1960a) provided a reproducible method for the precise quantification of minute amounts of insulin in biological fluids. However, since the initial description of the technique, a number of improvements have been introduced to facilitate the routine usage of the method in both clinical and investigative endocrinology (Ditschuneit & Faulhaber, 1975). Besides careful selection of cross-reacting antisera (Yalow & Berson, 1961 a, b), the major refinements of the technique concern the preparation of iodinated insulin and the separation of free and antibody bound hormone. Thus the development of alternative iodination procedures, based on modifications (Samols & Williams, 1961; Springell, 1961; Glover et al., 1967) of the original iodine monochloride method (McFarlane, 1958) or the chloramine-T procedure (Bannerjee & Gibson, 1962; Jørgensen & Binder, 1966) first described by Hunter and Greenwood (1962), has greatly facilitated the preparation of high specific activity tracer on both the commercial and small laboratory scale. However, the most significant development in assuring widespread acceptance of the technique was undoubtedly the development of numerous simple and reliable methods of separation (Grodsky & Forsham, 1960; Hales & Randle, 1963 a, b; Morgan & Lazarow, 1963; Herbert et al., 1965; Heding, 1966b) which has in turn prompted the marketing of various radioimmunoassay kits.

The development of new technological methods for the radioimmunoassay of insulin and other antigens represents a rapidly expanding area of clinical chemistry (Antoniades, 1976). Recent years have witnessed numerous advances including the emergence of immunoradiometric and solid phase techniques for the measurement of plasma insulin (Miles & Hales, 1969; Wide, 1971; Hales, 1972; Beck & Hales, 1975). The present chapter describes the establishment of a sensitive and precise radio-immunoassay for insulin relying on coated charcoal for the separation of the free and bound moieties. The general principles and practical guide lines upon which the method was developed have been discussed previously.

PREPARATION OF REAGENTS

ANTIBODY

1. INTRODUCTION

Some years after the isolation of insulin by Banting and Best (1922), Banting and colleagues (1938) demonstrated that the repeated administration of insulin for therapeutic purposes was associated with the gradual production of insulin resistance. The presence of insulin-binding antibodies in the sera of virtually all insulin-treated diabetics was later confirmed by Berson and colleagues (1956b; 1957a), who went on to utilise human immune serum as the basis for the first radioimmunoassay (Berson & Yalow, 1958). The application of the technique to the determination of human insulin was however impeded by the species specificity of the available antisera (Berson & Yalow, 1959a). Following extensive immunisation trials (Morse, 1959), sensitive radioimmunoassay techniques for the determination of human plasma insulin were established using guinea pig antibovine (Yalow & Berson, 1959, 1960a) and the superior antiporcine insulin antiserum (Yalow & Berson, 1961a, b).

Guinea pig serum containing antibodies to mammalian species of insulin is essential for current methods for insulin immunoassay, and for the induction of insulin deficiency in experimental animals (Wright, 1960; 1965). Thus, although insulin administered in adjuvant is antigenic in many different species (Moloney & Goldsmith, 1957; Moloney, 1960), the production of potent anti-insulin serum is best achieved by immunisation of the guinea pig (Morse, 1959; Pope, 1966). The enhanced responsiveness of this animal to the antigenic action of the hormone may well be related to the differences in the amino acid sequence of the molecule (Arquilla et al., 1972). Accordingly, Smith (1966) has demonstrated that bovine, porcine and human insulins differ by only 1-3 amino acid residues, whereas there exist 17 differences in the amino acid sequence of guinea pig insulin as compared to porcine insulin (figure 23).

Similarly, the cross reactivity between antisera produced against insulin derived from one species with that of a different species is believed to be partly related to the differences in the primary structure of the two molecules (Yalow, 1976). In the studies to be described, guinea pigs were immunised with insulin of bovine and porcine origin and the resulting antisera were screened for use in the radioimmunoassay of insulin from various animal species. The procedure adopted provided large volumes of high affinity insulin antisera suitable for use in both metabolic studies and radioimmunoassay.

2. MATERIALS AND METHODS

Guinea pigs

Male albino guinea pigs weighing 300-400 g were obtained from a commercial supplier (Christopher Hill, Poole, Dorset). The animals were kept in groups of five and maintained on a standard pellet diet (Heygates diet TR2, L.A.Pilsbury, Birmingham) supplemented with fine meadow hay. Ascorbic acid (0.4g/l) was added to the drinking water to prevent the occurence of scurvy (Peterson, 1966). Two separate batches of animals were used.

Insulins for Immunisation

Crystalline ox insulin (lot no. 24C-3130, 24.3 IU/mg) was obtained from Sigma Chemical Company Ltd., London. Ten times recrystallised pork insulin (Novo Research Institute, lot no. S4769, 23.5 IU/mg) was generously donated by Miss Catherine Borthwick of Martindale Pharmaceuticals Ltd., Romford, Essex.

Insulins for cross reactivity studies

In addition to using the insulin preparations described above, the cross reactivity of the antisera was tested with human and mouse insulins. Freeze dried human insulin (MRC, lot no. 66/304, 23.5 IU/mg) was provided by WHO International Laboratory for Biological Standards, Holly Hill, Hampstead. Lyophilised mouse insulin (lot no. M20169, 22.4 IU/mg) was

purchased from Novo Research Institute, Bagsvaerd, Denmark.

Inocula

The preparation of insulin as a water-in-oil emulsion followed the method described by Herbert (1973) with minor modifications. Crystalline insulin was dissolved in 0.3M hydrochloric acid at a concentration of 4 mg/ml. The solution was emulsified with three volumes of Freund's adjuvant as suggested by Hurn and Landon (1971). The oil phase was prepared by thoroughly mixing 7.5 ml Arlacel A and 42.5 ml paraffin oil. Complete adjuvant contained 0.5 mg/ml dried, heat killed Mycobacterium butyricum. Freund's complete and incomplete adjuvants were obtained from Miles Laboratories Ltd., Stoke Poges, Slough. Emulsions were usually prepared immediately prior to immunisation.

Immunisation procedure

The procedure adopted was similar to that originally described by Moloney and Coval (1955). Two groups each containing five guinea pigs were injected subcutaneously close to the midabdominal line. Each animal received 0.5 ml of an emulsion containing 500 µg of either bovine insulin (group A) or porcine insulin (group B) in Freund's adjuvant. At primary immunisation each animal received approximately 12 units of insulin in complete adjuvant. Secondary and subsequent injections were given at adjacent sites every four weeks using the same dose emulsified with incomplete adjuvant. Upon termination of a course of seven monthly injections, the guinea pigs in group A were rested for two months prior to a final booster injection. In order to avoid unnecessary hypoglycaemic reactions, the animals were always allowed free access to food both prior to, and following immunisation. The guinea pigs were given 10% glucose water ad libitum at the time of the primary injection.

Bleeding schedule and storage of antiserums

Guinea pigs fasted overnight were bled by cardiac puncture (usually 10-12 ml blood) under light either anaesthesia 12-14 days after the second booster and all subsequent injections. The blood was allowed to clot at room temperature and following separation, the serum was aliquoted into volumes of 50µl (one aliquot only) and 1-2 ml. The serum of each animal was stored separately at -20°C in tightly capped polystyrene tubes (Luckhams Ltd., Burgess Hill, Sussex).

Antibody assessment

At the time of analysis, the 50 µl aliquot of each serum was thawed and diluted with assay diluent to give a convenient working dilution of 1:10. A small volume of this solution was employed in the preliminary assessment and the remaining stock was stored at -20°C for use in subsequent tests.

The presence of insulin binding antibodies was evaluated by incubating increasing dilutions of each serum with 100 pg iodinated insulin in a final volume of 400 µl. Antibody dilution curves were constructed in duplicate for eight dilutions covering a ten thousand fold range of concentrations starting at 1:400. Following 48 hours of incubation at 4°C, free and antibody bound insulin were separated by the addition of coated charcoal and the distribution of the radioactivity between the two moieties was determined. The 50% maximum binding titre was evaluated graphically for each serum.

To assess the suitability of the antisera for use in radioimmunoassay, standard curves were prepared using mouse insulin and either beef (group A) or pig insulin (group B). Each antiserum was taken at a convenient dilution, binding 35-45% of the total radioactivity of the 100 pg tracer, and incubated as previously described. At the same time, the cross reactivity of every antiserum was tested using human, porcine, bovine and mouse insulins. This was achieved by a simplified method in which

125 pg of cold insulin was added to compete with the iodinated ox insulin for the antibody binding sites. To facilitate direct comparison of binding displacement data, the results of these experiments have been expressed as an index given by the equation $[100(Bo - B)/Bo]$. For similar reasons B/Bo has been adopted as the response metameter for the graphical illustration of the standard curves.

3. RESULTS

Guinea pigs were immunised for a period of up to eight months with eigher bovine (group A) or porcine insulin (group B) emulsified in Freund's adjuvant. One animal immunised with bovine insulin developed hypoglycaemia and died following the primary injection. However, no further losses due to hypoglycaemia were observed and subsequent deaths were either undiagnosed (group A) or associated with the anaesthetic regime. The mortality rates calculated from the percentage of animals lost and the number of cardiac punctures performed were 3.75 and 1.81 for groups A and B respectively.

At the end of the first ten weeks, and after one inoculation of complete adjuvant and two booster injections without added mycobacteria, all animals developed insulin binding antibodies (figure 24). The titre of sera obtained from individual animals within each of the two groups (expressed in the figure following log-inverse transformation) displayed wide variation. During the course of treatment, the concentration of serum antibodies continued to rise, reaching enormous values by the 22nd week of immunisation. By this time, high-titre antisera appeared in both groups, but only bovine insulin retained the capacity to evoke any further increase in antibody production. Thus following 26 weeks of immunisation, the binding capacity of serum obtained from the high producer in group A (GPA2) was sufficient to enable five million radioimmunological estimations per millilitre. Resting the guinea pigs

in group A for two months prior to administration of the final booster injection resulted in an approximate five-fold reduction in antibody titre (data not illustrated).

Standard curves prepared using antisera taken from two individual guinea pigs during the course of immunisation with either bovine (GPA2) or porcine insulin (GPB4) are illustrated in figures 25 and 26 respectively. These curves, which are essentially representative of the time course changes observed with other animals, give an indication of the cross-reactivity, working range and the effective affinity constant of each antiserum. The shape of the response curves prepared using antisera derived from either of the two guinea pigs displayed marked variation throughout the period of immunisation. Each serum contained high affinity antibodies and was eminently suitable for use in an homologous assay system. However, the energy of reaction with mouse insulin was much reduced, thereby restricting the full potential of each antiserum in the radioimmunological estimation of plasma insulin in the mouse.

These findings are further substantiated in figures 27 and 28 which briefly summarise the results of the inhibition curves for each guinea pig in the two groups. Despite variations in the response of individuals throughout the course of treatment, there was a general trend towards increased displacement of tracer as immunisation progressed, reaching near maximal values some 18-22 weeks after the primary inoculation. Attempts to increase antibody affinity by employing a long rest period prior to the final booster injection (Hurn & Landon, 1971) led to an approximate two-fold decrease in displacement (data not illustrated). When assessed using homologous antigen, inhibition was usually greater for antisera produced against porcine insulin. Displacement caused by an equivalent dose of mouse insulin was consistently less than that observed in either of the two groups, and despite minor changes in the

degree of cross reactivity between consecutive serum collections, rarely exceeded 50%.

The species specificity of the antisera was similar for both groups of animals. The mean percentage cross reaction for sera obtained by repeated immunisation with beef insulin (group A) was 66 (49-82), 64 (36-81) and 54 (40-70) for human, porcine and mouse insulin respectively. Antisera raised against porcine insulin (group B) exhibited 68 (44-96), 61 (43-98) and 53 (26-86) percentage cross reaction with human, bovine and mouse insulin.

Upon the basis of the binding data and cross reaction studies, one antiserum from each group was selected for further assessment. The sera which exhibited highest displacement values with mouse insulin were GPA3/4 (56%) and GPB1/3 (67%). Both sera showed good cross reaction with other species of insulin and percentages calculated from the relative displacement values for human, bovine, porcine and mouse insulin were 82, 100, 80 and 70 for GPA3/4, and 96, 98, 100 and 86 for the serum raised against porcine insulin, GPB1/3. Standard curves for these two antisera are illustrated in figure 29. Although both sera were characterised by high affinity constants, the binding data suggest that mouse insulin predominantly cross reacts with the high affinity component in the anti-porcine insulin antiserum GPB1/3.

4. DISCUSSION

The results of the present experiments agree with those of Moloney and Coval (1955) that a consistent immune response may be obtained by monthly inoculation of guinea-pigs with a water-in-oil emulsion containing crystalline insulin. The procedure adopted here, and used successfully in modified form by others (Robinson & Wright, 1961; Hurn & Landon, 1971), yielded a spectrum of antisera with binding properties suitable for use in both metabolic studies and radioimmunoassay. In contrast to

previous reports (Robinson & Wright, 1961; Wright & Norman, 1966), the present régime involving a single injection of Freund's adjuvant followed by monthly injections without added mycobacteria resulted in the production of high capacity sera capable of binding approximately 9 U insulin per milliliter by the 22nd week of immunisation. The most active sera, which compared favourably with those produced using a bacterial adjuvant containing H. pertussis vaccine (Wright et al., 1968a), were occasionally characterised by high displacement values, but unlike the studies of Hollins and Himsworth (1976) no significant correlation was observed. The sensitivity of the antisera, as assessed from the inhibition data, was frequently four times superior to that reported by Hurn and Landon (1971) using an almost identical immunisation schedule. However attempts to increase displacement further by resting animals prior to administration of a final booster injection (Hurn, 1971; Hurn & Landon, 1971) resulted in a dramatic decrease in sensitivity, and therefore confirm the latter results of Lader, Hurn and Court (1974).

Observations concerning the relative immunogenicity of bovine and porcine insulin were made difficulty by the mortalities in each of the two groups. On the basis of available data, the responses of guinea pigs to either antigen were similar, although bovine insulin appeared to possess an enhanced capacity to evoke long term proliferation of antibody forming cells. This conclusion is in general agreement with the studies of Wright and colleagues (Wright et al., 1968a; Makulu & Wright, 1971), who were able to demonstrate a small difference between the abilities of these two species of insulin to induce antibody production in the guinea pig. In contrast to the minor difference in immunogenicity, there was a marked divergence between the displacement values, and hence affinity, observed using antibodies directed towards the two species of insulin. The increased sensitivity of anti-porcine insulin antisera to a homologous antigen could result from the preferential stimulation of

high affinity antibody producing cells (Parker, 1971). Alternatively, the phenomenon may have resulted from the utilisation of radioiodinated beef insulin as tracer in the assay system. Ekins and colleagues (Ekins et al., 1968; Ekins & Newman, 1970) have demonstrated that a tracer with an affinity constant lower than that of the test compound will increase sensitivity.

During the course of treatment with either bovine or porcine insulin, there was a gradual trend towards increased displacement values as immunisation progressed. The tendency towards increased sensitivity and presumably high affinity was however subject to some degree of fluctuation. Such variation may reflect dynamic changes in the relative concentration of different orders of antibody binding sites each characterised by a specific affinity constant. Since each sub-population of antibodies can display different species specificity (Berson & Yalow, 1957; Arquilla et al., 1976), alterations in the cross reactivity of consecutive sera with mouse insulin may also occur by this mechanism.

Cross reaction studies with human, bovine, porcine and mouse insulin demonstrated a spectrum of different degress of species specificity in both groups of antisera. Despite variations between the degree of cross reactivity observed with different sera, the order of displacement was remarkably constant. In all cases, antisera displayed highest affinity for the homologous antigen and reacted 26-86% less strongly with mouse insulin. The degree of cross reaction with other species of insulin was intermediate between these two extremes, and human insulin generally exhibited a slightly greater avidity for the antibody binding sites than did the heterologous ungulate species. Although a certain degree of caution is warranted in interpreting these findings (Berson & Yalow, 1963; Hales, 1965), the relative cross reactivity of these antigens may be a result of differences in the primary structure of the insulin molecule (Berson & Yalow, 1959a). As illustrated in figure 23, mouse insulin

differs from human, bovine and porcine insulin by an average of four, six and four amino acid residues respectively. The observation that mouse insulin cross reacts less than either of the other antigens with bovine or porcine antisera is therefore not without theoretical justification. A further difficulty of interpretation arises from the identification of two types of rodent insulin differing from each other at B9 and B29 (Markussen, 1971; Humbel et al., 1972). It is envisaged that antisera discriminating between human and porcine insulin which differ only at B30, will display different affinities for the two types of mouse insulin.

Despite the greater similarity between the amino acid composition of porcine insulin and the structure of other insulin molecules, the species specificity of antisera was generally the same in each of the two groups. However, in accordance with the classical observations of Yalow and Berson (1961b), porcine insulin was occasionally capable of stimulating the production of non-discriminating antisera. The value of such an antiserum is demonstrated in the present study by consideration of the binding characteristics of GPA3/4 and GPB1/3. These sera contained antibodies of comparable affinity when assessed in an homologous assay system. However, using mouse insulin as the displacing antigen, GPB1/3 retained considerably more of its potential binding energy and consequently enabled the detection of minute concentrations of insulin in highly diluted mouse plasma. The differences in cross reaction observed with these two antisera are probably related to the region of the insulin molecule involved in antibody combination. Analysis of the specificities of these antisera with a range of mammalian insulins suggests that while GPA3/4 combined in the species variable region A4 - A9 and the neighbouring area of the B chain, the reaction with GPB1/3 was restricted to the N-terminal region of the B chain. These conclusions which are necessarily based on a limited amount of binding data, suggest that the

energy of the binding reaction with either antiserum was not adversely influenced by iodination of the tyrosine residues located at the solvent surface of the insulin molecule (Van Doesburgh & Havinga, 1964; Freedlender & Cathou, 1971).

LABELLED ANTIGEN

1. INTRODUCTION

Since the original description of the radioimmunoassay for insulin (Yalow & Berson, 1959), a variety of procedures have been developed for the preparation of radiolabelled insulin of high specific activity (Hughes, 1966; Chervu & Murty, 1975). Iodinated insulins have found widespread usage in immunoassay and as tracers for metabolic studies (Roth, 1973; Yalow & Berson, 1973; Starr & Rubenstein, 1974; Izzo, 1975 a, b), and have consequently been extensively characterised with respect to both biological and immunological properties (Brunfeldt et al., 1968; Arquilla et al., 1972; Blundell et al., 1972; Garratt et al., 1972).

The substutition reaction between cationic iodine and the anionic phenolate groups of the protein molecule was initially performed under a variety of conditions utilising either iodine in potassium iodide solution (Hughes & Straessle, 1950); iodide oxidised to a reactive state with nitrous acid (Pressman & Eisen, 1950); iodine liberated by acidification of a mixture of iodine and iodate (Francis et al., 1951) or iodine produced by the reaction of iodide with hydrogen peroxide (McFarlane, 1956). These methods which were implicit in the preparation of tracer hormone for many of the early studies (Berson et al., 1956a; Grodsky & Forsham, 1960; Yalow & Berson, 1960a) have been totally superseded in recent years by the development of numerous highly efficient methods for the production of undamaged radioiodinated proteins (Hughes, 1966; Hunter, 1974; Chervu & Murty, 1975). Thus insulin has

been iodinated to high specific activity by chemical oxidation with iodine monochloride (Samols & Williams, 1961; Springell, 1961) or chloramine-T (Bannerjee & Gibson, 1962), by enzymatic oxidation using lactoperoxidase (Thorell & Johansson, 1971) or horseradish peroxidase (Lambert & Jacquemin, 1973), and by electrolytic oxidation (Rosa et al., 1967). Despite the undoubted success of non-chemical methods in yielding high specific activity tracer with minimal damage (Ooms & Arquilla, 1966; Rosa et al., 1966a; Hamlin & Arquilla, 1974; Lambert & Jacquemin, 1974), neither enzymatic nor electrolytic iodination has been widely adopted. Accordingly, radioiodinated insulins prepared by modification of the original methods described by McFarlane (1958) and Hunter and Greenwood (1962) have been extensively used in both analytical and metabolic studies (Steiner & Freinkel, 1972; Hasselblatt & Bruchhausen, 1975). Although the iodine monochloride method has enjoyed a period of considerable popularity (Hales & Randle, 1963a; Izzo et al., 1964a; Arquilla et al., 1968), and remains the method of choice for at least one commercial supplier (Glover et al., 1967), there has been during the past decade, an increasing trend towards the small laboratory scale iodination using chloramine-T. This method which under appropriate conditions permits the strict control of the degree of iodination (Brunfeldt et al., 1968; Garratt et al., 1972) is currently employed for the preparation of iodoinsulins containing one or less atoms of iodine per molecule for use in both radioimmunoassay (Yalow & Berson, 1973; Starr & Rubenstein, 1974; Schneider et al., 1976) and radioreceptor studies (Cuatrecasas, 1971; Freychet et al., 1971; Gavin et al., 1972).

The relationship between the degree of iodination of insulin and its biological and immunological properties has been the subject of much investigation. These studies have demonstrated that although the biological activity of the insulin molecule was relatively unaltered by substitution with an average of one or less atoms of iodine (Arquilla

et al., 1968; Cuatrecasas, 1971; Freychet et al., 1971; Sodoyez et al., 1975), there was a progressive loss in hormonal activity with increasing degrees of substitution (Izzo et al., 1964; Rosa et al., 1966b) which became maximal above the trisubstituted derivative (Garratt, 1964; Rosa et al., 1967). In contrast to the marked changes in biological competence associated with the introduction of iodine into the insulin molecule was the subtle change in the immunological properties of heavily substituted preparations containing an average of six atoms of iodine per molecule (Izzo et al., 1964; Brunfeldt et al., 1968; Glover et al., 1967). However, in spite of the indifference of many antisera to high levels of substitution, the specific activity of iodinated insulin used in radioimmunoassay rarely exceeds 350 μCi/μg corresponding to monoiodinated insulin (Yalow, 1976). Indeed, to assure that the major fraction of radioactivity is not associated with more highly iodinated species, thereby minimising 'decay catastrophe' (Berson & Yalow, 1966, 1973; Yalow & Berson, 1969b) and consequently prolonging the shelf life of the preparation (Schneider et al., 1976), an average level of approximately 0.5 atoms of iodine per molecule of insulin is often regarded as optimal (Starr & Rubenstein, 1974; Schneider et al., 1976). The present section describes the preparation of iodinated insulin suitable for routine use in the radioimmunoassay of plasma insulin. Iodination by a modification of the chloramine-T procedure originally described by Hunter and Greenwood (1962), followed by purification using gel filtration yielded ^{125}I-insulin with a specific activity in the range 100-180 μCi/μg, corresponding to approximately 0.3-0.5 atoms of radio-iodine per molecule of insulin.

2. MATERIALS AND METHODS

Insulin for iodination

Crystalline ox insulin (lot no. 24C-3130, 24.3 IU/mg) was obtained from Sigma Chemical Company Ltd., London.

Insulin for displacement studies

Inhibition curves were prepared using lyophilised mouse insulin (lot no. M20 169, 22.4 IU/mg) purchased from Novo Research Institute, Bagsvaerd, Denmark.

[^{125}I] - Iodide

Sodium iodide [^{125}I] for protein iodination (code No. IMS 30) was supplied at a specific activity of 11-17 mCi/μg iodine by The Radiochemical Centre, Amersham. The material was distributed at a radioactive concentration of 100 mCi/ml in sodium hydroxide, pH 8-11, free from reducing agent and had a minimum isotopic abundance of 98% with less than 1% ^{126}I.

Preliminary studies with [^{127}I] - Iodide

In a series of preliminary experiments, insulin was iodinated by a modification of the standard procedure (see below) using Na^{127}I plus a trace amount of Na^{125}I (molar ratio approximately 10,000:1). Critical variables including the amount of chloramine-T (0.39-200 μg), the reaction time (2-480 seconds) and the pH (6.5-8.5) were examined with respect to trichloroacetic acid precipitable radioactivity and binding to excess insulin antiserum. Following iodination, the reaction volume was made up to 500 μl by the addition of ice cold 0.4% bovine serum albumin (Pentex, fraction V, Miles Laboratories Ltd., Stoke Poges). 50 μl aliquots of the resulting solution were taken for analysis of radioactivity, precipitation with trichloroacetic acid and following dilution (1:5) with assay diluent, for determination of charcoal binding and immunological competence. For estimation of trichloroacetic acid

precipitable radioactivity, the 50 µl aliquot was diluted with 450 µl 0.2% bovine serum albumin and following the addition of an equal volume of cold 10% trichloroacetic acid, the protein pellet was separated by centrifugation for ten minutes at 1,500 g using a refrigerated centrifuge. The supernatant was discarded and the precipitate was resuspended in 1 ml 5% trichloroacetic acid prior to an additional sequence of centrifugation and decantation. The radioactivity associated with the protein pellet was counted using a well-type crystal scintillation counter. Charcoal adsorption and immunological competence of each preparation was assessed by a minor modification of the procedure described for the routine radioimmunoassay of insulin. In brief, a 50 µl aliquot of the diluted reaction mixture was incubated at 4°C with 100 µl undiluted insulin antiserum (GPA2/2) or an equivalent amount of normal guinea pig serum in a final volume of 400 µl. Following 48 hours of incubation, the free and antibody bound moieties were separated by the addition of 10 mg coated charcoal and the radioactivity of the charcoal pellet was determined. The antiserum selected for these studies was capable of binding approximately 300 µg bovine insulin per millilitre serum.

Standard iodination procedure

Radioiodination was performed by a modification of the chloramine-T procedure (Hunter & Greenwood, 1962; Hunter, 1974). The following reactants were mixed at room temperature (approximately 20°C) in a small (1.6 ml) glass tube:

Sodium phosphate buffer pH 7.4 (0.4M), 10 µl

Insulin (2.5 µg) in hydrochloric acid (0.01M), 10 µl

Na ^{125}I (1 mCi), 10 µl

Chloramine-T (50 µg), 10 µl

The reaction was allowed to proceed for 10 seconds with thorough mixing and was terminated by the

addition of sodium metabisulphite (120 µg), 50 µl.

Mixing was continued for a further 45 seconds

prior to dilution of the remaining radioactive

iodide with potassium iodide (1 mg), 100 µl.

All reactants were weighed and diluted to the required concentration immediately before use. Chloramine-T, sodium metabisulphite and potassium iodide (Hopkin and Williams Ltd., Chadwell Heath, Essex) were dissolved in freshly prepared sodium phosphate buffer, pH 7.4 (0.04M).

Purification and storage of ^{125}I-insulin

Iodinated insulin was separated from damaged protein, unreacted iodide and other low molecular weight reactants by gel filtration. Thus upon completion of iodination, the reaction mixture was diluted with 100 µl 5% bovine serum albumin and the resulting solution was transferred to a 45 x 1 cm glass column of Sephadex G50 Fine (Pharmacia Fine Chemicals, Uppsala, Sweden) which had been previously equilibriated with eluent. The column was eluted at 5-10°C with phosphate buffer pH 7.4, 0.04M, 0.5% bovine serum albumin, using gravity feed under a constant head of pressure. One-ml fractions were collected in polystyrene tubes (Luckhams Ltd., Burgess Hill, Sussex) using a fraction collector (type 7000, LKB, South Croydon), and after counting, the peak tubes were selected for use in radioimmunoassay. The radioiodinated insulin was diluted with assay diluent, aliquoted and stored frozen at -20°C until required. Alternatively the pooled fraction was diluted with an equal volume of acid ethanol (1.5 ml concentrated hydrochloric acid, 75 ml ethanol), thoroughly mixed and stored at -20°C in aliquots each containing approximately 2 ml labelled insulin.

On a single occasion the elution profile of immunoreactive material was studied in greater detail using various fractions distributed between the void volume and the salt peak. In this instance, comparison of the

physico-chemical and immunological properties of the radioactive material enabled direct evaluation of the value of trichloroacetic acid precipitation and charcoal adsorption as indices of incubation damage. Selected fractions were stored overnight at 4°C prior to testing. The column was meanwhile calibrated with dextran blue (Pharmacia Fine Chemicals, Uppsala, Sweden) and a trace of sodium ^{125}iodide.

^{125}I insulin assessment

In a preliminary study, selected fractions distributed between the void volume and the salt peak were examined with respect to trichloroacetic acid precipitable radioactivity, charcoal adsorption and binding to excess insulin antiserum. The methods which were analogous to those already described were used to assess the distribution of radioactivity between the various constituents of each fraction. Trichloroacetic acid precipitable radioactivity was determined using a 10 µl aliquot which had previously been diluted to a final protein concentration of 2 mg/ml with 490 µl 0.2% bovine serum albumin. Charcoal adsorption was monitored using a similar volume made up to 400 µl with assay diluent, and immunological binding was assessed using a 100 µl aliquot containing approximately 12,000 counts per minute. The immunoreactive content of each fraction was determined following incubation of the diluted sample for 48 hours at 4°C in the presence or absence of excess insulin antiserum (1:40,000, GPB1/3). The percentage adsorption to charcoal in the absence of added antiserum was independent of the dilution used for analysis.

Routine evaluation of radioiodinated insulin was achieved by incubating 100 pg tracer, 10,000-20,000 counts per minute depending on specific activity, with increasing dilutions of insulin antiserum (GPB1/3) in a final volume of 400 µl. Antibody dilution curves were

usually prepared in triplicate for seven dilutions covering a twenty fold concentration range starting at 1:40,000. Following 48 hours of incubation at 4°C, free and antibody bound insulin were separated by the addition of coated charcoal and the distribution of radioactivity between the two moieties was determined. Radioactivity associated with protein was determined at the same time by analysis of the material precipitated by the addition of 500 µl ice cold 10% trichloroacetic acid to an equal volume of buffer containing 200 pg/ml labelled insulin and 2 mg/ml bovine serum albumin. A good tracer was characterised by > 85% binding to excess antibody, > 90% adsorption by coated charcoal and > 95% precipitation with trichloroacetic acid.

The iodinated preparation was further evaluated for routine use in radioimmunoassay by its ability to produce a sensitive standard curve. Inhibition curves were prepared in triplicate with doubling dilutions of mouse insulin ranging from 20 ng/ml to 0.019 ng/ml and the 50% maximum binding titre (1:160,000-1:240,000, GPB1/2). Incubation was carried out for 24 hours at 4°C before addition of the labelled species. The reaction was allowed to continue for a further 48 hours prior to separation. Under these conditions, the binding of a high quality tracer would be reduced by 10% on the addition of approximately 15 pg cold insulin.

3. RESULTS

Radiodinated beef insulin was prepared by modification of the chloramine-T procedure originally described by Hunter and Greenwood (1962). Iodination of 2.5 µg insulin with 1 mCi sodium ^{125}iodide at pH 7.4 using 50 µg chloramine-T followed by purification on Sephadex G50 Fine, yielded high quality tracer which stored under appropriate conditions remained suitable for use in radioimmunoassay for up to three months. The method resulted in the incorporation of approximately

0.4 atoms of iodine per molecule of insulin, corresponding to a specific activity of 140 μCi/μg.

Preliminary iodination experiments carried out using ^{127}iodide together with a negligible amount of ^{125}iodide demonstrated an intimate relationship between both the amount and duration of exposure to the oxidising agent and the percentage incorporation of radioactivity together with the immunological property of the protein fraction. No such interdependence was observed between any of the parameters monitored and the pH of the reaction over the range studied. Stepwise increments in the amount of chloramine-T (0.39-200 μg) obtained by doubling the concentration used in the reaction resulted in a progressive increase in the incorporation of radioactivity into trichloroacetic acid precipitable protein which reached near maximal values on the addition of 25 μg of the salt. Immunological damage assessed using excess insulin antiserum became apparent at amounts exceeding 12 μg reagent and steadily increased until approximately 50% of the radioactivity incorporated into protein in the presence of 200 μg chloramine-T was immunologically lame. In the light of the complex inverse relationship between the immunological competence of the product and both the amount of chloramine-T and the percentage incorporation of iodine into protein, an optimal amount of oxidising agent corresponding to 50 μg chloramine-T was selected for use in further studies. In contrast to the marked and variable effect of modulating the amount of chloramine-T used for iodination, the influence of increasing the reaction time (2-480 seconds) on the parameters tested was much less pronounced. Trichloroacetic acid precipitable radioactivity which had reached near maximal levels following a brief 2 second exposure to the oxidising agent attained peak values at approximately 30 seconds, and gradually declined thereafter to yield some 70% of the original count rate by 480 seconds. Prolonged exposure to chloramine-T was associated with a mild increase

in the extent of immunological damage. However, when assessed relative to the change in the incorporation of radioactivity into protein, the immunological potency of the product was unaltered. Consequently, conditions corresponding to maximum yield were chosen, and for routine iodination a reaction time of approximately 10 to 15 seconds was used.

Purification of a standard iodination mixture by gel filtration on a 45 x 1 cm column of Sephadex G50 Fine is illustrated in figure 30. The column which was presaturated with protein to minimise adsorption of the labelled insulin was eluted at a constant flow rate of approximately 0.75 ml per minute, permitting 65% recovery of radioactivity as iodinated insulin. The chromatogram which is representative of other iodinations shows good separation between the central peak containing undamaged insulin and both the high molecular weight components eluting close to the void volume (19 ml) and the unreacted sodium ^{125}iodide associated with the salt peak (46 ml). The specific activity of the preparation calculated according to the method described by Hunter and colleages (Greenwood et al., 1963; Hunter, 1973) was 139 μCi/μg corresponding to an average substitution of 0.38 atoms of iodine per molecule of insulin. The top five fractions (32-36) were selected for use in radioimmunoassay. The pool which contained 0.36 μg/ml iodinated insulin, sufficient for approximately 18,000 radioimmunological determinations, exhibited 84.5% binding to excess insulin antiserum, 93.5% adsorption to coated charcoal and 98% precipitation by trichloro-acetic acid. This preparation was capable of producing a 10% fall in bound counts on the addition of 17.8 pg cold insulin when incubated under optimal assay conditions with a 1:260,000 dilution of guinea pig antiporcine insulin antiserum. The tracer stored well at -20°C in either acid ethanol (0.18 μg/ml) or assay diluent (0.036 μg/ml) and was used in radioimmunoassay for a period of eight weeks.

The physicochemical and immunological properties of various fractions distributed between the void volume and the salt peak are shown in figure 31. The lower section of the figure illustrates the elution of radioactivity and its approximate distribution between the three major components. The profile of immunoreactive material closely followed the elution of radioactivity in the central area of the chromatogram corresponding to the insulin peak. Its distribution was skewed as a result of immunologically damaged high molecular weight aggregates of insulin eluting close to the void volume, and maximal binding was observed in the fraction corresponding to peak radioactivity. The adsorption of eluted radioactivity to coated charcoal demonstrated the avid uptake of both undamaged insulin and sodium iodide. In the absence of significant amounts of the radioactive salt, the adsorbent displayed excellent discrimination between immunologically competent insulin and the damaged hormone. In contrast to charcoal adsorption which under appropriate conditions was capable of monitoring the extent of iodination damage, trichloroacetic acid precipitation permitted accurate determination of the percentage incorporation of iodine into protein. The distribution of precipitable radioactivity mimicked the elution of immunoreactive material and low values were consistently observed with fractions associated with the salt peak. The reduced ability of trichloroacetic acid to precipitate radioactivity in fractions approximating to the void volume demonstrates the enhanced rate of liberation of free iodide by the damaged protein.

The relationship between the physico-chemical and immunological properties of the radioactivity eluted during the course of purification is illustrated in figure 32. A significant correlation was observed between adsorption to charcoal and binding to excess insulin antiserum in those fractions containing small amounts of free iodide. Similarly trichloroacetic acid precipitable radioactivity was significantly

correlated to the degree of immunological competence in the latter fractions. The association of these parameters with the degree of immunological competence demonstrates that when used in combination these two simple tests can be used to quickly assess both the damage and free iodide content of the radioiodinated insulin preparation.

4. DISCUSSION

The present study describes the preparation of radiolabelled insulin suitable for use in routine radioimmunoassay. Iodination of bovine insulin with carrier free sodium ^{125}iodide in mildly alkaline aqueous solution using chloramine-T and subsequent purification by gel filtration yielded high quality tracer with a specific activity of approximately 140 μCi/μg corresponding to an average incorporation of 0.4 atoms of iodine per molecule of insulin. The degree of substitution which coincides with the preferred specific activity for use in both radioimmunoassay and radioreceptor studies (Cuatrecasas, 1971; Schneider et al., 1976) represents a compromise between the exquisite sensitivity attainable at high specific activity and the greater stability and shelf life of less highly iodinated species of insulin (Berson & Yalow, 1966, 1973; Van Orden, 1972). Indeed, one can predict from the results of Monte Carlo simulation studies performed by Schneider and colleagues (1976) that even at an average of 0.4 atoms of radio-iodine per molecule approximately 25% of the radioactivity will be associated with the di-substituted insulin derivative. Although the degree of iodination directly influences the storage properties of iodinated insulin (Berson & Yalow, 1966, 1973; Schneider et al., 1976), the biological and immunological characteristics of the tracer preparation are largely dependent upon the precise location of the reacting tyrosine residues (Arquilla et al., 1966, 1976; Bihler & Morris, 1972; Lambert et al., 1972b). Despite variations in the position

of the substituent group observed using different conditions for iodination (Massaglia et al., 1969; Garratt et al., 1972; Lambert et al., 1972a; Lambert & Jacquemin, 1974), available evidence suggests that at an average of less than one atom of iodine per molecule of insulin, reaction in aqueous solution leads to a characteristic dominance of substitution at the two A chain tyrosine residues (De Zoeten & De Bruin, 1961; De Zoeten & Havinga, 1961; Springell, 1961; Van Doesburgh & Havinga, 1964; Schneider et al., 1976). Thus using chloramine-T as oxidising agent, Freedlender and Cathou (1971) demonstrated that at an average of 0.49 atoms iodine per molecule of insulin the approximate distribution of iodine at the four tyrosine residues A14, A19 and (B16 + B26) was 86, 10 and 4 percent respectively.

In accordance with observations made employing other proteins (Greenwood et al., 1963; Midgley & Jaffe, 1966; Hunter, 1973; Desbuquois, 1975), the present study demonstrated a complex and intimate relationship between both the amount and duration of the reaction with chloramine-T and the percentage incorporation of iodine together with the immunological property of the radiolabelled product. Exposure of insulin to large quantities of chloramine-T for prolonged periods of time resulted in a progressive decline in immunological competence of the iodinated derivative which was related to the amount of chloramine-T and to a lesser extent the duration of the substitution reaction. The exact relationship between the reagent and the degree of damage incurred under these conditions is complex. Immunological competence of radio-labelled insulin preparations is dependent upon many factors including the degree of iodination (Ooms & Arquilla, 1966; Brunfeldt et al., 1968), the location of the substituting group (Freedlender & Cathou, 1971; Bihler & Morris, 1972), the epitope involved in the binding reaction (Arquilla et al., 1966, 1968) and the ability of the antiserum to recognise small changes in the structure of the insulin molecule (Arquilla et al.,

1976; Hollins & Himsworth, 1976). However, since changes in both the extent and position of substitution are unlikely to be significant under the reaction conditions employed (Hamlin & Arquilla, 1974; Schneider et al., 1976), alterations in the immune binding of iodinated insulin preparations not associated with a reduction in trichloroacetic acid precipitable radioactivity, probably resulted from a direct effect of chloramine-T on the immunological competence of the insulin molecule. A deleterious effect of the reagent has been reported for peptide hormones (Berson & Yalow, 1967; Stagg et al., 1970; Butt, 1972; Rogol & Rosen, 1974; Shima et al., 1975). However, by selecting an optimal concentration of chloramine-T these subtle changes in the structure of the insulin molecule have been essentially eliminated. Accordingly, exposure of insulin to a small amount of chloramine-T for only a short period of time, thereby minimising both the extent of chemical oxidation and the adsorption of the peptide by glassware, has been shown to be a prerequisite to the successful production of high quality tracer for use in radioimmunoassay and radioreceptor studies (Gavin et al., 1972: Berson & Yalow, 1973: Roth, 1973: Schneider et al., 1976).

Radioiodinated insulin has been purified by numerous methods including dialysis (Berson et al., 1956a), adsorption chromatography with powdered cellulose (Yalow & Berson, 1960a), gel filtration (Bannerjee & Gibson, 1962), starch gel electrophoresis (Berson & Yalow, 1966) and more recently ion exchange chromatography (Freychet et al., 1971; Sodoyez et al., 1975). In the present study, gel filtration using Sephadex G50 Fine provided a rapid and convenient method for the efficient separation of iodinated insulin from both damaged protein and unreacted sodium iodide. Although reduction in the flow rate to approximately one-fifth of the value reported resulted in better separation of the constituent peaks, it failed to improve the performance

of the tracer in immunoassay and was accordingly judged as being both time consuming and unnecessary for routine purposes. Analysis of the physico-chemical and immunological properties of the eluted radioactivity provided important information concerning the potential usage of each fraction in radioimmunoassay. In accordance with the observations of Sönksen and Refetoff (1971), charcoal adsorption constituted an accurate and reliable means of assessing the immunological competence of radioiodinated insulin in the absence of significant amounts of free iodide. However, by combining the simple test with a measure of trichloroacetic acid precipitable radioactivity, both the degree of damage and the free iodide content can be quickly and reliably evaluated for any radioiodinated insulin preparation.

Various factors have been shown to influence the stability of iodinated insulin preparations during storage (Berson & Yalow, 1966; Van Orden, 1972). As illustrated by the present investigation, radioiodinated insulin stored at -20°C in either assay diluent or acid ethanol could be used repeatedly in radioimmunoassay without further purification until the level of remaining radioactivity became prohibitive. The success of the present storage régime in minimising damage to the tracer results from the selection of optimal variables including temperature, pH, bovine serum albumin and the concentration in terms of both radioactivity and iodinated peptide (Van Orden, 1972). Storage in acid ethanol was particularly convenient since in addition to avoiding freezing, thereby removing the necessity of preparing multiple aliquots, the solvent efficiently prevented both glassware adsorption and proteolytic destruction of the labelled hormone. However, two of the most important factors concerned in the long term preservation of the immunological competence of radioiodinated insulin relate to the degree of chemical substitution and the extent of freedom from contamination with high molecular weight components produced during iodination (Berson & Yalow, 1966, 1973; Yalow & Berson, 1969b; Schneider et al., 1976).

STANDARD

1. INTRODUCTION

Since the original studies of Berson and Yalow on the species specificity of insulin antisera (1958, 1959a), the need for homologous standards in the radioimmunoassay of peptide hormones has become increasingly apparent (Bangham & Cotes, 1971, 1974; Cotes, 1974).

Early attempts to obviate the necessity of using human insulin standards in the radioimmunoassay of this peptide hormone in man were based on the ability of certain antisera to react identically with a species of insulin which was readily available in pure form (Berson & Yalow, 1959b; Yalow & Berson, 1961b). The selection of cross reacting antisera which was initially made on an empirical basis is still necessary for rare species of insulin (Falkmer & Patent, 1972; Thorpe & Ince, 1976). However, in these instances the provision of suitable antisera is greatly assisted by our current knowledge of amino acid sequences (Blundell et al., 1972; Humbel et al., 1972) and the nature of the binding process (Arquilla et al., 1972, 1976).

Much has been achieved in recent years concerning the production and distribution of reference preparations suitable for use as standards in the radioimmunoassay of insulin (Soeldner et al., 1974; Bangham, 1976). Highly purified human insulin is readily supplied by the World Health Organisation and standard insulin preparations for a wide range of mammalian species are available from a number of commercial suppliers. The present section describes the biological potency of the reference preparation employed in current studies, the preferred method of storage, the procedure used to prepare daily working standards and some preliminary tests designed to assess its potential usage as a valid reference preparation in the radioimmunoassay of mouse insulin.

2. MATERIALS AND METHODS

Standard insulin preparation

Twice recrystallised mouse insulin (lot no. M20169) was purchased from Novo Research Institute, Bagsvaerd, Denmark. The biological potency of the preparation determined by bioassay using the mouse convulsion test was 22.4 (19.2 - 26.1) IU/mg. The figures in parentheses give the 95% confidence intervals.

Storage

Upon receipt of the preparation, the contents of the vial which consisted of 100 µg freezedried mouse insulin and 1 mg human albumin were dissolved in 1,000 µl of sterile double distilled deionised water to give a concentrated stock of 100 µg/ml containing 1 mg/ml human albumin. The solution was split into aliquots of approximately 120 µl and stored at -20°C in tightly capped polystyrene tubes. Every 2-4 months a working stock of 1 µg/ml was prepared from this in assay diluent. The resulting solution (10 ml final volume) was divided into aliquots of approximately 200 µl and similarly stored in the deep freeze.

Preparation of daily working standards

On the day of the assay daily working standards were prepared from the 1 µg/ml working stock. The top insulin standard was prepared on the addition of 100 µl of the working stock to 4.9 ml assay diluent. Thereafter doubling dilutions were made such that the final range of daily working standards was 20 ng/ml to 0.019 ng/ml mouse insulin. Strict ice temperature was observed throughout the procedure.

Validation of radioimmunoassay

The immunochemical identity of standards and unknowns was assessed from the ability of serial dilutions of pooled plasma samples containing unknown concentrations of endogenous mouse insulin to yield inhibition curves parallel to the standard response curve when both were plotted in terms of the logarithm of the insulin concentration. In addition, the validity of the method was tested by determination of the relationship between the measured insulin concentration and the plasma dilution factor. Accordingly, 100 µl aliquots of pooled plasma samples derived from five fasted obese hyperglycaemic mice or a corresponding number of fed lean animals were analysed in triplicate under routine assay conditions undiluted or diluted 1:2, 1:5 or 1:10 with charcoal treated insulin free mouse plasma. Standard incubation mixtures and control tubes contained insulin free plasma at a final concentration of 1:4.

3. RESULTS

Standard mouse insulin was stored in aliquots at -20°C as either a concentrated stock at 100 µg/ml or a working stock at 1 µg/ml. At regular intervals the working stock was prepared using screened assay diluent and this was successfully utilised in the preparation of the daily working standards. Freezing and thawing of the preparation was minimised by the technique, and throughout an 18 month period of constant usage, during which time five different batches of the working stock were employed, no evidence was found to suggest that the standard had deteriorated during storage.

The suitability of the reference preparation supplied by the Novo Research Institute as a valid standard in the present radioimmunoassay system was assessed for both the inhibition curves produced using multiple dilutions of mouse plasma and the ability of the observed insulin concentration in the unknown plasma to fall

linearly with dilution. As illustrated in figure 33, serial dilution of endogenous insulin present in the pooled plasma samples derived from obese hyperglycaemic mice or from lean mice gave inhibition curves which were parallel to the standard curve on log hormone concentration plots. A significant correlation was observed between the measured insulin concentration, expressed as a percentage of the concentration in the undiluted sample, and the plasma dilution factor of each pool (figure 34), thereby confirming the immunochemical identity of the reacting species. The plasma insulin concentration of the two pools, calculated from the mean of the individual estimates, was 3.60 ± 0.10 ng/ml for fasted obese hyperglycaemic mice and 1.07 ± 0.08 ng/ml for fed lean mice.

4. DISCUSSION

A standard preparation suitable for use in radioimmunoassay should contain a single molecular species, identical to the substance to be measured, which remains stable throughout storage and all subsequent manipulative procedures (Bangham & Cotes, 1971, 1974). The requirement for a homologous standard in the present studies stems from the structural dissimilarity between mouse insulin and the species of insulin readily available in pure form (figure 23). Thus employment of bovine, porcine or other closely related species of insulin as a standard in the radioimmunoassay of mouse insulin would be associated with a progressive underestimation of hormone concentration, which would vary according to both the specificity of the antiserum and the degree of parallelism between the two response curves (figures 25-29).

Freezedried insulin preparations remain stable for considerable periods of time when stored at reduced temperature (Storring et al., 1975). However, once the ampoule has been opened the responsibility for the stability of its contents is entirely in the hands of the user (Bangham &

Cotes, 1971). The stability of insulin in solution is dependent upon numerous factors including concentration, temperature, pH, protein content of the diluent and the duration of storage (Krogh & Hemmingsen, 1928; Berson & Yalow, 1966; Storvick & Henry, 1968; Pingel & Vølund, 1972). Despite the importance of the immunochemical integrity for the validation of radioimmunoassay, a single set of optimal storage conditions remains to be defined. The present régime based on the general recommendations of Bangham and Cotes (1974) involved storage of multiple aliquots of concentrated insulin diluted with an enzyme-free protein buffer at -20°C in the dark. Freezing and thawing was minimised and daily working standards prepared by serial dilution from five different lots of working stock, spanning a period of 18 months, showed no sign of deterioration, thereby confirming the stability of the standard preparation during storage.

The most important criterion for the validation of radioimmunoassay concerns the immunological identity of the standard and unknowns (Yalow & Berson, 1968; Ekins et al., 1968; Berson & Yalow, 1973). Even repeatedly recrystallised preparations of insulin are not homogeneous (Mirsky & Kawamura, 1966; Dillon & Romans, 1967), and contain variable amounts of glucagon, arginine insulins, deaminoinsulins, proinsulin and other contaminants (Staub et al., 1955; Craig et al., 1960; Mirsky & Kawamura, 1966; Steiner, 1968). Thus reference insulin preparations distributed for use as standards in both bioassay and radioimmunoassay have invariably contained small amounts of the precursor hormone (Lubetzki, 1969; Rolando & Torroba, 1972; Sönksen, 1976), thereby leading to non-parity of inter-laboratory assay results (Sönksen, 1971; Soeldner et al., 1974). Although the identity and homogeneity of standard mouse insulin supplied by the Novo Research Institute was not specified, the reference preparation was immunochemically identical to endogenous mouse insulin when analysed under routine assay conditions.

The ability of multiple dilutions of pooled plasma samples to yield inhibition curves parallel to the standard response curve, plus the fact that the observed insulin concentration of the unknown plasma fell linearly with dilution, not only justified the use of the standard preparation but also conferred a high degree of validity upon the individual components of the assay system (Yalow & Berson, 1960a; Albano & Ekins, 1970).

It is well established that laboratory rodents such as the rat and mouse are unique in possessing two types of insulin (Clark & Steiner, 1969; Markussen, 1971; Humbel et al., 1972), differing from each other at residues B9 and B29 (figure 23). The immunochemical identity of the standard and unknown in the assay system suggests that the antiserum employed failed to discriminate between the two types of mouse insulin. In addition, the similarity between the dilution curves obtained using plasma derived from obese hyperglycaemic mice and their lean counterparts indicates that the hyperinsulinaemia observed in the obese animals represents a true elevation of circulating insulin and is not associated with the presence of pathological insulin-like components.

RADIOIMMUNOASSAY

1. INTRODUCTION

The design and optimisation of a sensitive radioimmunoassay for insulin is dependent upon the binding characteristics of the antiserum and both the physico-chemical and immunological properties of the iodinated and native insulin antigens (Yalow & Berson, 1968, 1970b; Albano & Ekins, 1970; Albano et al., 1972). However, despite the fundamental role of the basic reagents in determining the main criteria upon which a reliable method is judged, the sensitivity, specificity and accuracy of radioimmunoassay are subject to modification during the stages of

immunoassay (Berson & Yalow, 1964; Yalow & Berson, 1969b; Albano et al., 1972). Factors inherent in the incubation medium or introduced in the unknown sample can adversely affect both the insulin-antibody reaction and the efficiency of the separation technique, giving rise to non-specific artifacts which drastically reduce the accuracy of the method (Morgan et al., 1964a; Heding, 1966 a, b , 1972; Henderson, 1970; Szabo & Mahler, 1970; Brodal, 1971a), and if not eliminated lead to disparate assay results (Adams et al., 1971; Cotes et al., 1969; Marschner et al., 1974).

Since the conception of a radioimmunological technique for the estimation of insulin (Berson & Yalow, 1957) and its description some two years later (Yalow & Berson, 1959), a large number of improvements and other less desirable modifications have been introduced to facilitate the routine application of the method in both clinical and investigative endocrinology (Albano & Ekins, 1970; Buchanan & McCarroll, 1971). By far the majority of variations relate to the critical stage of separation which was originally performed by chromatoelectrophoresis (Yalow & Berson, 1960a). Thus free and antibody bound insulin have been separated by a wide range of physicochemical and immunological techniques including wick chromatography (Ørskov, 1967), gel filtration (Genuth et al., 1965), solid phase coupling (Miles & Hales, 1968b; Colt et al., 1971; Velasco et al., 1973; Beck & Hales, 1975; Davis et al., 1976), selective adsorption (Meade & Klitgaard, 1962; Herbert et al., 1965; Rosselin et al., 1966; Zaharko & Beck, 1968; Coffey et al., 1974) and both chemical (Grodsky & Forsham, 1960; Heding, 1966b; Mitchell & Byron, 1967; Desbuquois & Aurbach, 1971) and immunochemical precipitation (Hales & Randle, 1963 a, b; Morgan & Lazarow, 1963; Quabbe, 1969). Although each of these techniques carries potential advantages and disadvantages in terms of cost and convenience (Logsdon & Green, 1971; Raptis, 1972; Kagan, 1975; Antoniades, 1976), the most important

property in determining the potential usage of any separation procedure is its ability to effect a rapid and efficient separation without interference from non-specific factors introduced in the unknown sample (Albano et al., 1972; Malvano et al., 1974a; Starr & Rubenstein, 1974). Accordingly, popular methods employed in the radioimmunoassay of insulin including double antibody (Hales & Randle, 1963a; Morgan & Lazarow, 1963), ethanol precipitation (Heding, 1966b; Wright et al., 1968b) and charcoal adsorption (Herbert et al., 1965) offer a good combination of practicability and reliability for the routine determination of insulin concentration. However, as has been illustrated on numerous occasions (Morgan et al., 1964 a, b; Buchanan & McCarroll, 1971; Wright et al., 1971; Raptis, 1972), separation techniques differ in their susceptibility to non-specific interference and although each method undoubtedly constitutes a valid procedure under optimal working conditions (Hales & Randle, 1963 a, b; Albano et al., 1972; Heding, 1972; Wright, 1976), only the charcoal separation technique enables these non-specific effects to be monitored and accordingly is particularly useful as a routine method for separation in the radioimmunoassay of insulin (Albano & Ekins, 1970; Buchanan & McCarroll, 1971; Palmieri et al., 1971; Albano et al., 1972; Malvano et al., 1974a).

The purpose of the present section is to describe the application of the precepts outlined in the previous chapter to the development of a sensitive and precise radioimmunoassay for insulin relying on horse serum and dextran coated charcoal for the separation of free and antibody bound hormone moieties. The method which was optimised using the traditional approach to radioimmunoassay design developed by Yalow and Berson (Berson & Yalow, 1964; Yalow & Berson, 1968, 1969a, 1970b) permitted the accurate determination of fasting insulin concentration in 10 µl peripheral plasma. The following account affords a detailed description of the routine radioimmunoassay technique together with some observations concerning its methodological validation.

2. MATERIALS AND METHODS

Assay diluent

A sodium phosphate buffer (0.04M, pH 7.4) with bovine serum albumin (0.5%), NaCl (0.3%) and sodium ethyl mercurithiosalicylate (0.02%) was used as a routine assay diluent. The buffer was prepared by dissolving 23 g Na_2HPO_4; 5.975 g $NaH_2PO_4 . 2H_2O$; 15 g NaCl; and 1 g sodium ethyl mercurithiosalicylate in 5 litres of double distilled water and stored in bulk for 3-4 months at 4°C. Bovine serum albumin (fraction V, lot 259, code 81-003-3, Miles Laboratories Ltd., Stoke Poges) was added to the buffer immediately prior to use.

Antiserum

Guinea pig antiporcine insulin antiserum (GPB1/3) was stored at -20°C in 120 µl aliquots at a dilution of 1:100. On the day of assay, a 100 µl aliquot was diluted with an appropriate volume of assay diluent to give the working 50% maximum binding titre (approximately 1:50,000 depending on the quality of the tracer). A commercially available antiserum (Wellcome Reagents Ltd., Beckenham, Kent) also gave excellent results in the present assay system. The preparation was stored as supplied at 4°C or frozen in 2 ml aliquots at a dilution of 1:4000.

Labelled insulin

^{125}I bovine insulin prepared by modification of the chloramine-T method (Hunter & Greenwood, 1962) was stored at -20°C diluted in acid ethanol. Aliquots were diluted to the required concentration with assay diluent immediately prior to use. For routine purposes a solution containing 1 ng/ml corresponding to a tracer dose of 100 pg ^{125}I-insulin was employed. However, depending on the target antigen concentration solutions ranging from 2.5 ng/ml to 0.4 ng/ml were also utilised. Radioiodinated insulin prepared by the iodine monochloride technique (Glover et al., 1967) was also employed with considerable success in

the present studies. The tracer (IM38, Radiochemical Centre, Amersham) supplied at a concentration of 20 ng/ml with a specific activity of approximately 50 μCi/μg was stored at 4°C until required. Both preparations gave consistently good results in radioimmunoassay and in a comparative study, antibody dilution curves and dose response curves prepared with these reagents were practically superimposable.

Standard

Standard mouse insulin (lot no. M20169, Novo Research Institute, Bagsvaerd, Denmark) was stored in aliquots at -20°C as either a concentrated stock at 100 μg/ml or a working stock at 1 μg/ml. At 2-4 monthly intervals the working stock was prepared using assay diluent and this was utilised in the preparation of the daily working standards. On the day of the assay, working standards were prepared by serial dilution such that the final concentration range was 20 ng/ml to 0.019 ng/ml mouse insulin.

Preparation of plasma samples for immunoassay

Blood was collected in small polythene tubes (250 μl capacity) that had been prewashed with a solution of heparin (500 U/ml) and dried in air. The samples were briefly centrifuged using a Microfuge (Beckman Ltd., Eastfield Industrial Estate, Fife) and 10 μl aliquots of unhaemolysed plasma were stored in tightly capped immunoassay tubes at -20°C.

Insulin free plasma

In order to minimise the effect of non-specific factors introduced with the unknown plasma samples, an equivalent volume of insulin free plasma was added to the standard incubation mixtures in the routine plasma insulin assay. Insulin free plasma was prepared by mixing pooled fasting rat plasma with charcoal (50 ml : 5 g Sigma activated charcoal) for six hours at room temperature, followed by ultracentrifugation for

40 minutes at 4°C using an MSE Superspeed 50 refrigerated centrifuge (Measuring and Scientific Equipment Ltd., London) at 30,000 g to remove the bulk of the charcoal. The plasma was decanted and filtered through progressively smaller bacterial filters (smallest pore size 0.1 μ, Millipore (U.K.) Ltd., London) until completely free of charcoal. The charcoal treated plasma was stored in 500 μl aliquots at -20°C.

Charcoal suspension

Dextran coated charcoal was stored as a 5% stock suspension of activated charcoal (lot no. 93C-0360, Sigma Chemical Company Ltd., London) coated 1:10 with dextran T70 (Pharmacia Great Britain Ltd., London) in sodium phosphate buffer (0.04M, pH 7.4) at 4°C. The suspension was diluted to a final concentration of 1% approximately thirty minutes prior to separation using the same buffer supplemented with 12.5% (v/v) sterile horse serum (No. 5, Wellcome Reagents Ltd., Beckenham, Kent).

Immunoassay procedure

Radioimmunoassay was performed in 63.5 x 9.5 mm polystyrene tubes fitted with polythene stoppers (Luckhams Ltd., Burgess Hill, Sussex). All additions were made using Gilson variable micropipettes equipped with interchangeable plastic tips (Anachem Ltd., Luton, Bedfordshire). The reactants were mixed without foaming using a variable speed rota-mixer (Hook and Tucker Ltd., London). Incubations and all subsequent manipulations were performed in a cold room at approximately 4°C.

The stages involved in the routine radioimmunoassay of plasma insulin are shown in table 1. Each assay consisted of triplicate sets of mouse insulin standards (20 ng/ml to 0.019 ng/ml), four zeros, four non-specifics and four total count tubes, followed by up to 531 unknowns. Non-specific tubes included with the standards contained assay diluent in place of antiserum to enable a correction to be made for non-specific binding and incubation damage. On occasion, non-specific tubes were also

included for each unknown plasma sample. However, this step was later considered to be unnecessary since little variation was found between the non-specific effects produced by different plasma samples.

The initial reactants were mixed and incubated for 24 hours at 4°C prior to the addition of the tracer hormone. Following the first incubation, ^{125}I-labelled bovine insulin was introduced, the tubes were mixed and the reaction was allowed to proceed for a further 48 hours at 4°C. Quadruplicate aliquots of iodinated insulin were also added to empty tubes, and retained at 4°C without further additions. These tubes were used to determine the 'total count' of tracer hormone added.

After the second incubation period, free and antibody bound insulin were separated by the addition of 1000 µl of the horse serum and dextran coated charcoal suspension. The contents were allowed to stand for 30 minutes in the cold room before centrifugation for 20 minutes at 4°C using an MSE Mistral 4L refrigerated centrifuge (Measuring and Scientific Equipment Ltd., London) at 1,500 g. The supernatant containing the bound hormone moiety was removed by decantation and the final drop of fluid remaining on the lip of the tube was aspirated with a pasteur pipette connected to a water vacuum pump.

The radioactivity associated with the charcoal pellet was counted simultaneously in two channels for a fixed period of time using a well-type crystal scintillation counter ICN Tracer Lab Gamma Set 500 (ICN Pharmaceuticals, Walton-on-Thames, London), connected directly to a teletype machine (model 33 ASR, Teletype Corporation, Illinois, USA) with a paper tape perforator (type ASCII). The counting time employed varied according to the specific activity of the tracer and the initial level of binding in the absence of mouse insulin. However, in all instances, a minimum of 10,000 counts were accumulated for each tube.

In order to facilitate the processing of a large number of samples, an automated and computerised method was employed for the routine analysis of radioimmunoassay data. The count rate of individual tubes was recorded directly onto standard 8 track punched paper tape. This was transferred to an ICL 1904 S computer and processed according to the programme described in Chapter 4. In brief, the method used logit and log transformation to obtain a linear response curve, followed by unweighted and iterated weighted least squares regression analysis. The standard curve was plotted in five different co-ordinate systems by computer and the potency estimates, corrected for dilution, were obtained for the unknown samples. The method converged rapidly and only two or three iterations were necessary.

Alternative separation techniques

In order to validate the use of charcoal separation in the present assay system, a comparative study was undertaken to assess the degree of disruption caused by each of the most commonly employed separation techniques (see Landon, 1971a). Radioimmunoassays were performed simultaneously under optimal incubation conditions using identical reagents. Separation was performed as described above, or by modification of the classical methods developed by Heding (1966b) or by Hales and Randle (1963a).

Ethanol: the incubation mixture and procedure was exactly the same as for the charcoal method. Free and antibody bound hormone moieties were separated by the addition of 1.6 ml ice cold ethanol. After mixing, centrifugation (1,500 g) and one wash with 80% ethanol, antibody bound radioactivity was counted in the precipitate.

Post-precipitated double antibody: the incubation mixture consisted of 100 µl standard, 100 µl diluent supplemented with EDTA (0.04M) and normal rabbit serum (1:200, v/v), 100 µl insulin antiserum and 100 µl iodinated insulin. After the second incubation period, 100 µl of suitably diluted rabbit anti-guinea pig globulin antiserum (Wellcome Reagents Ltd., Beckenham, Kent) containing EDTA (0.04M) was added. The reaction was allowed to proceed for 24 hours at 4°C prior to separation by centrifugation (3,000 g). The supernatant was decanted and the antibody precipitate washed with assay diluent. Following an additional sequence of centrifugation and decantation, the radioactivity associated with the bound hormone moiety was determined.

Pre-precipitated double antibody: insulin binding reagent was prepared in bulk by incubating equal volumes of half optimal dilutions of insulin antiserum and rabbit anti-guinea pig globulin antiserum (Wellcome Reagents Ltd., Beckenham, Kent) in assay diluent supplemented with EDTA (0.04M). Following 24 hours at 4°C, 100 µl aliquots of the pre-precipitated antiserum were removed for use in radioimmunoassay. Upon termination of the second incubation period, the radioactivity associated with the immune complex was sedimented by centrifugation (3,000 g), washed with assay diluent and counted.

3. METHODOLOGICAL VALIDATION

Assay diluent

The selection of an appropriate assay diluent constitutes an important factor in the successful development of radioimmunoassay (Heding, 1966a). A particular problem associated with small peptides such as insulin is their tendency to adsorb non-specifically to glass and other inert surfaces when present in dilute solution (Ferrebee et al., 1951; Newerly & Berson, 1957; Hill, 1959). This potential source of error and variation in radioimmunoassay can be minimised by the

inclusion of exogenous protein in the buffer, however care must be taken to avoid the introduction of proteolytic enzymes, insulin-like components or other interfering substances which may be present in certain albumin preparations (Sönksen et al., 1965; Heding, 1972). As illustrated in figure 35, assay buffer supplemented with a range of commercial albumin preparations (0.5%, w/v) yielded essentially superimposable antibody dilution curves in the present assay system, thereby confirming the absence of interfering substances. Pentex bovine serum albumin was selected for routine use in the insulin radioimmunoassay. Assay diluent prepared using this protein was particularly easy to pipette, it reduced glassware adsorption to approximately two percent and was completely free from cross-reacting substances when analysed against charcoal treated assay diluent.

Insulin-free plasma as a constituent of the standard incubation mixture

Small differences in the composition of incubation mixtures can adversely effect both the insulin-antibody reaction and the efficiency of the separation technique giving rise to non-specific artefacts which drastically reduce the accuracy of the method (figure 36). To minimise the effect of non-specific factors introduced in the unknown plasma samples, an equivalent volume of insulin free plasma was added to the standard incubation mixtures in the routine plasma insulin assay, thereby assuring 100% recovery of endogenous insulin (figure 34). Failure to equalise the protein content of all incubation mixtures was invariably associated with low, and in certain instances negative, estimates for unknown plasma insulin concentration. The present study therefore confirms the original observations of Albano and Ekins (1970) that the addition of charcoal treated plasma to the standard tubes enables a correction to be made for non-specific binding and incubation damage, thereby yielding accurate assay results.

Incubation conditions

The temperature and time of incubation can be adjusted to suit the requirements of a particular assay. However the effect of these variables on the sensitivity of the assay is predominantly determined by the affinity constant of the antiserum and the relative contribution of the enthalpy and entropy components to the standard free energy change (Berson & Yalow, 1959d; Keane et al., 1976). The rate of the reaction between insulin and antibody was markedly influenced by temperature (figure 6). However, since the affinity constant of this antiserum also displayed temperature dependence, both the sensitivity potential of the antiserum and maximal precision of the assay could only be exploited at reduced temperature. Indeed the reduced rate of dissociation of the immune complex at $4^{\circ}C$ permitted a considerable enhancement of assay sensitivity following delayed addition of the labelled species (figure 7).

Concentration of reactants

The concentration of reactants employed in radioimmunoassay is predominantly determined by the required assay sensitivity and the target antigen concentration (Ekins, 1974a). Thus variation in the amount of iodinated insulin employed for incubation has a marked effect on sensitivity and the effective working range of the assay (figure 11). The theoretical principles governing the attainment of highest assay sensitivity and precision as defined by Yalow and Berson (1968, 1969a) and by Ekins and colleagues (1968, 1969) has been fully discussed in the preceeding chapter.

Separation procedure

Several factors affect the suitability of charcoal for the separation of free and antibody bound insulin in radioimmunoassay (Albano & Ekins, 1970; Albano et al., 1972). One of the most critical factors concerns the selection of an optimal concentration of charcoal which adsorbs the

free insulin but leaves the antibody bound hormone moiety in solution (Buchanan & McCarroll, 1971). The optimal amount of charcoal used for separation must be determined empirically since the efficiency of different charcoals to effect complete separation varies not only with respect to their adsorptive properties but also according to the coating applied (Albano et al., 1972; Cresto et al., 1972; Frayn, 1976). In the present assay system 10 mg horse serum and dextran coated charcoal enabled complete separation of free and antibody bound insulin, regardless of hormone concentration up to 100 ng/ml insulin. As illustrated in figure 37, approximately 50% free insulin was immediately adsorbed onto charcoal, however the adsorption process did not plateau for a further 30 minutes.

Considerable controversy has arisen concerning the selection of an appropriate coat for the charcoal particle (Ekins, 1969a; Herbert, 1969a; Palmieri et al., 1971; Herbert & Bleicher, 1976). As illustrated in figure 38, untreated charcoal or charcoal applied with a variety of coats can effect the efficient separation of free and antibody bound insulin under optimal conditions in the absence of added plasma. However, as illustrated in figure 39, separation based on horse serum and dextran coated charcoal was particularly advantageous since its use essentially eliminated non-specific effects on charcoal adsorption, minimised the experimental error in the determination of the response metameter, and thereby removed the requirement for non-specific tubes of individual plasma samples. The addition of horse serum also displaced reactants adsorbed to glassware, led to the production of a firm charcoal pellet and accordingly resulted in the efficient separation of the bound hormone moiety.

The charcoal separation technique yielded identical dose response curves to those prepared by alternative methods relying on either ethanol or post-precipitation with double antibody for the separation of free and antibody bound insulin (figure 19). In accordance with previous

observations (Albano & Ekins, 1970; Malvano et al., 1974) pre-precipitation was associated with a modification of binding characteristics.

4. RESULTS

A sensitive and precise radioimmunoassay for insulin relying on horse serum and dextran coated charcoal for the separation of free and antibody bound hormone moieties has been developed. The technique which was routinely employed in conjunction with a fully automated and computerised radioimmunoassay data processing system permitted the accurate determination of fasting insulin concentration in 10 µl peripheral plasma.

Assay sensitivity

Although maximal assay sensitivity is a function of the affinity constant of the antiserum and the quality of the tracer hormone employed (Ekins et al., 1968; Yalow & Berson, 1968), it is possible, by manipulation of assay variables to enable the precise estimation over a wide range of hormone concentrations (Ekins et al., 1970; Ekins, 1974a). In the present study, the sensitivity and working range of the assay were adjusted such that the precision in the measurement of a set of unknown samples with a defined target concentration was maximal. Such a practice enabled the estimation of insulin in both plasma and pancreatic extracts and was usually achieved by alteration of the incubation times (figure 7). However in those instances where high sensitivity of the plasma insulin assay was required, the delayed addition of a reduced dose of tracer hormone was most successful (figure 11).

Assay sensitivity has been defined by Ekins and colleagues (Ekins et al., 1968; Ekins & Newman, 1970) as the standard deviation of the response metameter at zero hormone concentration divided by the slope of the response curve at this point (figure 9). In a typical routine plasma insulin assay, performed under optimal incubation conditions,

the coefficient of variation in the measurement of the response metameter at zero concentration (including the statistical error of counting) was 1.9%. The slope of the response curve at this point was approximately 1.2% per pg/ml incubation mixture. These values yield a formal detection limit of 1.6 pg/ml assuming a single determination of each assay point, and 0.9 pg/ml when each point represents the mean response of triplicate incubation tubes. The sensitivity attained in buffer was slightly greater than that observed in the presence of plasma, and the formal sensitivity of the response curve prepared in the absence of insulin free plasma was increased from 1.6 pg/ml to 1.3 pg/ml of incubation mixture.

Maximal sensitivity achieved in the present assay system was 0.5 pg/ml insulin in the final incubation mixture, corresponding to a 10% fall in bound counts on the addition of 9.2 pg cold insulin. The effective affinity constant of the predominating antibody binding sites was 8.1×10^{13} litres/mole resulting in a standard free energy change of -82.97 KJ/mole.

Assay precision

Assay precision is related to the error in the measurement of the response metameter and the slope of the response curve (Ekins & Newman, 1970; Ekins, 1974a). Accordingly, the precision of measurement of any target insulin concentration is dependent upon the statistical sum of the experimental errors and its position on the response curve relative to the working range or midpoint of the assay. In a typical routine plasma insulin assay, the coefficient of variation was less than 3% for concentrations up to 1.25 ng/ml, and extended from 2.3 to 9.6% over the entire range of concentrations studied. The slope of the response curve at the individual points was approximately 0.9% per pg/ml incubation mixture in the most sensitive region of the response curve, and was 0.04% per pg/ml at the end of the concentration scale.

This assay was able to discriminate changes of approximately 6 pg/ml at concentrations less than 1.25 ng/ml, differences of 100 pg/ml at concentrations between 1.25 and 5 ng/ml, and alterations in the region of 300 pg/ml in the higher concentration range extending to 20 ng/ml insulin.

Formal studies of assay precision were performed both within and between assays using four plasma samples containing between 0.1 and 24.5 ng/ml insulin. Analysis within single assay runs gave an inter-assay coefficient of variation between 1.3 and 4.2%. Between assay precision was slightly inferior to that observed within a single assay, and the intra-assay coefficient of variation for duplicate determinations ranged from 1.4 to 7.6% for concentrations less than 5 ng/ml, and was 12.4% for the highest insulin concentration. Charcoal treated plasma yielded insulin values indistinguishable from zero hormone concentration.

Assay specificity

The most important criterion for the validation of radioimmunoassay concerns the immunological identity of the standard and unknowns (Ekins et al., 1968; Berson & Yalow, 1973). The ability of multiple dilutions of pooled plasma to yield inhibition curves parallel to the standard response curve (figure 33), plus the fact that the observed insulin concentration of the unknown plasma fell linearly with dilution (figure 34), conferred a high degree of validity upon the radioimmunoassay system (Yalow, 1976).

Standard curve

An important feature of the present radioimmunoassay is the application of a fully automated and computerised data processing system to the routine handling of assay results. The programme was written in ALGOL 60 according to the precepts defined by Rodbard and colleagues (Rodbard & Lewald, 1970; Rodbard, 1971, 1974; Rodbard &

Frazier, 1975). The method which employed logit and log transformation followed by unweighted and iterated weighted least squares regression analysis provided excellent linearisation, thereby facilitating dose interpolation over the entire concentration range. As illustrated in table 2 which summarises the essential output data from a number of consecutive insulin assays, the method converged rapidly. Following five iterations a high degree of linear fit was observed.

A simplified flow chart illustrating the essential features of the programme is shown in figure 56. In addition to providing potency estimates for the unknown samples and full details concerning the functions involved in the calculation of the response metameter together with statistical analysis of goodness of fit, the programme afforded a thorough and complete analysis of each assay in terms of its most fundamental properties. Scratchard analysis was performed to estimate the effective affinity constant, the binding capacity of the various classes of antibody combining sites present and the standard free energy change for the reaction. An optimisation routine similar to that of Ekins and colleagues (1968, 1970) permitted the prediction of the concentration of labelled hormone and antibody to provide optimal sensitivity. Data from a typical computer output for the routine radioimmunoassay of plasma insulin is presented in figure 40.

5. DISCUSSION

Although many separation techniques have been applied to the radioimmunoassay of insulin, much evidence has been accumulated to suggest that failure to control non-specific interference during the penultimate stage of radioimmunoassay represents a common source of error which invariably leads to the production of disparate assay results (Cotes et al., 1969; Marschner et al., 1974; Costantini et al., 1975). Methods currently employed for the separation of free and

antibody bound insulin vary in their susceptibility to non-specific interference (Buchanan & McCarroll, 1971; Silbert & Sawin, 1975). However in accordance with the observarions of Albano and colleagues (Albano & Ekins, 1970; Albano et al., 1972), the present study illustrates the potential of coated charcoal to enable the reproducible separation of the two hormone moieties under carefully defined incubation conditions. The charcoal separation system itself is relatively sensitive to changes in protein concentration in the incubation mixture (Keane et al., 1968; Cresto et al., 1972; Frayn, 1976). However, problems stemming from this source have been successfully eliminated in the present method by the application of a horse serum and dextran coat to the charcoal particles and by the addition of hormone free plasma to the standard incubation mixtures.

The attainment of high sensitivity in radioimmunoassay offers considerable advantages in terms of both the detection limit and precision of the method (Ekins & Newman, 1970; Ekins, 1974b). The present assay system which has a formal detection limit of approximately 1.0 pg/ml enabled the routine estimation of insulin concentration in highly diluted plasma samples, thereby reducing the concentration of non-specific factors likely to interfere with either the insulin antibody reaction or the efficiency of the separation technique. Indeed the only factor found to be adversely affect assay performance was associated with badly haemolysed plasma samples which invariably led to spuriously low estimates of insulin concentration. However, since this form of interference has been reported to occur in a variety of assay systems relying on different separation techniques (Brodal, 1971a; Albano et al., 1972; Cantrell et al., 1972), it is likely that the haemolysate exerts its effect indirectly by enzymatic degradation of the insulin molecule itself (Brodal, 1971b).

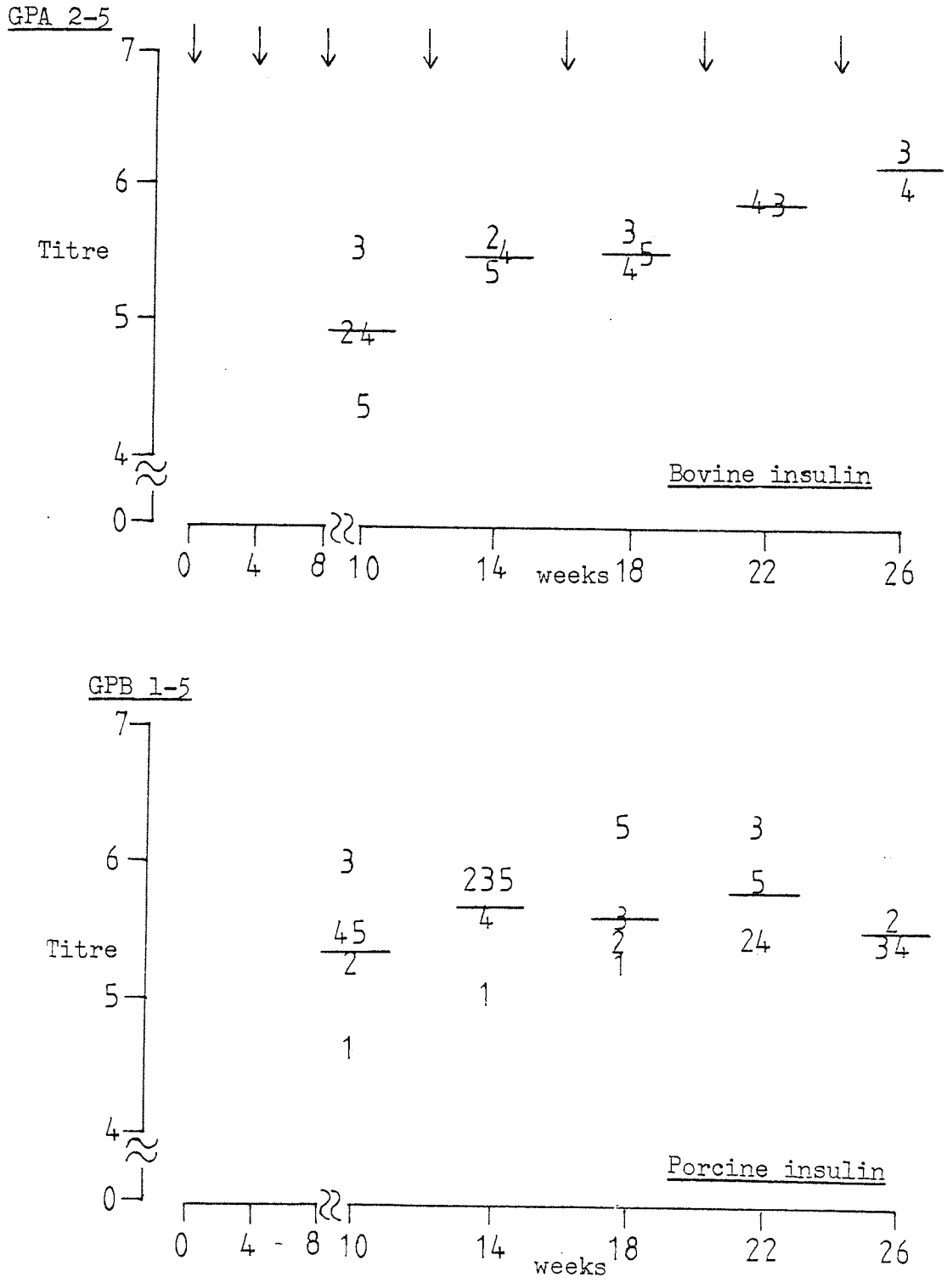
A further feature of the present radioimmunoassay system is the application of a fully automated and computerised data processing system to the routine handling of assay results. Although numerous methods are available for routine data analysis (Rodbard, 1974), the logit function introduced by Berkson (1944) and applied to radioimmunoassay by Rodbard and colleagues (1968) has found widespread use in the routine data processing of numerous competitive protein binding assays (Midgley et al., 1969a; Rodbard & Lewald, 1970; Rodbard & Frazier, 1972; Rodbard & Hutt, 1972). The present study describes the successful application of the technique to the radioimmunoassay of insulin, and serves to illustrate the potential advantages of computerised methods over more conventional approaches employed to obtain assay results. Graphical methods alone or linear interpolation between adjacent points on the dose response curve do not provide efficient utilisation of data, do not afford estimates of precision, are subject to erratic behaviour and subjective biases, forfeit important information about the assay system and accordingly contribute to the inaccurate estimation of insulin concentration observed in collaborative studies (Cotes et al., 1969; Marschner et al., 1974; Costantini et al., 1975).

Figure 23 The amino acid sequences of mouse, human, bovine, porcine and guinea pig insulins.

A CHAIN

RESIDUE	MOUSE (1 & 2)	HUMAN	PORCINE	BOVINE	GUINEA PIG
1	Gly				
2	Ile				
3	Val				
4	Asp	Glu	Glu	Glu	
5	Gln				
6	Cys				
7	Cys				
8	Thr			Ala	
9	Ser				Gly
10	Ile			Val	Thr
11	Cys				
12	Ser				Thr
13	Leu				Arg
14	Tyr				His
15	Gln				
16	Leu				
17	Glu				
18	Asn				Ser
19	Tyr				
20	Cys				
21	Asn				

Figure 24 Development of titre during immunisation of guinea pigs with crystalline bovine (GPA 2-5) and porcine (GPB 1-5) insulins$^{\phi}$.



$^{\phi}$Titre is expressed as the reciprocal of the final working dilution of the serum that will bind 50% of the labelled antigen.
Arrows indicate the time of injections.

Figure 25 Binding characteristics of insulin antisera derived from a single guinea pig (GPA2) during the course of immunisation with crystalline bovine insulin. Assessment with bovine (×) and mouse insulins (•)

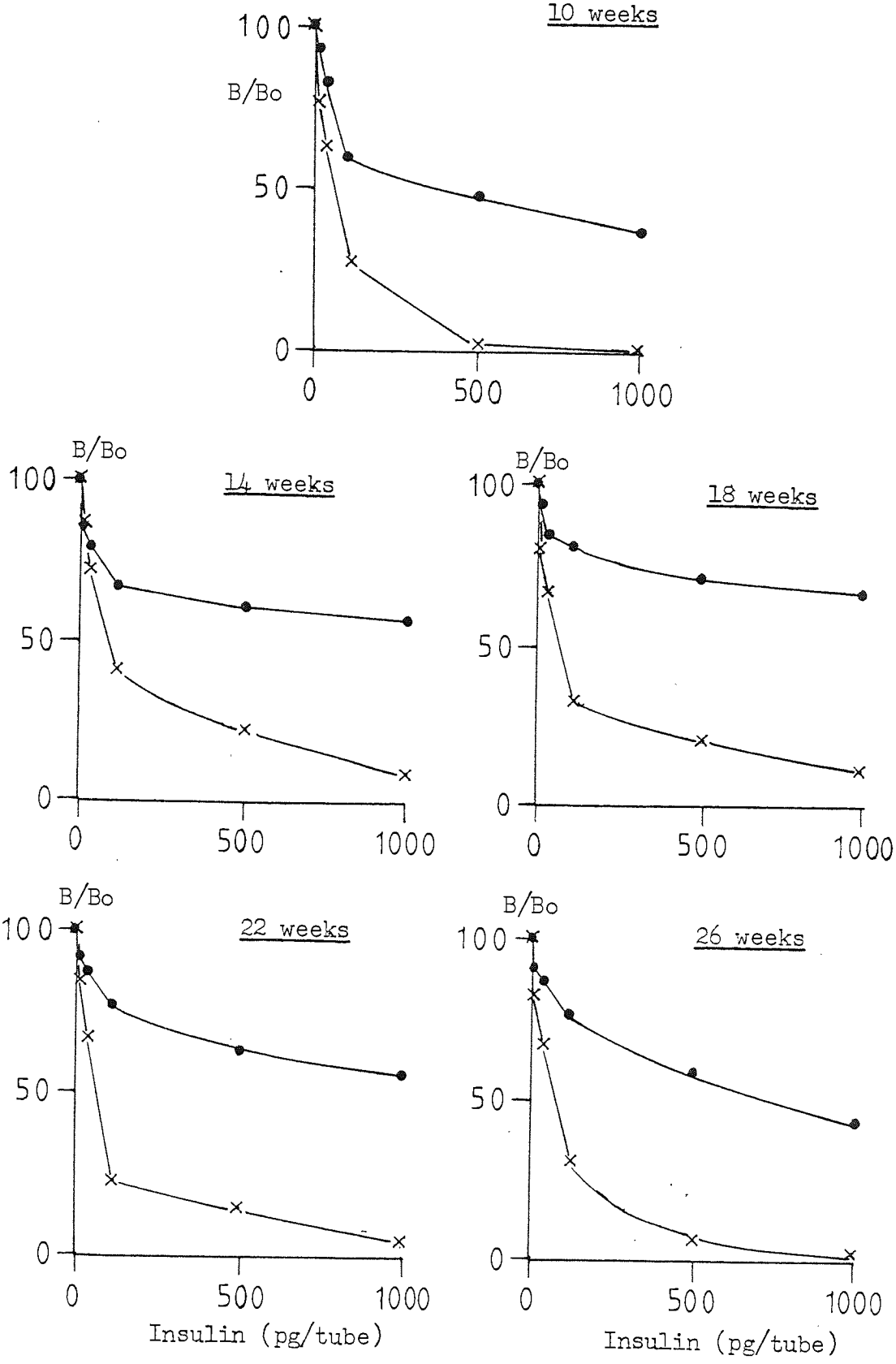


Figure 26 Binding characteristics of insulin antisera derived from a single guinea pig (GPB4) during the course of immunisation with crystalline porcine insulin. Assessment with porcine (×) and mouse insulins (•).

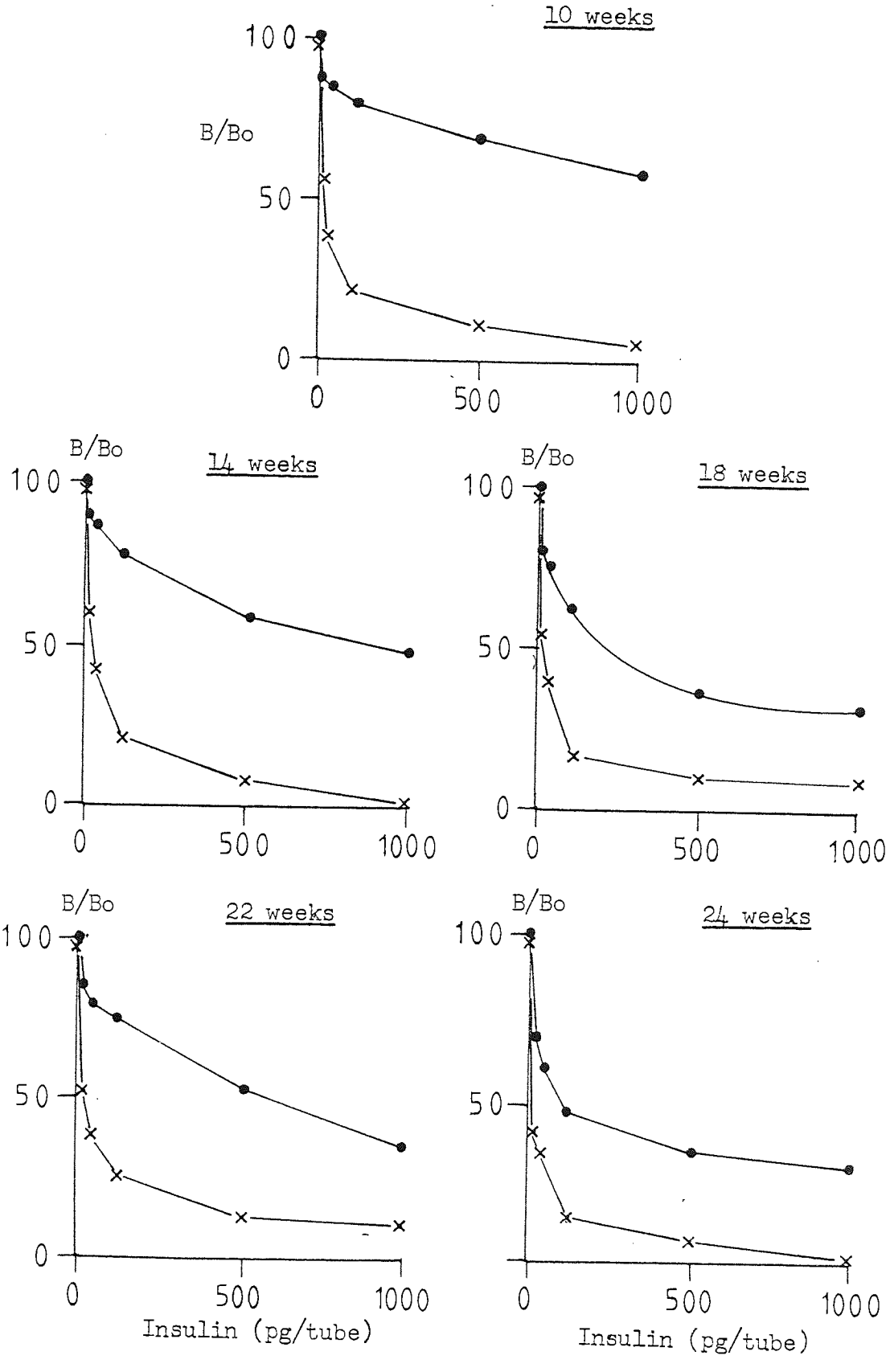
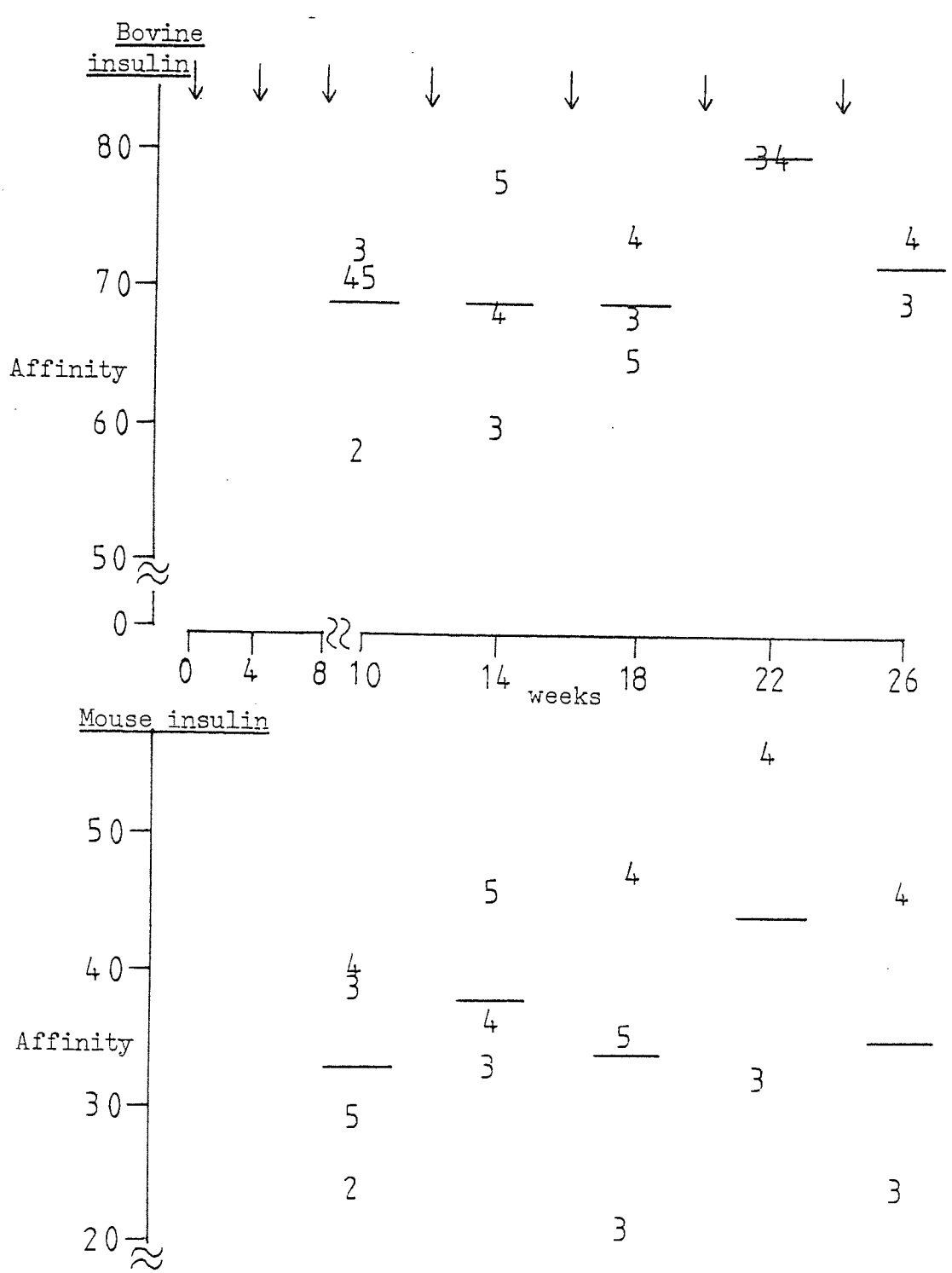
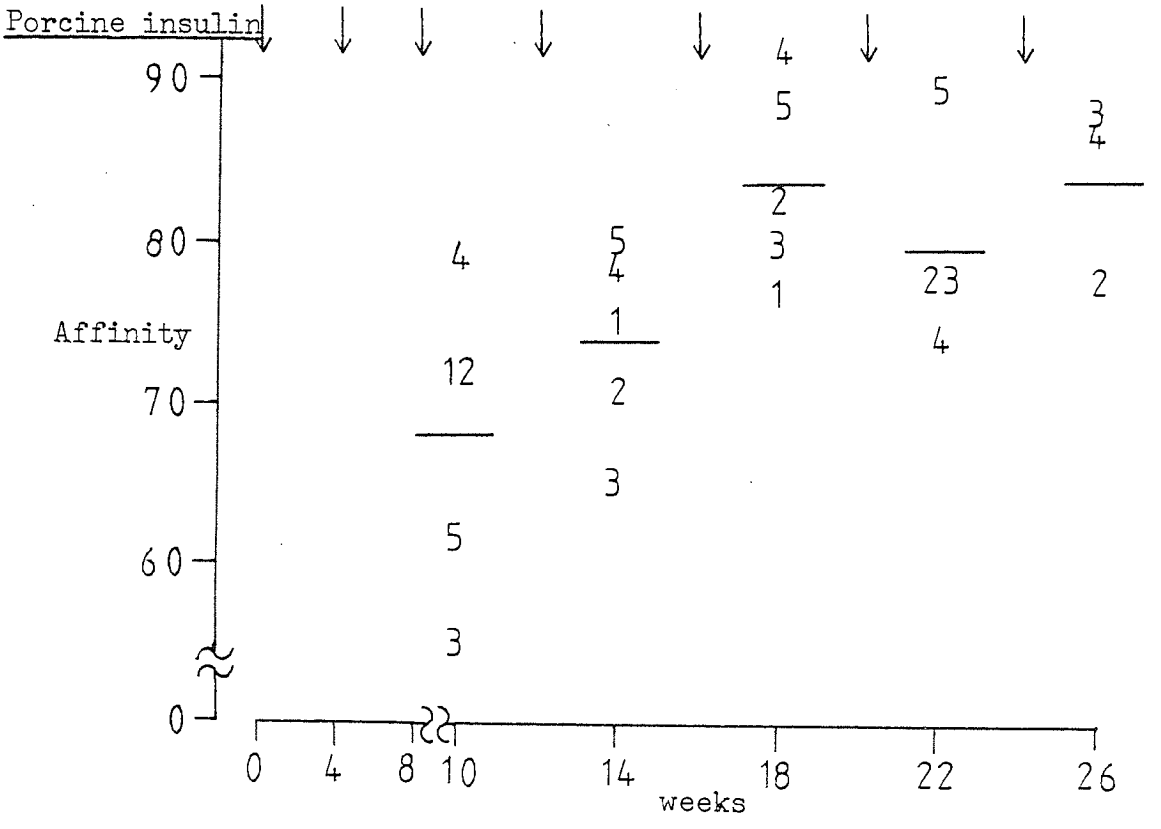


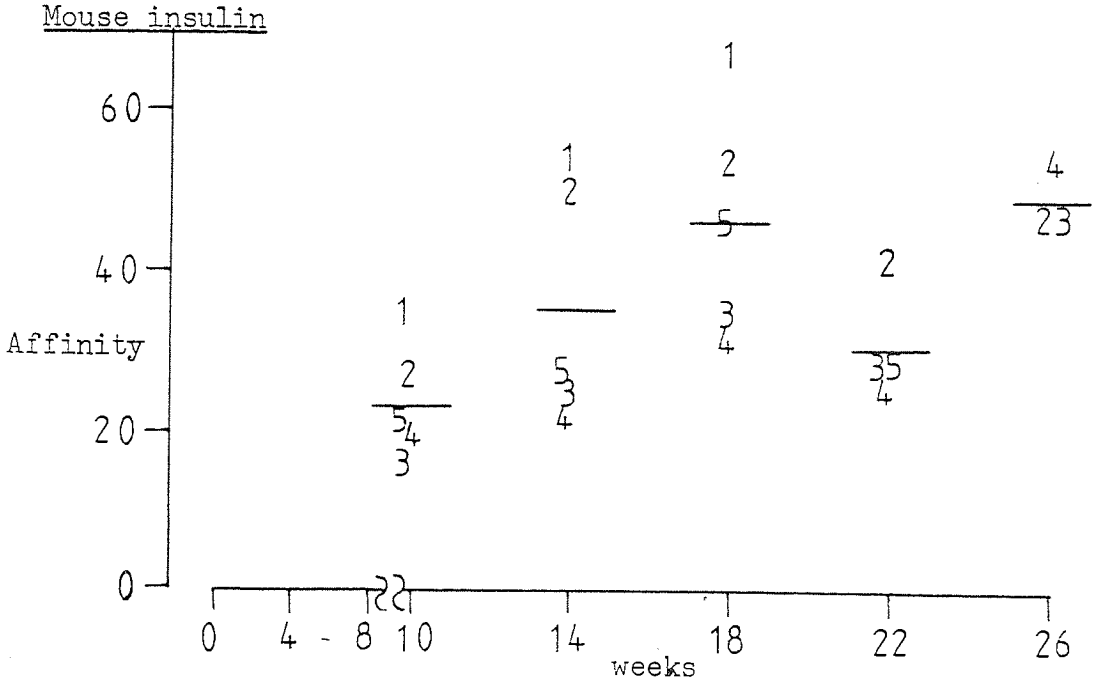
Figure 27 Development of affinity during immunisation of guinea pigs (GPA 2-5) with crystalline bovine insulin: Assessment with bovine and mouse insulins[ø].



[ø]Affinity is expressed as the % fall in bound counts caused by 125 pg standard insulin: Sensitivity index (displacement) = $(Bo - B_{125})/Bo \times 100$. Arrows indicate the time of injections.

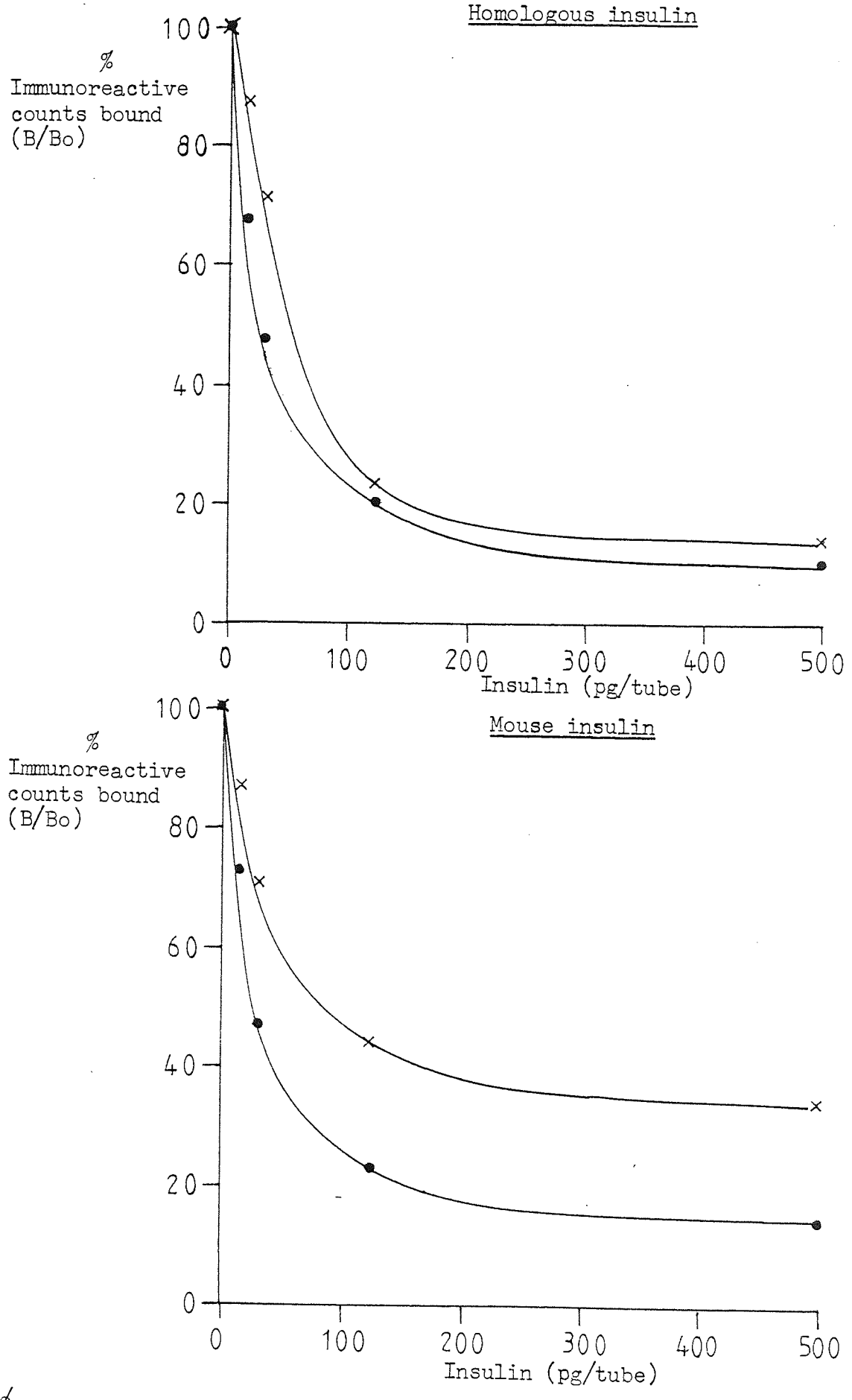
Figure 28 Development of affinity during immunisation of guinea pigs (GPB 1-5) with crystalline porcine insulin: Assessment with porcine and mouse insulins[ø].





[ø]Affinity is expressed as the % fall in bound counts caused by 125 pg standard insulin: Sensitivity index (displacement) = $(Bo - B_{125})/Bo \times 100$. Arrows indicate the time of injections.

Figure 29 Reaction of guinea pig insulin antisera (GPA3/4, ×——× and GPB1/3, ●——●), with homologous insulin and mouse insulin$^{\phi}$.



$^{\phi}$The immunogen (homologous antigen) for GPA3/4 and GPB1/3 was bovine insulin and porcine insulin respectively.

Figure 30 Purification of a standard insulin iodination mixture by gel filtration on a 45 x 1 cm column of Sephadex G50-fine. One ml fractions were collected at a flow rate of 45 ml/hour.

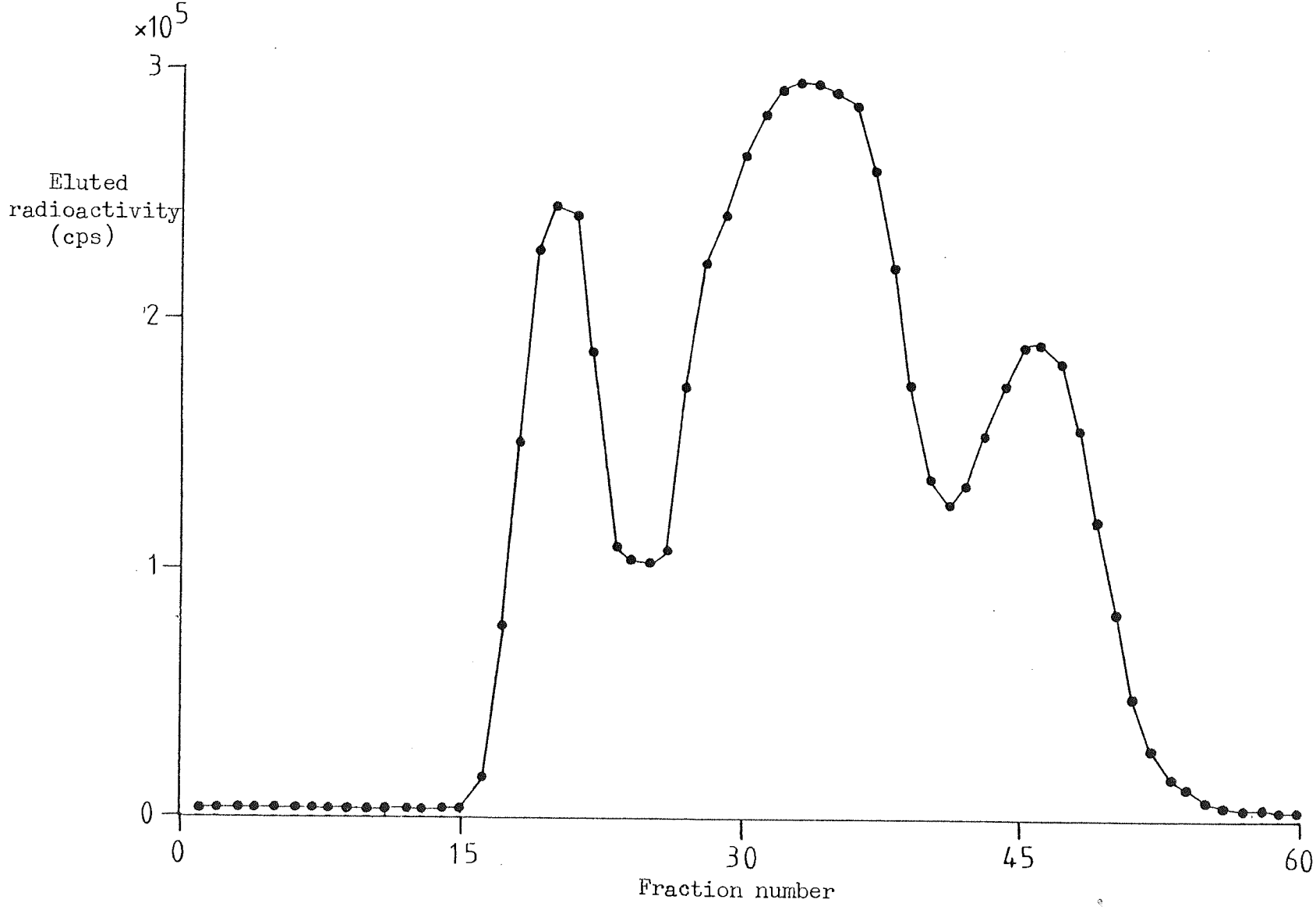


Figure 31 Physicochemical and immunochemical properties of radioactivity eluted during the purification of ^{125}I-insulin.

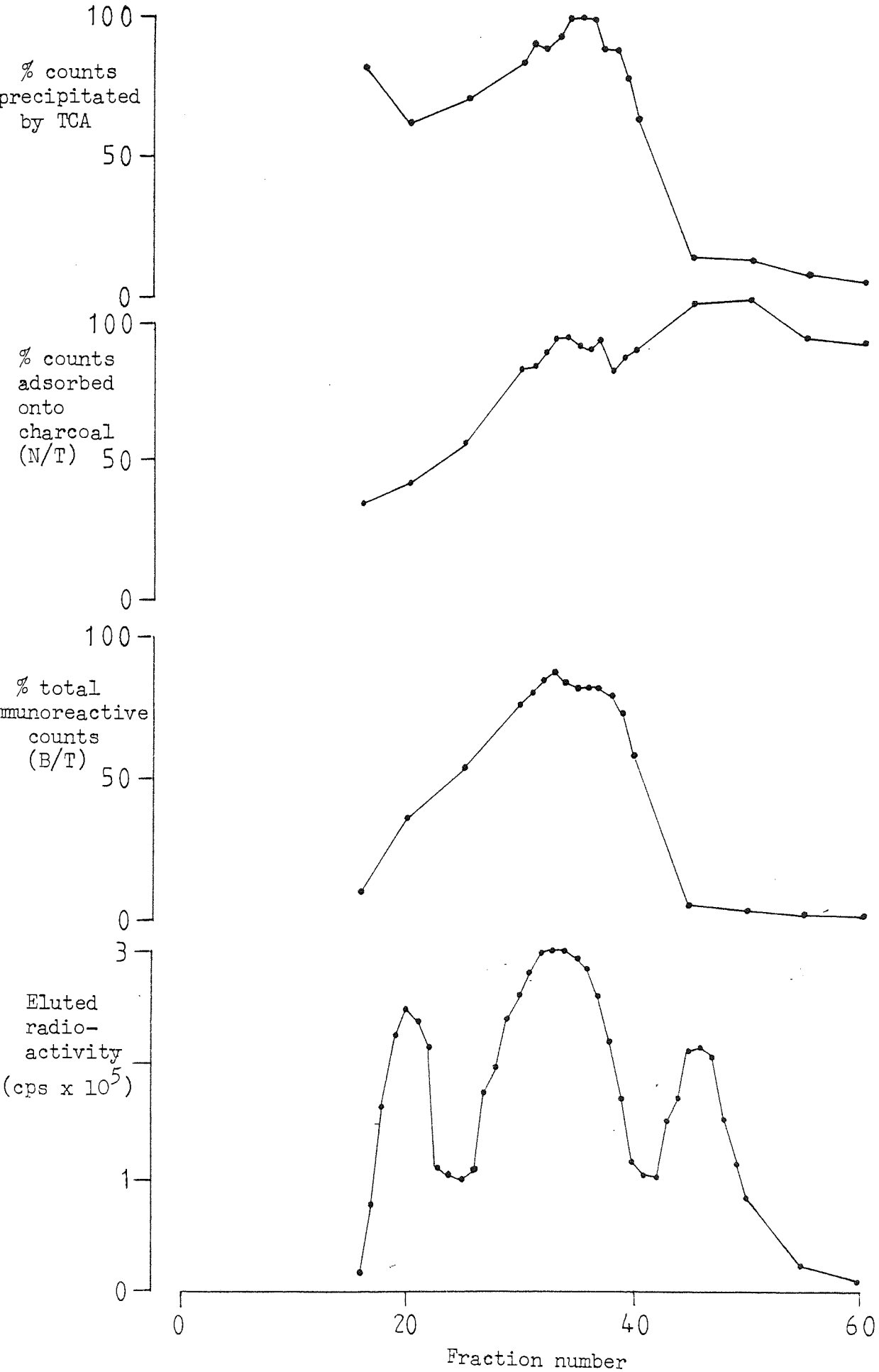
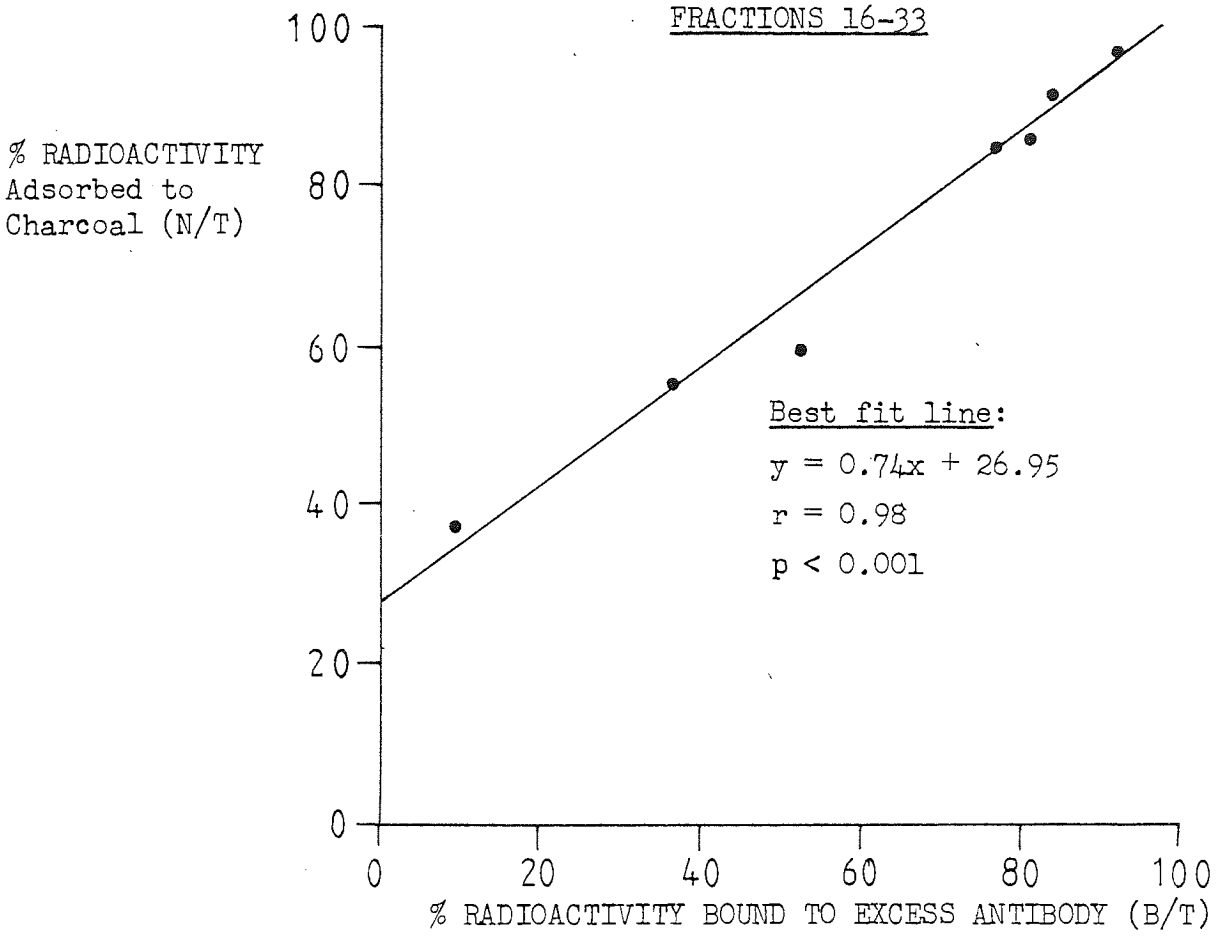


Figure 32 Relationship between the physico-chemical and immunochemical properties of the radioactivity eluted during purification of an insulin iodination mixture by gel chromatography.



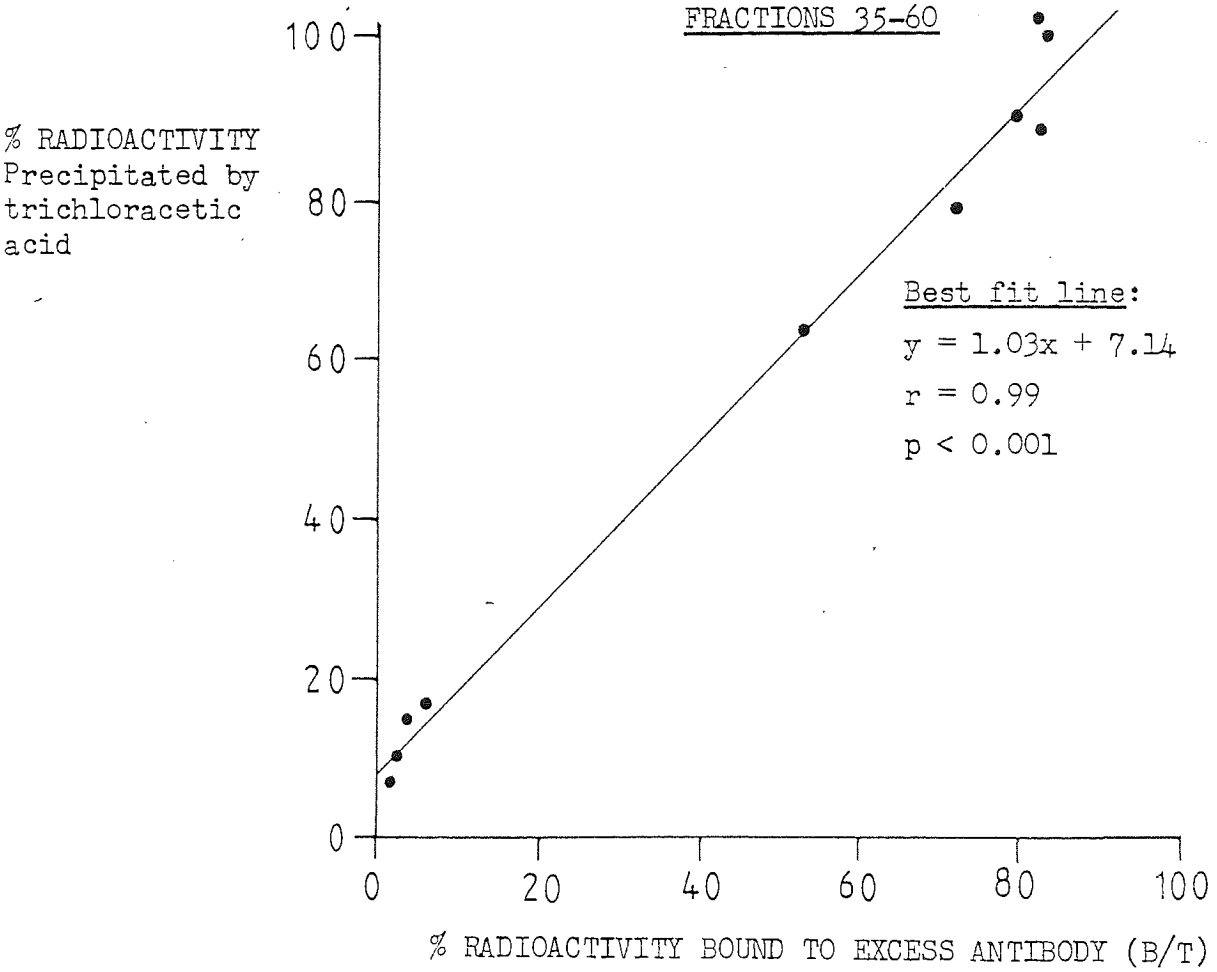


Figure 33 Dilution curves for standard and endogenous plasma mouse insulin.

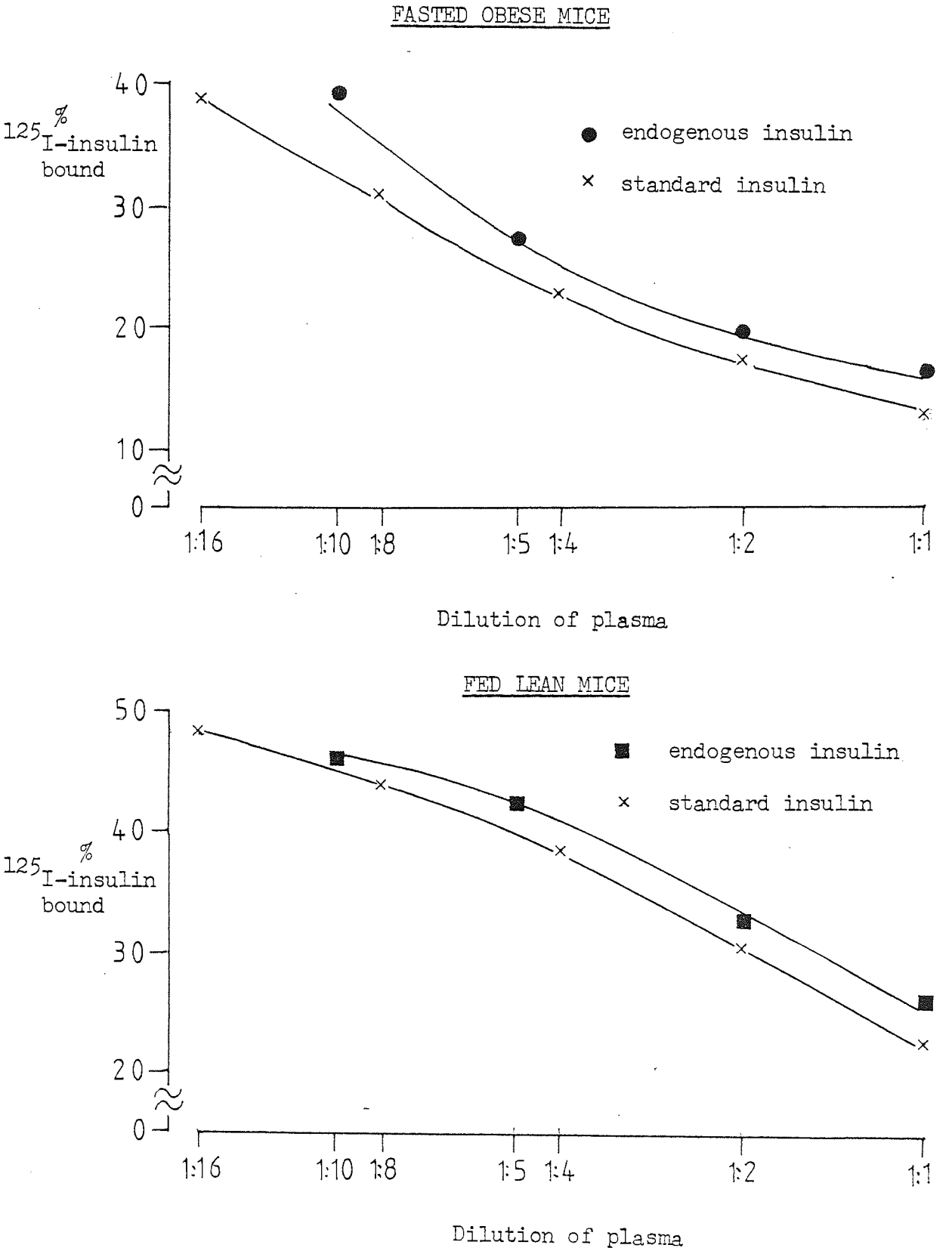
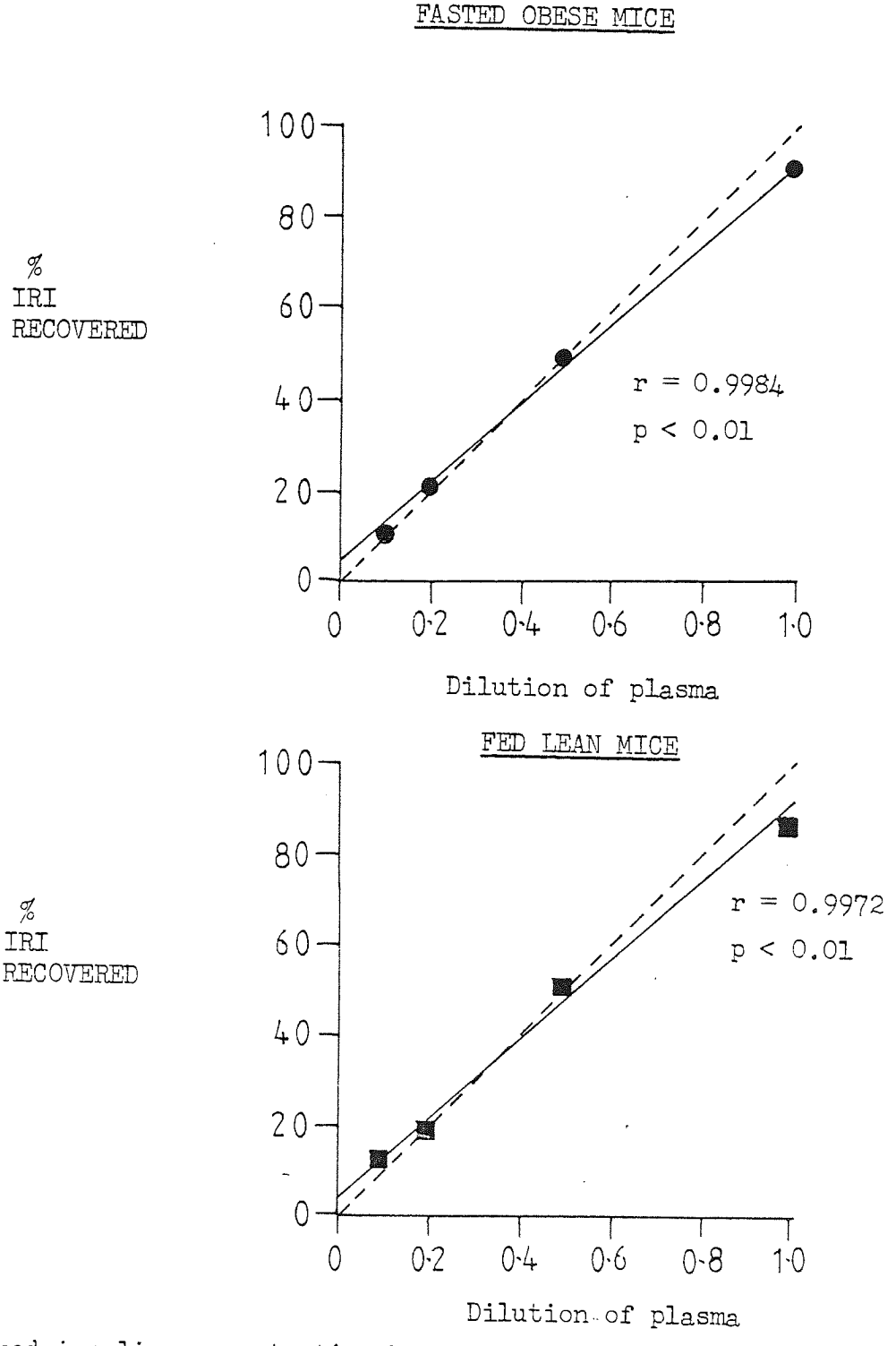
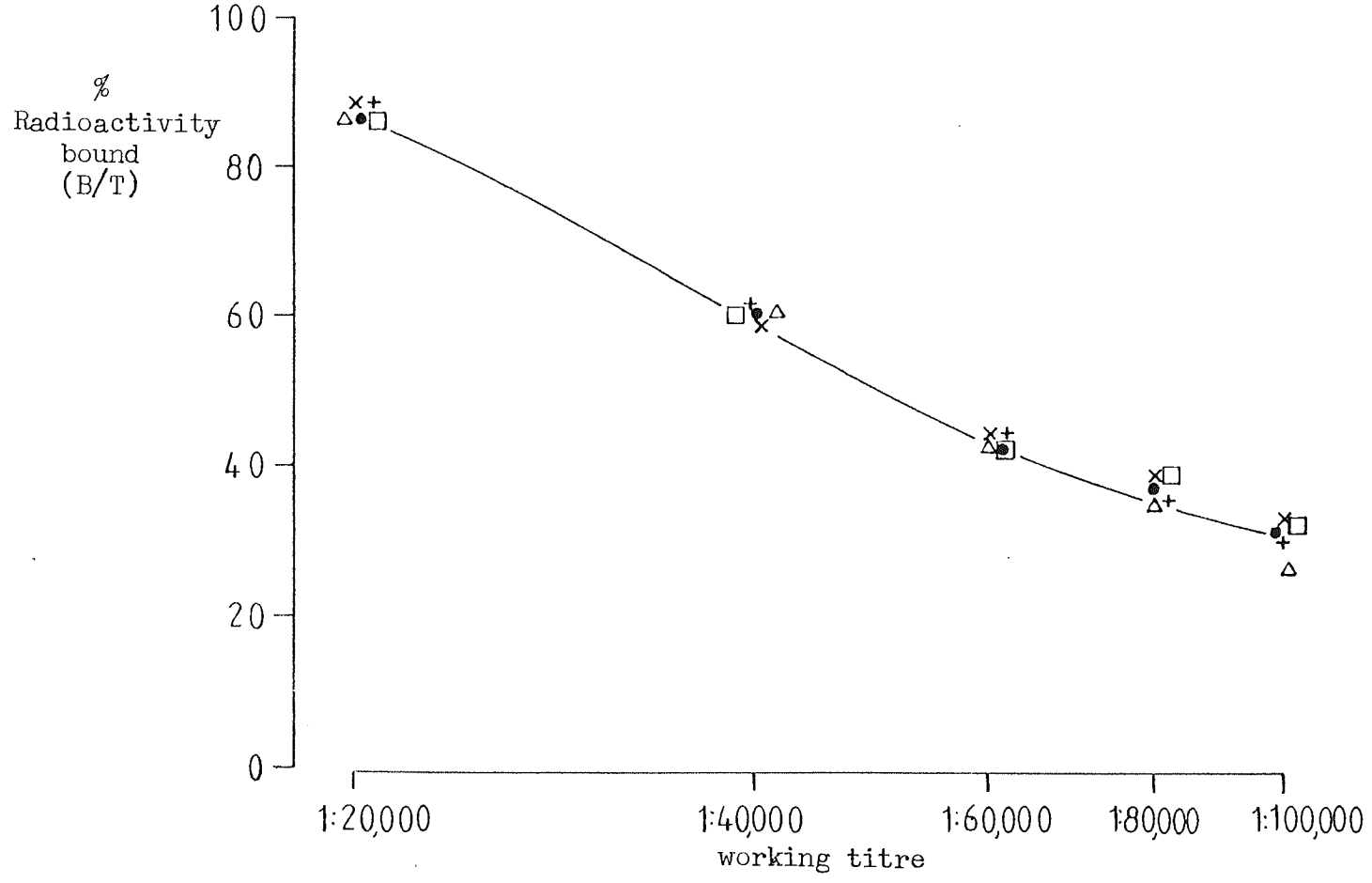


Figure 34 Effect of dilution of pooled mouse plasma on the measured endogenous insulin concentration$^{\phi}$



$^{\phi}$The measured insulin concentration has been expressed as a percentage of the mean estimated concentration in the undiluted sample. The plasma insulin concentration of the two pools was 3.60 ± 0.10 ng/ml for fasted obese mice and 1.07 ± 0.08 ng/ml for fed lean mice.

Figure 35 Insulin antibody dilution curves prepared using buffers supplemented (0.5% w/v) with five different albumin preparations$^{\phi}$.



$^{\phi}$Albumins: x Armour BPA WA1073; △ Armour BPA XC2671; ● Pentex BSA 246/18003; + Sigma BSA 81C/13028.
□ Sigma HSA 1230-1120.

Figure 36 Effect of insulin free plasma on the insulin dose response curve. Dextran coated charcoal separation.

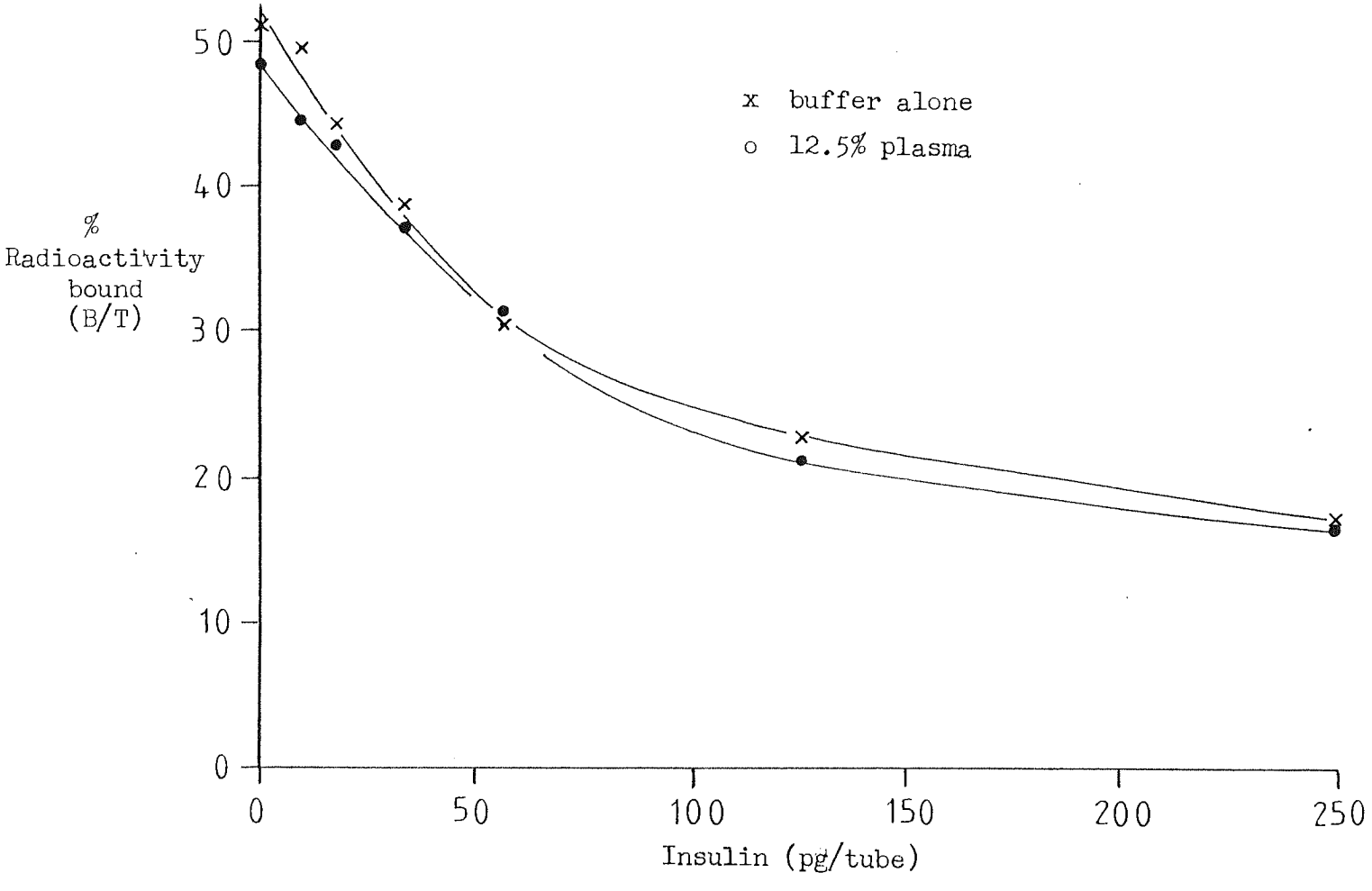


Figure 37 The effect of time on the adsorption of ^{125}I-insulin onto horse serum and dextran coated charcoal at 4°C.

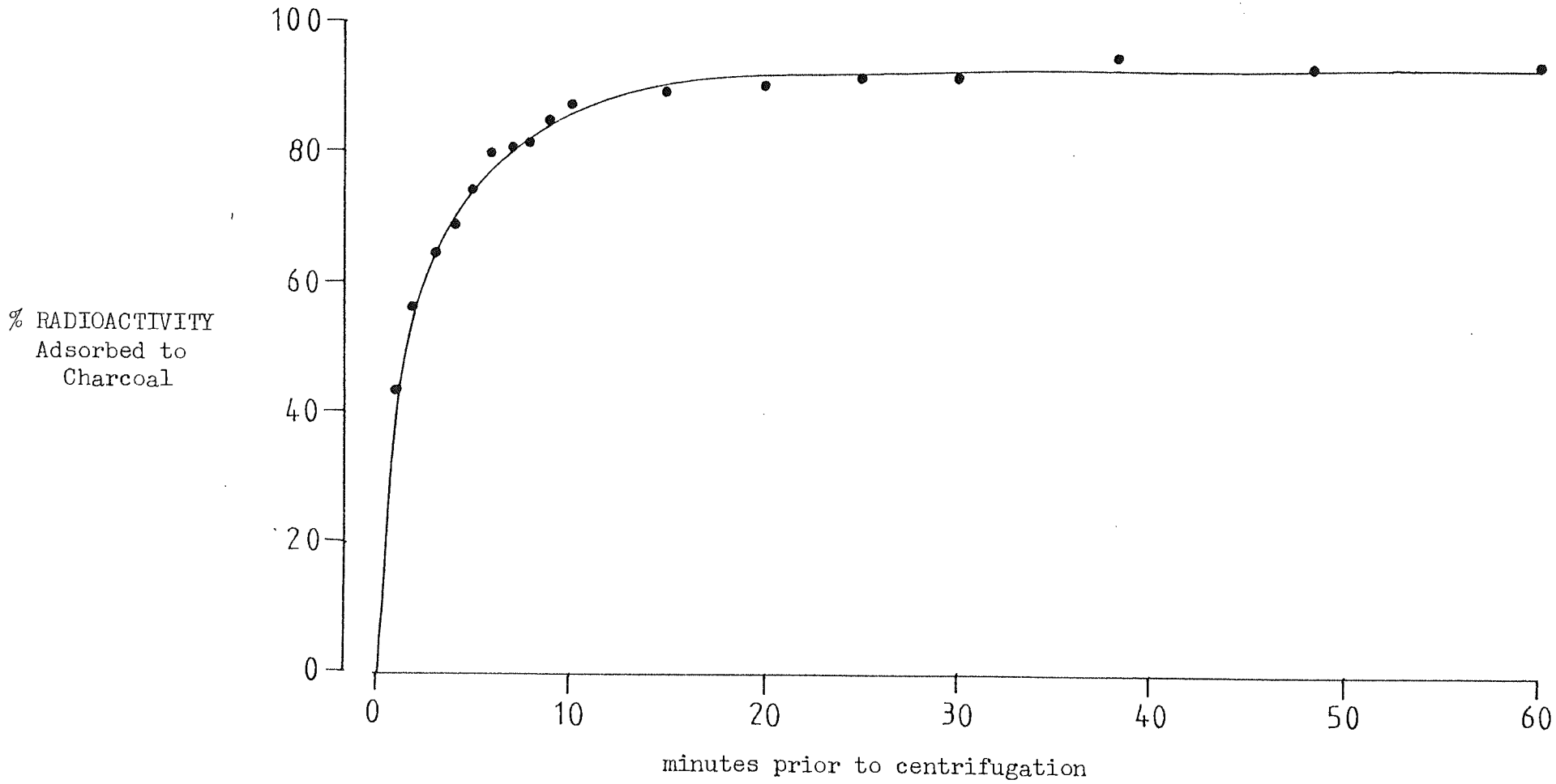
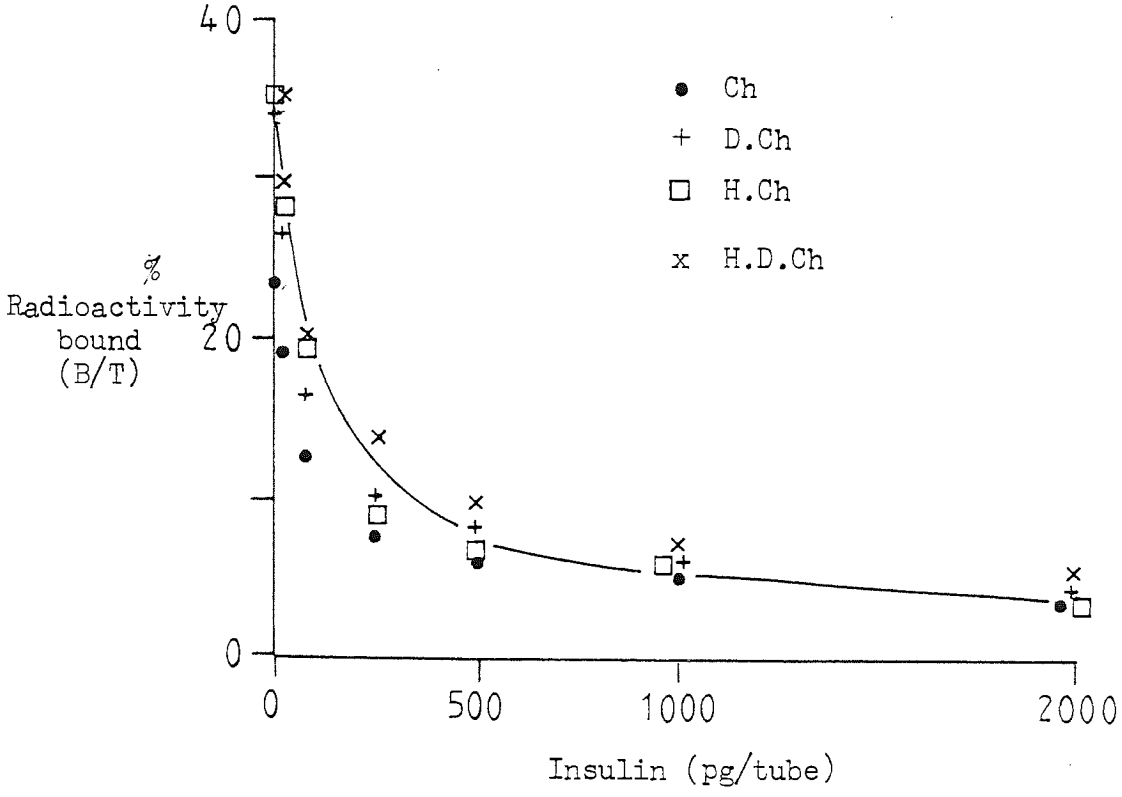


Figure 38 Effect of different protein coats on the separation of free and antibody bound insulin by charcoal[ø].



[ø]Abbreviations: Ch, Charcoal; D.Ch, dextran-charcoal (1:10, w/w); H.Ch, horse serum-charcoal (1:10, v/w); H.D.Ch, horse serum-dextran-charcoal (1:1:10, v/w/w).

Figure 39 Effect of plasma concentration on the efficiency of dextran-charcoal and horse serum-dextran-charcoal separation of free and antibody bound ^{125}I-insulin.

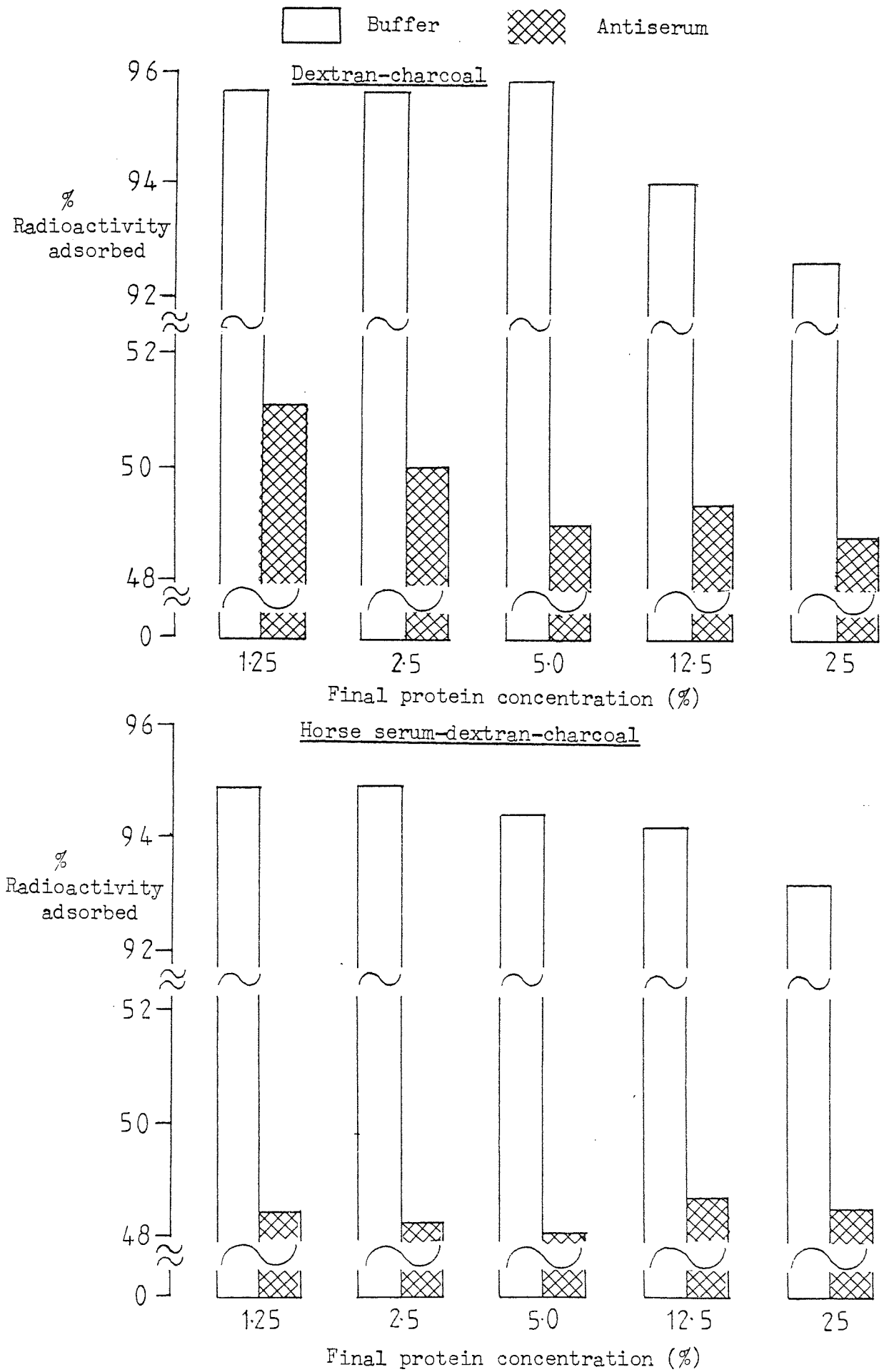


FIGURE 40

SAMPLE COMPUTER OUTPUT FOR INSULIN
RADIOIMMUNOASSAY DATA PROCESSING

INSULIN RIA
PLASMA INSULIN (PLASMA CURVE)
STANDARDS AND 266 UNKNOWNS

LOW STANDARDS OMITTED FROM ASSAY: 0 LOW STANDARDS IGNORED IN CALCULATION: 0

RATIO OF CHANNEL 1 : CHANNEL 2 (CR) = 1.08917

STANDARDS

MODE	CASSETTE	TUBE	TIME MIN X 0.01	CHANNEL 1	CHANNEL 2	CHANNEL 2 X CR.	COUNTS PER MINUTE CHANNEL 1	(CHANNEL 2 X CR) CHANNEL 2	MEAN	S.D.	OUTLIERS OUTSIDE MEAN +/- 1.5 X SD	CONCN. PG/ML.
1	121	1	300	23357	21495	23411	7785.67	7803.56				
1	121	2	300	22858	21040	22915	7619.33	7638.38				
1	121	3	300	23857	21949	23905	7952.33	7968.38	7794.61	148.57		20000
1	121	4	300	22612	20840	22697	7537.33	7565.77				
1	121	5	300	22373	20615	22452	7457.67	7484.09				
1	121	6	300	23498	21639	23589	7832.67	7863.10	7623.44	178.24		10000
1	121	7	300	22615	20909	22772	7538.33	7590.82				
1	121	8	300	22320	20526	22355	7440.00	7451.78				
1	121	9	300	22718	20944	22811	7572.67	7603.53	7532.85	70.94		5000
1	121	10	300	21027	19387	21115	7009.00	7038.27				
1	147	1	300	21407	19792	21556	7135.67	7185.30				
1	147	2	300	21588	19870	21641	7196.00	7213.62	7129.64	86.59		2500
1	147	3	300	19982	18405	20045	6660.67	6681.77				
1	147	4	300	20403	18780	20454	6801.00	6817.91				
1	147	5	300	20040	18551	20204	6680.00	6734.77	6729.35	66.97		1250
1	147	6	300	19628	18016	19622	6542.67	6540.54				
1	147	7	300	19101	17629	19200	6367.00	6400.05				
1	147	8	300	18488	17071	18592	6162.67	6197.47	6368.40	162.82		625
1	147	9	300	17443	16081	17514	5814.33	5838.06				
1	147	10	300	16584	15254	16613	5528.00	5537.82				
1	111	1	300	16203	14841	16164	5401.00	5387.89	5584.52	197.39		313
1	111	2	300	15405	14119	15377	5135.00	5125.77				
1	111	3	300	15126	13861	15096	5042.00	5032.11				
1	111	4	300	16079	14801	16120	5359.67	5373.37	5177.99	152.01		156
1	111	5	300	14806	13691	14911	4935.33	4970.39				
1	111	6	300	14304	13207	14384	4768.00	4794.68				
1	111	7	300	14001	12897	14046	4667.00	4682.14	4802.92	126.44		78
1	111	8	300	13618	12538	13655	4539.33	4551.81				
1	111	9	300	13775	12740	13875	4591.67	4625.14				
1	111	10	300	14544	13329	14517	4848.00	4838.97	4665.82	140.94		39
1	399	1	300	14078	12997	14155	4692.00	4718.44				
1	399	2	300	14285	13173	14347	4761.67	4782.34				
1	399	3	300	13965	12830	13973	4655.00	4657.81	4711.21	52.98		20

1	399	4	300	13568	12492	13605	4522,67	4535,11			
1	399	5	300	13260	12214	13303	4420,00	4434,18			
1	399	6	300	12757	11685	12726	4252,33	4242,13			
1	399	7	300	12655	11649	12687	4218,33	4229,06	4356,73	135,67	ZERO
1	399	8	300	25552	23449	25539	8517,33	8512,94			
1	399	9	300	24280	22429	24428	8093,33	8142,64			
1	399	10	300	25081	23184	25250	8360,33	8416,74			
1	130	1	300	25587	23549	25648	8529,00	8549,75	8390,20	180,00	8093,33
							RECALCULATION		8432,61	144,96	8142,64
							RECALCULATION		8480,93	74,82	RNS
1	130	2	300	27179	24931	27153	9059,67	9050,97			
1	130	3	300	26785	24500	26683	8928,33	8894,50			
1	130	4	300	27212	24905	27125	9070,67	9041,53			
1	130	5	300	26995	24725	26926	8998,33	8975,46	9002,43	65,14	TC

VALUES OF TC AND RNS RESPECTIVELY ARE 9002,43 8480,93 DIFFERENCE = 521,50 RATIO = 0,9421 BO = 4124,21

MEAN TOTAL COUNT = 27007,30 S.D. = 195,415 PIPETTING ERROR = NEGLIGIBLE MEAN BO/T = 0,45812

CONCN.	B	MEAN B	(MEAN B)/T	B/BO	MEAN B/BO	VARIANCE	C.OF V. (SD/MEAN X 100)	ASSAY ** PRECISION (PG/ML)
20000	695,27			0,169				
	677,37			0,164				
	861,60			0,209				
	842,55			0,204				
	528,60			0,128				
	512,55	686,72	0,07624	0,124	0,16641	1,2988 -3	21,647	> 500
10000	943,60			0,229				
	915,16			0,222				
	1023,27			0,248				
	996,85			0,242				
	648,27			0,157				
	617,83	857,50	0,09525	0,150	0,20792	1,8688 -3	20,787	> 500
5000	942,60			0,229				
	890,11			0,216				
	1040,93			0,252				
	1029,16			0,250				
	908,27			0,220				
	877,41	948,08	0,10531	0,213	0,22988	2,9598 -4	7,483	> 500
2500	1471,93			0,357				
	1442,66			0,350				
	1345,27			0,326				
	1295,63			0,314				
	1284,93			0,312				
	1267,31	1351,29	0,15010	0,307	0,32765	4,4098 -4	6,408	141,82
1250	1820,27			0,441				
	1799,17			0,436				
	1679,93			0,407				
	1663,03			0,403				

	1800,93			0,437					
	1746,16	1751,58	0,19457	0,423	0,42471	2,6378 -4	3,823		55,39
625	1938,27			0,470					
	1940,39			0,470					
	2113,93			0,513					
	2080,89			0,505					
	2318,27			0,562					
	2283,46	2112,53	0,23466	0,554	0,51223	1,5598 -3	7,707		74,48
313	2666,60			0,647					
	2642,87			0,641					
	2952,93			0,716					
	2943,11			0,714					
	3079,93			0,747					
	3093,04	2894,42	0,32174	0,750	0,70230	2,2918 -3	4,815		27,43
156	3345,93			0,811					
	3355,16			0,814					
	3438,93			0,834					
	3448,82			0,836					
	3121,27			0,757					
	3107,57	3302,95	0,36689	0,753	0,80087	1,3588 -3	4,402		15,75
78	3545,60			0,860					
	3510,54			0,851					
	3712,93			0,900					
	3686,25			0,894					
	3813,93			0,925					
	3798,80	3678,01	0,40856	0,921	0,89184	9,3998 -4	3,438		7,10
39	3941,60			0,956					
	3929,13			0,953					
	3889,27			0,943					
	3855,79			0,935					
	3632,93			0,881					
	3641,96	3815,11	0,42379	0,883	0,92505	1,1688 -3	3,694		10,33
20	3788,93			0,919					
	3762,49			0,912					
	3719,27			0,902					
	3698,60			0,897					
	3825,93			0,928					
	3823,12	3769,72	0,41874	0,927	0,91405	1,6518 -4	1,406		5,64

AT ZERO CONCENTRATION (= SENSITIVITY): 2,0208

** (NOTE: "ASSAY PRECISION" BASED ON C.V. OF RAW COUNT RATES) THE TWO HIGHEST CONCENTRATIONS USE THE SAME SLOPE)

FINAL VOLUME 400 UL MOLECULAR WEIGHT 6000 TRACER CONCENTRATION 1000 PG/ML

SCATCHARD PLOT: VALUE OF P* = 41,667

B/T(P* + P):	66,71	43,66	26,33	21,89	18,24	15,89	17,59	17,68	18,35	18,35	17,79
B/T/(1-B/T):	0,08	0,11	0,12	0,18	0,24	0,31	0,47	0,58	0,69	0,74	0,72

SLOPE M = -0,0100 INTERCEPT C = 0,6415 COEFFICIENT R = -0,6004

T-VALUE = 2,2524 9 DEGREES OF FREEDOM

INVERSE OF SLOPE (L/PMOLE) = -99,969 X-INTERCEPT (PMOLE/L) = 64,150 FREE ENERGY CHANGE (J/MOLE) = 83488,2

OPTIMISATION: P-OPT = 399.8768 Q-OPT = 299.9076 P-OPT/P0 = 9.5970 Q-OPT/Q0 = 4.6766

CONCENTRATION:	20000	10000	5000	2500	1250	625	313	156	78	39	20
B/T VALUES	0.076	0.095	0.105	0.150	0.195	0.235	0.322	0.367	0.409	0.424	0.419
B/B0 VALUES:	0.1664	0.2079	0.2299	0.3276	0.4247	0.5122	0.7023	0.8009	0.8918	0.9251	0.9140
LOG CONCN.:	4.3010	4.0000	3.6990	3.3979	3.0969	2.7959	2.4948	2.1938	1.8928	1.5918	1.2907
LOGIT TERM:	-1.6113	-1.3375	-1.2090	-0.7188	-0.3035	0.0489	0.8583	1.3917	2.1094	2.5131	2.3641

SLOPE M = -1.5286 INTERCEPT C = 4.6470 COEFFICIENT R = -0.9861 SUM OF WEIGHTS = 11.0000

GOODNESS OF FIT VALUES OF SX, SY, SXY, SXQ, SYQ: 30.75 4.11 -3.76 95.93 25.49
S.S.D.(FROM CORRELATION COEFFICIENT) = 0.6615 (FROM SLOPE) = 0.6615 M.SQ.DEV. FROM REGRESSION = 0.0735
S.S.D.FROM REGRESSION = 0.2711 S.S.D.OF REGRESSION COEFFICIENT = 0.0859 T-STATISTIC = -17.8013 9 D.F.

ITERATIONS REQUIRED: 5 A0 = 0.000800 AI = 0.001080

ITERATION 1

LOG CONCN.	YHI	EXP(YHI)	YH	YH(1-YH)	WKG.LOGIT	WEIGHT	THIS Y	NEXT Y
4.3010	-1.9276	0.1455	0.1270	0.1109	-1.5723	13.1199	0.1664	0.1270
4.0000	-1.4674	0.2305	0.1873	0.1522	-1.3322	23.1238	0.2079	0.1873
3.6990	-1.0073	0.3652	0.2675	0.1960	-1.1993	35.2618	0.2299	0.2675
3.3979	-0.5471	0.5786	0.3665	0.2322	-0.7146	45.0815	0.3276	0.3665
3.0969	-0.0869	0.9167	0.4783	0.2495	-0.3016	47.2939	0.4247	0.4783
2.7959	0.3732	1.4524	0.5922	0.2415	0.0419	40.5099	0.5122	0.5922
2.4948	0.8334	2.3011	0.6971	0.2112	0.8581	28.7155	0.7023	0.6971
2.1938	1.2935	3.6456	0.7847	0.1689	1.3890	17.3195	0.8009	0.7847
1.8928	1.7537	5.7758	0.8524	0.1258	2.0668	9.1980	0.8918	0.8524
1.5918	2.2138	9.1508	0.9015	0.0888	2.4792	4.4469	0.9251	0.9015
1.2907	2.6740	14.4978	0.9355	0.0604	2.3190	2.0127	0.9140	0.9355

LOGIT TERM:	-1.5723	-1.3322	-1.1993	-0.7146	-0.3016	0.0419	0.8581	1.3890	2.0668	2.4792	2.3190

SLOPE M = -1.5224 INTERCEPT C = 4.5359 COEFFICIENT R = -0.9802 SUM OF WEIGHTS = 266.0836

ITERATION 2

LOG CONCN.	YHI	EXP(YHI)	YH	YH(1-YH)	WKG.LOGIT	WEIGHT	THIS Y	NEXT Y
4.3010	-2.0119	0.1337	0.1180	0.1040	-1.9248	11.6733	0.1270	0.1180
4.0000	-1.5536	0.2115	0.1746	0.1441	-1.4650	21.0039	0.1873	0.1746
3.6990	-1.0953	0.3344	0.2506	0.1878	-1.0053	32.9441	0.2675	0.2506
3.3979	-0.6370	0.5289	0.3459	0.2263	-0.5459	43.6207	0.3665	0.3459
3.0969	-0.1788	0.8363	0.4554	0.2480	-0.0866	47.6139	0.4783	0.4554
2.7959	0.2795	1.3225	0.5694	0.2452	0.3725	42.6833	0.5922	0.5694
2.4948	0.7378	2.0913	0.6765	0.2188	0.8317	31.2891	0.6971	0.6765
2.1938	1.1961	3.3071	0.7678	0.1783	1.2910	19.5059	0.7847	0.7678
1.8928	1.6544	5.2297	0.8395	0.1348	1.7504	10.6401	0.8524	0.8395
1.5918	2.1126	8.2700	0.8921	0.0962	2.2099	5.2320	0.9015	0.8921
1.2907	2.5709	13.0777	0.9290	0.0660	2.6695	2.4148	0.9355	0.9290

LOGIT TERM:	-1.9248	-1.4650	-1.0053	-0.5459	-0.0866	0.3725	0.8317	1.2910	1.7504	2.2099	2.6695

SLOPE M = -1.5259 INTERCEPT C = 4.6387 COEFFICIENT R = -1.0000 SUM OF WEIGHTS = 268.4409

ITERATION 3

LOG CONCN.	YHI	EXP(YHI)	YH	YH(1-YH)	WKG.LOGIT	WEIGHT	THIS Y	NEXT Y
4.3010	-1.9241	0.1460	0.1274	0.1112	-2.0090	13.1816	0.1180	0.1274
4.0000	-1.4648	0.2311	0.1877	0.1525	-1.5511	23.1896	0.1746	0.1877
3.6990	-1.0055	0.3659	0.2679	0.1961	-1.0934	35.3083	0.2506	0.2679
3.3979	-0.5461	0.5792	0.3668	0.2322	-0.6359	45.0987	0.3459	0.3668
3.0969	-0.0868	0.9169	0.4783	0.2495	-0.1785	47.2930	0.4554	0.4783
2.7959	0.3725	1.4514	0.5921	0.2415	0.2788	40.5250	0.5694	0.5921
2.4948	0.8319	2.2976	0.6968	0.2113	0.7361	28.7558	0.6765	0.6968
2.1938	1.2912	3.6372	0.7844	0.1691	1.1935	17.3608	0.7678	0.7844
1.8928	1.7505	5.7577	0.8520	0.1261	1.6511	9.2412	0.8395	0.8520
1.5918	2.2099	9.1146	0.9011	0.0891	2.1088	4.4763	0.8921	0.9011
1.2907	2.6692	14.4285	0.9352	0.0606	2.5666	2.0299	0.9290	0.9352

LOGIT TERMS -2.0090 -1.5511 -1.0934 -0.6359 -0.1785 0.2788 0.7361 1.1935 1.6511 2.1088 2.5666

SLOPE M = -1.5196 INTERCEPT C = 4.5276 COEFFICIENT R = -1.0000 SUM OF WEIGHTS = 266.6660

ITERATION 4

LOG CONCN.	YHI	EXP(YHI)	YH	YH(1-YH)	WKG.LOGIT	WEIGHT	THIS Y	NEXT Y
4.3010	-2.0085	0.1342	0.1183	0.1043	-1.9214	11.7292	0.1274	0.1183
4.0000	-1.5510	0.2120	0.1749	0.1443	-1.4624	21.0659	0.1877	0.1749
3.6990	-1.0936	0.3350	0.2509	0.1880	-1.0035	32.9909	0.2679	0.2509
3.3979	-0.6361	0.5294	0.3661	0.2263	-0.5449	43.6374	0.3668	0.3661
3.0969	-0.1786	0.8364	0.4555	0.2480	-0.0865	47.6137	0.4783	0.4555
2.7959	0.2788	1.3216	0.5693	0.2452	0.3719	42.4972	0.5921	0.5693
2.4948	0.7363	2.0881	0.6762	0.2190	0.8302	31.3301	0.6968	0.6762
2.1938	1.1937	3.2994	0.7674	0.1785	1.2887	19.5603	0.7844	0.7674
1.8928	1.6512	5.2132	0.8391	0.1350	1.7472	10.6887	0.8520	0.8391
1.5918	2.1086	8.2371	0.8917	0.0965	2.2059	5.2861	0.9011	0.8917
1.2907	2.5661	13.0150	0.9286	0.0663	2.6648	2.4352	0.9352	0.9286

LOGIT TERMS -1.9214 -1.4624 -1.0035 -0.5449 -0.0865 0.3719 0.8302 1.2887 1.7472 2.2059 2.6648

SLOPE M = -1.5231 INTERCEPT C = 4.6306 COEFFICIENT R = -1.0000 SUM OF WEIGHTS = 268.8346

ITERATION 5

LOG CONCN.	YHI	EXP(YHI)	YH	YH(1-YH)	WKG.LOGIT	WEIGHT	THIS Y	NEXT Y
4.3010	-1.9207	0.1465	0.1278	0.1115	-2.0056	13.2434	0.1183	0.1278
4.0000	-1.4622	0.2317	0.1881	0.1527	-1.5486	23.2354	0.1749	0.1881
3.6990	-1.0037	0.3665	0.2682	0.1963	-1.0917	35.3547	0.2509	0.2682
3.3979	-0.5452	0.5798	0.3670	0.2323	-0.6350	45.1098	0.3461	0.3670
3.0969	-0.0866	0.9170	0.4784	0.2495	-0.1784	47.2922	0.4555	0.4784
2.7959	0.3719	1.4504	0.5919	0.2416	0.2781	40.5400	0.5693	0.5919
2.4948	0.8304	2.2942	0.6964	0.2114	0.7346	28.7960	0.6762	0.6964
2.1938	1.2889	3.6287	0.7840	0.1694	1.1912	17.4201	0.7674	0.7840
1.8928	1.7474	5.7397	0.8516	0.1264	1.6479	9.2844	0.8391	0.8516
1.5918	2.2059	9.0785	0.9008	0.0894	2.1048	4.5058	0.8917	0.9008
1.2907	2.6644	14.3596	0.9349	0.0609	2.5618	2.0472	0.9286	0.9349

LOGIT TERMS -2.0056 -1.5486 -1.0917 -0.6350 -0.1784 0.2781 0.7346 1.1912 1.6479 2.1048 2.5618

SLOPE M = -1.5169 INTERCEPT C = 4.5192 COEFFICIENT R = -1.0000 SUM OF WEIGHTS = 266.8489

GOODNESS OF FIT VALUES OF SX, SY, SXY, SXQ, SYQ: 831.29 -55.04 -343.87 2703.27 272.87

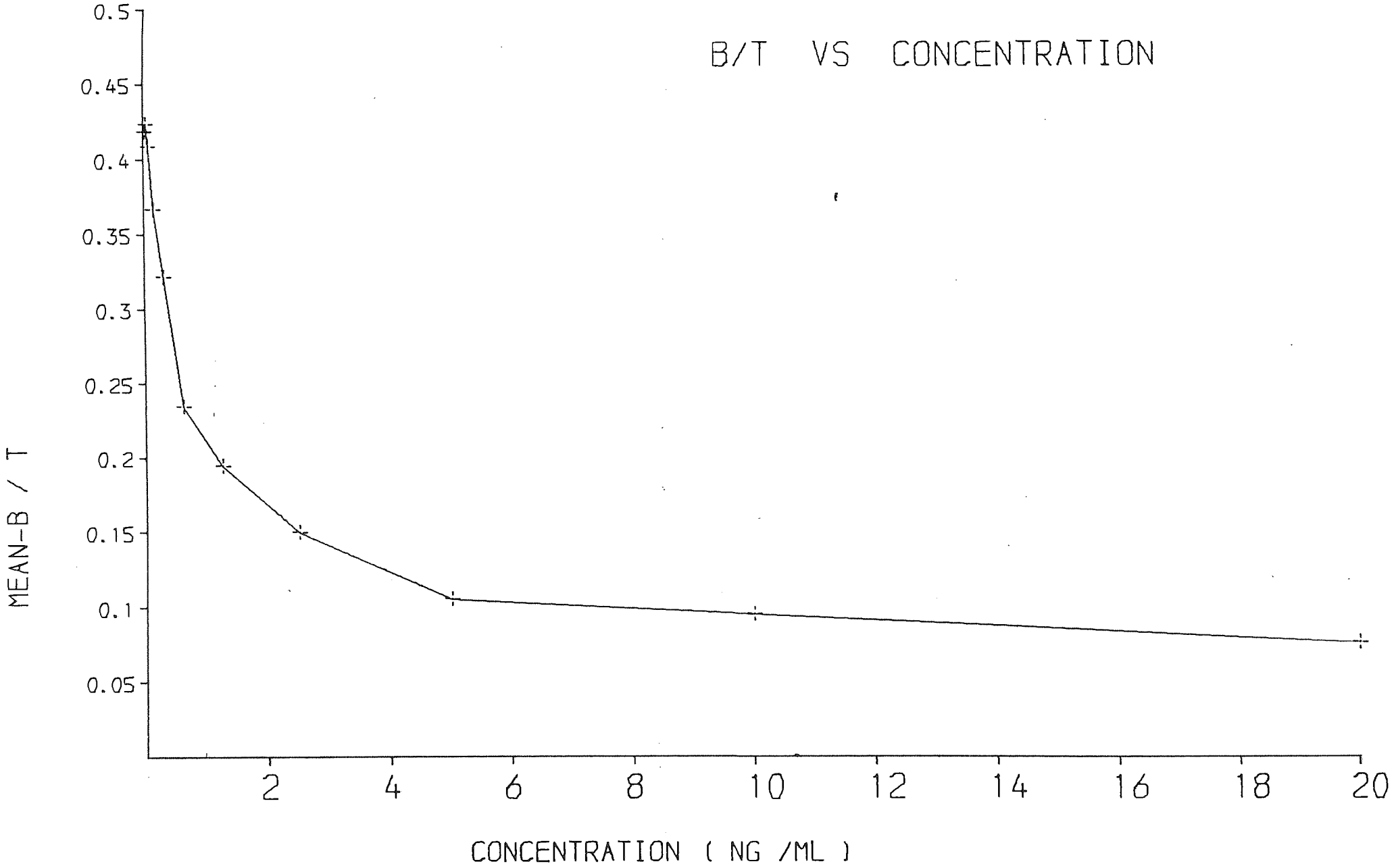
S.S.D.(FROM CORRELATION COEFFICIENT) = 0.0000 (FROM SLOPE) = 0.0000 M.SQ.DEV. FROM REGRESSION = 0.0000

S.S.D.FROM REGRESSION = 0.0011 S.S.D.OF REGRESSION COEFFICIENT = 0.0001 T-STATISTIC = -14933.5354 9 D.F.

UNKNOWNS

DILUTION = 10.00 NUMBER = 270

MODE	CASSETTE	TUBE	TIME MIN X 0.01	CHANNEL 1	CHANNEL 2	CHANNEL 2 X CR.	COUNTS PER MINUTE(CHANNEL 1	CHANNEL 2	MEAN	CONCN. PG/ML.	FINAL CONC. PG/ML.
1	130	6	300	15679	14391	15674	5226.33	5224.52	5225.43	128.32	1283.24
1	130	7	300	15750	14444	15731	5250.00	5243.76	5246.88	134.51	1345.09
1	130	8	300	15629	14317	15593	5209.67	5197.65	5203.66	122.23	1222.33
1	130	9	300	16800	15430	16805	5600.00	5601.72	5600.86	266.61	2666.11
1	130	10	300	15729	14425	15711	5243.00	5236.86	5239.93	132.49	1324.86
1	143	1	300	15629	14370	15651	5209.67	5214.90	5213.28	124.90	1249.03
1	143	2	300	16348	15106	16452	5449.33	5484.09	5466.71	209.24	2092.45
1	143	3	300	15974	14689	15998	5324.67	5332.71	5328.69	159.81	1598.08
1	143	4	300	16390	15135	16484	5463.33	5494.62	5478.98	214.08	2140.85
1	143	5	300	18181	16713	18203	6060.33	6067.50	6063.92	562.36	5623.63
1	143	6	300	14928	13689	14909	4976.00	4969.67	4972.83	68.01	680.06
1	143	7	300	13983	12870	14017	4661.00	4672.33	4666.67	21.11	211.09
1	143	8	300	14503	13299	14484	4834.33	4828.08	4831.21	43.08	430.79
1	143	9	300	14777	13598	14810	4925.67	4934.63	4931.15	60.06	600.59
1	143	10	300	16000	14676	15984	5333.33	5327.99	5330.66	160.45	1604.54
1	146	1	300	15172	13891	15129	5057.33	5043.00	5050.17	84.18	841.77
1	146	2	300	15822	14487	15778	5274.00	5259.37	5266.69	140.38	1403.80
1	146	3	300	14491	13272	14564	4830.33	4854.58	4842.46	44.85	448.49
1	146	4	300	15652	14370	15651	5217.33	5216.90	5217.11	125.98	1259.76
1	146	5	300	16386	14983	16318	5462.00	5439.44	5450.72	203.05	2030.48
1	146	6	300	15379	14120	15378	5126.33	5126.14	5126.23	102.00	1019.98
1	146	7	300	15045	13793	15022	5015.00	5007.42	5011.21	75.79	757.94
1	146	8	300	14255	13089	14256	4751.67	4751.84	4751.75	31.57	315.69
1	146	9	300	16486	15094	16439	5495.33	5479.74	5487.54	217.51	2175.08
1	146	10	300	14246	13030	14191	4748.67	4730.42	4739.54	29.95	299.50
1	121	1	300	15025	13831	15064	5008.33	5021.22	5014.78	76.54	765.41
1	121	2	300	16271	14960	16293	5423.67	5431.09	5427.38	194.23	1942.32
1	121	3	300	15288	14037	15288	5096.00	5096.00	5096.00	94.68	946.79
1	121	4	300	15081	13871	15107	5027.00	5035.74	5031.37	80.07	800.71
1	121	5	300	15875	14539	15835	5291.67	5278.25	5284.96	145.94	1459.39
1	121	6	300	14695	13539	14746	4898.33	4915.21	4906.77	55.65	556.52
1	121	7	300	15341	14038	15289	5113.67	5096.37	5105.02	96.83	968.29
1	121	8	300	15764	14533	15828	5254.67	5276.07	5265.37	139.98	1399.85
1	121	9	300	14620	13425	14621	4873.33	4873.82	4873.58	49.93	499.29
1	121	10	300	15726	14304	15797	5242.00	5265.34	5253.77	136.53	1365.34
1	141	1	300	14127	12965	14120	4709.00	4706.82	4707.91	25.94	259.42
1	141	2	300	14432	13229	14408	4810.67	4802.67	4806.67	39.34	393.41
1	141	3	300	14649	13427	14624	4883.00	4874.55	4878.77	50.80	508.04
1	141	4	300	14701	13307	14711	4900.33	4903.59	4901.96	54.80	548.03
1	141	5	300	14292	13111	14279	4764.00	4759.83	4761.91	32.95	329.45
1	141	6	300	16029	14693	16002	5343.00	5334.16	5338.58	163.06	1630.60
1	141	7	300	14408	13164	14337	4802.67	4779.07	4790.87	37.02	370.21
1	141	8	300	14238	13013	14173	4746.00	4724.25	4735.12	29.37	293.75
1	141	9	300	15173	13890	15128	5057.67	5042.64	5050.15	84.17	841.74
1	141	10	300	15324	14018	15267	5108.00	5089.11	5098.55	95.28	952.85
1	127	1	300	13932	12763	13900	4644.00	4633.49	4638.74	18.09	180.89
1	127	2	300	14579	13371	14563	4859.67	4854.22	4856.94	47.18	471.79
1	127	3	300	14706	13456	14655	4902.00	4885.08	4893.54	53.33	533.32
1	127	4	300	14359	13201	14378	4786.33	4792.50	4789.42	36.81	368.11
1	127	5	300	13886	12775	13914	4628.67	4637.85	4633.26	17.52	175.20
1	127	6	300	13780	12664	13793	4593.33	4597.55	4595.44	13.81	138.08
1	127	7	300	17258	15793	17201	5752.67	5733.50	5743.08	339.33	3393.28
1	127	8	300	16855	15428	16803	5611.67	5600.99	5606.33	269.17	2691.68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MEAN-B / T
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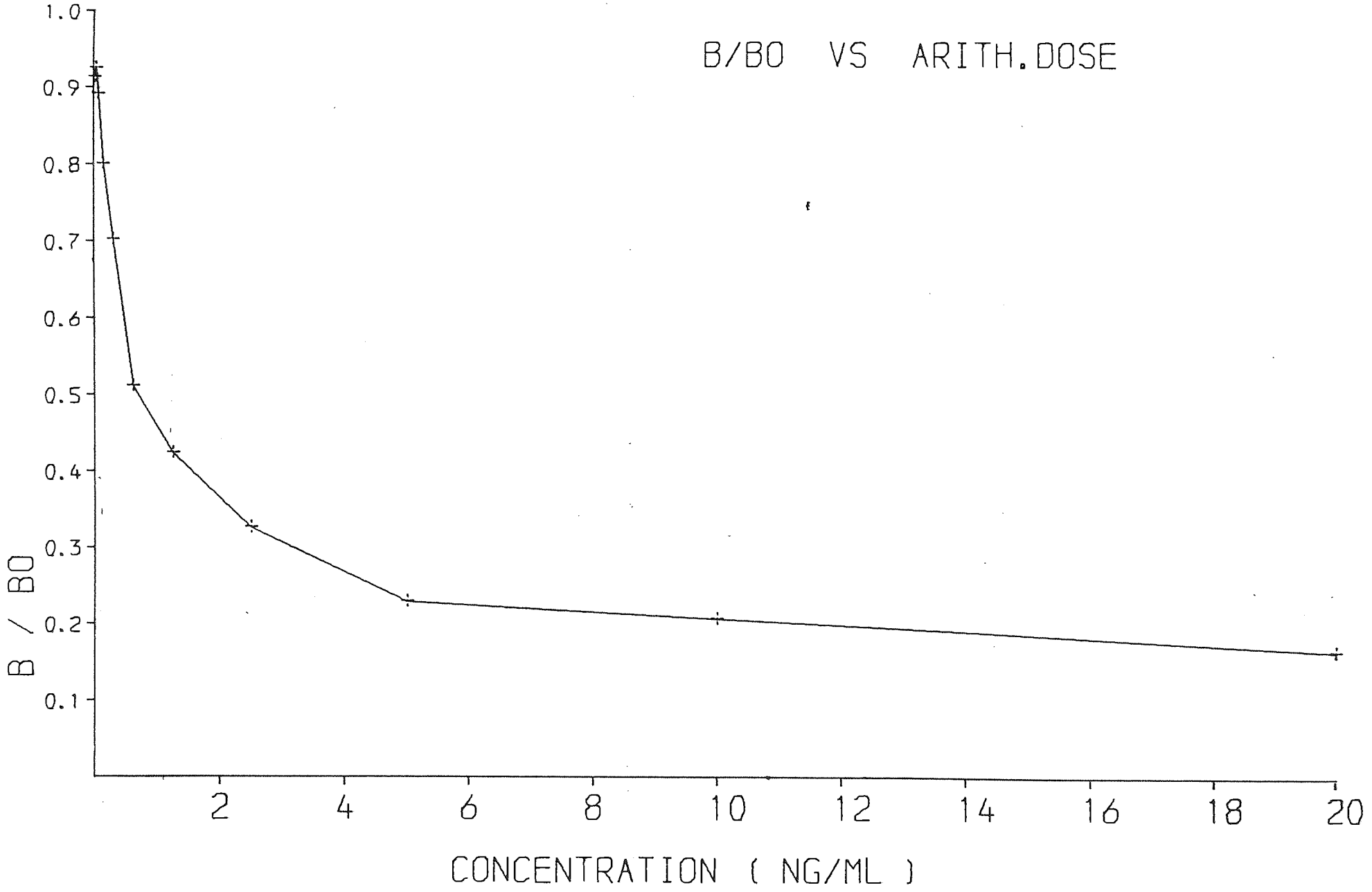
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
2
4
6
8
10
12
14
16
18
20
CONCENTRATION (NG /ML)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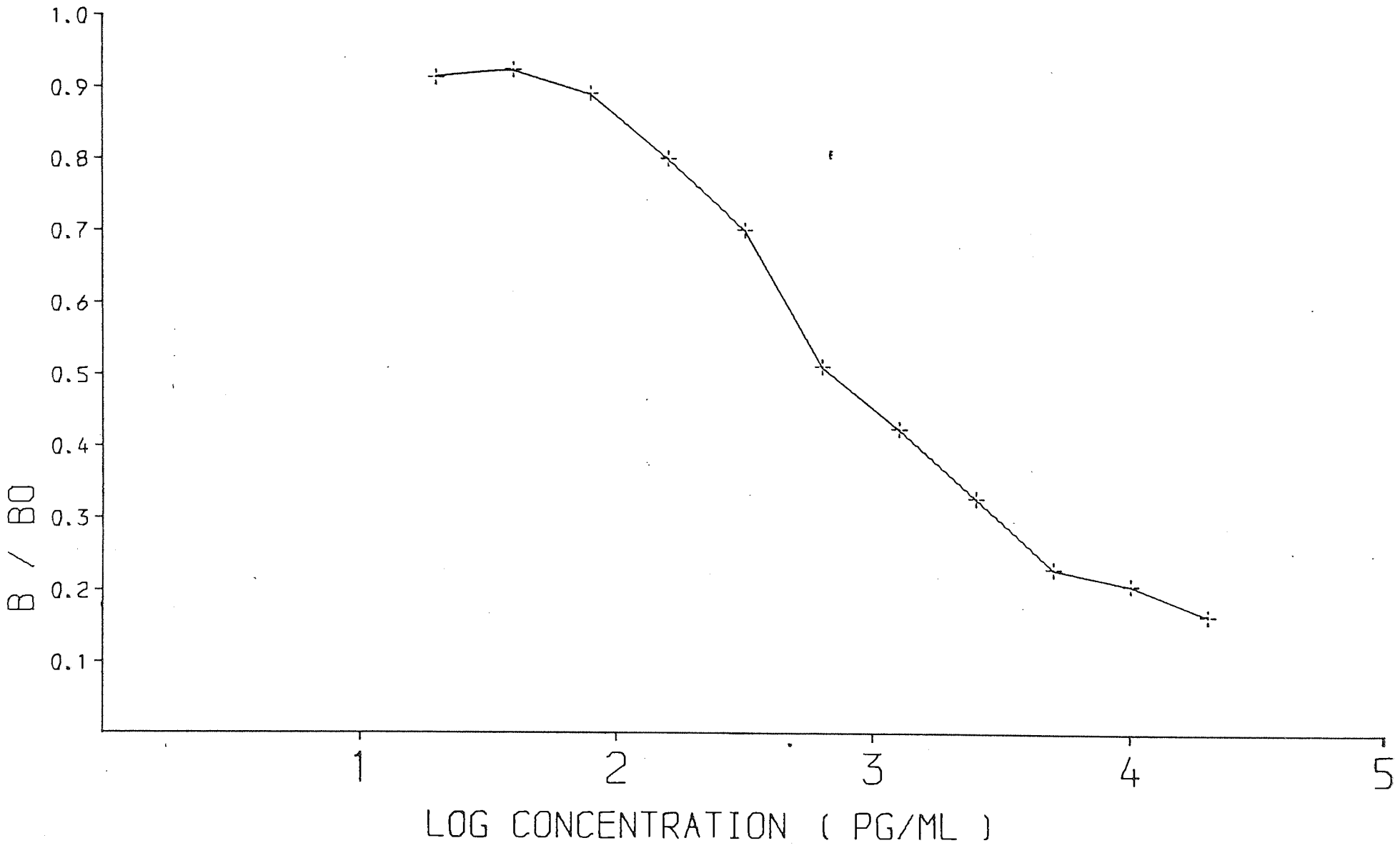
0.7
0.6
0.5
0.4
0.3
0.2
0.1
B / BO
2
4
6
8
10
12
14
16
18
20
CONCENTRATION (NG/ML)


B/BO VS LOG.DOSE
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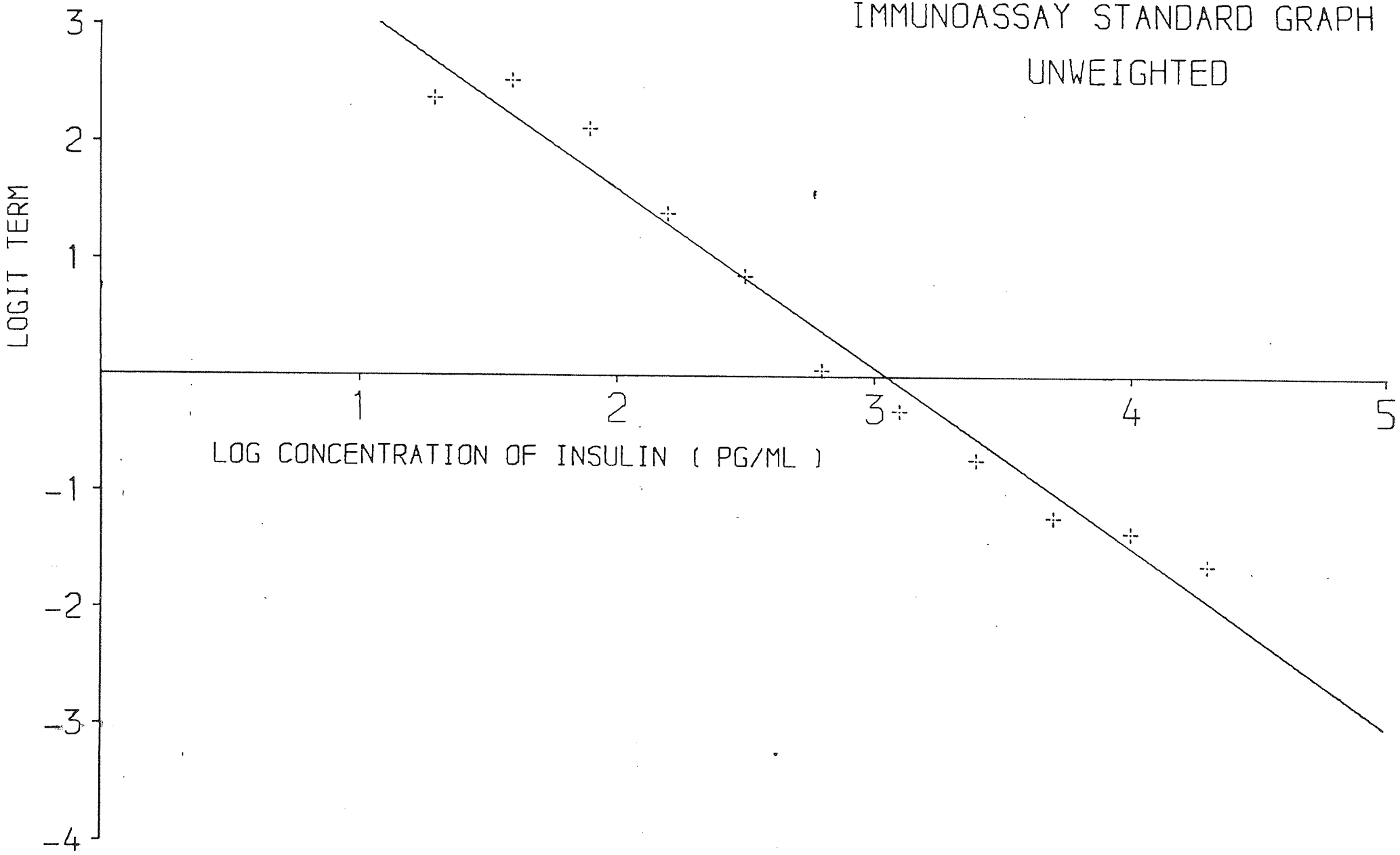
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
B / BO
1
2
3
4
5
LOG CONCENTRATION (PG/ML)


IMMUNOASSAY STANDARD GRAPH
UNWEIGHTED
LOGIT TERM
3
2
1
-1
-2
-3
-4
1
2
3
4
5
LOG CONCENTRATION OF INSULIN (PG/ML)

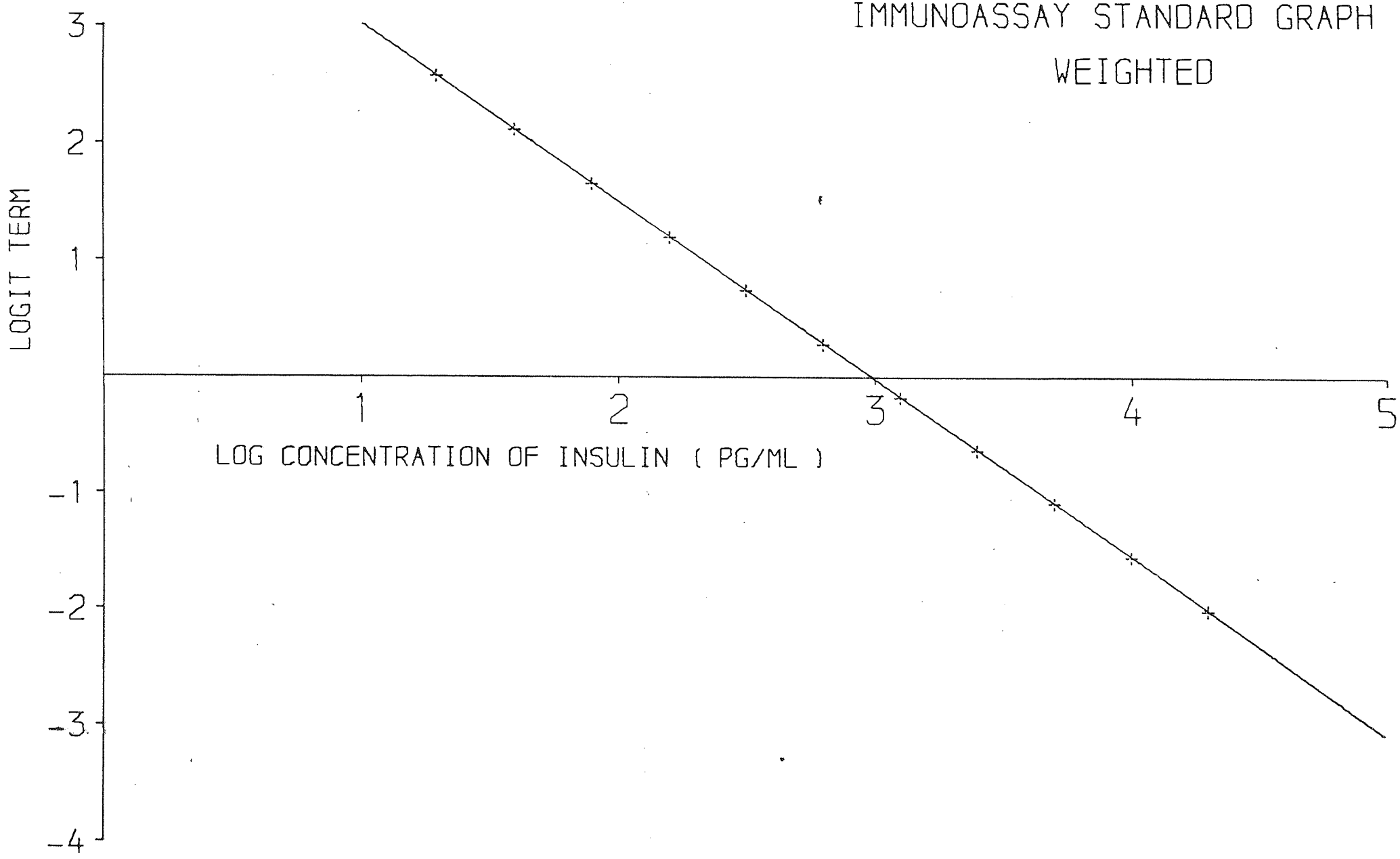

IMMUNOASSAY STANDARD GRAPH
WEIGHTED
LOGIT TERM
3
2
1
-1
-2
-3
-4
1
2
3
4
5
LOG CONCENTRATION OF INSULIN (PG/ML)

Table 1 Protocol for the routine radioimmunoassay of plasma insulin.

	STAGE 1 Insulin free plasma µl added	STAGE 2 Assay diluent µl added	STAGE 3 Standard insulin µl added	STAGE 4 Antiserum µl added	INSULIN pg/tube	STAGE 5 Labelled insulin µl added
STANDARD TUBES (3)	10	90	100	100	2000	100
					1000	
					500	
					250	
					125	
					62.5	
					31.3	
					15.6	
					7.8	
					3.9	
					1.9	
ZERO (4)		190	0	0	0	
NON-SPECIFIC (4)		290	0	0	0	
TOTAL COUNT (4)	0	0	0	0	0	
	Test plasma					
UNKNOWN TUBES						
TEST	10	190	0	100		100
NON-SPECIFIC	10	290	0	0		100

Table 2 Essential computer output from the logit-log analysis of insulin radioimmunoassay data.

ASSAY	B/T	N/T	UNWEIGHTED REGRESSION					WEIGHTED REGRESSION				
			SLOPE (m)	INTERCEPT (c)	CORRELATION COEFFICIENT (r)	t-value	ΣW	SLOPE (m)	INTERCEPT (c)	CORRELATION COEFFICIENT (r)	t-value	ΣW
1	0.45	0.91	-1.89	5.93	0.98	20	11	-1.75	5.38	1.00	771	221
2	0.50	0.95	-2.04	6.23	0.99	22	11	-1.74	5.09	0.99	212	207
3	0.23	0.92	-1.35	4.99	0.99	21	11	-1.39	5.13	1.00	8984	244
4	0.43	0.94	-1.90	5.56	0.97	13	11	-1.67	4.64	0.99	263	227
5	0.43	0.95	-1.69	5.24	0.98	15	11	-1.38	4.06	0.99	165	252
6	0.43	0.95	-1.86	5.92	0.97	13	11	-1.61	4.92	0.99	270	225
7	0.45	0.94	-1.52	4.64	0.98	18	11	-1.51	4.51	1.00	9000	266
8	0.44	0.92	-1.69	5.18	0.98	20	11	-1.71	5.14	1.00	4449	244
9	0.43	0.92	-1.91	6.20	0.97	13	11	-1.61	5.01	0.99	212	220
10	0.41	0.87	-2.00	6.11	0.98	16	11	-1.84	5.48	1.00	578	212
11	0.47	0.94	-1.87	5.63	0.98	16	11	-1.95	5.77	1.00	1442	224
12	0.50	0.93	-2.00	6.51	0.98	17	11	-1.47	4.58	0.99	90	221
13	0.47	0.95	-1.88	5.98	0.97	14	11	-1.74	5.34	1.00	553	223
14	0.42	0.90	-2.13	6.58	0.99	24	11	-1.94	5.78	1.00	404	201

CHAPTER 3

RADIOIMMUNOASSAY OF GLUCAGON

GENERAL INTRODUCTION

Since the discovery of a hyperglycaemic factor present in crude pancreatic extracts of insulin (Murlin et al., 1923) and subsequent purification (Staub et al., 1953) and characterisation of a 29 amino acid single chain polypeptide (Bromer et al., 1957), considerable effort has been applied to the elucidation of the physiology and pathophysiology of glucagon (Bloom, 1974a; Felig et al., 1976; Unger & Orci, 1976; Buchanan, 1977). Initial studies relied on biological properties for quantitative purposes (Sokal, 1972; Luyckx et Lefebvre, 1976). However, the hormonal status of the peptide was uncertain prior to the advent of the glucagon radioimmunoassay (Unger et al., 1959) which enabled the estimation of small amounts of glucagon present in body tissues (Unger et al., 1961, 1962; Aguilar-Parada et al., 1969b).

Glucagon was the second hormone to be measured by radioimmunoassay and came as the first 'spin-off' of the monumental methodological breakthrough of Berson and Yalow (1958, 1959b) which was to reshape diabetes research and biomedical science in general (Unger, 1973, 1976a). However, the development of a method of sufficient sensitivity and specificity to measure the concentration of glucagon in peripheral plasma proved to be considerably more difficult than that for insulin (Marks, 1969). The problem was not a result of species specificity of antisera, since the primary structure of glucagon unlike that of insulin is identical in all mammals studied so far (Bromer et al., 1957, 1971; Sundby & Markussen, 1971, 1972; Thomsen et al., 1972). It was a consequence of the poor stability and weak immunogenicity of the peptide and more important still, the persistent cross reactivity of glucagon antisera with various molecular weight proteins distributed within the pancreas and throughout the gastrointestinal tract (Moody, 1972; Faloona & Unger, 1974b; Buchanan, 1976; Unger, 1976b).

Many of the technical problems which bedevilled early attempts to establish a reliable radioimmunoassay for plasma glucagon have now been recognised and essentially eliminated (Assan et al., 1971; Luyckx, 1972; Faloona & Unger, 1974a; Luyckx & Lefebvre, 1976). Proteolytic destruction of glucagon which occurs during processing and incubation of the plasma sample has been minimised either by the use of proteolytic enzyme inhibitors (Eisentraut et al., 1968; Ensinck et al., 1972), or by employing plasma extraction techniques (Heding, 1971; Buchanan, 1973). Non-specific interference in general has been controlled by ensuring that the standard incubation mixtures are identical to the unknowns in terms of sample composition (Weir et al., 1973; Alford et al., 1977). Iodination damage has been avoided by trace labelling, which employed in combination with purification by ion exchange chromatography yields monoiodinated tyrosinated glucagon of exceptionally high quality (Jørgensen & Larsen, 1972). However, perhaps the most critical factor involved in the establishment of the glucagon radioimmunoassay, and which undoubtedly assures the long term availability of the technique, has been the successful production of high affinity glucagon antisera against glucagon-protein conjugates (Heding, 1972a; Holst & Aasted, 1974). The antisera, which although less potent in terms of binding energy than those directed towards a good immunogen such as insulin, have enabled the attainment of sufficiently high sensitivity in radioimmunoassay to permit the precise estimation of minute physiological concentrations of glucagon present in small volumes of peripheral plasma (Bloom, 1971; Heding, 1971; Buchanan, 1973; Faloona & Unger, 1974a).

Unfortunately the problem of specificity and hence accuracy persists in the glucagon radioimmunoassay (Buchanan, 1977). Although much has been achieved to improve the yield of high affinity glucagon antisera (Holst & Aasted, 1974; Sperling et al., 1974; Garaud et al., 1976), thereby increasing the incidence of antisera suitable for use in

radioimmunoassay, the majority of immune sera react with the N-terminal sequences of the glucagon molecule and consequently display strong cross-reaction with extracts of gut (Buchanan, 1976, a, b). Occasionally an antiserum may be produced which reacts with the C-terminal sequences of the glucagon molecule and shows low cross-reactivity with some species of gut GLI (Heding, 1972a). Such antisera traditionally termed 'pancreatic glucagon specific' have been used extensively in the study of glucagon physiology and pathophysiology (Bloom, 1974a; Unger & Orci, 1976; Buchanan, 1977). However, since even C-terminal reactive antisera react with various species of GLI present in the stomach, small intestine, colon, pancreas and plasma (Vranic et al., 1974; Valverde et al., 1975; Buchanan, 1976a; Recant et al., 1976), the interpretation of all glucagon radioimmunoassays must be made with extreme caution (Heding, 1971; Unger, 1976b; Buchanan, 1977).

A particular problem associated with the variable cross-reactivity of glucagon antisera with potentially heterogeneous forms of glucagon present in the pancreas and other uncharacterised peptides distributed throughout the entire gastrointestinal tract concerns the expression of assay results (Buchanan, 1976a; Field, 1976; Unger, 1976b). In order to clarify this somewhat controversial area, Buchanan (1976a, 1977) has suggested that the material which is measurable by antibodies which react with N-terminal sequences of glucagon be described as N-terminal reactive GLI and that which is measurably by antibodies which react with the C-terminal sequences of glucagon be described as C-terminal reactive GLI. From the structure-function studies of Assan and Slusher (1972) it could then be implied that C-terminal reactive GLI might have the biological activity of glucagon, whereas N-terminal reactive GLI without C-terminal reactivity may not have the biological activity of glucagon.

The general principles involved in the development of radioimmunoassays for insulin and glucagon are identical. However, as described above,

certain fundamental differences exist between the practical approaches which lead to the development of suitable methods for the estimation of these two pancreatic hormones. The present chapter is designed to illustrate these differences and describes the establishment of a reliable radioimmunoassay for glucagon using coated charcoal for the separation of the free and bound hormone moieties. For details of the methodology common to the two techniques, the reader is asked to refer to the appropriate sections in the previous chapter.

PREPARATION OF REAGENTS

ANTIBODY

1. INTRODUCTION

A particular problem associated with the development of a reliable radioimmunoassay for glucagon relates to the preparation of an antiserum of sufficiently high affinity and specificity to enable the accurate determination of minute concentrations of glucagon present in biological fluids (Heding, 1972a). Glucagon, like other members of the secretin-glucagon family of peptides (Bloom, 1974; Rayford et al., 1976a), is a particularly poor immunogen (Heding, 1972a), and this factor alone prevented the estimation of endogenous glucagon in peripheral plasma until some ten years after the initial description of the technique by Unger and colleagues (1959).

Early attempts to produce glucagon antisera by long term immunisation of crystalline glucagon emulsified in Freund's complete adjuvant, in a manner analogous to that employed for insulin (Moloney & Coval, 1955), were relatively unsuccessful (Grodsky et al., 1961; Unger et al., 1961). The antisera produced were characterised by poor binding capacities and although such sera have occasionally been used in radioimmunoassays it appears from the standard curves that the antibodies were of low affinity (Lawrence, 1966; Schopman et al., 1967; Hazzard et al., 1968). A land-

mark in the production of high quality antisera for use in the glucagon radioimmunoassay was the observation that the immunogenicity of glucagon could be considerably enhanced by association of the peptide with larger proteinic or non-proteinic molecules (Assan et al., 1965; Weinges, 1966). Thus glucagon antisera have been raised against various types of immunogens including alum precipitated mixtures of glucagon and egg albumin (Assan et al., 1965; Probst & Colwell, 1966), glucagon adsorbed to polyvinyl-pyrrolidone (Shima & Foà, 1968; Nonaka & Foà, 1969; Edwards et al., 1970a) and glucagon covalently coupled to protein (Assan et al., 1965; Heding, 1969). Each technique has met with a varying degree of success and although two of Unger's most distinguished antisera 30K and G-58 were prepared using alum precipitated and polyvinyl-pyrrolidone adsorbed glucagon respectively (Faloona & Unger, 1974a), recent studies suggest that most consistent results are obtained with chemically prepared protein conjugates (Buchanan, 1971a; Heding, 1972a).

The use of protein conjugated glucagon was first reported by Assan and colleagues (1965) who coupled the peptide to serum albumin using a water soluble carbodiimide (Goodfriend et al., 1964). Since that time, high affinity glucagon antisera have been successfully produced in rabbits and guinea pigs using a variety of conjugates as immunogen, including glucagon polymerised by carbodiimide activation (Heding, 1969; Ruitton & Frederich, 1975; Luyckx & Lefebvre, 1976) and glucagon coupled to serum albumin, thyroglobin, hemocyanin or poly-L-lysine using carbodiimide (Goldfine & Ryan, 1970; Cuatrecasas et al., 1972; Holst & Aasted, 1974; Sperling et al., 1974), glutaraldehyde (Frohman et al., 1970; Garaud et al., 1976), difluorodinitrobenzene (McEvoy et al., 1977; Tager et al., 1977), diethylmalomidate (Grey et al., 1970) or bis-diazo benzidine (Senyk et al., 1972). Carbodiimide has proved to be a particularly useful coupling reagent and the majority of glucagon antisera currently employed in radioimmunoassay, with the important exception of 30K, have been raised against either glucagon polymers or

glucagon-serum albumin conjugates prepared by the carbodiimide condensation reaction (Leclercq-Meyer et al., 1970; Bloom, 1971; Heding, 1971; Buchanan, 1973; Ruitton & Frederich, 1975; Luyckx & Lefebvre, 1976; Alford et al., 1977).

There is evidence to suggest that the coupling procedure employed may influence the specificity of antisera prepared against glucagon-serum albumin conjugates (Garaud et al., 1976). In the studies to be described rabbits and guinea pigs were immunised with glucagon-bovine plasma albumin conjugates prepared using either carbodiimide or glutaraldehyde as the coupling reagent. The resulting antisera were assessed in terms of titre, specificity and suitability for use in the radioimmunoassay of glucagon.

2. MATERIALS AND METHODS

Animals

Female New Zealand White rabbits weighing 2-3 kg. were obtained from a commercial supplier (Hylyne Rabbit Farms, Northwich, Cheshire). The animals were housed individually and maintained on a standard pellet diet (Heygates diet TR2, L.A. Pilsbury, Birmingham) supplemented with fine meadow hay. Male albino guinea pigs were obtained from the usual source at 100-200 g.

Glucagons for immunisation

Two types of crystalline glucagon were used for the preparation of immunogen. Crystalline beef glucagon (lot no. 502277) was obtained from Calbiochem Ltd., London. Twice recrystallised pork glucagon (lot no. G201174) was purchased from Novo Research Institute, Bagsvaerd, Denmark. Both preparations contained less than 0.005% insulin as determined by radioimmunoassay.

Standard glucagon preparation

Freeze dried procine glucagon (MRC, lot no. 69/194, 0.99 IU/mg) provided by WHO International Laboratory for Biological Standards, Holly Hill, Hampstead was used as a reference preparation.

Gut GLI for cross reactivity studies

In addition to using the glucagon preparation described above, the cross reactivity of the antisera was tested with a crude acid alcohol extract of mouse gut enriched with GLI. The extract which was prepared from the entire gastrointestinal tract according to the method described by Kenny (1955), separated into three major peaks of immunoreactivity on a 50 x 1 cm. column of Sephadex G-50 Superfine (Pharmacia Great Britain Ltd., London) corresponding to proteins in the 40,000, 9,000 and 3,500 molecular weight range. The preparation was calibrated using an N-terminal reactive glucagon antiserum (R59, generously donated by Professor K. D. Buchanan, Queen's University, Belfast), and diluted to 2.5 ng equiv/ml glucagon with assay diluent. This antiserum gave identical dilution curves for gut GLI and glucagon.

Preparation of immunogens

Carbodiimide: crystalline beef glucagon was conjugated to bovine plasma albumin (fraction V, batch XC 2671, Armour Pharmaceutical Company Ltd., Eastbourne, Sussex) by modification of the method described by Goodfriend and colleagues (1964). The immunogen was prepared as follows: 50 mg crystalline beef glucagon was dissolved in 0.3 M hydrochloric acid. To this was added 200 pg ^{125}I-glucagon in 1 ml of distilled water supplemented with 2.5 mg bovine plasma albumin, followed by a further 60 mg of albumin in 1.5 ml of distilled water. The solution was made up to 16 ml and 300 mg 1-ethyl-3 (3-dimethyl-aminopropyl)-carbodiimide hydrochloride (Sigma Chemical Company Ltd., London) was added dropwise in 1 ml of distilled water. This mixture was stirred at room temperature

for 24 hours after which the preparation was extensively dialysed against sodium phosphate buffer (0.01M, pH 7.4) containing 0.15 M sodium chloride using 8/32" Visking dialysis tubing (Scientific Instruments Centre Ltd., London). The extent of coupling was 84%, yielding a molar ratio of glucagon to albumin of about 12:1. The conjugate was diluted to 2.5 mg/ml glucagon and stored at -20°C in 500 µl aliquots.

Glutaraldehyde: crystalline pork glucagon was conjugated to bovine plasma albumin (fraction V, batch XC 2671 , Armour Pharmaceutical Company Ltd., Eastbourne, Sussex) by minor modification of the method described by Garaud and colleagues (1976). The immunogen was prepared as follows: 10 mg crystalline pork glucagon, 200 pg ^{125}I-glucagon and 22.5 mg bovine plasma albumin were mixed in 4 ml of sodium phosphate buffer (0.2M, pH 7.3). To this suspension 4 ml 5% glutaraldehyde was added and the mixture was stirred at room temperature (20°C) for 24 hours prior to dialysis as previously described. The extent of coupling was 73%, yielding a molar ratio of glucagon to albumin of approximately 7:1. The conjugate was diluted to 1 mg/ml glucagon, aliquoted and stored at -20°C.

Inocula

The inocula were prepared as water-in-oil emulsions using Freund's complete and incomplete adjuvants. For immunisation of rabbits, the conjugate was thoroughly emulsified with three volumes of adjuvant. A less viscuous emulsion prepared using equal volumes of the two phases was employed for the inoculation of guinea pigs.

Immunisation procedure

Three groups each containing five animals were inoculated with the glucagon-bovine plasma albumin conjugates using a common immunisation scheme. Primary injections were given in complete adjuvant. Secondary

and subsequent injections were given at adjacent sites every four weeks using the same doses of immunogen emulsified with incomplete adjuvant.

A single group of rabbits received multiple intramuscular and subcutaneous injections of the glucagon-bovine plasma albumin conjugate prepared using carbodiimide. Each animal was inoculated with an equivalent of 1.25 mg glucagon in a volume of 1.5 ml distributed equally between 4 intramuscular sites and 7-15 different subcutaneous sites on the back. Immunisation of guinea pigs was performed as previously described, each animal receiving approximately 0.5 ml of an emulsion containing an equivalent of either 500 µg glucagon conjugated using carbodiimide (group C) or 300 µg glucagon coupled by reaction with glutaraldehyde (group D).

Bleeding schedule and storage of antiserum

All animals were bled between 12 and 14 days after the second booster and all subsequent injections. Apart from the bleeding of rabbits which was performed by diagonal incision of the marginal ear vein (Herbert, 1973a), all procedures were identical to those already described.

Antibody assessment

The presence of glucagon binding antibodies was evaluated by incubating increasing dilutions of each serum with 10 pg iodinated glucagon in a final volume of 450 µl containing 125 KIU trasylol (Bayer U.K. Ltd., Haywards Heath, West Sussex). Antibody dilution curves were constructed in duplicate for five dilutions covering a five hundred fold range of concentrations starting at 1:450. Following 48 hours of incubation at 4°C, free and antibody bound glucagon were separated by the addition of coated charcoal, and the distribution of the radioactivity between the two hormone moieties was determined. The 50% maximum binding titre was evaluated graphically.

In order to assess the suitability of the antisera for use in

radioimmunoassay, standard curves were prepared using pork glucagon. Each antiserum was taken at a convenient dilution, binding 35÷45% of the total radioactivity of the 10 pg tracer, and incubated as described in the presence of between 350 and 3.9 pg cold glucagon. At the same time, the cross reactivity of a single antiserum derived from each animal was tested using equivalent amounts of gut GLI, and a fixed quantity (100 ng) of crude cholecystokinin (Sigma Chemical Company Ltd., London), gastrin pentapeptide (Calbiochem Ltd., London) and secretin (Sigma Chemical Company Ltd., London).

Expression of results

In order to facilitate direct comparison of binding displacement data, the results have invariably been expressed as an index given by the equation $[100(Bo - B)/Bo]$, where Bo and B represent counts bound in the absence and presence of 125 pg cold glucagon respectively. This approach, which provides an estimate of antibody affinity, not only serves to eliminate small differences in the initial level of binding but also permits evaluation of displacement in terms of the midpoint of the assay. Antisera commonly employed in the plasma glucagon radioimmunoassay have 50% displacement values in the region of 50-125 pg cold glucagon (Heding, 1971; Buchanan, 1973; Faloona & Unger, 1974a; Luyckx & Lefebvre, 1976). The ratio of displacement between glucagon and gut GLI was used as an estimate of antiserum specificity. Thus a ratio of one would indicate an antiserum reacting equally well with glucagon and gut GLI, while very high values would indicate a high degree of specificity for glucagon.

3. RESULTS

Rabbits and guinea pigs were immunised for a period of seven months with glucagon-bovine plasma albumin conjugates. All animals remained healthy throughout the period of immunisation, and deaths which occurred in the two groups of guinea pigs inoculated with either glucagon conjugated using carbodiimide (group C) or glucagon coupled by reaction with glutaraldehyde (group D) were associated with the bleeding régime. The mortality rate calculated from the percentage of animals lost and the number of cardiac punctures performed was 2.10 and 0.95 for groups C and D respectively.

As illustrated in figure 41a, all animals immunised with the glucagon-bovine plasma albumin conjugate prepared using carbodiimide had developed glucagon antibodies by the tenth week of treatment. The titre of sera obtained from individual animals (expressed in the figure following log-inverse transformation) displayed wide variation and low producers were found in each of the two groups. Despite the extent of variation between the members of each group, consistently higher titres were observed in guinea pigs and, unlike rabbits where the immune response remained fairly constant throughout the course of treatment, enormous surges of antibody production were recorded in the majority of animals. In sharp contrast to the success of the conjugate coupled by carbodiimide condensation in eliciting antibody production in both species tested, the glucagon-bovine plasma albumin preparation synthesised using glutaraldehyde was only weakly immunogenic in guinea pigs (data not illustrated). Thus of the five animals inoculated, only one responded by producing glucagon antibodies, and a maximal titre of 1:9000 (log-inverse titre of 3.95) was observed following administration of the fourth booster injection to this animal.

Antibody affinity, here defined as the ability of small amounts of unlabelled glucagon (125 pg) to compete with the tracer hormone for the

antibody binding sites is plotted against immunisation time for the groups receiving the conjugate prepared using carbodiimide in figure 41b High affinity antisera were found in both groups, but again only guinea pigs exhibited a trend towards increased displacement values as immunisation progressed. Indeed, the mean displacement value of glucagon antisera taken from guinea pigs after the fourth booster injection had risen to 75% corresponding to a midpoint of less than 80 pg glucagon. Slightly lower affinities were observed with the antisera derived from the guinea pig successfully immunised with the glucagon-bovine plasma albumin conjugate prepared using glutaraldehyde (data not illustrated), and the displacement values remained remarkably constant at 56-61% throughout the period of treatment.

The final results of seven months of immunisation are given in table 3, which lists the mean titre, mean affinity and specificity of the glucagon antisera derived from each animal. A total of eight animals, including three rabbits, yielded antisera with displacement values greater than 40% which could be used at titres in excess of 1:3000 (log-inverse 3.48), and were accordingly suitable for use in radioimmunoassay. Such antisera exhibited a wide range of binding characteristics and although no evidence was found to suggest interference from any of the crude gastrointestinal preparations tested, namely cholecystokinin, gastrin and secretin, variable degrees of cross reaction were observed with gut GLI. The rabbits produced large quantities of high affinity antibodies which in two out of three instances reacted poorly with gut GLI. Guinea pigs immunised with the conjugate synthesised using carbodiimide (group C) yielded glucagon antisera comparable in quality to that produced against a good immunogen such as insulin. Such antisera exhibited a high cross reaction with gut GLI. In contrast, poor results were obtained following repeated inoculation of guinea pigs with the conjugate prepared using glutaraldehyde (group D). The animal which

responded to immunisation did however yield high affinity glucagon antisera which displayed partial discrimination of gut GLI.

Standard curves illustrating the reaction of glucagon and gut GLI with selected rabbit and guinea pig glucagon antisera prepared using the glucagon-bovine plasma albumin conjugates are shown in figures 42 and 43. Rabbit glucagon antisera, ROG1/2 and ROG2/3, reacted weakly with gut GLI, corresponding to 6.6 and 3.2% cross reaction at 250 pg equivalents of glucagon. These sera which have formal detection limits of 3.0 and 7.5 pg/ml incubation media respectively will yield reasonably accurate estimates of glucagon concentration. Guinea pig glucagon antiserum GPC1/2 however displayed a strong cross reaction with the gut extract, and yielded essentially superimposable dilution curves for glucagon and gut GLI. This phenomenon suggests that the antiserum which has a formal detection limit of 2.7 pg/ml incubation media, would accurately quantify the sum of glucagon immunoreactivity in a given sample. As illustrated in figure 43, the remaining guinea pig antisera cross reacted to varying degrees with gut GLI, and accordingly can only provide true estimates of glucagon concentration in systems free from contamination with gastrointestinal factors. Such sera contained high affinity glucagon antibodies which provided a formal detection limit in the region of 2 pg/ml incubation media. The partially discriminating glucagon antiserum GPC4/3 was particularly potent yielding a detection limit of 0.9 pg/ml and a midpoint value of 40 pg/ml in radioimmunoassay.

4. DISCUSSION

It has been stated (Hurn & London, 1971; Hurn, 1972) that the successful production of antibodies for radioimmunoassay is more of an art than a science. In this context, success, defined in terms of titre, affinity and specificity, has been particularly difficult to achieve in connection with the production of glucagon antibodies suitable for use

in radioimmunoassay (Heding, 1972a). Thus, although the yield of potentially useful glucagon antisera has been dramatically increased in recent years by employing glucagon-albumin conjugates as immunogens (Buchanan, 1971a), the quality of antisera produced and the resulting sensitivity achieved in radioimmunoassay remain extremely variable (Garaud et al., 1976; Lundqvist et al., 1976; Luyckx & Lefebvre, 1976; Alford et al., 1977; McEvoy et al., 1977; Tager et al., 1977).

The results of the present study confirm the original report of Assan and colleagues (1965) that a consistent immune response may be obtained by repeated immunisation of rabbits and guinea pigs with a water-in-oil emulsion containing a glucagon-albumin conjugate prepared by reaction with carbodiimide. However, unlike many of the early studies (Assan et al., 1965; Frohman et al., 1970; Goldfine & Ryan, 1970) which employed conjugates containing 2-4 moles of glucagon per mole of albumin, the present immunogen, prepared at a molar ratio of 12:1, evoked the production of large quantities of high affinity glucagon antibodies eminently suitable for use in both metabolic studies and radioimmunoassay. Indeed, there has been a general trend in recent years towards the use of conjugates containing approximately 10 haptenic groups per mole of protein (Parker, 1976), and the most successful immunisation programmes with respect to the production of glucagon antisera have employed conjugates containing 10-12 moles of glucagon per mole of albumin (Holst & Aasted, 1974; Garaud et al., 1976; Tager et al., 1977). Although the conjugate prepared by reaction with glutaraldehyde had a lower molar ratio of 7:1, the lack of immunogenicity of the preparation in the majority of guinea pigs inoculated was unexpected. Recent studies performed by Garaud and colleagues (1976) with a similar preparation suggested that glucagon-glutaraldehyde-albumin conjugates evoked the production of C-terminal reactive glucagon antibodies in the rabbit. The reasons for the lack of success in guinea pigs is unclear,

and the sole responder in this group yielded high affinity antibodies which, in accordance with the experience of Frohman and colleagues (1970), displayed only partial discrimination between glucagon and gut GLI.

Repeated immunisation of rabbits and guinea pigs with a glucagon-bovine plasma albumin conjugate prepared by reaction with carbodiimide yielded high quality glucagon antisera suitable for radioimmunoassay which exhibited a wide range of binding characteristics. The response of rabbits to the immunogen was variable, and like previous studies (Goldfine & Ryan, 1970; Holst & Aasted, 1974), some of the animals inoculated failed to produce sufficient quantities of high affinity antibodies to yield potentially useful antisera. The remaining rabbits however displayed a good immune response which in accordance with the observations of Holst and Aasted (1974) using a bimonthly immunisation scheme, remained fairly constant throughout the period of treatment. The resulting glucagon antisera exhibited extremely high affinity (Heding, 1972a) as early as the tenth week of immunisation, further illustrating the potential of glucagon-protein conjugates to stimulate rapid proliferation of high affinity B-cells (Heding, 1969; Goldfine & Ryan, 1970; Tager et al., 1977). Indeed, in contrast to previous reports (Goldfine & Ryan, 1970; Grey et al., 1970), the present conjugate displayed even greater immunogenicity in guinea pigs, and antibody titres which were initially five fold higher than those observed in rabbits, continued to rise as immunisation progressed reaching levels comparable to those recently observed by Tager and colleagues (1977). Surprisingly the affinity of the antibodies was not reduced by the massive levels of synthesis, which presumably represent the products of an essentially inexhaustable clone of high affinity antibody forming cells (Parker, 1971). Thus, in accordance with the observations of Heding (1972a) using protamine zinc glucagon or alum precipitated glucagon, antibody affinity continued to rise progressively throughout the course of immunisation,

and, in the present study, particularly potent glucagon antisera exhibiting midpoint values in the region of 40 pg with detection limits of approximately 1 pg/ml were consistently produced. The reasons for the marked response of guinea pigs to the glucagon-bovine plasma albumin conjugate is not clear since there is no evidence to suggest any inverse correlation between the size of the animal and the titre of the antiserum it produces (Hurn, 1971). The primary structure of the glucagon molecule is identical in all mammalian species thus far studied including pig (Bromer et al., 1957), ox (Bromer et al., 1971), rat (Sundby & Markussen, 1971), man (Thomsen et al., 1972) and rabbit (Sundby & Markussen, 1972). However, Assan and colleagues (1969) have reported that glucagon extracted from the pancreas of the guinea pig, alone of all mammals, deviated in its reaction with anti-beef/pork glucagon antibodies from that derived from any of the aforementioned species. These studies raise the possibility that guinea pig glucagon may differ with respect to either its primary or secondary structure from other species of mammalian glucagon, and although Heding (1972a) has discounted such differences as insignificant, the present studies suggest that even minor anomalies may play an important role in determining the immunogenicity of glucagon-albumin conjugates prepared by reaction with carbodiimide.

Cross reaction studies designed to evaluate the specificity of glucagon antisera, failed to reveal any interference from cholecystokinin, gastrin, secretin, or GIP, VIP and motilin which were presumably present in the crude preparations as impurities (Brown & Parkes, 1967; Brown et al., 1970; Said & Mutt, 1970). However, despite the lack of interference from members of the secretin-glucagon family of peptides (Rayford et al., 1976a), variable degrees of cross reactivity were observed with the yet uncharacterised group of proteins collectively termed gut GLI (Sundby et al., 1976; Buchanan, 1977). The diversity of the antisera with respect

to specificity may be related to the immunogen, since Holst and Aasted (1974) have noticed that the one-step reaction with carbodiimide yields a variety of coupled products. Nevertheless, this incongruity is implicit in all current immunisation schemes (Heding, 1972a), and as yet no method has been devised for the production of glucagon specific antibodies. Indeed, antisera currently employed for the radioimmunoassay cannot be regarded as glucagon specific (Buchanan et al., 1974; Buchanan, 1976a, 1977), since all sera examined in detail including 30K, invariably cross react to a small but significant extent with gut extracts (Heding, 1971; Flanagan et al,, 1974; Sperling et al., 1974; Ruitton & Frederich, 1975; Luyckx & Lefebvre, 1976; Alford et al., 1977). The phenomenon of residual cross reactivity with C-terminal reactive antisera may be a consequence of glucagon containing cells in the gut (Sasak et al., 1975), and the present cross reaction studies were particularly austere because an extract prepared from the entire gastro-intestinal tract, containing significant amounts of the 3,500 molecular weight GLI, was used to assess antibody specificity. In spite of the severity of this test, two rabbit antisera reacted very weakly with gut GLI, corresponding to approximately 5% cross reaction, thereby confirming the apparent tendency for glucagon-albumin conjugates to yield a high proportion of antisera with restricted specificity in this species (Garaud et al., 1976; Tager et al., 1977). Guinea pig glucagon antisera, however, displayed strong cross reaction with the gut extract, and one particular antiserum (GPC1/2) yielded essentially superimposable dilution curves for glucagon and gut GLI. The simultaneous assay of plasma samples with such two antisera of different and well characterised specificity enables one to perform the subtraction radioimmunoassay for glucagon and gut GLI, first described by Heding (1971).

In conclusion, it has been demonstrated that the method of immunisation using a glucagon-bovine plasma albumin conjugate coupled at a molar ratio

of 12:1 using 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide resulted in the development of glucagon antisera suitable for radioimmunoassay in a large number of animals. Such antisera exhibited a broad spectrum of specificity, ranging from those which were relatively specific for glucagon to others which exhibited an identical reaction with glucagon and gut GLI. However, in view of the problems associated with the identification of gut GLI (Samols et al., 1966; Unger, 1966; Buchanan et al., 1967), together with the difficulties posed by marked heterogeneity of glucagon-like peptides in pancreas (O'Connor & Lazarus, 1976), gut (Murphy et al., 1973, Conlon et al., 1975; Holst, 1977) and consequently plasma (Valverde et al., 1974, 1975; Flanagan et al., 1975; Weir et al., 1975), the interpretation of all glucagon radioimmunoassays must be made with extreme caution (Heding, 1971; Unger, 1976b; Buchanan, 1977). Although there is no evidence to suggest that the conclusions of physiological or clinical studies in man or animals based on carefully selected antisera, are qualitatively invalidated by these components (Unger, 1976b), the heterogeneity and diversity of glucagon-like peptides in both the pancreas and gut revealed using cross reacting antisera such as GPC1/2 (figure 44), illustrates the enormous practical, and indeed conceptual problems associated with the use of less well characterised glucagon antisera.

LABELLED ANTIGEN

1. INTRODUCTION

Conventional chloramine-T iodination (Hunter & Greenwood, 1962) is now almost universally employed for the preparation of radioactive iodine-labelled proteins and peptides of high specific activity and has accordingly been used extensively in connection with the synthesis of iodinated glucagon for use in radioimmunoassay (for review see Luyckx & Lefebvre, 1976). Glucagon, unlike the majority of peptides, is however very susceptible to iodination damage which severely reduces the immunological

potency of the labelled species (Schopman et al., 1967; Desbuquois, 1975; Shima et al., 1975). This form of damage leads to rapid spontaneous degradation of the tracer hormone during storage (Faloona & Unger, 1974a), thereby restricting the shelf life of the preparation and markedly reducing assay sensitivity (Luyckx, 1972). Such changes which are commonly minimised by repeated purification of the labelled peptide (Faloona & Unger, 1974a; Luyckx & Lefebvre, 1976), are undoubtedly related to the extent of iodination, since the immunoreactivity of iodinated glucagon is dramatically reduced at levels of substitution involving the introduction of more than one atom of iodine per molecule of glucagon (Desbuquois, 1975). However, Shima and colleagues (1975) have presented convincing evidence to suggest that more subtle forms of damage, resulting from side reactions initiated during iodination with chloramine-T and involving the oxidation of tryptophan and methionine residues of the glucagon molecule, might also have deleterious effects on the energy of binding with C-terminal reactive antisera.

A major advance in the preparation of high quality iodinated glucagon for radioimmunoassay was the introduction of the trace labelling technique (Jørgensen & Larsen, 1972). The reaction which uses a large quantity of glucagon relative to radioactive iodine and a moderate amount of chloramine-T yields an iodinated product of low specific activity corresponding to an average substitution in the region of 0.07 atoms of iodine per molecule of glucagon. Subsequent purification by ion exchange chromatography however, allows separation of the iodinated hormone from both unreacted iodide and the non-labelled peptide, and also permits separation of mono-iodinated-tyrosinated derivatives from the damaged diiodo-tyrosinated glucagon derivatives. The mono-iodinated tyrosinated product is stable, varies little in quality from one iodination to the next and has a specific activity sufficient to permit full exploitation of the sensitivity potential of current glucagon antisera (Jørgensen &

& Larsen, 1972; Bloom, 1974; Desbuquois, 1975; Alford et al., 1977; Buchanan, 1977).

Despite the obvious advantages of the trace labelling technique (Jørgensen & Larsen, 1972) for the preparation of high quality iodinated glucagon for use in radioimmunoassay, the method has thus far failed to gain widespread recognition (Faloona & Unger, 1974a; Luyckx & Lefebvre, 1976). This is undoubtedly due to the unwillingness of individuals and commercial suppliers to change from conventional chloramine-T methods which have been used with moderate, and no doubt in some instances considerable success over a period of years. The present section describes the preparation of iodinated glucagon suitable for routine use in radioimmunoassay. Iodination by minor modification of the method described by Jørgensen and Larsen (1972), followed by purification using anion exchange chromatography yielded high quality ^{125}I-glucagon with a minimum specific activity of 465 μCi/μg. The method has been briefly compared with the conventional chloramine-T procedure employed for the iodination of insulin.

2. MATERIALS AND METHODS

Glucagon for iodination

Highly purified crystalline pork glucagon (lot no. 258-V016-36) was generously donated by Doctor Mary A. Root of Lilly Research Laboratories, Indianapolis, Indiana, U.S.A.

Glucagon for displacement studies

Freeze dried procine glucagon (MRC, lot no. 69/194, 0.99 IU/mg) was provided by WHO International Laboratory for Biological Standards, Holly Hill, Hampstead.

$[^{125}I]$ - Iodide

Sodium iodide $[^{125}I]$ for protein iodination (code no. IMS 30) was supplied as previously described at a specific activity of 11-17 mCi/μg iodine by the Radiochemical Centre, Amersham.

Studies with $[^{127}I]$ - Iodide

In a series of experiments, strictly analogous to those previously described for insulin, glucagon was iodinated by modification of the conventional chloramine-T procedure (see below) using $Na^{127}I$ plus a trace amount of $Na^{125}I$. Critical variables including the amount of chloramine-T (0.39-200 μg) and the reaction time (2-480 seconds) were examined with respect to trichloroacetic acid precipitable radioactivity and binding to excess glucagon antiserum (ROG2/1).

Conventional Chloramine-T procedure

Glucagon was iodinated by modification of the traditional chloramine-T procedure (Hunter & Greenwood, 1962) described in the previous chapter. Where appropriate, the products of the reaction were separated by gel filtration using a 50 x 1 cm column of Sephadex G-50 Superfine (Pharmacea Great Britain Ltd., London). The column was eluted at a constant flow rate of 8.5 ml/hr and 1.25 ml fractions were collected.

Routine iodination procedure

Preparation of high quality radioiodinated glucagon for routine use in radioimmunoassay was performed by minor modification of the trace labelling technique introduced by Jørgensen and Larsen (1972). The following reactants were mixed at room temperature (approximately 20°C) in a small (1.6 ml) glass tube:

Sodium phosphate buffer pH 7.2 (0.4M), 25 μl

Glucagon (10 μg) in sodium hydroxide (0.01M), 25 μl

$Na^{125}I$ (0.5 mCi), 5 μl

Chloramine-T (20μg), 5 μl

The reaction was allowed to proceed for 10 seconds with thorough mixing and was terminated by the addition of sodium metabisulphite (48μg), 20μl. Mixing was continued for a further 45 seconds prior to dilution of the remaining radioactive iodide with potassium iodide (500 μg), 50 μl.

All reactants were weighed and diluted to the required concentration immediately before use. Chloramine-T, sodium metabisulphite and potassium iodide (Hopkin and Williams Ltd., Chadwell Heath, Essex) were dissolved in freshly prepared sodium phosphate buffer, pH 7.2 (0.04M).

Purification and storage of ^{125}I-glucagon suitable for use in radioimmunoassay

Mono-iodinated tyrosinated glucagon was separated from diiodo-tyrosinated glucagon, native glucagon, unreacted iodide and other low molecular weight reactants by ion exchange chromatography (Jørgensen & Larsen, 1972). Thus, upon completion of iodination a further 5 μl of 2M TRIS and 0.5 ml of eluent were added to the reaction mixture, and the resulting solution was transferred to a 28 x 1 cm glass column of QAE Sephadex A-25 (Pharmacea Great Britain Ltd., London) which had been previously equilibriated with eluent. The column was eluted at 5-10°C with 0.08M TRIS, 0.08M NaCl, 0.02M HCl (pH 8.9) containing 0.5% bovine plasma albumin and 50 KIU/ml trasylol, at a constant flow rate of 9ml/hr maintained by a peristaltic pump (MHRE 88, Watson & Marlow Ltd., Falmouth). One-ml fractions were collected in polystyrene tubes (Luckhams Ltd., Burgess Hill, Sussex) using a fraction collector (type 7000, LKB, South Croydon), and after counting, the peak tubes were selected for use in radioimmunoassay. The pooled fraction was diluted with an equal volume of acid ethanol (1.5 ml concentrated hydrochloric acid, 75 ml ethanol), thoroughly mixed and stored at -20°C in aliquots each containing

approximately 2 ml radioiodinated glucagon.

^{125}I-glucagon assessment

In a preliminary study, fractions distributed throughout the entire elution profile of radioactivity were examined with respect to trichloroacetic acid precipitable radioactivity, charcoal adsorption and binding to glucagon antiserum (YY118, partially discriminating N-terminal reactive antiserum, generously donated by Professor K.D.Buchanan, Queen's University, Belfast). The methods which were analogous to those already described for insulin can be used to assess the distribution of radioactivity between the various constituents of each fraction.

Routine evaluation of radioiodinated glucagon was however achieved by analysis of both antibody dilution and inhibition curves prepared under standard assay conditions using 10 pg amounts of the iodinated product. A good tracer was characterised by > 85% binding to excess antibody, > 90% adsorption by coated charcoal, > 95% precipitation with trichloracetic acid and was capable of producing > 10% fall in bound counts on the addition of 30 pg cold glucagon when used in conjunction with a high titre of glucagon antiserum.

3. RESULTS

Radioiodinated pork glucagon was prepared by minor modification of the trace labelling technique described by Jørgensen and Larsen (1972). Iodination of 10 µg glucagon with 0.5 mCi sodium ^{125}iodide at pH 7.2 using 20 µg chloramine-T followed by purification using QAE Sephadex A-25, yielded high quality tracer which stored under appropriate conditions in acid ethanol remained suitable for use in radioimmunoassay for at least three months. The reaction resulted in the incorporation of approximately 0.04 atoms of iodine per molecule of glucagon and the mono-iodinated tyrosinated glucagon component, purified out by high resolution ion

exchange chromatography, had a specific activity of approximately 500 μCi/μg.

Iodination experiments performed by modification of the traditional chloramine-T procedure (Hunter & Greenwood, 1962) with ^{127}iodide together with a trace amount of ^{125} iodide demonstrated an intimate relationship between both the amount and duration of exposure to the oxidising agent and the percentage incorporation of radioactivity together with the immunoreactivity of the product. Although only approximate percentage transfers were obtained due to adsorption of glucagon to the reaction vial and pipette, stepwise increments in the amount of chloramine-T resulted in a progressive increase in the incorporation of radioactivity into trichloroacetic acid precipitable protein which reached maximal values, corresponding to an average substitution in the region of 0.3 atoms of iodine per molecule of glucagon, on the addition of 25 μg of the salt (figure 16). The immunological competence of the product, however, was reduced at amounts exceeding 1.5 μg and steadily decreased until approximately half of the radioactivity incorporated into protein in the presence of 200 μg chloramine-T was immunologically lame. Accordingly, the influence of increasing the reaction time (2-480 seconds) in the presence of 50 μg of chloramine-T had a marked and somewhat variable effect on the parameters studied (data not illustrated). Trichloroacetic acid precipitable radioactivity was maximal following a brief 5 second exposure to the oxidising agent and, together with the immunological competence of the product, slowly declined thereafter reaching values some 75% of the initial count rate by 480 seconds. The results of an iodination procedure based on the conventional chloramine-T method using a 10 second exposure to 50 μg chloramine-T in the presence of 1 mCi sodium ^{125}iodide, as successfully employed for the preparation of radioiodinated insulin, are presented in figure 45. Purification of the iodination mixture by

gel filtration on a 50 x 1 cm column of Sephadex G50 Superfine yielded iodinated glucagon (peak A) with an estimated specific activity of 84 μCi/μg. The preparation which could be used at a minimal practical dose corresponding to 75 pg ^{125}I-glucagon was bound to the extent of 63% by excess glucagon antibodies and produced less than a 1% fall in bound counts on the addition of 31 pg cold glucagon when evaluated for use in radioimmunoassay.

Radioiodinated pork glucagon of exceptionally high quality was prepared for routine use in radioimmunoassay by minor modification of the trace labelling technique described by Jørgensen and Larsen (1972). Purification of a standard iodination mixture by ion exchange chromatography using a 28 x 1 cm column of QAE Sephadex A-25 is illustrated in figure 46. The column which was presaturated with albumin to minimise adsorption of the labelled glucagon was eluted at a constant flow rate of 9 ml per hour, permitting 62% recovery of radioactivity as monoiodinated tyrosinated glucagon. A high proportion, some 26%, of the applied radioactivity remained in the column. The chromatogram which is representative of other iodinations shows two major peaks of radioactivity corresponding to monoiodo-and diiodo-tyrosinated glucagons respectively. The location of the major glucagon peak relative to native glucagon which elutes at fraction ten (casual observation) indicates that the specific activity of the preparation was considerably higher than that of the starting material. As illustrated in figure 47, which shows the physicochemical and immunological properties of the eluted radioactivity, the profile of immunoreactive material, measured using the approximate 50% maximum binding titre of glucagon antiserum, separated into two shallow peaks corresponding to the distribution of radioactivity. Damage assessed from the adsorption of radioactivity to coated charcoal, was greater in the second peak illustrating the potential problems associated with the presence of the diiodo-tyrosinated glucagon derivative. Trichloroacetic

acid precipitable radioactivity remained consistently high, and peak values were associated with the major iodinated glucagon peak which exhibited excellent binding characteristics with excess glucagon antiserum. The top twenty-one fractions (60-80) were selected for use in radioimmunoassay and the pool which contained 8 ng/ml iodinated glucagon, corresponding to an estimated specific activity of 600 μCi/μg, was sufficient for more than 300,000 radioimmunological determinations. This preparation exhibited 88.1% binding to excess glucagon antiserum, 97.6% adsorption to coated charcoal, 99.1% precipitation by trichloroacetic acid and was capable of producing a 12.3% fall in bound counts on the addition of 31 pg cold glucagon when incubated under optimal assay conditions using a high titre of glucagon antiserum. The tracer stored exceptionally well at -20°C in acid ethanol (5-8 ng/ml) and was used in radioimmunoassay for approximately three months. In a single study, using a similar glucagon preparation the percentage binding to excess glucagon antiserum which was initially 86% five days after iodination was 90% at day 14, 88% at day 32, 85% at day 42 and had fallen to 84% by 60 days after labelling.

4. DISCUSSION

The introduction of iodine into the glucagon molecule is associated with marked changes in both the immunological and biological activity of the hormone (Bromer et al., 1973; Desbuquois, 1975). These changes, which are strongly dependent upon the extent of iodination (Desbuquois, 1975), have until recently prevented the preparation of high quality tracer with adequate stability for repeated use in radioimmunoassay (Schopman et al., 1967; Faloona & Unger, 1974a; Luyckx & Lefebvre, 1976). The immunological competence of iodoglucagon is markedly reduced following the introduction of more than one atom of iodine into the glucagon molecule which otherwise occurs with equal frequency at the two tyrosine residues

located at positions 10 and 13 respectively (Desbuquois, 1975). Thus even at an average level of substitution corresponding to 1.01 g atoms/mol, approximately 18% of incorporated radioactivity is associated with species other than the monoiodo-tyrosinated derivative and approximately 83 and 17% of this radioactivity is present in diiodotyrosyl and monoiodohistidyl residues respectively (Desbuquois, 1975). At higher levels of substitution, iodine is predominantly incorporated into these residues, thereby causing further decreases in immunological competence, displacement values and presumably stability of iodinated glucagon (Desbuquois, 1975). To assure the substitution of a single atom of iodine into either of the tyrosine residues a much lower specific activity must be accepted. However, such a compromise is difficult to accept in the context of the glucagon radioimmunoassay where, in order to reach sufficient sensitivity to enable the estimation of physiological concentrations of the hormone, a maximum practical specific activity has often been sought (Luyckx & Lefebvre, 1976).

A further problem associated with the successful iodination of glucagon concerns the subtle damage incurred during exposure of the peptide to chloramine-T (Desbuquois, 1975; Shima et al., 1975). The problems associated with this form of chemical damage are exemplified in the present study which illustrates a dramatic decrease in the immunological competence of glucagon iodinated in the presence of amounts of chloramine-T exceeding 1.5 µg. Thus, under constant reaction conditions, stepwise increments in the quantity of chloramine-T used for iodination resulted in a considerable increase in immunochemical damage which cannot be explained solely on the basis of the relatively minor changes in the extent of substitution. Prolonged exposure to the oxidising agent was also associated with a gradual decline in the immunological competence of iodinated glucagon, but these changes were relatively minor in comparison with the marked effects observed with even small increments

in the amount of chloramine-T. Such subtle forms of damage, which presumably result from side reactions initiated during iodination leading to the oxidation of various amino acid residues, including methionine at position 27 (Shima et al., 1975), can be minimised by accepting a lower iodination yield associated with the reduction in the amount of chloramine-T to optimal quantities. Indeed, Goldfine and colleagues (1972) have prepared iodinated glucagon for use in receptor studies using as little as 0.6 μg chloramine-T. However in the majority of instances considerably larger quantities of the reagent have been employed for iodination (Rodbell et al., 1971; Desbuquois et al., 1974; Shima et al., 1975).

In contrast to the conventional chloramine-T iodination procedure (Hunter & Greenwood, 1962) which in the present study yielded low specific activity iodinated glucagon of relatively low quality, the trace labelling technique introduced by Jøregensen and Larsen (1972) enabled the preparation of monoiodinated tyrosinated glucagon of exceptionally high quality. The success of the method undoubtedly results from the use of large quantities of glucagon relative to small amounts of sodium ^{125}iodide and chloramine-T, thereby reducing both iodination and subtle forms of damage to minimal levels. Purification of the iodinated product, which in the present studies initially contained approximately 0.05 atoms of iodine per molecule of glucagon by anion exchange chromatography, allows complete separation of the monoiodo-tyrosinated glucagon species from unreacted iodide, non labelled peptide and the damaged diiodo-tyrosinated derivatives. The mono-iodinated tyrosinated glucagon product isolated was stable, varied little from one iodination to the next and had a specific activity in the region of 600 μCi/μg, sufficient to enable full exploitation of the sensitivity potential of each glucagon antiserum. The present study therefore confirms the contention (Jørgensen & Larsen, 1972; Bloom, 1974;

Alford et al., 1977; Buchanan, 1977) that iodoglucagon prepared by the trace labelling technique serves as a high quality reagent for use in the glucagon radioimmunoassay.

STANDARD

1. INTRODUCTION

Since the primary structure of the glucagon molecule is identical in all mammalian species thus far studied including pig (Bromer et al., 1957), ox (Bromer et al., 1971), rat (Sundby & Markussen, 1971), man (Thomsen et al., 1972) and rabbit (Sundby & Markussen, 1972), the need for a homologous standard in the radioimmunoassay of glucagon in mammals is probably unnecessary (Luyckx, 1972). Indeed, Assan and colleagues (1969) have reported that glucagon extracted from the pancreas of a wide range of animals including pig, ox, rat, man, rabbit, dog, sheep and mouse yielded identical dilution curves in a radioimmunoassay system using anti beef/pork glucagon antibodies. The only mammal whose glucagon deviated in its binding characteristics with this antiserum was the guinea pig. A major problem associated with the provision of a suitable reference preparation for use as a standard in radioimmunoassay arises from the poor stability of the glucagon molecule (Bromer, 1972 a, b), and the presence of precursor and other potentially heterogeneous forms of glucagon within the pancreas (Unger & Orci, 1976). However, much has been achieved in recent years with respect to this goal and as a result of an extensive international collaborative study, the first International Standard for Glucagon has been established (Annable et al., 1974). Although the provision of such a preparation represents in itself a remarkable achievement, the variable specificity of current glucagon antisera makes absolute comparison of inter-laboratory assay results relatively meaningless (Alford et al., 1977). The present section describes the nature of the preparation, the method of storage, the

preparation of daily working standards and a preliminary test designed to assess the validity of the radioimmunoassay system described.

2. MATERIALS AND METHODS

Standard glucagon preparation

The first International Reference Preparation of glucagon, porcine for immunoassay (lot no. 69/194) was provided by WHO International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Holly Hill, Hampstead, London. The biological potency of the preparation which serves as the International Standard for bioassay (Annable et al., 1974) is 0.99 (0.97-1.03) IU/mg glucagon. The figures in parentheses give the 95% confidence intervals. The glucagon provided was essentially homogeneous as assessed by electrophoresis and by isoelectric focusing in polyacrylamide gel, and no evidence was found to suggest the presence of either desamidoglucagon or glucagon aggregates (Annable et al., 1974).

Storage and preparation of daily working standards

The storage régime and the method employed for the preparation of daily working standards were strictly analogous to those already described for insulin. In brief, glucagon was stored at -20°C in acid alcohol (Kenny, 1955), in the form of two stock solutions. The concentrated stock of 25 µg/ml was used every 2-4 months to prepare a working stock of 0.25 µg/ml which was employed for the preparation of daily working glucagon standards. The top glucagon standard was prepared in assay diluent and thereafter doubling dilutions were made such that the final range of concentrations was 5 ng/ml to 0.019 ng/ml porcine glucagon. Strict ice temperature was observed throughout all manipulative procedures.

Validation of radioimmunoassay

The immunochemical identity of standards and unknowns was assessed from the ability of serial dilutions of an acid ethanol extract of mouse pancreas to yield estimated glucagon concentrations which fall linearly with dilution. Accordingly, 100 µl aliquots of the extract previously diluted to cover a thousand fold range of concentrations starting at 1:1,000, and corresponding to an absolute addition of between 100 nl and 0.1 nl of acid ethanol, were analysed in triplicate under routine assay conditions using a C-terminal reactive glucagon antiserum (ROG1/2) which displays little cross reaction with gut GLI.

3. RESULTS

Standard pork glucagon was stored in aliquots of acid alcohol at $-20^{\circ}C$ as either a concentrated stock at 25 µg/ml or a working stock at 0.25 µg/ml. At regular intervals the working stock was prepared and this was successfully employed for the preparation of daily working standards. Freezing and thawing, proteolytic degradation and glassware adsorption were either completely avoided or reduced to negligible values by this technique and, during the entire period of usage, no evidence was found to suggest that the standard had deteriorated during storage.

The degree of validation of the present radioimmunoassay system was assessed from the ability of the observed glucagon concentration of an acid alcohol extract of mouse pancreas to fall linearly with dilution. As illustrated in figure 48, a significant correlation was observed between the glucagon concentration measured with the C-terminal reactive antiserum and the volume of extract analysed, thereby confirming the immunochemical identity of the reacting species. The apparent glucagon concentration of the extract, calculated from the mean of the individual estimates was 95.03 ± 9.35 µg/ml.

4. DISCUSSION

The most important criterion for the validation of radioimmunoassay concerns the immunochemical identity of the standard and unknowns (Ekins et al., 1968; Yalow & Berson, 1968; Berson & Yalow, 1973). Efforts directed towards the establishment of a valid glucagon radioimmunoassay have until recently been foiled by the lack of control of non specific interference, which together with the variable cross reactivity of glucagon antisera and the heterogeneity of reference preparations has led to wide variations in the estimation of plasma glucagon concentration (Heding, 1971; Weir et al., 1975; Luyckx & Lefebvre, 1976; Alford et al., 1977). Accordingly the provision of a suitable reference glucagon preparation constitutes only one of several essential requirements governing the accuracy of current radioimmunological techniques (Buchanan, 1977). The demonstration of immunological identity between the standard and an acid alcohol extract containing mouse glucagon, therefore confers a high degree of validity upon the method which employs this particular glucagon antiserum.

The pork glucagon adopted as standard in the present studies is currently distributed as an International Reference Preparation for immunoassay by the WHO International Laboratory for Biological Standards in London. The glucagon present in each of these ampoules has been cautiously defined 'essentially homogeneous', as assessed by electrophoresis and isoelectric focusing in polyacrylamide gel, but the preparation is free from contamination with either desamidoglucagon or glucagon aggregates (Annable et al., 1974). A major problem associated with the stability of glucagon during storage concerns the prevention of chemical and physical changes which otherwise serve to reduce the potency of dilute solutions of glucagon (Bromer, 1972 a, b). In the present study, glucagon was successfully stored for extended periods of time in acid alcohol at -20°C. Buchanan (1973) also found standard glucagon

solutions to store well in this solvent system which serves to prevent potential losses of glucagon associated with freezing and thawing, proteolytic destruction and glassware adsorption.

RADIOIMMUNOASSAY

1. INTRODUCTION

Although glucagon was the second hormone to be measured by radioimmunoassay (Unger et al., 1959, 1961; Grodsky et al., 1961), the development of a method of sufficient sensitivity and specificity to enable the accurate quantification of the minute circulating concentrations of glucagon in peripheral plasma has been hampered by numerous hitherto unforeseen technical difficulties (Heding, 1971; Unger, 1973). Problems associated with both the production of high affinity antisera relatively specific for glucagon and the preparation of a good quality tracer hormone have largely been surpassed in recent years, thereby permitting a vast improvement in assay sensitivity and specificity (Bloom, 1974; Luyckx & Lefebvre, 1976). Nevertheless, the level of accuracy attained using current radioimmunoassay systems remains questionable due to the quantitative interference of non specific factors introduced in the unknown sample, which due to the close association between target hormone concentration and the detection limit of the assay, invariably constitutes a large proportion of the total incubation mixture (Weir et al., 1973; Alford et al., 1977). Thus factors introduced in the unknown sample, or indeed inherent in the incubation medium itself, have been shown to have marked deleterious effects on both the fundamental immunochemical reaction and the efficiency of the separation technique (Buchanan & McCarroll, 1971; Heding, 1971). To control the errors associated with the presence of non specific factors, certain steps have now been recognised as essential for the execution of a reliable glucagon radioimmunoassay (Luyckx & Lefebvre, 1976; Buchanan, 1977). Proteolytic

destruction of glucagon which occurs during processing and incubation of the plasma sample must be minimised by the use of either proteolytic enzyme inhibitors (Eisentraut et al., 1968; Assan, 1972; Ensinck et al., 1972) or plasma extraction techniques (Heding, 1971; Palmer et al., 1973). The latter approach introduced by Heding (1971) to avoid the variable degradation of glucagon observed in the presence of large quantities of trasylol, is particularly elegant in that not only does it eliminate the proteolytic enzymes but it also leads to the precipitation of endogenous plasma proteins which would otherwise interfere during the separation stage of the immunoassay. However, even under these stringent conditions, every effort must be made to ensure that all incubation mixtures are identical in all respects other than hormone concentration, and the addition of an equivalent volume of hormone free plasma or indeed plasma extract, to the standard tubes plays a vital role in assuring the reliability of the technique (Weir et al., 1973; Alford et al., 1977).

A considerable number of methods are currently available for the separation of the two hormone moieties on termination of the incubation (Hunter & Ganguli, 1971; Ratcliffe, 1974; Antoniades, 1976), and most of these techniques have at some time been employed in connection with the glucagon radioimmunoassay (Luyckx & Lefebvre, 1976). Thus free and antibody bound glucagon have been separated by a wide variety of physico-chemical and immunological techniques including chromatoelectrophoresis (Unger et al., 1961), paper chromatography (Lawrence, 1966; Assan et al., 1967; Øskov et al., 1968), solid phase coupling (Beck & Hales, 1975; Lundqvist et al., 1976), selective adsorption (Weinges, 1966; Aguilar-Parada et al., 1969a; Nonaka & Foà, 1969; Leclercq-Meyer et al., 1970) and both chemical (Grodsky et al., 1961; Edwards et al., 1970a; Heding, 1971; Henquin et al., 1974) and immunochemical precipitation (Shima & Foà, 1968; Hazzard et al., 1968). Despite this array of methodology, virtually all current glucagon radioimmunoassay systems

rely on charcoal to effect a rapid and efficient separation of free and bound hormone moieties (Buchanan & McCarroll, 1971; Faloona & Unger, 1974a; Leclercq-Meyer et al., 1975; Luyckx & Lefebvre, 1976; Alford et al., 1977). The use of adsorbents such as charcoal, offer a unique advantage in terms of the glucagon radioimmunoassay since non specific effects associated with the analysis of plasma samples can be detected and essentially eliminated (Buchanan & McCarroll, 1971; Weir et al., 1973; Alford et al., 1977).

The present section describes the development of a sensitive and precise radioimmunoassay for glucagon which relies on horse serum and dextran coated charcoal for the separation of free and antibody bound hormone moieties. Although the technique was established in accordance with the precepts outlined in the previous chapters, certain fundamental differences exist between the practical approaches which lead to the development of suitable methods for the estimation of physiological concentrations of insulin and glucagon. The following account emphasises such differences and affords a full description of the routine radioimmunoassay technique together with numerous observations concerning its methodological validation.

2. MATERIALS AND METHODS

Assay diluent

A sodium phosphate buffer (0.04M, pH 7.4) with bovine plasma albumin (0.5%), NaCl (0.3%) and sodium ethyl mercurithiosalicylate (0.02%) was used as a routine assay diluent. The albumin preparation employed (fraction V, batch XC 2671, kindly made available by Mr. B. Gilmour of Armour Pharmaceutical Company Ltd., Eastbourne, Sussex) represented one of many such preparations screened for potential use in radioimmunoassay. This particular batch was judged to be free of interfering substances and enabled the attainment of high sensitivity in the routine assay.

Storage and preparation of reagents

Antiserum, tracer hormone and standard were stored as outlined in the previous sections. The reagents were diluted immediately prior to use with assay diluent to give the working 50% maximum binding titre of glucagon antiserum, 100 pg/ml iodinated glucagon and a range of standard concentrations from 5 ng/ml to 0.019 ng/ml pork glucagon.

Preparation of plasma samples for immunoassay

Blood was collected in small (0.6 ml) polystyrene tubes (Luckhams Ltd., Burgess Hill, Sussex) that had been prewashed with a solution of heparin (500 U/ml) and dried in air. The samples were centrifuged at 4°C and aliquots of plasma were extracted without further delay by a micromodification of the method described by Heding (1971). Strict ice temperature was observed throughout the entire procedure. Plasma extracts were prepared by adding 400 µl of 74% ethanol to 100 µl of plasma, followed by vigorous mixing, centrifugation and decantation of the supernatant into immunoassay tubes containing 20 µl sodium ethyl mercurithiosalicylate solution (0.5 mg/ml). The extracts were evaporated to dryness overnight under negative pressure in a desiccator containing fused porous lumps of anhydrous calcium chloride and stored at -20°C. At the time of assay, the extracts were reconstituted to their original volume by addition of assay buffer (without protein).

Glucagon free plasma extract

In order to minimise the effect of non specific factors introduced in the unknown plasma extracts, glucagon standards were prepared using a reconstituted glucagon free plasma extract. Glucagon free human plasma, prepared as described previously was extracted in bulk with alcohol, aliquoted into amounts equivalent to 5 ml original plasma and blown dry under a stream of air. The extract was stored at -20°C.

Charcoal suspension

Dextran coated charcoal was stored as a 1.0% stock suspension of activated charcoal coated 1:10 with dextran T-70 in sodium phosphate buffer (0.04M, pH 7.4) at 4°C. The suspension was diluted to a final concentration of 0.25% using the same buffer supplemented with 12.5% (v/v) sterile horse serum and mixed with a magnetic stirrer at 4°C for at least 20 minutes prior to use.

Immunoassay procedure

Radioimmunoassay was performed in a manner strictly analogous to that employed for the routine radioimmunoassay of insulin. In brief, standards and unknowns were preincubated for 24 hours at 4°C in 350 µl assay diluent containing 250 KIU trasylol (Bayer U.K. Ltd., Haywards Heath, West Sussex) and the appropriate dilution of glucagon antiserum required to bind approximately 50% of the tracer hormone. Upon completion of the first incubation period, 100 µl diluent containing 10 pg iodinated glucagon was added to each tube and the reaction was allowed to proceed for a further 48 hours prior to separation. Free and antibody bound hormone moieties were separated by the addition of 1000 µl of horse serum and dextran coated charcoal, and the radioactivity associated with the charcoal pellet was determined. The count rate of individual tubes was recorded directly onto paper tape, which was transferred to an ICL 1904S computer and processed according to the program developed by Flatt and Thornburn (1976).

3. METHODOLOGICAL VALIDATION

Assay diluent

The selection of an appropriate assay diluent is of fundamental importance in determining both the sensitivity and reliability of any given glucagon radioimmunoassay system (Luyckx, 1972; Faloona & Unger, 1974a). As illustrated in figure 18, both ionic strength and pH of the

buffer can influence the binding reaction. However, such changes are relatively minor in comparison with the dramatic reduction in assay sensitivity associated with the use of certain albumin preparations. Marked variation in the proteolytic activity of different protein solutions has previously been noted (Buchanan, 1971b; Heding, 1971; Hunter, 1973), and the importance of screening a variety of albumin preparations prior to the adoption of any one for routine usage in radioimmunoassay is illustrated in table 4, which shows the results of antibody dilution curves prepared under standard incubation conditions using assay buffer supplemented (0.5%, w/v) with five different commercial products. A wide range of binding data were obtained under identical conditions, and the quality of each preparation with regard to freedom from contamination with interfering substances and the ability to prevent glassware adsorption could not be related to the source of the protein or to the supplier. Non specific interference as indicated by a reduction in the 50% maximum binding titre, was particularly apparent using Pentex bovine serum albumin, fraction V (lot 246, code 81-003). The reduced level of binding observed in the presence of low concentrations of antibody could be related to the enzyme content of the albumin, however, other forms of interference arising from contamination with glucagon-like components or other less well defined substances cannot be discounted on the basis of these studies. In contrast to the poor results obtained with certain of these albumins, the antibody dilution curve prepared using Sigma crystalline bovine serum albumin (lot no. 1230-1120) displayed considerably enhanced binding characteristics. Almost identical results were obtained with the relatively inexpensive Armour bovine plasma albumin preparation (fraction V, batch XC 2671). On the basis of these studies and subsequent tests designed to evaluate assay sensitivity (figure 18), batch XC 2671 was selected for use in the glucagon radioimmunoassay. More recent analysis of this preparation against

Behringwerke human serum albumin (lot D.351/E.1674/21) with an electrophoretic purity of 100%, has confirmed the exceptional performance of batch XC 2671 in connection with the routine glucagon radioimmunoassay.

Trasylol as a constituent of the standard and unknown incubation mixtures

The susceptibility of iodinated glucagon to incubation damage during radioimmunoassay has been recognised since the initial studies of Ungar and colleagues (1961, 1963). This type of interference which invariably resulted in grossly inaccurate estimates of glucagon concentration in physiological fluids, has been avoided in more recent years by the introduction of proteolytic enzyme inhibitors (Assan et al., 1971). Thus, the addition of trasylol to both standard and unknown incubation mixtures largely eliminates proteolytic degradation (Eisentraut et al., 1968), and, unlike other inhibitors such as benzamidine (Ensinck et al., 1972), does not adversely affect antibody binding (Luyckx & Lefebvre, 1976). To eliminate this potential source of error in the present studies, 250 KIU trasylol was routinely added to all incubation mixtures. As illustrated in figure 49, stepwise increments in the amount of trasylol added had relatively little effect on the shape of the standard curve, confirming the contention that the albumin preparation incorporated in the assay diluent was enzyme-free. Although this system was successfully employed for the analysis of extracts and unknowns which could be diluted several fold prior to incubation, the addition of 100 µl glucagon-free plasma to each of the standard incubation mixtures was associated with a gross reduction in the extent of binding (data not illustrated).

Extraction of glucagon from plasma

Although proteolytic destruction of glucagon which occurs during processing and incubation of plasma can be markedly reduced by the use of trasylol, thereby vastly improving the accuracy of current radioimmunoassay methods, considerable variation in the extent of damage incurred with individual plasma samples, even in the presence of large quantities of trasylol, constitutes an unsolved problem (Heding, 1971). Such discrepancies, together with those resulting from other forms of non specific interference, can be minimised by preparing an individual standard curve in an aliquot of the test plasma which has previously been stripped of glucagon using either charcoal (Weir et al., 1973) or a specific immunosorbent (Alford et al., 1977). These methods are unsuitable in the majority of instances, and an alternative approach, involving the extraction of glucagon from plasma by micromodification of the ethanol technique described by Heding (1971), was adopted in the present studies. The recovery of iodinated glucagon and insulin added to hormone free plasma (approximately 100 pg/ml) was determined after extraction at various ethanol concentrations. As illustrated in figure 50, the recovery of both hormones was greater than 85% at any ethanol concentration less than 65%. A final concentration of 60% was selected for routine extraction. This concentration of ethanol allowed more than 97% of the glucagon to be recovered in the supernatant and permitted the formation of a heavy precipitate of plasma proteins. The recovery of iodinated insulin was 92%.

Concentration of reagents and standard incubation conditions

Although both the concentration of reagents and the incubation conditions can be chosen to meet the requirements of each assay in terms of sensitivity and precision, the attainment of high sensitivity in the glucagon radioimmunoassay is associated with considerable advantages (Buchanan, 1977). The ability to detect minute amounts of the hormone

is indeed a prerequisite to the estimation of physiological concentrations. However, in many instances, high sensitivity also permits considerable dilution of the unknown samples, thereby reducing potential errors associated with non specific interference. Accordingly, optimal assay conditions were employed throughout the present study, enabling full exploitation of the sensitivity potential of each antiserum. The theoretical principles governing the attainment of high sensitivity and precision have been previously discussed.

Separation procedure

In theory, charcoal, coated with molecules of appropriate molecular size and configuration, may be used for a virtually instantaneous separation of any free agent from the same agent bound to a carrier, provided there is a significant difference in molecular size and configuration between the two moieties (Herbert, 1969; Herbert & Bleicher, 1976). One of the most critical factors associated with the use of charcoal for this purpose concerns the selection of an optimal concentration which adsorbs the free agent but leaves the complexed species in solution (Buchanan & McCarroll, 1971; Luyckx, 1972). In the present assay system 2.5 mg horse serum and dextran coated charcoal enabled the efficient separation of free and antibody bound glucagon regardless of hormone concentration up to 100 ng/ml glucagon. As illustrated in figure 51, this amount of charcoal represents an optimum concentration below which the adsorption of iodinated glucagon is incomplete and above which adsorption of bound glucagon occurs. The latter effect is particularly apparent at low titres of glucagon antiserum.

4. RESULTS

A sensitive and precise radioimmunoassay for glucagon has been developed. The technique which relies on horse serum and dextran coated charcoal for the separation of free and antibody bound hormone moieties was routinely employed in conjunction with a fully automated and computerised radioimmunoassay data processing system.

Assay sensitivity and precision

Although attainment of high sensitivity is an essential prerequisite to the development of a reliable radioimmunoassay for glucagon, many antisera characterised by high affinity constants prove unsuitable for routine use due to a lack of specificity (figures 42 & 43). In the present studies, two antisera, exhibiting a weak cross reaction with proteins of gastrointestinal origin, were used for the routine estimation of glucagon concentration. Antiserum ROG1/2, with a detection limit in the region of 3 pg/ml was used for the analysis of glucagon derived from pancreatic origin. However, a well characterised antiserum such as YY89 was employed for routine estimation of glucagon in peripheral plasma. This antiserum which is currently employed for the quantification of circulating glucagon (Buchanan, 1973) reacts with C-terminal fragments of the glucagon molecule and is characterised by a detection limit of approximately 0.9 pg/ml glucagon. Both antisera could discriminate changes of approximately 10 pg/ml at concentrations less than 1000 pg/ml. Intra- and inter-assay reproducibility expressed as a coefficient of variation for concentrations falling within this range were in the region of 5% and 12% respectively.

Maximal sensitivity achieved in the present assay system was observed using the partially discriminating guinea pig antiserum GPC4/3. This antiserum could detect as little as 0.8 pg/ml glucagon in the final incubation mixture, corresponding to a 10% fall in bound counts on the addition of 8.2 pg cold glucagon.

Assay specificity

The most important criterion for the validation of the glucagon radioimmunoassay concerns the specificity of the antiserum employed (Heding, 1971). Rabbit antiserum ROG1/2 cross reacts less than 7% with gut GLI as assessed in the present studies (figure 44), and permits quantitative estimation of glucagon derived from pancreatic extracts (figure 48). Glucagon antiserum YY89 which was employed for the estimation of physiological concentrations of the hormone in peripheral plasma has been extensively characterised by Buchanan and colleagues (Buchanan, 1973; Buchanan et al., 1974; Flanagan et al., 1974, 1975; Conlon et al., 1975). This antiserum reacts with C-terminal fragments of the glucagon molecule and usually shows less than 2% cross reaction with extracts of both procine and human small and large intestine. In human plasma, the antiserum detects a heterogeneity of at least four GLI species including one of similar molecular weight to glucagon, and a large molecular weight component, possibly the size of a globulin, which is excluded by extraction with ethanol. The remaining species are of intermediate size, the predominant one possessing a molecular weight of approximately 12,000 daltons. Neither antiserum cross reacts with any other known pancreatic or gastrointestinal hormone, including secretin, VIP and GIP.

Standard curve

An important feature of the present radioimmunoassay is the application of a fully automated and computerised data processing system to the routine handling of assay results. The programme which was written in ALGOL 60 and is described elsewhere in this thesis, was developed according to the precepts defined by Rodbard and colleagues (Rodbard & Lewald, 1970; Rodbard, 1971, 1974; Rodbard & Frazier, 1975). The method converged rapidly, and following five iterations, a highly significant degree of linear fit was observed. Data from a typical computer output are presented as figure 52.

5. DISCUSSION

Despite the widespread application of radioimmunoassay in the field of biomedical sciences, the development of a sensitive and reliable radioimmunoassay for glucagon has proven considerably more difficult than might at first have been anticipated (Marks, 1969; Unger, 1973; Buchanan, 1977).

A critical factor in determining the validity of the method concerns the production and selection of a suitable antiserum, specific for glucagon, with an affinity constant sufficiently high to enable the quantification of minute concentrations of the hormone (Heding, 1972a). To date, no such antiserum has been produced and antibodies routinely employed in current radioimmunoassay systems invariably cross react, although in some instances only to a small extent, with uncharacterised proteins of gastrointestinal origin (Buchanan, 1977). It is therefore imperative that only well characterised antisera be used to estimate glucagon concentrations in systems contaminated with intestinal factors (Flanagan et al., 1974). The use of antisera regardless of their specificity will result in confusing and spurious estimates (Heding, 1971; Buchanan, 1976a).

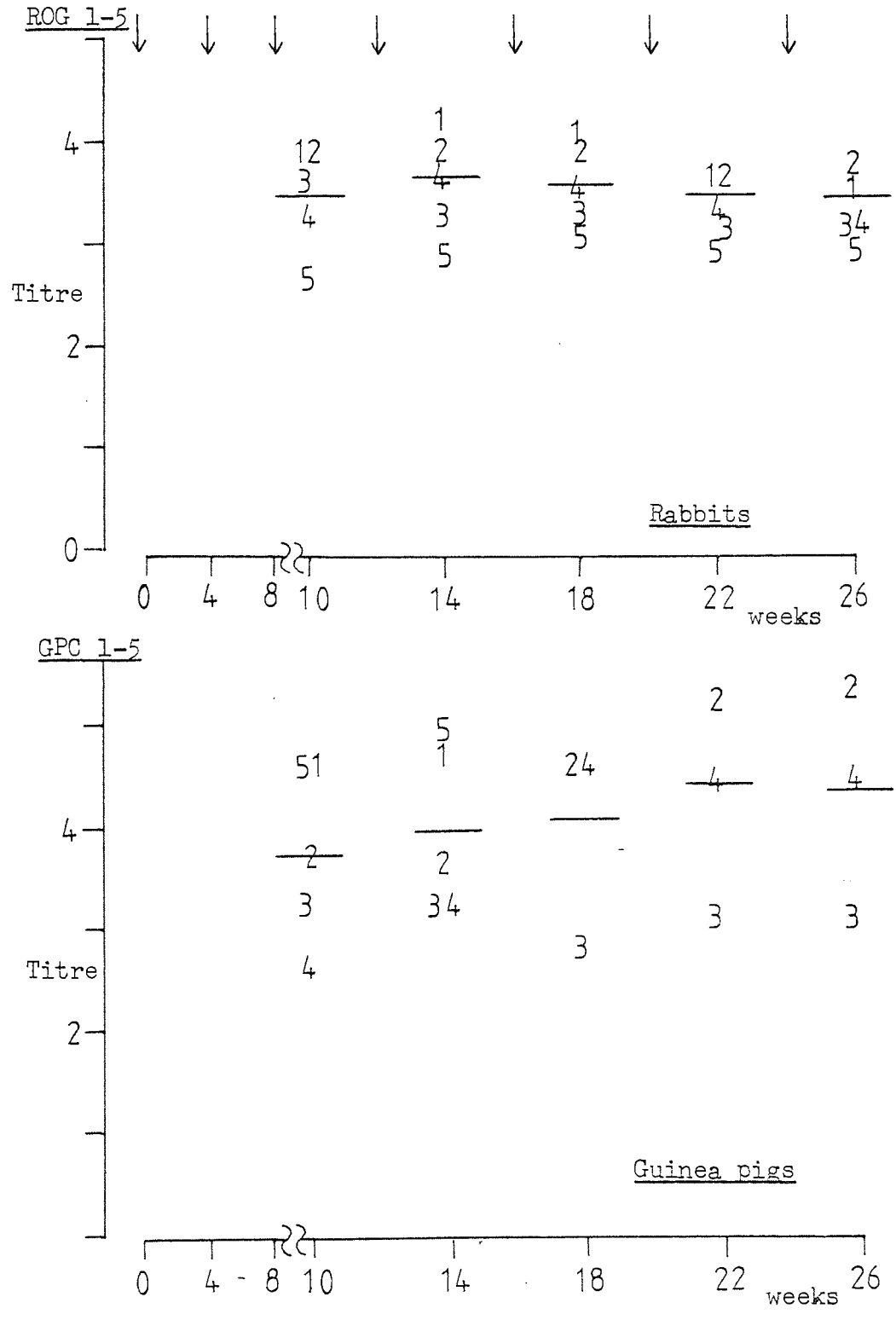
Specificity remains as a major problem in radioimmunoassay, but it is by no means the only factor which governs the accuracy of glucagon estimations. Failure to control non specific interference constitutes an additional source of error which by action on both the primary immunological reaction and the separation stage can markedly affect the precision of each determination (Weir et al., 1973). To minimise this particular form of interference, standard and unknown incubation mixtures must be identical in all respects other than hormone concentration (Buchanan, 1977). The glucagon content of each plasma can be measured against a standard curve prepared in an aliquot of the same plasma previously stripped of glucagon (Weir et al., 1973; Alford et al., 1977). However, an alternative approach adopted with considerable success in the

present study involves the analysis of ethanol extracts. Such an approach minimises individual variation between plasma samples which can accordingly be quantified using a single standard curve prepared in a reconstituted extract derived from a pool of plasma previously treated with charcoal. This method pioneered by Heding (1971), not only serves to eliminate proteolytic enzymes and plasma proteins which would otherwise interfere in the radioimmunoassay, but also excludes certain high molecular weight GLI components which would constitute an additional source of error (Heding, 1971; Buchanan, 1977).

Regardless of the steps taken to reduce non specific interference, it is a considerable advantage, in the context of the glucagon radioimmunoassay, to employ a separation technique which permits detection and correction of non specific effects (Buchanan & McCarroll, 1971). The charcoal separation technique has been widely adopted for this purpose (Faloona & Unger, 1974a; Luyckx & Lefebvre, 1976) and the present study illustrates the potential of horse serum and dextran coated charcoal to effect an efficient and highly reliable separation of free and antibody bound glucagon under routine assay conditions.

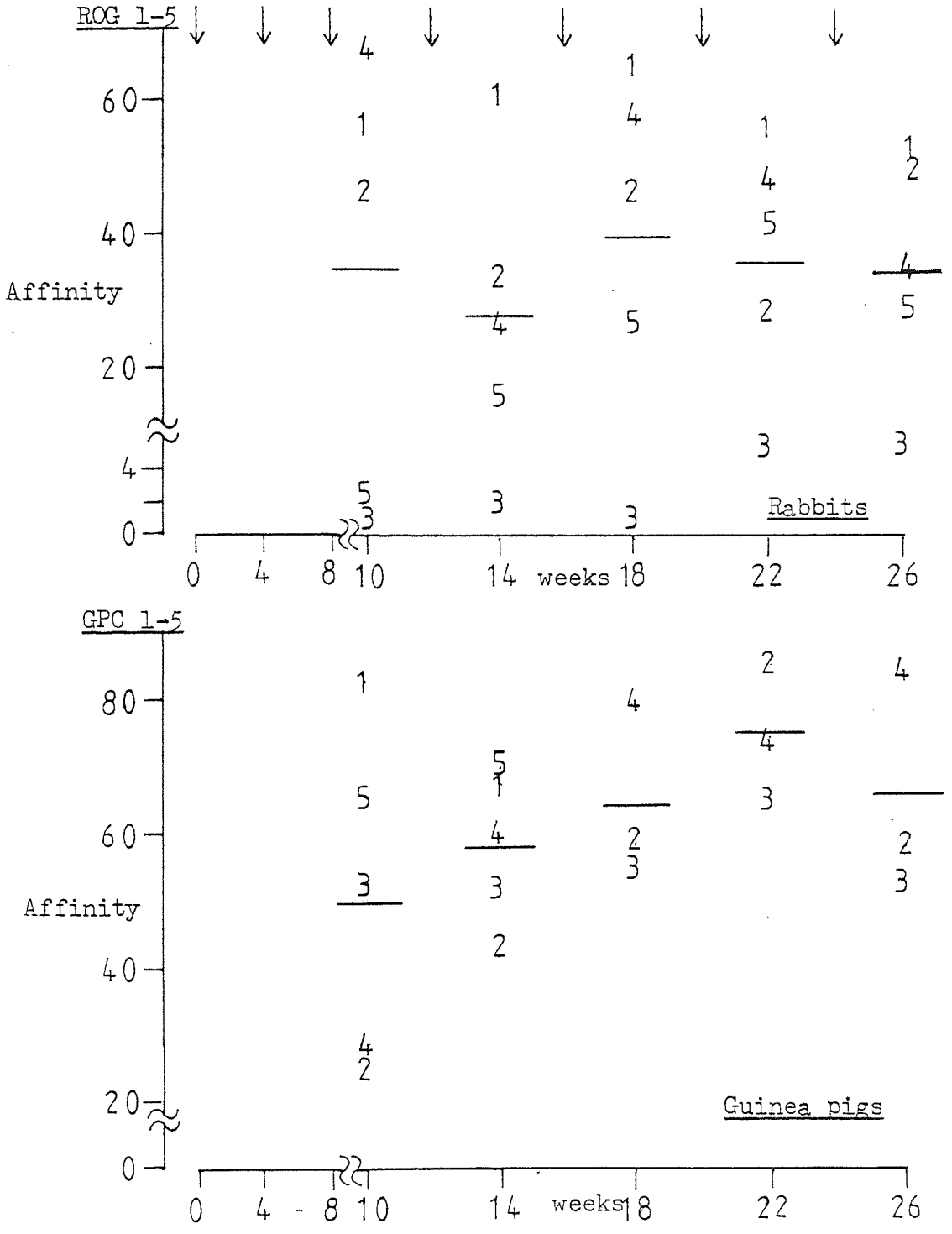
The radioimmunoassay system described in the present section was used in conjunction with a fully automated and computerised data processing system, thereby demonstrating the enormous potential of the logit-log method with respect to routine handling of assay results in both the insulin and glucagon radioimmunoassay.

Figure 4la Development of titre during immunisation of rabbits (ROG 1-5) and guinea pigs (GPC 1-5) with a glucagon bovine plasma albumin conjugate prepared using carbodiimide$^{\phi}$.



$^{\phi}$Titre is expressed as the reciprocal of the final working dilution of serum that will bind 50% of the labelled antigen. Arrows indicate the time of injections.

Figure 41b Development of affinity during immunisation of rabbits (ROG 1-5) and guinea pigs (GPC 1-5) with a glucagon bovine plasma albumin conjugate prepared using carbodiimide ϕ.



ϕ Affinity is expressed as the % fall in bound counts caused by 125 pg porcine glucagon: sensitivity index (displacement) = $(Bo - B_{125})/Bo \times 100$. Arrows indicate the time of injections.

Figure 42 Reaction of discriminating rabbit antisera (ROG 1/2 and ROG 2/3) and the non-discriminating guinea pig antiserum (GPC 1/2) with glucagon (×) and gut GLI (◊).

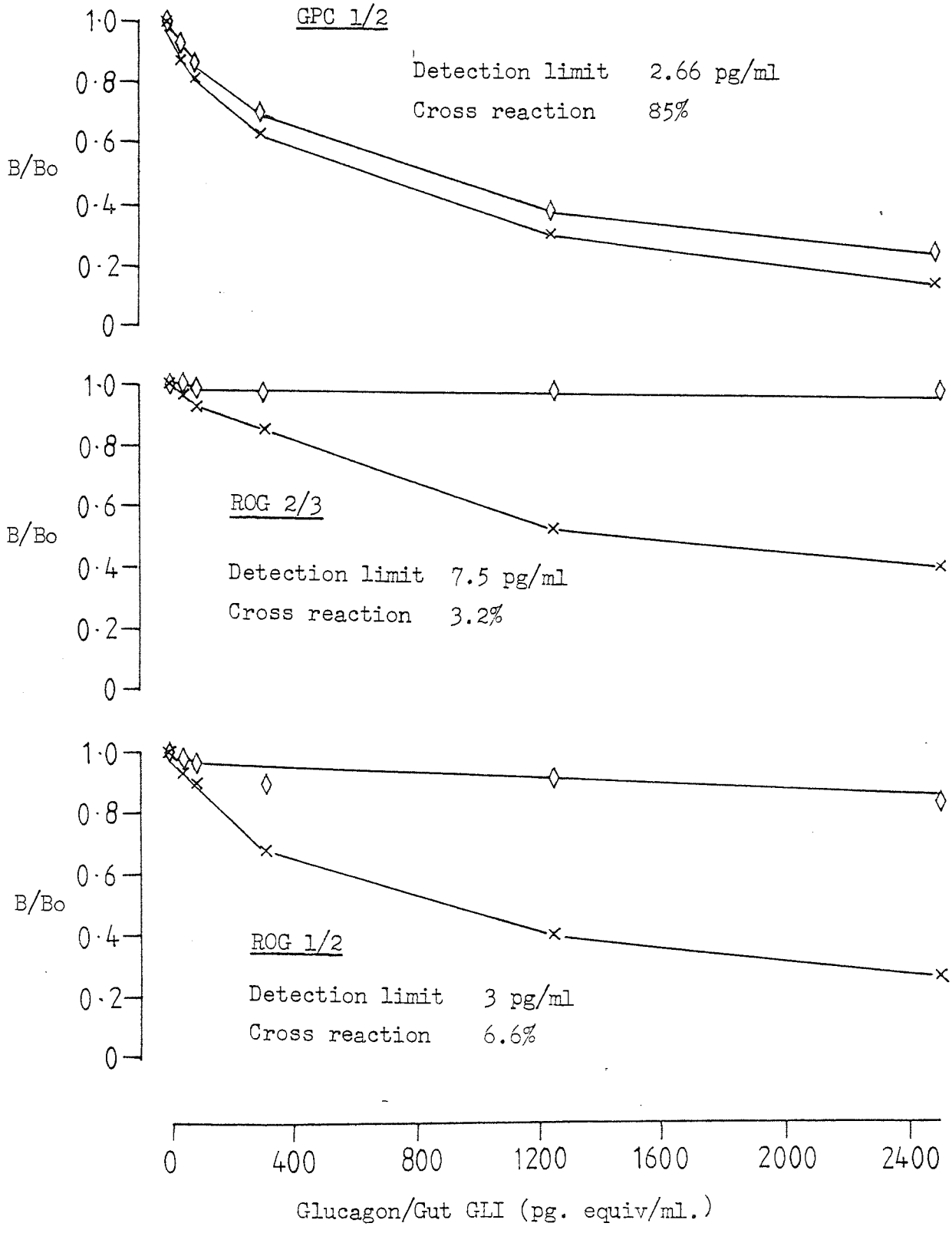


Figure 43 Reaction of partially discriminating guinea pig antisera (GPC4/3, GPC5/2 and GPD4/3) with glucagon (×) and gut GLI (◊).

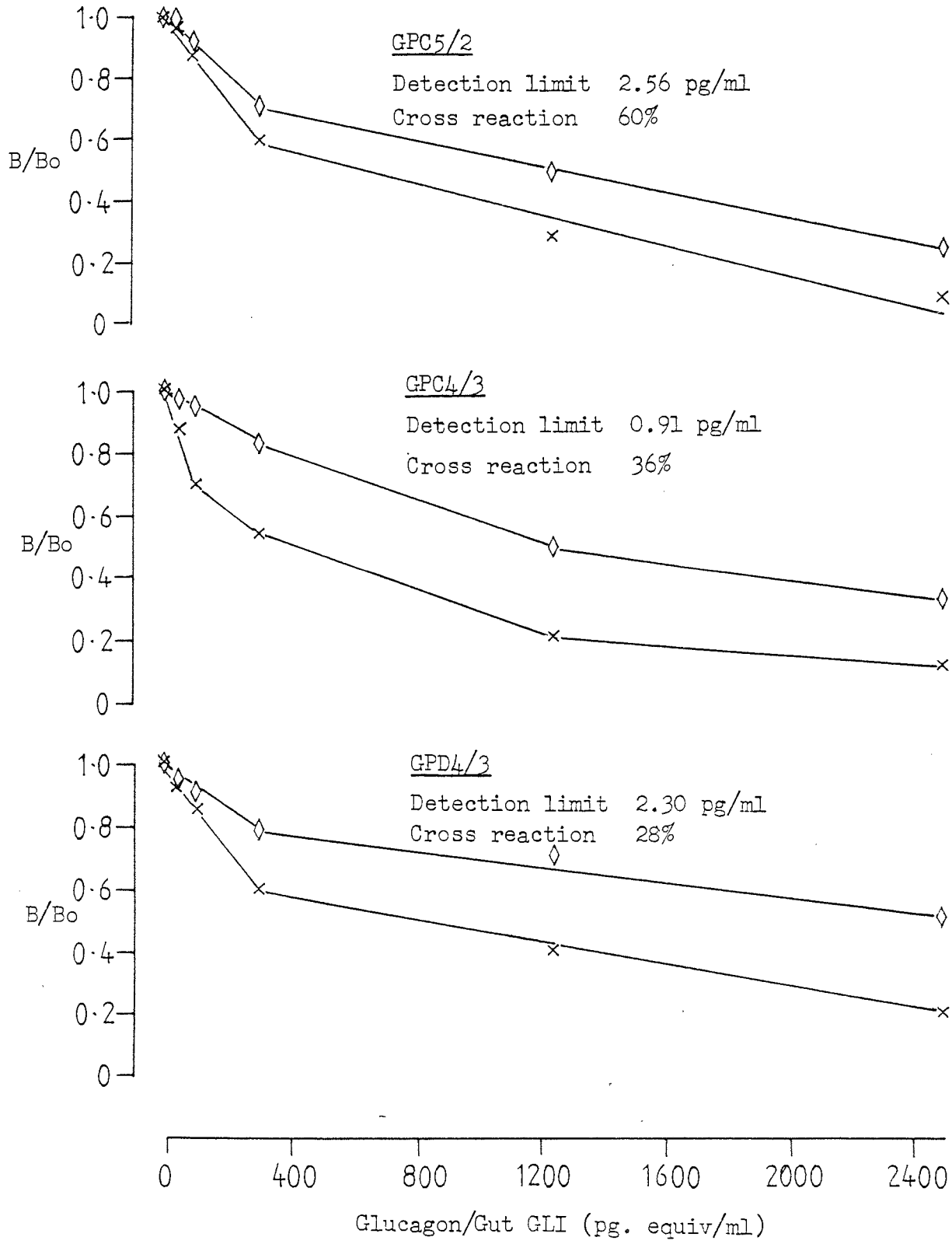


Figure 44 Glucagon-like immunoreactivity profiles of mouse pancreatic and duodenal-ileal extracts on Sephadex G-50 as assessed with an N-terminal reactive antiserum (GPC1/2). Calibration markers: vo, void volume; c, cytochrome-C; i, insulin; g, glucagon; s/p, salt peak.

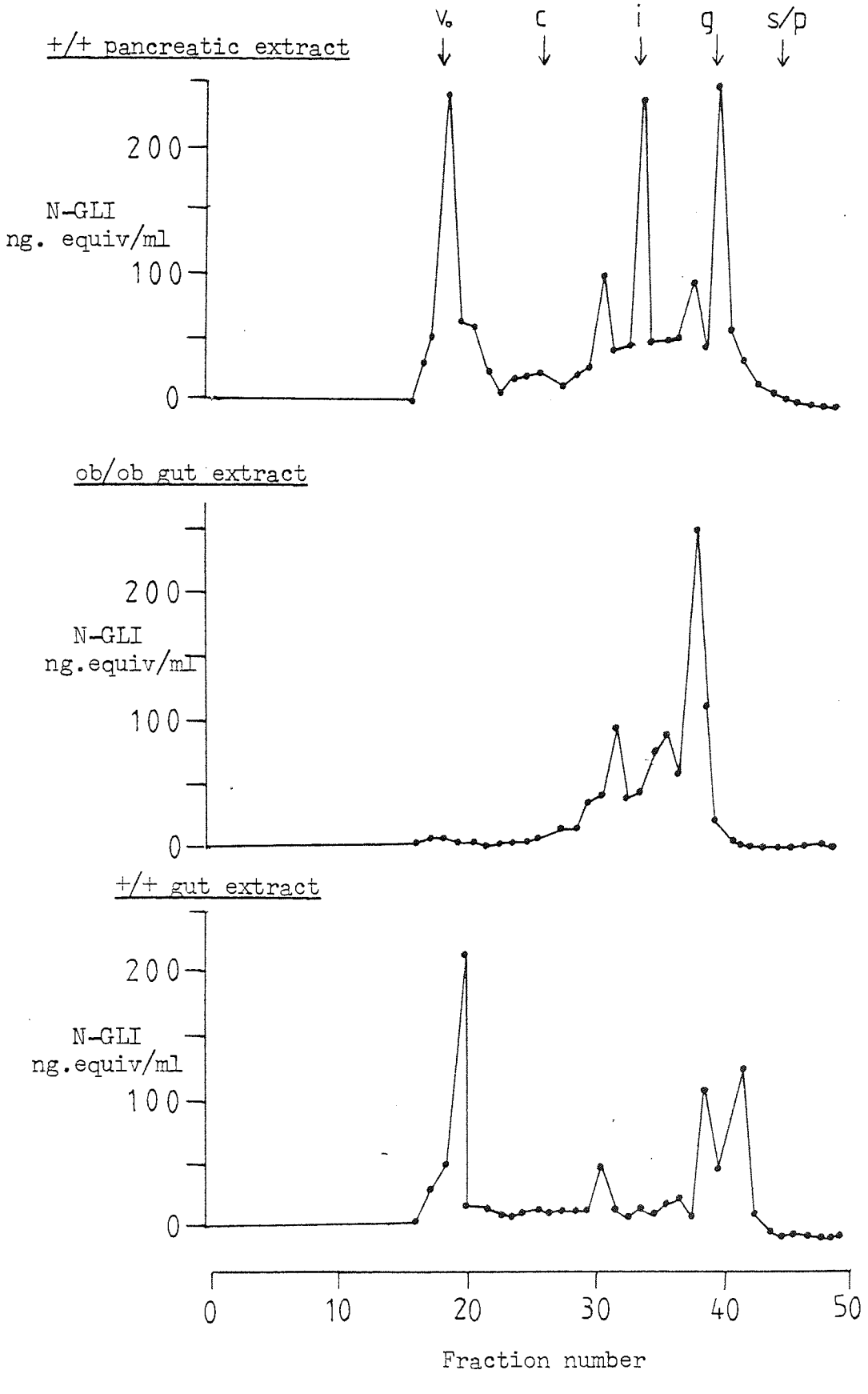
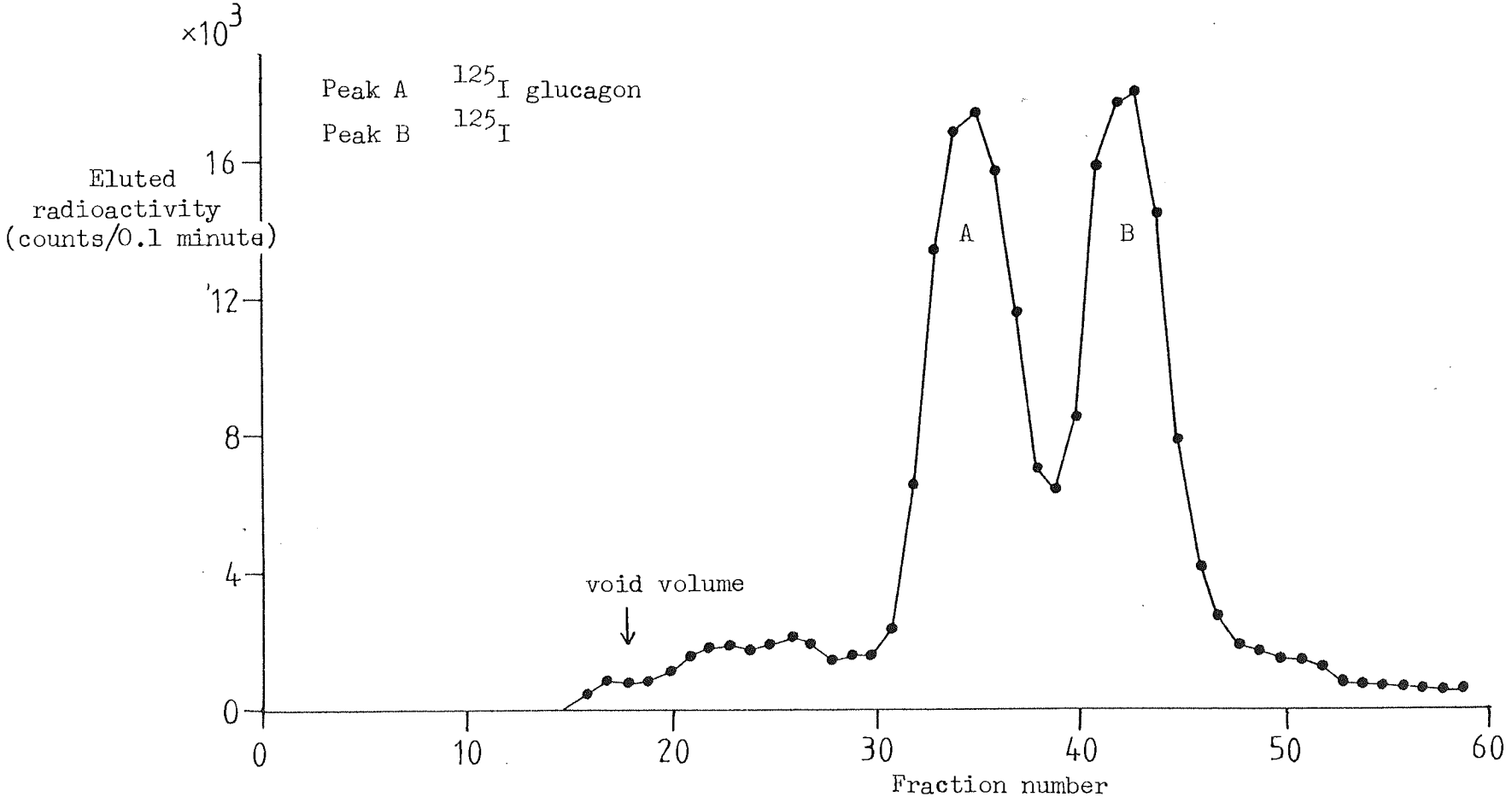


Figure 45 Purification of a conventional glucagon iodination mixture by gel chromatography using a 50 x 1 cm column of Sephadex G-50 Superfine$^{\phi}$.



$^{\phi}$1.25 ml fractions were collected at a flow rate of 8.0 ml/hour.

Figure 46 Purification of a trace labelling glucagon iodination mixture by anion exchange chromatography using a 28 x 1 cm column of QAE Sephadex A25. One ml fractions were collected at a flow rate of 9 ml/hour.

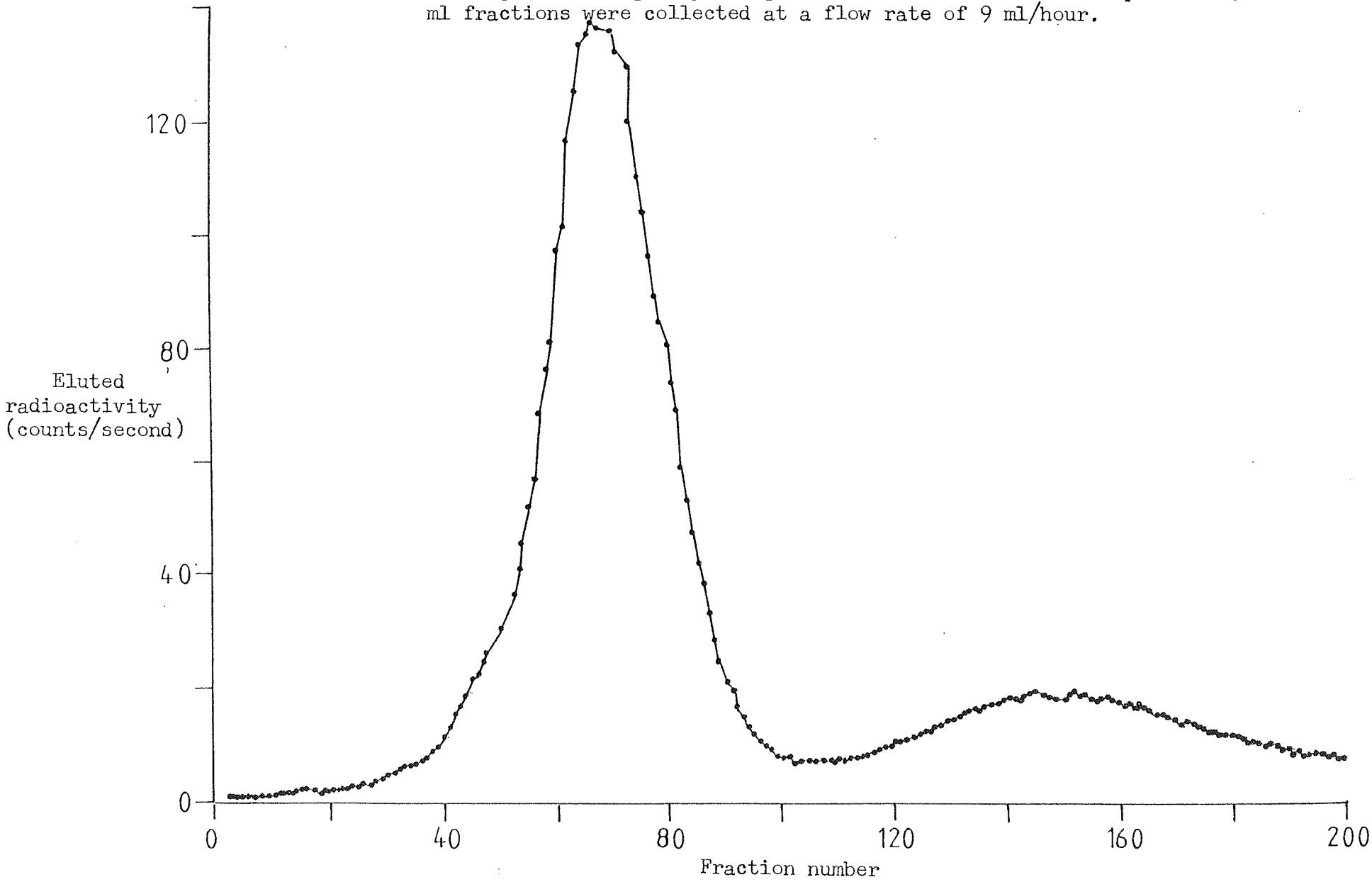


Figure 47 Physicochemical and immunochemical properties of radioactivity eluted during purification of a trace labelling glucagon iodination mixture by anion exchange chromatography.

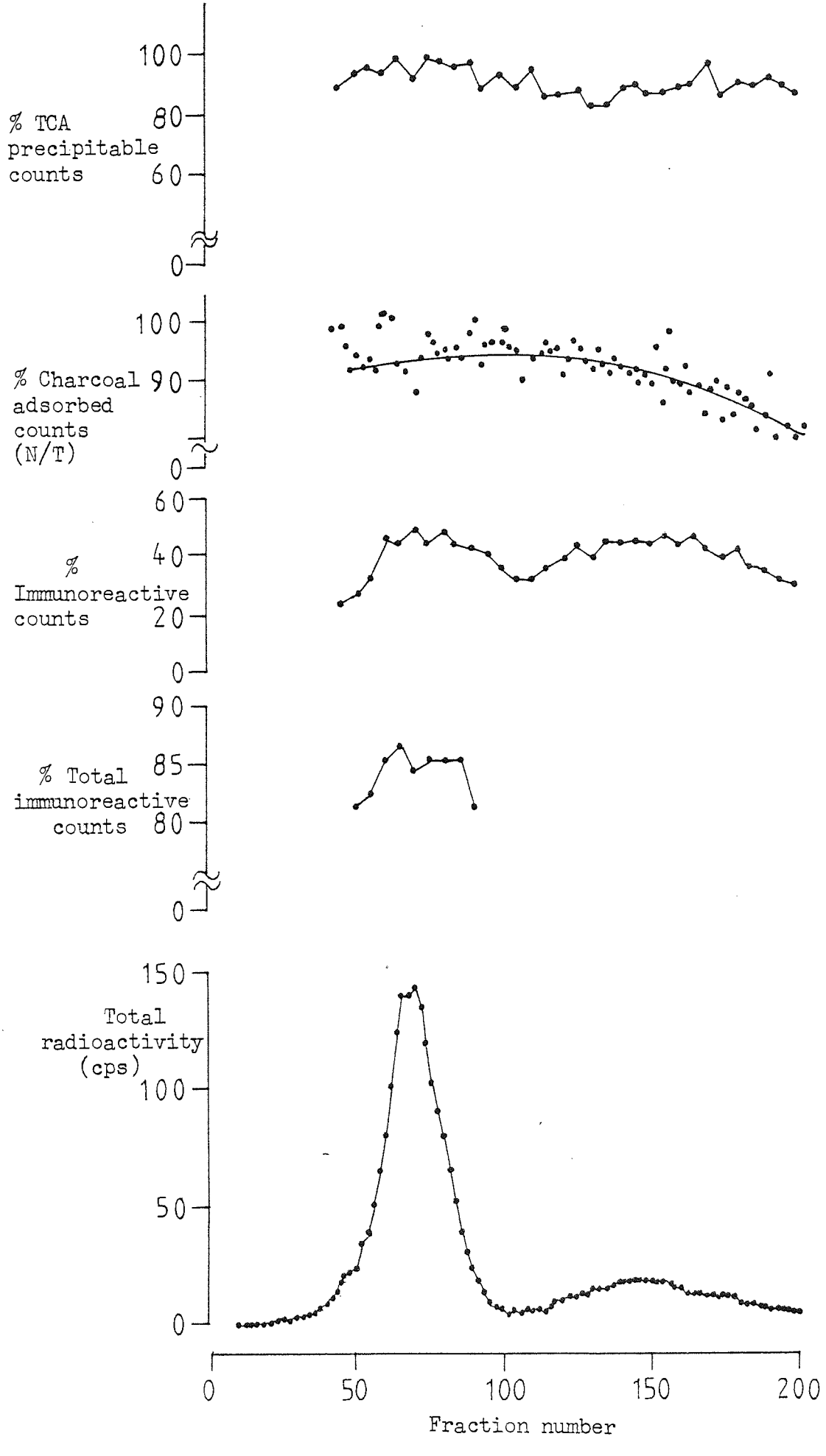


Figure 48 Effect of dilution of a mouse pancreatic extract on the concentration of GLI measured with a C-terminal reactive antiserum (ROG 1/2).

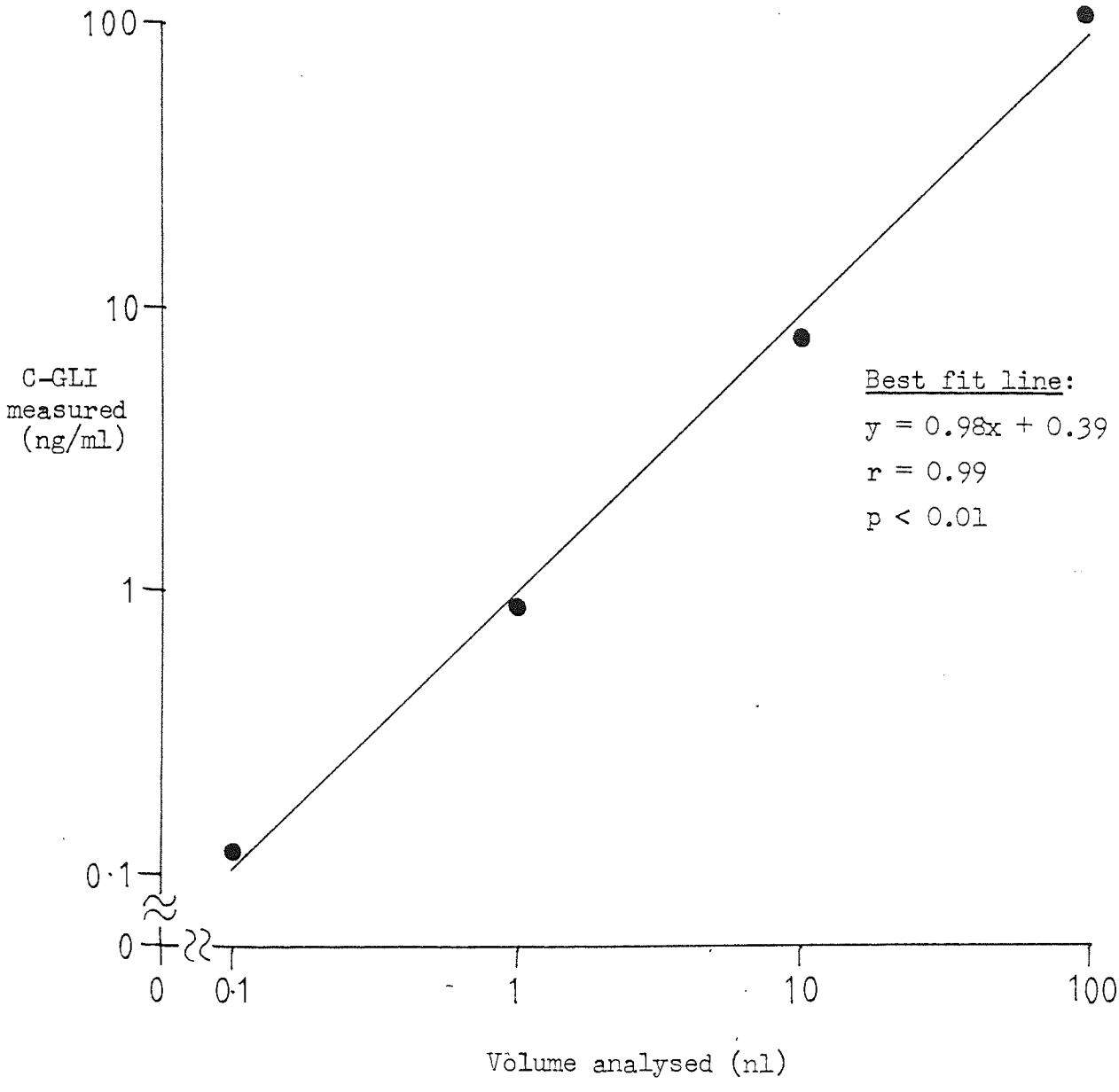


Figure 49 Effect of trasylol on the glucagon standard curve.

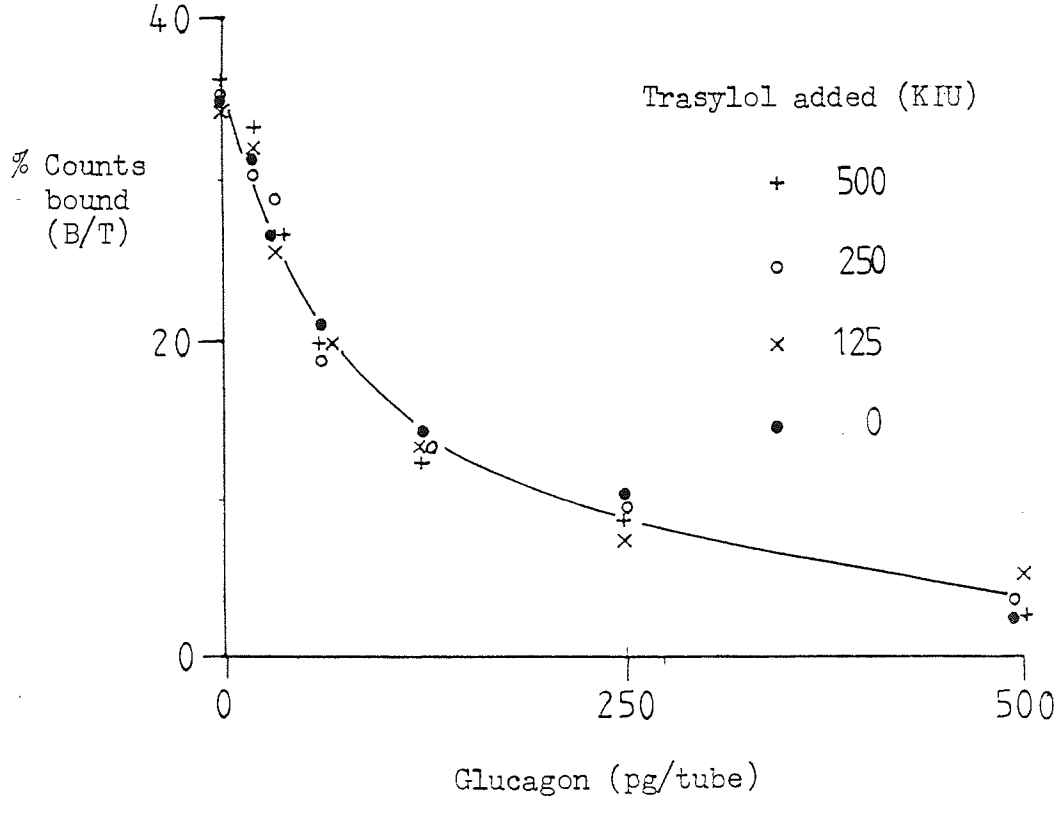


Figure 50 Recovery of ^{125}I-glucagon and ^{125}I-insulin (100 pg/ml) from charcoal treated plasma at various final ethanol concentrations.

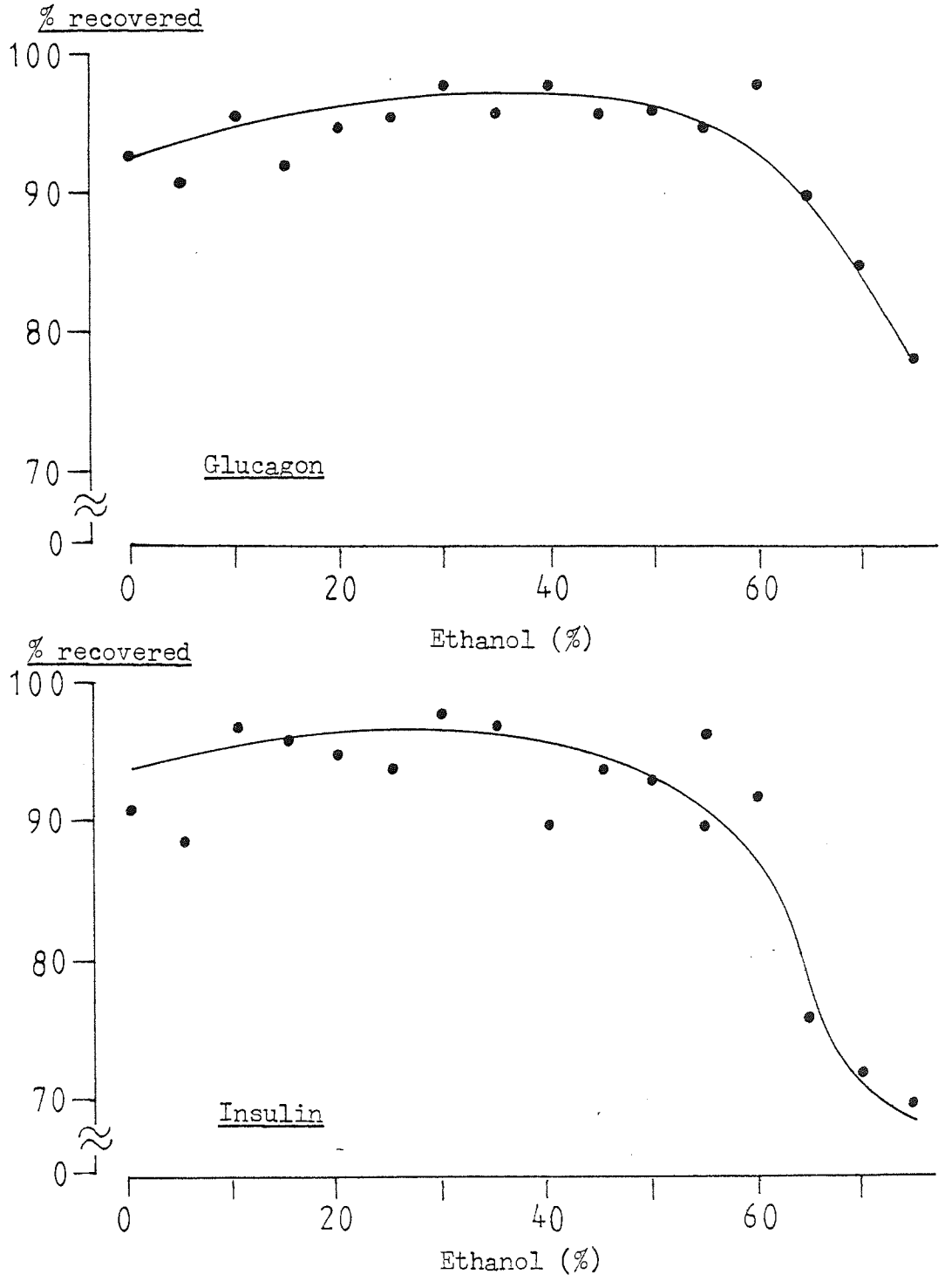


Figure 51 Effect of concentration of coated charcoal on the separation of free and antibody bound ^{125}I-glucagon in the presence and absence of added antiserum.

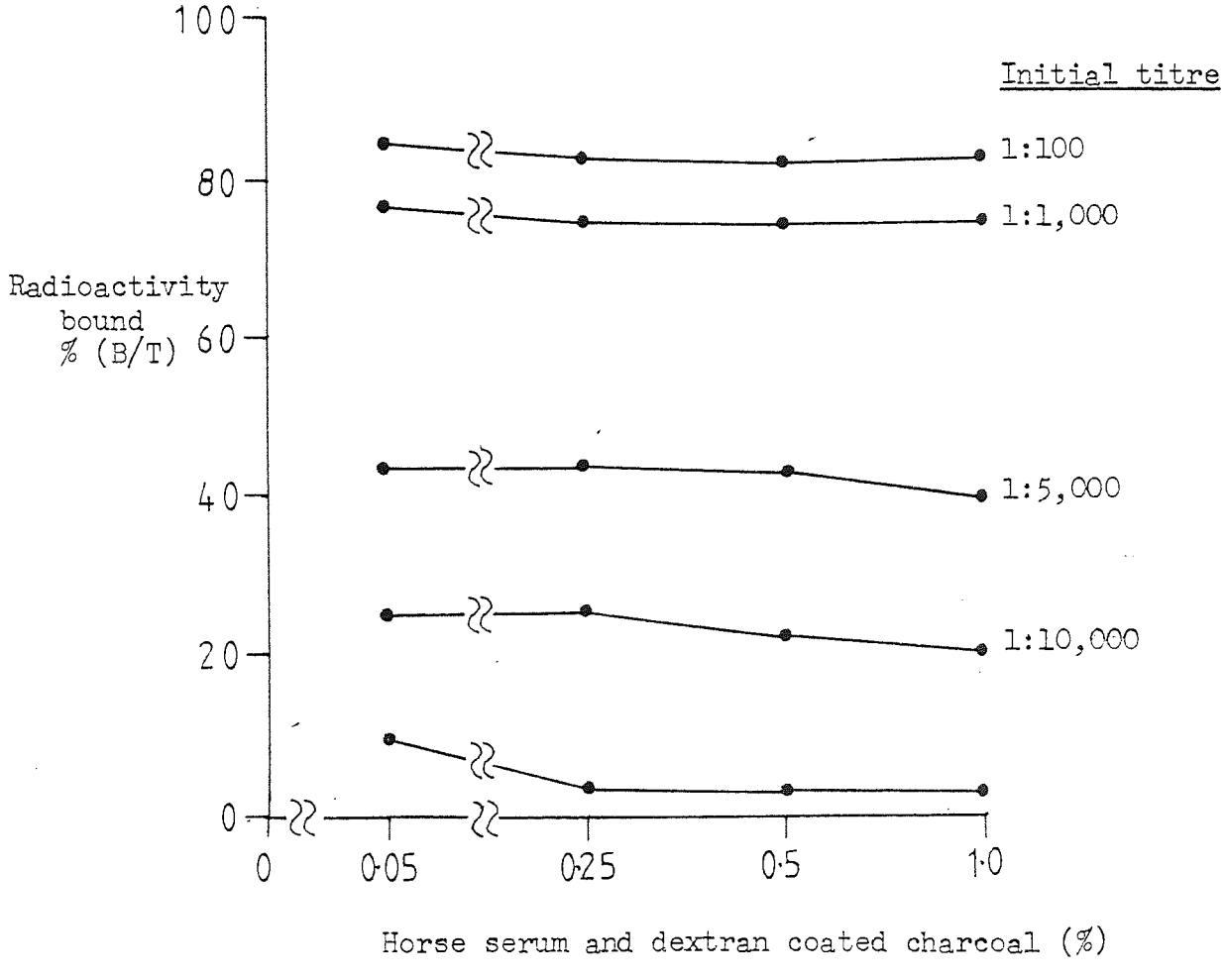


FIGURE 52

SAMPLE COMPUTER OUTPUT FOR GLUCAGON
RADIOIMMUNOASSAY DATA PROCESSING

FILENAME PIRG2
DATA INPUT 6.4.77
GLI RIA
STATICS (SYM/CHOL)
STANDARDS AND 290 UNKNOWNS

LOW STANDARDS OMITTED FROM ASSAY: 0 LOW STANDARDS IGNORED IN CALCULATION: 2

RATIO OF CHANNEL 1 : CHANNEL 2 (CR) = 1.04234

STANDARDS

MODE	CASSETTE	TUBE	TIME MIN X 0.01	CHANNEL 1	CHANNEL 2	CHANNEL 2 x CR.	COUNTS PER MINUTE(CHANNEL 2 x Cp) CHANNEL 1	CHANNEL 2	MEAN	S.D.	OUTLIERS OUTSIDE MEAN +/- 1.5 x SD	CONCN. PG/ML.
1	118	1	400	17527	16866	17580	4381.75	4395.02				
1	118	2	400	18314	17607	18352	4578.50	4588.12				
1	118	3	400	17879	17161	17888	4469.75	4471.89	4480.64	87.67		5000
1	118	4	400	16864	16218	16905	4216.00	4226.16				
1	118	5	400	16777	16159	16843	4194.25	4210.79				
1	118	6	400	16720	16080	16761	4180.00	4190.20	4202.90	17.52		2500
1	118	7	400	14627	14062	14657	3656.75	3664.34				
1	118	8	400	14707	14169	14769	3676.75	3692.23				
1	118	9	400	14834	14334	14941	3708.50	3735.22	3688.96	29.41		1250
1	118	10	400	13025	12563	13095	3256.25	3273.73				
1	3	1	400	13041	12558	13090	3260.25	3272.42				
1	3	2	400	13157	12650	13186	3289.25	3296.40	3274.72	15.73		625
1	3	3	400	11724	11293	11771	2931.00	2942.78				
1	3	4	400	12307	11813	12313	3076.75	3078.29				
1	3	5	400	12117	11641	12134	3029.25	3033.47	3015.26	64.24		313
1	3	6	400	11315	10849	11308	2828.75	2827.08				
1	3	7	400	11225	10801	11258	2806.25	2814.58				
1	3	8	400	11597	11143	11615	2899.25	2903.70	2846.60	43.32		156
1	3	9	400	10738	10280	10721	2684.50	2680.37				
1	3	10	400	11381	10921	11383	2845.25	2845.85				
1	143	1	400	11796	11311	11790	2949.00	2947.47	2825.41	119.90		78
1	143	2	400	10714	10286	10721	2678.50	2680.37				
1	143	3	400	10924	10511	10956	2731.00	2739.01				
1	143	4	400	11430	11004	11470	2857.50	2867.47	2758.98	84.04		39
1	143	5	400	10853	10483	10929	2713.25	2732.23				
1	143	6	400	11193	10744	11199	2798.25	2799.72				
1	143	7	400	11038	10617	11067	2759.50	2766.63	2761.60	34.72		20
1	142	4	400	10451	10054	10480	2612.75	2619.92				
1	142	5	400	10718	10281	10716	2679.50	2679.07				
1	142	6	400	11310	10858	11318	2827.50	2829.43				
1	142	7	400	11235	10812	11270	2808.75	2817.44	2734.30	95.69		ZERO
1	142	8	400	20001	19191	20004	5000.25	5000.88				
1	142	9	400	19525	18747	19541	4881.25	4885.18				

1	142	10	400	19850	19076	19886	4962.50	4971.44			
1	152	1	400	19603	18764	19558	4900.75	4889.61	4936.48	52.47	BNS
1	152	2	400	20440	19582	20411	5110.00	5102.77			
1	152	3	400	20762	19897	20739	5190.50	5184.85			
1	152	4	400	20244	19406	20230	5061.00	5057.43			
1	152	5	400	20606	19729	20564	5151.50	5141.08	5124.89	51.01	TC

VALUES OF TC AND BNS RESPECTIVELY ARE 5124.89 4936.48 DIFFERENCE = 188.41 RATIO = 0.9632 B0 = 2202.19

MEAN TOTAL COUNT = 20499.56 S.D. = 204.042 PIPETTING ERROR = 101.535 MEAN B0/T = 0.42970

CONCN.	B	MEAN B	(MEAN B)/T	B/B0	MEAN B/B0	VARIANCE	C.OF V. (SD/MEAN X 100)	ASSAY ** PRECISION (PG/ML)
5000	554.73			0.252				
	541.46			0.246				
	357.98			0.163				
	348.37			0.158				
	466.73			0.212				
	464.59	455.64	0.08891	0.211	0.20691	1.5858 -3	19.241	175.24
2500	720.48			0.327				
	710.32			0.323				
	742.23			0.337				
	725.69			0.330				
	756.48			0.344				
	746.28	733.58	0.14314	0.339	0.33312	6.3328 -5	2.389	37.54
1250	1279.73			0.581				
	1272.14			0.578				
	1259.73			0.572				
	1244.26			0.565				
	1227.98			0.558				
	1201.26	1247.52	0.24342	0.545	0.56649	1.7848 -4	2.358	18.11
625	1680.23			0.763				
	1662.76			0.755				
	1676.23			0.761				
	1664.06			0.756				
	1647.23			0.748				
	1640.09	1661.77	0.32425	0.745	0.75460	5.1058 -5	0.947	5.94
313	2005.48			0.911				
	1993.70			0.905				
	1859.73			0.844				
	1858.20			0.844				
	1907.23			0.866				
	1903.02	1921.23	0.37488	0.864	0.87242	8.5008 -4	3.344	18.67
156	2107.73			0.957				
	2109.40			0.958				
	2130.23			0.967				
	2121.91			0.964				
	2037.23			0.925				
	2032.79	2089.88	0.40779	0.923	0.94900	3.8704 -4	2.073	9.45
78	2251.98			1.023				

	2256,11			1.024					
	2091,23			0.950					
	2090,64			0.949					
	1987,48			0.903					
	1989,01	2111,08	0,41193	0.903	0,95863	2,9648 -3	5,679		98,95
30	2237,98			1.025					
	2256,11			1.024					
	2205,48			1.001					
	2197,46			0.998					
	2078,98			0.944					
	2069,01	2177,51	0,42489	0.940	0,98879	1,4568 -3	3,860		11,25
20	2223,23			1.010					
	2204,25			1.001					
	2138,23			0.971					
	2136,76			0.970					
	2176,98			0.989					
	2169,85	2174,89	0,42438	0.985	0,98760	2,4858 -4	1,596		57,44

AT ZERO CONCENTRATION (= SENSITIVITY): 15,365

** (NOTE: "ASSAY PRECISION" BASED ON C.V. OF RAW COUNT RATES). THE TWO HIGHEST CONCENTRATIONS USE THE SAME SLOPE)

FINAL VOLUME 450 UL MOLECULAR WEIGHT 3500 TRACER CONCENTRATION 100 PG/ML

SCATCHARD PLOT: VALUE OF P* = 6,349								
B/T(P* + P):	28,79	23,63	20,86	14,93	9,82	6,63	4,66	3,75	3,27
B/T/(1-B/T):	0,10	0,17	0,32	0,48	0,60	0,69	0,70	0,74	0,74

SLOPE M = -0,0261 INTERCEPT C = 0,8410 COEFFICIENT R = -0,9938
T-VALUE = 23,6971 7 DEGREES OF FREEDOM
INVERSE OF SLOPE (L/PMOLE) = -38,278 X-INTERCEPT (PMOLE/L) = 32,193 FREE ENERGY CHANGE (J/MOLE) = 81277,4

OPTIMISATION: P*OPT = 153,1126 Q-OPT = 114,8344 P*-OPT/P* = 24,1152 Q-OPT/Q = 3,5471

CONCENTRATION:	5000	2500	1250	625	313	156	78	39	20
B/T VALUES	0,089	0,143	0,243	0,324	0,375	0,408	0,412	0,425	0,424
B/BO VALUES:	0,2069	0,3331	0,5665	0,7546	0,8724	0,9490	0,9586	0,9888	0,9876
LOG CONCN.:	3,6990	3,3979	3,0969	2,7959	2,4948	2,1938	1,8928	1,5918	1,2907
LOGIT TERM:	-1,3437	-0,6941	0,2675	1,1233	1,9225	2,9236	3,1429	4,4799	4,3778

SLOPE M = -2,5445 INTERCEPT C = 8,1482 COEFFICIENT R = -0,9914 SUM OF WEIGHTS = 0,0000

GOODNESS OF FIT VALUES OF SX, SY, SXY, SXQ, SYQ:	22,45	16,20	26,58	61,46	64,98

S.S.D.(FROM CORRELATION COEFFICIENT) = 0,6137 (FROM SLOPE) = 0,6137 M.SQ.DEV. FROM REGRESSION = 0,0877
S.S.D.FROM REGRESSION = 0,2961 S.S.D.OF REGRESSION COEFFICIENT = 0,1270 T-STATISTIC = -20,0378 7 D.F.

ITERATIONS REQUIRED: 5 A0 = 0,000800 AI = 0,001080

ITERATION 1

LOG CONCN.	YHI	EXP(YHI)	YH	YH(1-YH)	WKG.LOGIT	WEIGHT	THIS Y	NEXT Y
3,6990	-1,2640	0,2825	0,2203	0,1718	-1,3419	28,4250	0,2069	0,2205
3,3979	-0,4980	0,6078	0,3780	0,2351	-0,6889	45,7530	0,3331	0,3780
3,0969	0,2680	1,3074	0,5066	0,2456	0,2675	42,7087	0,5665	0,5666

2,7959	1,0340	2,8123	0,7377	0,1935	1,1214	23,4508	0,7546	0,7377
2,4948	1,8000	6,0405	0,8581	0,1217	1,9172	8,5816	0,8724	0,8581
2,1938	2,5660	13,0130	0,9286	0,0663	2,8733	2,4358	0,9490	0,9286
1,8928	3,3319	27,9925	0,9655	0,0333	3,1253	0,6018	0,9586	0,9655
1,5918	4,0979	60,2148	0,9837	0,0161	4,4171	0,1586	0,9888	0,9837
1,2907	4,8639	129,5286	0,9923	0,0076	4,2409	0,0309	0,9876	0,0923

LOGIT TERM: -1,3419 -0,6889 0,2675 1,1214 1,9172 2,8733 3,1253 4,4171 4,2409

SLOPE M = -2,7786 INTERCEPT C = 8,8491 COEFFICIENT R = -0,9973 SUM OF WEIGHTS = 152,1263

ITERATION 2

LOG CONCN,	YHI	EXP(YHI)	YH	YH(1-YH)	WKG,LOGIT	WEIGHT	THIS Y	NEXT Y
3,6990	-1,4289	0,2396	0,1933	0,1559	-1,2556	24,1008	0,2203	0,1933
3,3979	-0,5924	0,5530	0,3561	0,2293	-0,4968	44,3814	0,3780	0,3561
3,0969	0,2440	1,2764	0,5607	0,2463	0,2680	43,1648	0,5666	0,5607
2,7959	1,0805	2,9461	0,7466	0,1892	1,0335	22,2841	0,7377	0,7466
2,4948	1,9169	6,8000	0,8718	0,1118	1,7948	7,1731	0,8581	0,8718
2,1938	2,7534	15,6954	0,9401	0,0563	2,5498	1,7467	0,9286	0,9401
1,8928	3,5898	36,2272	0,9731	0,0261	3,2979	0,3692	0,9655	0,9731
1,5918	4,4263	83,6175	0,9882	0,0117	4,0394	0,0730	0,9837	0,9882
1,2907	5,2627	193,0013	0,9948	0,0051	4,7739	0,0140	0,9923	0,9948

LOGIT TERM: -1,2556 -0,4968 0,2680 1,0335 1,7948 2,5498 3,2979 4,0394 4,7739

SLOPE M = -2,5336 INTERCEPT C = 8,1144 COEFFICIENT R = -1,0000 SUM OF WEIGHTS = 143,3071

ITERATION 3

LOG CONCN,	YHI	EXP(YHI)	YH	YH(1-YH)	WKG,LOGIT	WEIGHT	THIS Y	NEXT Y
3,6990	-1,2574	0,2844	0,2214	0,1724	-1,4207	28,6004	0,1933	0,2214
3,3979	-0,4947	0,6098	0,3788	0,2353	-0,5912	45,7944	0,3561	0,3788
3,0969	0,2680	1,3073	0,5666	0,2456	0,2440	42,7088	0,5607	0,5666
2,7959	1,0307	2,8030	0,7371	0,1938	1,0799	23,5343	0,7466	0,7371
2,4948	1,7934	6,0098	0,8573	0,1223	1,9116	8,6670	0,8718	0,8573
2,1938	2,5561	12,8854	0,9280	0,0668	2,7375	2,4783	0,9401	0,9280
1,8928	3,3188	27,6271	0,9651	0,0337	3,5582	0,6169	0,9731	0,9651
1,5918	4,0815	59,2340	0,9834	0,0163	4,3745	0,1431	0,9882	0,9834
1,2907	4,8442	127,0010	0,9922	0,0078	5,1871	0,0321	0,9948	0,9922

LOGIT TERM: -1,4207 -0,5912 0,2440 1,0799 1,9116 2,7375 3,5582 4,3745 5,1871

SLOPE M = -2,7671 INTERCEPT C = 8,8133 COEFFICIENT R = -1,0000 SUM OF WEIGHTS = 152,5754

ITERATION 4

LOG CONCN,	YHI	EXP(YHI)	YH	YH(1-YH)	WKG,LOGIT	WEIGHT	THIS Y	NEXT Y
3,6990	-1,4220	0,2412	0,1943	0,1566	-1,2491	24,2752	0,2214	0,1943
3,3979	-0,5891	0,5548	0,3569	0,2295	-0,4935	44,4557	0,3788	0,3569
3,0969	0,2439	1,2762	0,5607	0,2463	0,2680	43,1670	0,5666	0,5607
2,7959	1,0769	2,9355	0,7459	0,1895	1,0302	22,3733	0,7371	0,7459
2,4948	1,9099	6,7522	0,8710	0,1124	1,7883	7,2525	0,8573	0,8710
2,1938	2,7428	15,5310	0,9395	0,0568	2,5400	1,7799	0,9280	0,9395
1,8928	3,5758	35,7236	0,9728	0,0265	3,2851	0,3792	0,9651	0,9728
1,5918	4,4088	82,1696	0,9880	0,0119	4,0234	0,0756	0,9834	0,9880
1,2907	5,2418	189,0024	0,9947	0,0052	4,7548	0,0146	0,9922	0,9947

LOGIT TERM: -1.2491 -0.4935 0.2680 1.0302 1.7883 2.5400 3.2851 4.0234 4.7548

SLOPE M = -2.5228 INTERCEPT C = 8.0808 COEFFICIENT R = -1.0000 SUM OF WEIGHTS = 145.7528

ITERATION 5

LOG CONCN.	YHI	EXP(YHI)	YH	YH(1-YH)	WKG.LOGIT	WEIGHT	THIS Y	NEXT Y
3.6990	-1.2509	0.2863	0.2225	0.1730	-1.4139	28.7752	0.1943	0.2225
3.3979	-0.4914	0.6117	0.3796	0.2355	-0.5879	45.8352	0.3569	0.3796
3.0969	0.2680	1.3073	0.5666	0.2456	0.2439	42.7089	0.5607	0.5666
2.7959	1.0274	2.7939	0.7364	0.1941	1.0763	23.6175	0.7459	0.7364
2.4948	1.7869	5.9706	0.8565	0.1229	1.9046	8.7528	0.8710	0.8565
2.1938	2.5463	12.7596	0.9273	0.0674	2.7271	2.5212	0.9395	0.9273
1.8928	3.3057	27.2680	0.9646	0.0341	3.5444	0.6322	0.9728	0.9646
1.5918	4.0651	58.2734	0.9831	0.0166	4.3574	0.1478	0.9880	0.9831
1.2907	4.8246	124.5338	0.9920	0.0079	5.1666	0.0334	0.9947	0.9920

LOGIT TERM: -1.4139 -0.5879 0.2439 1.0763 1.9046 2.7271 3.5444 4.3574 5.1666

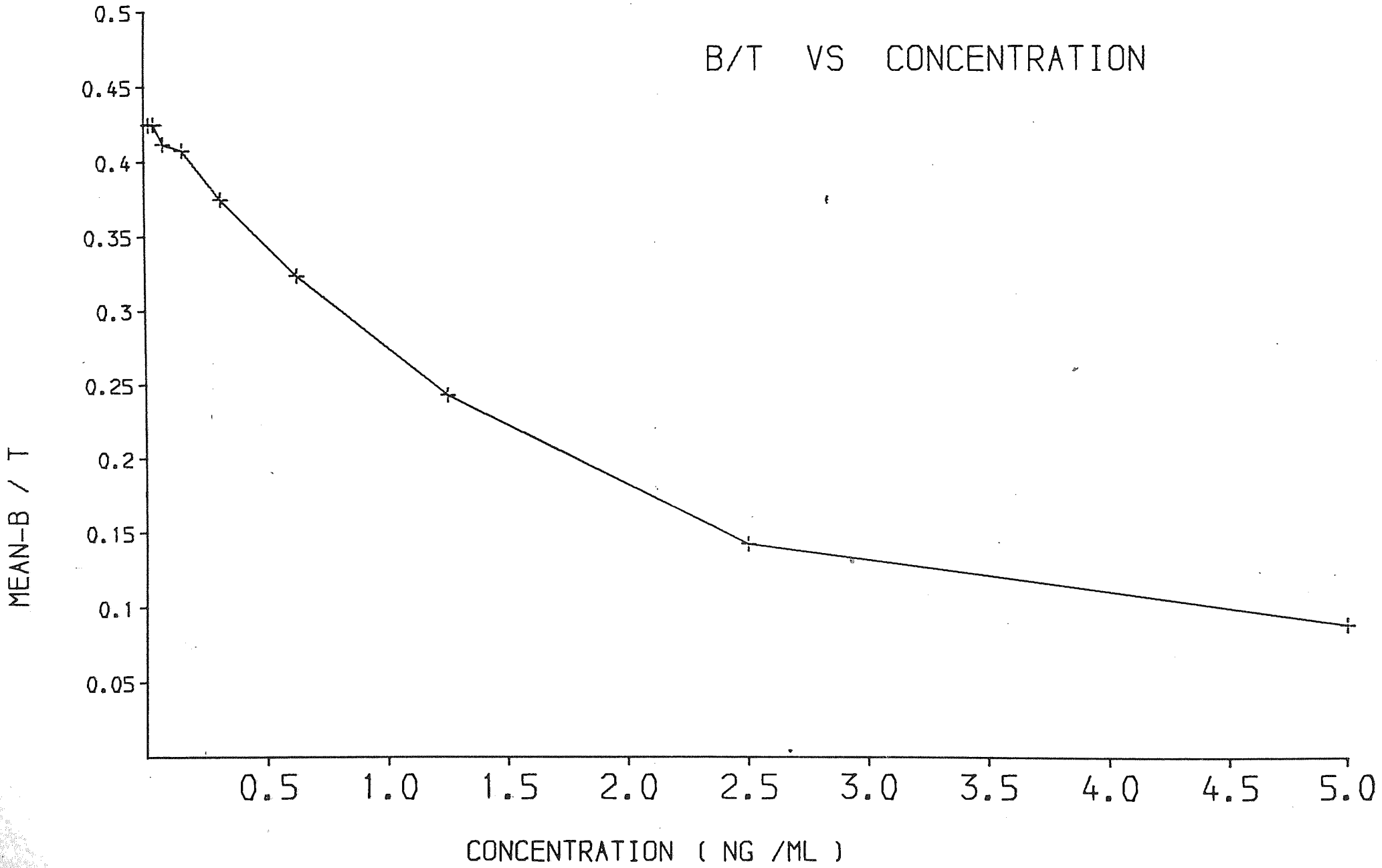
SLOPE M = -2.7556 INTERCEPT C = 8.7777 COEFFICIENT R = -1.0000 SUM OF WEIGHTS = 153.0242

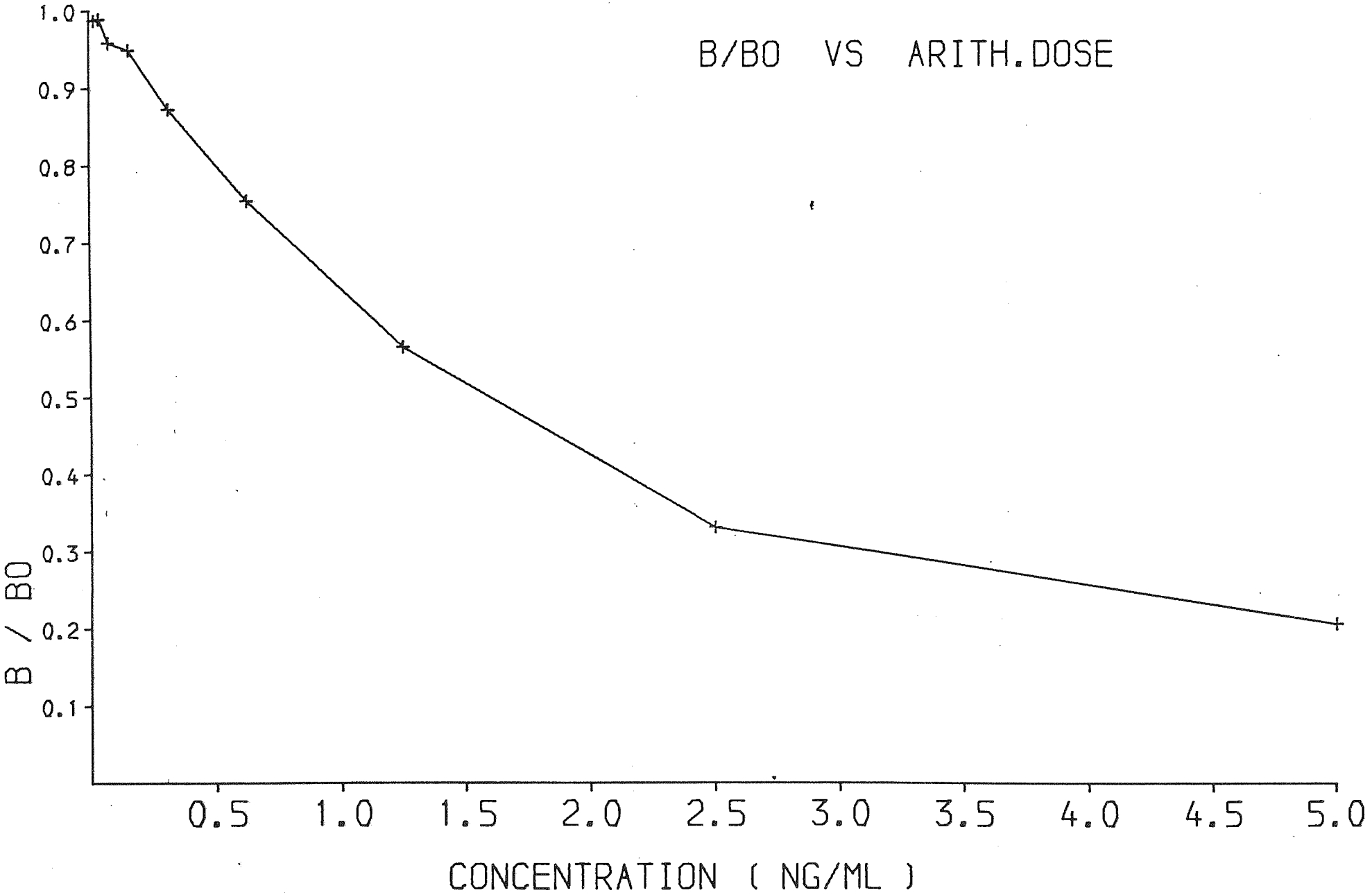
GOODNESS OF FIT VALUES OF SX, SY, SXY, SXQ, SYQ: 489.32 -5.19 -76.56 1586.46 168.40
S.S.D. (FROM CORRELATION COEFFICIENT) = 0.0010 (FROM SLOPE) = 0.0010 M.SQ.DEV. FROM REGRESSION = 0.0001
S.S.D. FROM REGRESSION = 0.0121 S.S.D. OF REGRESSION COEFFICIENT = 0.0026 T-STATISTIC = -1060.1118 7 D.F.

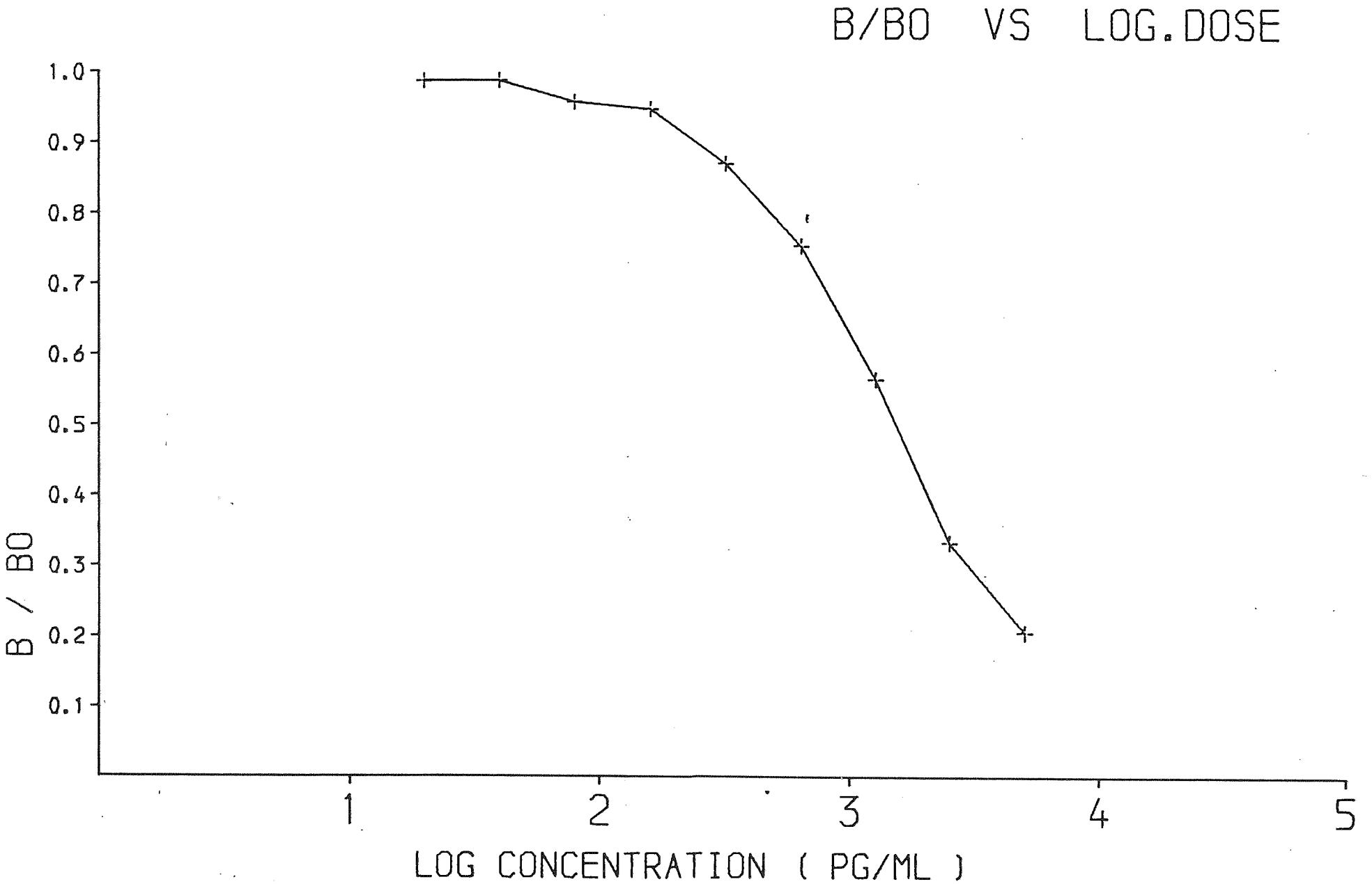
UNKNOWNS

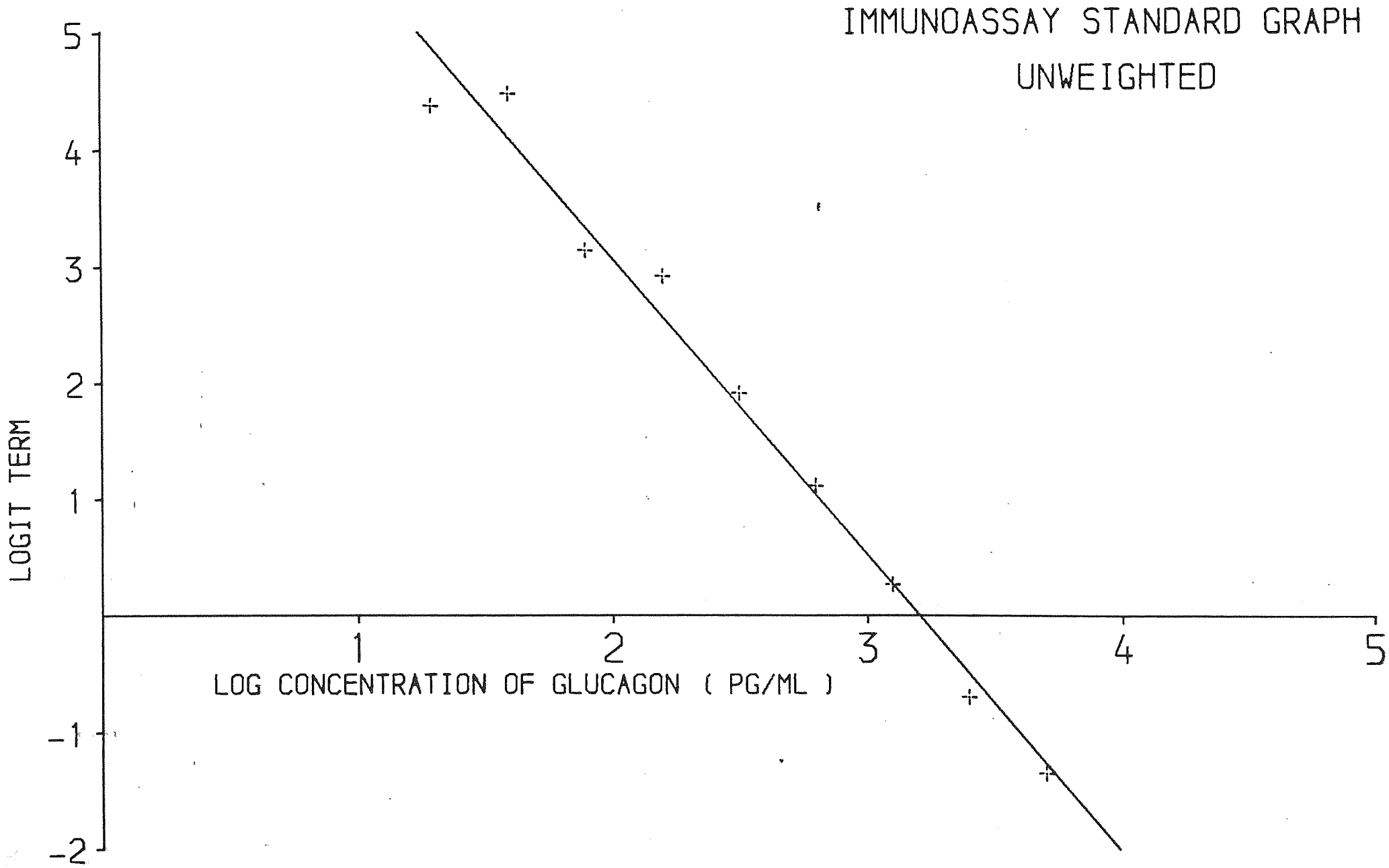
DILUTION = 10.00 NUMBER = 290

MODE	CASSETTE	TURE	TIME MIN X 0.01	CHANNEL 1	CHANNEL 2	CHANNEL 2 X CR.	COUNTS PER MINUTE(CHANNEL 2 X CR) CHANNEL 1	CHANNEL 2	MEAN	CONCN. PG/ML.	FINAL CONC. PG/ML.
1	152	6	400	4260	4054	4226	1065.00	1056.41	1060.71	.....	
1	152	7	400	4317	4139	4314	1079.25	1078.56	1078.91	.....	
1	152	8	400	17187	16575	17277	4296.75	4349.19	4307.97	3299.53	32995.32
1	152	9	400	15287	14702	15324	3821.75	3831.12	3826.43	1511.74	15117.41
1	152	10	400	16972	16280	16969	4243.00	4242.32	4242.66	2932.20	29321.97
1	123	1	400	12332	11809	12309	3083.00	3077.25	3080.12	376.32	3763.20
1	123	2	400	15163	14550	15166	3790.75	3791.51	3791.13	1432.82	14328.17
1	123	3	400	10657	10239	10673	2664.25	2668.13	2666.19	.....	
1	123	4	400	12115	11595	12086	3028.75	3021.48	3025.11	317.76	3177.56
1	123	5	400	12836	12306	12823	3209.00	3205.71	3207.36	518.82	5188.17
1	123	6	400	12015	11556	12045	3003.75	3011.32	3007.53	299.32	2993.24
1	123	7	400	11823	11353	11834	2955.75	2958.42	2957.08	247.00	2470.00
1	123	8	400	11532	11069	11538	2883.00	2884.41	2883.71	171.59	1715.94
1	123	9	400	12577	12050	12560	3144.25	3140.05	3142.15	444.58	4443.81
1	123	10	400	14386	13782	14366	3596.50	3591.38	3593.94	1055.85	10558.46
1	113	1	400	14779	14148	14747	3694.75	3686.75	3690.75	1228.82	12288.19
1	113	2	400	12846	12298	12819	3211.50	3204.67	3208.09	519.67	5196.68
1	113	3	400	15001	14455	15046	3770.25	3761.54	3765.90	1378.84	13788.36
1	113	4	400	15592	14945	15578	3898.00	3894.44	3896.22	1680.81	16805.09
1	113	5	400	15390	14771	15396	3847.50	3849.10	3848.30	1562.75	15627.53
1	113	6	400	16022	15425	16076	4005.50	4019.00	4012.25	2008.99	20089.84
1	113	7	400	14039	13478	14049	3509.75	3512.16	3510.96	922.56	9225.63
1	113	8	400	13640	13219	13779	3460.00	3444.67	3452.33	835.40	8353.95
1	113	9	400	16709	16155	16837	4199.75	4209.23	4204.49	2744.45	27444.51
1	113	10	400	12955	12446	12973	3238.75	3243.24	3240.99	558.56	5585.61
1	153	1	400	16856	16279	16968	4214.00	4242.06	4228.03	2858.14	28581.39
1	153	2	400	14153	13565	14139	3538.25	3534.83	3536.54	962.34	9623.44
1	153	3	400	12334	11759	12257	3083.50	3064.22	3073.86	369.57	3695.73
1	153	4	400	14098	13513	14085	3524.50	3521.28	3522.89	940.98	9409.82
1	153	5	400	13985	13391	13958	3496.25	3489.49	3492.87	895.10	8950.98
1	153	6	400	15745	15174	15816	3936.25	3954.11	3945.18	1811.51	18113.06


B/T VS CONCENTRATION
MEAN-B / T
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
CONCENTRATION (NG /ML)


B/BO VS ARITH.DOSE
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
B / BO
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
CONCENTRATION (NG/ML)


B/BO VS LOG.DOSE
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
B / BO
1
2
3
4
5
LOG CONCENTRATION (PG/ML)


IMMUNOASSAY STANDARD GRAPH
UNWEIGHTED
LOGIT TERM
5
4
3
2
1
-1
-2
1
2
3
4
5
LOG CONCENTRATION OF GLUCAGON (PG/ML)

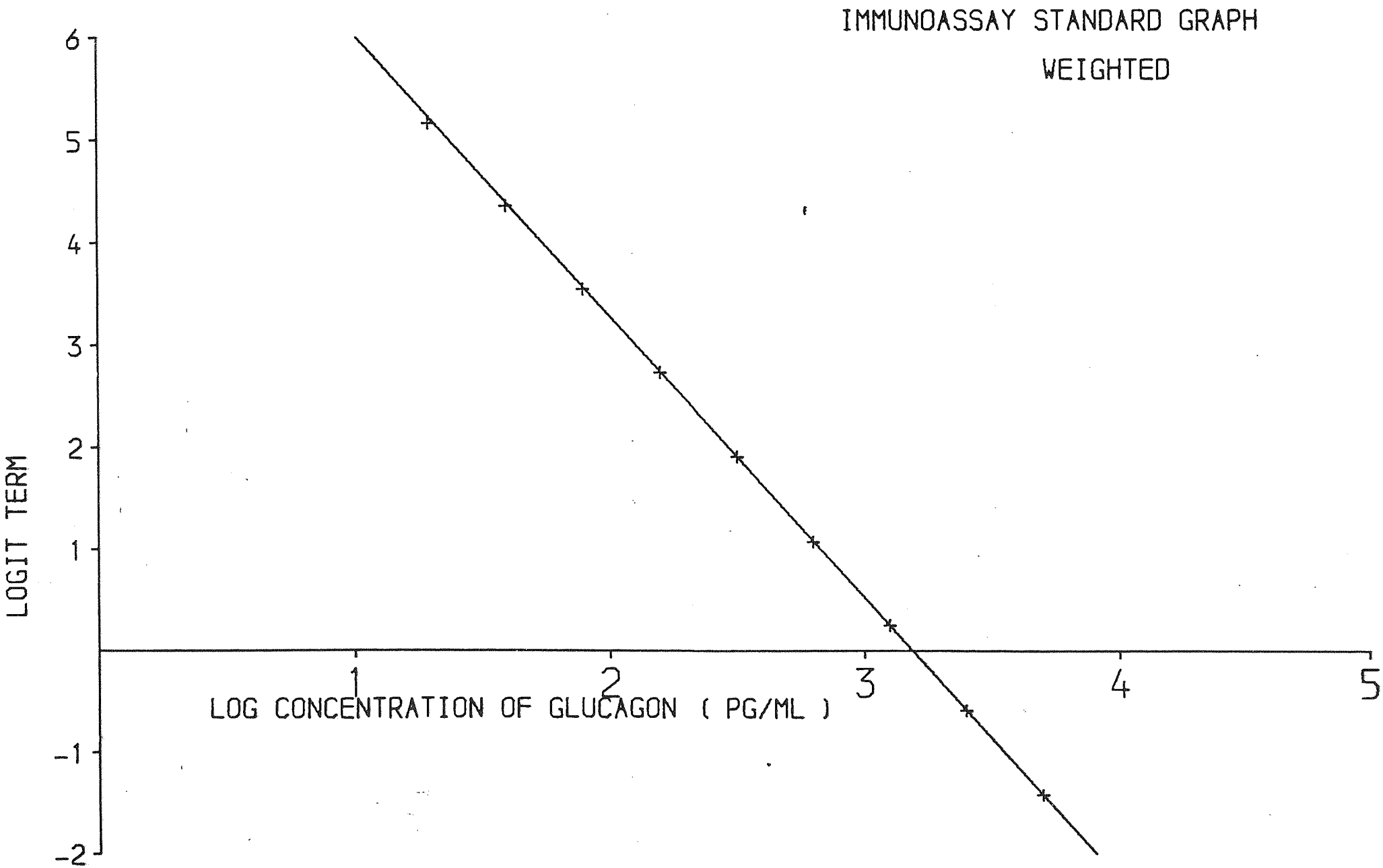

IMMUNOASSAY STANDARD GRAPH
WEIGHTED
LOGIT TERM
6
5
4
3
2
1
-1
-2
1
2
3
4
5
LOG CONCENTRATION OF GLUCAGON (PG/ML)

Table 3 Characterisation of sera from rabbits and guinea pigs immunised with glucagon-bovine plasma albumin conjugates[ϕ]

COUPLING AGENT	ANIMAL	MONTHS OF IMMUNISATION	NUMBER OF SERA	MEAN TITRE	AFFINITY MEAN DISPLACEMENT	SPECIFICITY
CARBODIIMIDE	ROG 1	7	5	10,000	58	8.03
	ROG 2	7	5	7,650	41	23.00
	ROG 3	7	5	2,790	2	-
	ROG 4	7	5	3,150	46	1.52
	ROG 5	7	5	945	22	3.71
CARBODIIMIDE	GPC 1	4	2	51,750	75	1.25
	GPC 2	7	5	146,970	54	2.07
	GPC 3	7	5	1,395	55	2.36
	GPC 4	7	5	12,150	66	1.58
	GPC 5	4	2	67,500	67	1.40
GLUTARALDEHYDE	GPD 1	2	1	-	-	-
	GPD 2	7	5	-	-	-
	GPD 3	7	5	-	-	-
	GPD 4	7	5	4,410	59	2.10
	GPD 5	7	5	-	-	-

[ϕ]Titre is expressed as the reciprocal of the final working dilution of the serum that will bind 50% of the labelled antigen in the assay system employed. Affinity is expressed as the % fall in bound counts caused by 125 pg porcine glucagon: D.G. = $(Bo - B_{125})/Bo \times 100$. Specificity is expressed as the ratio of displacement (D) of the tracer caused by 125 pg porcine glucagon and 125 pg equivalent gut GLI: D.G./D.GLI.

Table 4 The effect of different albumin-containing buffers on glucagon antibody dilution curves.

ANTIBODY DILUTION (WORKING TITRE)	% BINDING (B/T x 100) ALBUMINS$^{\phi}$				
	1	2	3	4	5
1:100	84	81	83	84	82
1:1,000	72	70	72	75	70
1:3,000	54	58	60	65	56
1:5,000	44	51	53	56	46
1:10,000	28	36	40	40	32
% ADSORBED BY CHARCOAL (N/T)	97	95	97	96	92
50% MAXIMUM BINDING INVERSE TITRE	5,600	7,600	9,600	9,800	6,400
% ADSORBED BY GLASSWARE	5	6	6	4	10

ϕ Albumins	Lot nos.
1. Pentex bovine serum albumin V	246/81-003
2. Armour bovine serum albumin V	WA 1073
3. Armour bovine serum albumin V	XC 2671
4. Sigma crystalline bovine serum albumin	81C-13028
5. Sigma human serum albumin	1230-1120

CHAPTER 4

COMPUTER ANALYSIS OF RADIOIMMUNOASSAY DATA

INTRODUCTION

The development of computer programmes for the analysis of radioimmunoassay data has attracted considerable attention as a consequence of the enormous sample throughput and the need for more rigorous and objective assessment of the validity of assay results (Rodbard, 1971; Ekins, 1976). Thus, during an era which has steadily moved towards automation together with a more general refinment of the technique (Ekins, 1974a; Ertingshausen et al., 1975; Young, 1976), numerous time saving routine radioimmunoassay data processing systems have emerged (Rodbard, 1974; Rodbard & Frazier, 1975; Rodbard & Rogers, 1976).

Statistical analysis of radioimmunoassay data has only recently diverted the attention of professional statisticians from their hitherto concern with bioassay techniques (Bliss, 1952; Finney, 1964). Although the fundamental problems involved are essentially similar in nature, customary methods for statistical analysis and processing of bioassay data are not directly applicable to the handling of radioimmunoassay results (Ekins, 1971; Rodbard, 1971).

The problems of primary concern in applying classical bioassay statistics to radioimmunoassay stem from the fact that radioimmunoassays invariably result in nonlinear standard calibration curves which display nonuniformity of variance (Midgley et al., 1969a; Rodbard, 1971). Numerous methods have been described to overcome these problems (Rodbard, 1971, 1974; Rodbard & Rogers, 1976), and computer programmes which have been published, differ in both the facilities they offer and the mathematical approach adopted towards fitting the standard dose-response curve (Rodbard & Frazier, 1975; Ekins, 1976). Despite considerable differences in the level of complexity of these programmes, the majority fall into one of the following mathematically defined categories: those based on the law of mass action with its accompanying

assumptions (Meinert & McHugh, 1968; Baulieu & Raynaud, 1970; McHugh & Meinert, 1970; Wilson et al., 1971; Feldman, 1972); those of the 'spline' type which depend on the semi-empirical joining of adjacent points on the standard curve (Brown et al., 1970a; Marschner et al., 1974a; Leclercq-Meyer et al., 1975; England & Cain, 1976; Shoucri et al., 1976); and those theoretically justified methods which rely on linearisation of the dose-response curve (Hales & Randle, 1963a; Rodbard & Lewald, 1970; Fernandez & Loeb, 1975; Ekins, 1976; Hatch et al., 1976).

Although each of the curve-fitting procedures mentioned above has its relative merits (Rodbard & Frazier, 1975; Ekins, 1976), linearisation of the standard dose-response curve offers considerable advantages, whether one uses either graphical or computerised methods for curve-fitting and dose interpolation (Rodbard, 1971, 1974). Linearisation can be achieved by employing one of a variety of unique, but algebraic equivalent, transformations of the original response metameter which acquire theoretical justification at saturation (Rodbard et al., 1969; Shaw et al., 1977). Such methods may be classified into three groups: the reciprocal hyperbolic methods first used by Hales and Randle (1963a), where 1/B was expressed as a linear function of the dose (Murphy, 1968; Rodbard et al., 1969; Bliss, 1970; Fernandez & Loeb, 1975; Ekins, 1976); a second method suggested by Yalow and Berson (1968), which used the relationship between log (B/T) and log ($p + p^*$); and the logit-log methods introduced by Rodbard and colleagues (1968), which relied upon the relationship between logit (B/Bo) and the logarithm of the dose (Rodbard & Lewald, 1970; Healy, 1972; Arrigucci et al., 1973; Rodbard & Frazier, 1975; Hatch et al., 1976). Of these approaches, the logit-log transformation has gained general acceptance as the basis for computer programmes (Ekins, 1971, 1976; Rodbard & Frazier, 1975). This method which affords linearisation over a wide range of concentrations

has been successfully applied to more than fifty different radioimmunoassay systems, covering all substances from proteins and hormonal peptides to steroids, drugs and cyclic nucleotides (Midgley et al., 1969a; Duddleson et al., 1972; Rodbard & Hutt, 1972; Rodbard & Frazier, 1975; Hatch et al., 1976; Martinez-Alonso et al., 1976; Smith et al., 1976; Woo & Cannon, 1976).

The logit-log method, introduced for the analysis of radioimmunoassay data by J. A. Cooper and further developed by Rodbard and colleagues (Rodbard et al., 1968, 1969, 1970; Rodbard & Cooper, 1970; Rodbard & Lewald, 1970; McBride & Rodbard, 1971; Rodbard, 1971; Rodbard & Frazier, 1975), was used as the basis for the present data processing system. The computational system was developed according to the precepts defined by Rodbard (1974) and was used routinely for the analysis of both insulin and glucagon radioimmunoassay data (see chapters 2 and 3). The essential features of the logit-log method and the computer programme are outlined in the following sections.

THE LOGIT LOG METHOD

The logit function was introduced by Berkson (1944) for the purpose of linear transformation of sigmoidal dose response curves:

$$Y = \frac{1}{1 + e^{-(c + mX)}} \quad , \qquad (32)$$

where Y represents a measurable quantity of a response variable dependent on a dose variable X, the base of the natural system of logarithms, e, and the constants c and m.

Rearranging this equation yields

$$\frac{Y}{1 - Y} = e^{(c + mX)} \qquad (33)$$

and the linear relationship

$$\log_e \left(\frac{Y}{1 - Y}\right) = c + mX \quad , \qquad (34)$$

where the logit function is defined as

$$\text{logit } Y = \log_e \left(\frac{Y}{1 - Y}\right) \quad . \qquad (35)$$

In radioimmunoassay, the sigmoidal curve of B/Bo vs. log (X) suggests that a linear dose-response curve might be obtained by use of a 'sigmoidal' or 'S-shaped' transformation of the response variable. This was confirmed by Rodbard and colleagues (1968, 1969) who described the dose-response curve by the linear equation

$$Y' = \text{logit } Y = c + m \log (X) \quad , \qquad (36)$$

where Y = B/Bo, X is the dose, and c and m are the intercept and slope, respectively.

Either the logit, probit, or arc-sine transformation can be used to linearise the semi-logarithmic dose-response curves obtained in radioimmunoassay (Finney, 1964, 1971; Rodbard et al., 1968; Vivian & LaBella, 1971; Healy, 1972). However, the logit transformation is preferable since it is the easiest to calculate, provides the simplest expressions for weighting, and is theoretically justified (Berkson, 1951; Rodbard et al., 1968, 1969; Rodbard & Cooper, 1970). This method has been extensively developed by Rodbard and colleagues (Rodbard & Cooper, 1970; Rodbard & Lewald, 1970; Rodbard & Hutt, 1972).

When the reciprocal-hyperbolic methods (described above) are successful, the logit-log method will also linearise, with a slope of either -2.303 or -1 depending on whether common or natural logarithms

are used on the abscissa (Rodbard et al., 1969; Ekins, 1971; Shaw et al., 1977). In addition, the logit-log method provides excellent linearisation in many cases where the reciprocal-hyperbolic methods fail, viz., whenever the slope of the logit-log dose response curve is significantly different from the values mentioned above (Rodbard et al., 1971; Ekins, 1974b; Rodbard & Frazier, 1975; Hatch et al., 1976).

Although the phenomenon of heteroscedasticity or nonuniformity of variance is inherent in radioimmunoassay (Rodbard, 1971; Ekins, 1976), it is exacerbated using both reciprocal plots and logit-log transformation (Midgley et al., 1969a; Rodbard et al., 1970; Walker, 1977), necessitating the use of weighted least squares regression analysis (Brownlee, 1965). Failure to recognise this problem, as indicated by the persistent use of unweighted linear regression with these methods, has resulted in the calculation of biased estimates of both the intercept and the slope (see table 2), leading to the production of inaccurate assay results (Rodbard, 1974; Woo & Cannon, 1976; Walker, 1977). The use of truncation (Vivian & LaBella, 1971) may be regarded as a sub-optimal method of weighting under these conditions (Rodbard & Hutt, 1972; Rodbard, 1974).

The major purpose of the unweighted regression in the logit-log method is to obtain initial estimates for subsequent calculation of an iterative weighted least squares linear regression using a maximum likelihood method (Rodbard & Lewald, 1970). This procedure is performed in a manner analogous to that described for the analysis of quantal bioassay data using either the logit or probit transforms (Bliss, 1952; Finney, 1964).

The need to perform iterative weighted regression analysis, in turn, requires a knowledge of the variance of $Y = B/Bo$ for any position on the dose-response curve. As presented by Rodbard and Cooper (1970),

the variance of the response variable can be described by a model incorporating both a constant and a Poisson-like component:

$$\text{Var}(Y) = ao + a_1 Y \quad . \qquad (37)$$

This two parameter model fits well with data from both radioimmunoassays (Midgley et al., 1969a; Rodbard & Lewald, 1970) and immunoradiometric assays (Rodbard & Hutt, 1972) and performs in a manner almost indistinguishable from the more extensive seven parameter model (Rodbard & Lewald, 1970; Rodbard, 1971). The magnitude of ao and a_1 depends on the total counts T; nonspecific counts N; Bo; pipetting error (Vp) and the misclassification errors (V1 and V2). Many workers have either assumed $a_1 = 0$ (Tälljedal & Wold, 1970; Wilson et al., 1971; Vivian & LaBella, 1971; Burger et al., 1972; Leclercq et al., 1972; Arriguci et al., 1973) or ao = 0 (Bliss, 1970; Duddleson et al., 1972). However by pooling estimates of the variance of Y for a total of 47 insulin and glucagon assays, and constructing a graph of Var (Y) vs. narrow regions of Y (e.g., for $0 \leq Y < .1$, $.1 \leq Y < .2$, etc.), the two parameter model of Rodbard and Cooper (1970) was found to provide the best fit (figure 53). The resulting estimates of ao and a_1, given by the intercept and slope, were 0.00080 and 0.00108, respectively. These values were used to calculate the parameters of equation 36.

In the statistical model used by Rodbard and colleagues (Rodbard & Cooper, 1970; Rodbard & Lewald, 1970), the reciprocals of the variance of logit Y are used as weights for the weighted least squares linear regression. The weight (W) assigned to each point on the dose-response curve is given by

$$W = \frac{1}{\text{Var.}(\text{logit } Y)} \quad , \qquad (38)$$

where

$$\text{Var.}(\text{logit } Y) = \frac{\text{Var.}(\hat{Y})}{\hat{Y}^2(1 - \hat{Y})^2} \qquad (39)$$

and

$$\text{Var.}(Y) = a_0 + a_1\hat{Y} \qquad (40)$$

Ideally, the weights should be expressed as a function of X instead of Y (Rodbard & Hutt, 1972). This is achieved by using a reiterative procedure such that the $\hat{Y}$ values on the right hand side of equations 39 and 40 represent Y values predicted for a given value of X on the basis of the previous iteration (Rodbard & Frazier, 1975). In all subsequent regression calculations a 'working logit', analogous to the 'working probit' (Finney, 1964), is used as the modified response variable (Rodbard & Lewald, 1970).

DESCRIPTION OF THE PROGRAMME

A computer programme was written in ALGOL 60 for detailed analysis of radioimmunoassay data. The method used logit and log transformations to obtain a linear dose-response curve, followed by unweighted and iterated weighted least squares regression analysis. The standard curve was plotted in five different co-ordinate systems by computer and the potency estimates, corrected for dilution, were obtained for the unknown samples. This programme formed the basis of a fully automated data processing system which in addition to facilitating the calculation of assay results, provided estimates of assay precision, together with Scratchard plot analysis and details of the concentrations of labelled antigen and antibody required to achieve optimal sensitivity (minimal least detectable dose).

The programme has been successfully applied to the routine analysis of both insulin and glucagon radioimmunoassay data (see chapters 2 and 3) and has provided information suitable for use as an empirical quality control system (table 2 and figure 54). Sample input and sample computer output for radioimmunoassay data processing are shown in figures 40 and 52.

APPLICABILITY

The present method was designed for use with radioimmunoassays of insulin and glucagon which rely on coated charcoal for the separation of free and bound hormone moieties. In these assays the radioactivity associated with the free fraction was counted for a fixed period of time, without automatic subtraction of non-specific counts (N).

However, with only minimal modification, the method can also be applied to assays in which either the bound, or both bound and free fractions are measured, or to assays in which 'preset counts' are used. The crystal scintillation counter was connected directly to a teletype machine (model 33 ASR) equipped with a paper tape perforator (type ASC II). Alternatively, the paper tape was prepared manually by use of the teletype in the 'local' mode.

COMPUTER AND LANGUAGE

The programme was written in ALGOL 60 for use with an ICL 1904S computer. The analysis was performed routinely overnight at the University of Aston Computer Centre at an approximate cost of 10p per 500 tube assay. A complete set of programme listings are shown in figure 55.

PROTOCOL

The order in which the assay tubes were placed in the counter was determined by the format of the programme. A standard curve was included at the beginning of each assay and consisted of 45 tubes terminating in quadruplicates for estimation of the initial amount bound Bo, nonspecific counts N and total counts T. The remaining tubes represented standard incubation mixtures covering an 11-fold range of doubling dilutions, starting at either 20 ng/ml or 5 ng/ml antigen. Although the top of the concentration scale was essentially fixed,

the absolute concentration range could be modified by omission of low dose standards.

Information from the scintillation counter was recorded directly onto standard 8 track punched paper tape; before counting commenced, the identifying text, terminated by a recognition symbol (*) was typed onto the tape using the teletype machine in manual (local) mode. This was followed by the additional input information required for preparing the standard curve, performing the statistical analysis and calculating the potency estimates for the unknown samples, i.e.,

1. Number of low standards omitted from the usual eleven.
2. Number of low standards to be ignored in the calculations.
3. Number of blocks of unknowns.
4. Dilution of unknowns in each block.
5. Number of unknowns in each block.
6. Tracer concentration, pg/ml.
7. Final incubation volume, µl.
8. Molecular weight of the antigen.
9. Incubation temperature, ^{o}A.
10. Number of iterations required.
11. Value of ao.
12. Value of a_1.

Data from the computer comprised six items per sample (tube) counted: mode; cassette number; tube number; time in hundredths of a minute; counts from channel one; and counts from channel two. Upon completion of counting, the input data were terminated by a second recognition symbol (////) and the paper tape was transferred to the computer which stored the data as a named file.

CALCULATIONS

After the data file had been loaded, the computer was instructed to run the appropriate programme and to draw the resulting standard graphs on which the calculation of the potency estimates for the unknown samples was based. A simplified flowchart of the programme is shown in figure 56.

The programme first read in the data and calculated the individual channel ratios together with the mean and standard deviation. Values lying outside 3 standard deviations of the mean were rejected, and the corrected value was carried forward for use in later calculations. As illustrated in figure 57, which shows a sample computer output, channel bias was 5.25% with channel one recording 94.75% of the counts detected by channel two. The bias was not constant and the mean channel ratio, calculated for individual assays over a period of 14 months, ranged from 109.6% to 94.6% .

Upon completion of the above calculations, the programme returned to the beginning of the data and proceeded to calculate the mean and standard deviation of the count rate for the tubes in the standard curve. Each set of tubes was corrected for channel bias and counting time, and the mean and standard deviation were adjusted to exclude aberrant values lying outside 1.5 standard deviations of the mean.

Once these analyses had been performed, the following parameters were calculated (a detailed list of the equations used in the programme is presented in a later section):

1. The pipetting error (Vp)

$$Vp = \sqrt{\frac{D^2 - T}{T}} \quad , \qquad (41)$$

where D is the standard deviation of the total counts and T is the total counts. In those instances where $D^2 \leq T$, the pipetting error was considered to be

2. The fraction of the total counts adsorbed to charcoal in the absence of added antiserum

$$N/T \quad , \qquad (42)$$

where N and T represent nonspecific counts and total counts, respectively. The value of N was substituted for that of T in all subsequent calculations.

3. The initial level of binding

$$(B/T)_o = Bo/N = \frac{N - Fo}{N} \quad , \qquad (43)$$

where Fo and Bo represent the free and the bound counts recorded in the absence of the standard antigen.

4. The individual and mean counts bound (B) for each set of standard incubation tubes, together with the mean fraction bound (B/T) at each of the dose levels.

5. The response variable (Y)

$$Y = B/Bo = \frac{N - F}{N - Fo} \quad , \qquad (44)$$

together with the mean, variance and coefficient of variation for each set of standard incubation tubes.

6. Assay precision ($\triangle$ p)

$$\triangle p = \frac{\triangle Rp}{\text{slope at } Rp} \quad , \qquad (45)$$

where $\triangle$ Rp is the precision (coefficient of variation) of measurement of the response metameter (F) at an antigen concentration, p.

Having completed the above calculations, the programme performed Scratchard plot analyses and determined the effective affinity constant (K), the binding capacity (q) of the various classes of antibody binding sites, and the standard free energy change (ΔF^{o}) of the antigen-antibody reaction. This was followed by estimation of the concentrations of antibody (3/K) and labelled antigen (4/K) predicted to provide optimal assay sensitivity (minimal least detectable dose). The radioimmunoassay dose-response curve was then plotted in co-ordinates of B/T vs. arithmetic dose (X), B/Bo vs. arithmetic dose (X), and B/Bo vs. logarithmic dose (X).

At this stage in the analysis, the programme commenced the first linear regression of logit (Y) vs. log (X). The (X) and (Y) values were modified by log and logit transformations:

$$X' = \log_{10}(X) \quad , \qquad (46)$$

$$Y' = \text{logit } (Y) \quad , \qquad (47)$$

where, as defined in equation (35)

$$\text{logit } Y = \log_e \left(\frac{Y}{1 - Y}\right) .$$

The unweighted least squares linear regression of Y' on X' was then calculated (ignoring any Y values outside the range $0 < Y < 1$), tested for 'goodness of fit' and the graph plotted. The values of the intercept, c, and slope, m, and values of $\hat{Y}$ predicted from the regression line were used in the calculation of a provisional set of weights (W), and the first weighted regression was performed. For this and all subsequent regression calculations, a 'working logit' was used as the ordinate:

$$\text{working logit} = y' = \frac{Y - \hat{Y}}{\hat{Y}(1 - \hat{Y})} + \hat{Y}' \quad . \qquad (48)$$

The slope and intercept of the first weighted regression were printed out, and estimates of $\hat{Y}$ for each value of X' on the standard curve were used for the estimation of a new set of weights and working logits. This process was reiterated as many times as was required for the method to converge, usually only two or three iterations being necessary. At this point, the programme provided a final table of X'; $\hat{Y}'$; $\hat{Y}/(1 - \hat{Y})$; $\hat{Y}$; $\hat{Y}(1 - \hat{Y})$; y'; W; Y and $\hat{Y}$, followed by the final weighted regression. The resulting equation was tested for 'goodness of fit', and the t-statistic with its corresponding degrees of freedom calculated. The final weighted regression was plotted in co-ordinates of logit Y vs. log(X).

The programme then read the unknown data, calculated the individual count rate and the transformed response variables Y and Y' (equations 35 and 44) and subsequently determined the logarithm of the potency estimate ($\hat{X}'$) from the standard curve:

$$\hat{X}' = \frac{Y' - c}{m} \quad . \qquad (49)$$

The corresponding dose, $\hat{X}$, was then determined:

$$\hat{X} = \exp(\hat{X}') \quad , \qquad (50)$$

and the potency estimate, corrected for dilution, was printed for each of the unknown samples.

ASSUMPTIONS

The following assumptions were implicit for the validation of the present method:

1. The standard and unknown antigen were assumed to be identical, not only in respect to the shape and position of the dose response curve, but also with respect to the errors.

2. The relationship between logit(Y) and log (X) was assumed to be linear. This relationship has substantial empirical and theoretical basis (Midgley et al., 1969a; Duddleson et al., 1972; Rodbard & Frazier, 1975; Martinez-Alonso et al., 1976; Woo & Cannon, 1976) and has been shown to be mathematically exact at or near the region of antibody saturation (Rodbard et al., 1969; Rodbard & Cooper, 1970; Shaw et al., 1977).

APPROXIMATIONS

In addition to the basic assumptions the method used a number of mathematical approximations (Rodbard & Cooper, 1970; Rodbard & Lewald, 1970). The most important of these was:

$$\text{Var.}(\text{logit } Y) \cong \frac{\text{Var.}(Y)}{Y^2(1 - Y)^2} \qquad (51)$$

This approximation remained valid so long as the square of the coefficient of variation was small. This was a satisfactory approximation over the range $0.05 < Y < 0.95$.

LIMITATIONS

The above assumptions and approximations represent the limitations of the present method. However, an additional factor which might have affected the success of the logit-log approach was the requirement for accurate and precise estimates of Bo and N which were regarded as constants in both the curve fitting and dose interpolation procedures (Rodbard & Lewald, 1970; Ekins, 1974b).

In the present study, reliable estimates of Bo and N were obtained by the use of replicates, and the errors incurred were considered to be negligible for practical purposes. However, since any serious error in

the determination of either parameters might result in significant nonlinearity of the logit-log plot (Hatch et al., 1976), it was thought advisable in certain instances, to employ end-point adjustment (Burger et al., 1972; Healy, 1972; Rodbard & Hutt, 1972; Hatch et al., 1976). Such an approach generalised the technique so that satisfactory results were obtained in special cases when the two parameter logit-log method failed (Rodbard & Frazier, 1975; Hatch et al., 1976).

EQUATIONS

The following equations were employed in the computer analysis of radioimmunoassay data (a list of nonstandard abbreviations used in this section has been provided at the beginning of this thesis):

1. LOGIT LOG ANALYSES

a. $Y = B/Bo = \frac{N - F}{N - Fo}$ — response variable.

b. $Y' = \text{logit } Y = \log_e \frac{Y}{1 - Y}$ — modified response variable.

c. $X' = \log_{10}(X)$ — modified (X) variable.

d. $Y' = c + m\,(X')$ — regression equation.

e. $m = \frac{\Sigma WX'Y' - (\Sigma WX')(\Sigma WY')/\Sigma W}{\Sigma WX'^2 - (\Sigma WX')^2/\Sigma W}$ — regression slope estimate.

f. $c = \bar{Y}' - m\bar{X}'$ — intercept estimate.

g. $\hat{Y} = \frac{1}{(1 + \exp(-\hat{Y}'))}$ — inverse logit transformation.

h. $y' = \frac{Y - \hat{Y}}{\hat{Y}(1 - \hat{Y})} + \hat{Y}'$ — working logit.

i. $\text{Var.}(Y) = ao + a,\hat{Y}$ — variance of Y in terms of $\hat{Y}$.

j. $\text{Var.}(Y') = \frac{\text{Var.}(Y)}{\hat{Y}^2(1 - \hat{Y})^2}$ — variance of Y' in terms of variance $\hat{Y}$.

k. $W = \frac{1}{\text{Var.}(Y')}$ — weight for individual point on the standard curve.

l. $\hat{X}' = \frac{Y' - c}{m}$ — reverse (inverse) regression equation.

m. $\hat{X} = \exp.(\hat{X}')$ — inverse (anti-) log transformation.

2. ADDITIONAL ANALYSES

n. $\Delta p = \frac{\Delta Rp}{\text{Slope (at Rp)}}$ — assay precision.

o. $\Delta po = \frac{\Delta Ro}{\text{Slope (at Ro)}}$ — assay sensitivity.

p. $B/F = K(q - B)$ — Scratchard equation.

q. $\Delta F^{o} = RT \text{ Log}_e K$ — standard free energy change.

r. $(p^*)opt. = 4/K$ — optimal concentration of labelled antigen.

s. $(q)opt. = 3/K$ — optimal concentration of antibody binding sites.

3. GOODNESS OF FIT

t. $r = \sqrt{\frac{\Sigma xy-(\Sigma x)(\Sigma y)/n}{\Sigma x^2-(\Sigma x)^2/n)(\Sigma y^2-(\Sigma y)^2/n)}}$ — regression slope estimate.

u. $\Sigma dy.x^2 = \Sigma y^2-(\Sigma y)^2/n - \frac{(\Sigma xy-(\Sigma x)(\Sigma y)/n)^2}{\Sigma x^2-(\Sigma x)^2/n}$ — sum of squares of deviations.

$\Sigma dy.x^2 = (1-r^2)(\Sigma y^2-(\Sigma y)^2/n)$ — sum of squares of deviations.

v. $sy.x^2 = \frac{\Sigma dy.x^2}{n - 2}$ — mean square deviation from regression.

w. $sy.x = \sqrt{sy.x^2}$ — sample standard deviation from regression.

y. $sb = \frac{sy.x}{\sqrt{\Sigma x^2 - (\Sigma x)^2/n}}$ — sample standard deviation of the regression coefficient.

z. $t = m/sb$ — student's t-value for (n-2) df.

Figure 53 Relationship between VAR.(B/Bo) and B/Bo for insulin and glucagon radioimmunoassays$^{\phi}$

$^{\phi}$The variance of B/Bo was calculated from triplicates at 10-12 dose levels for a combined total of 47 insulin and glucagon radioimmunoassays.

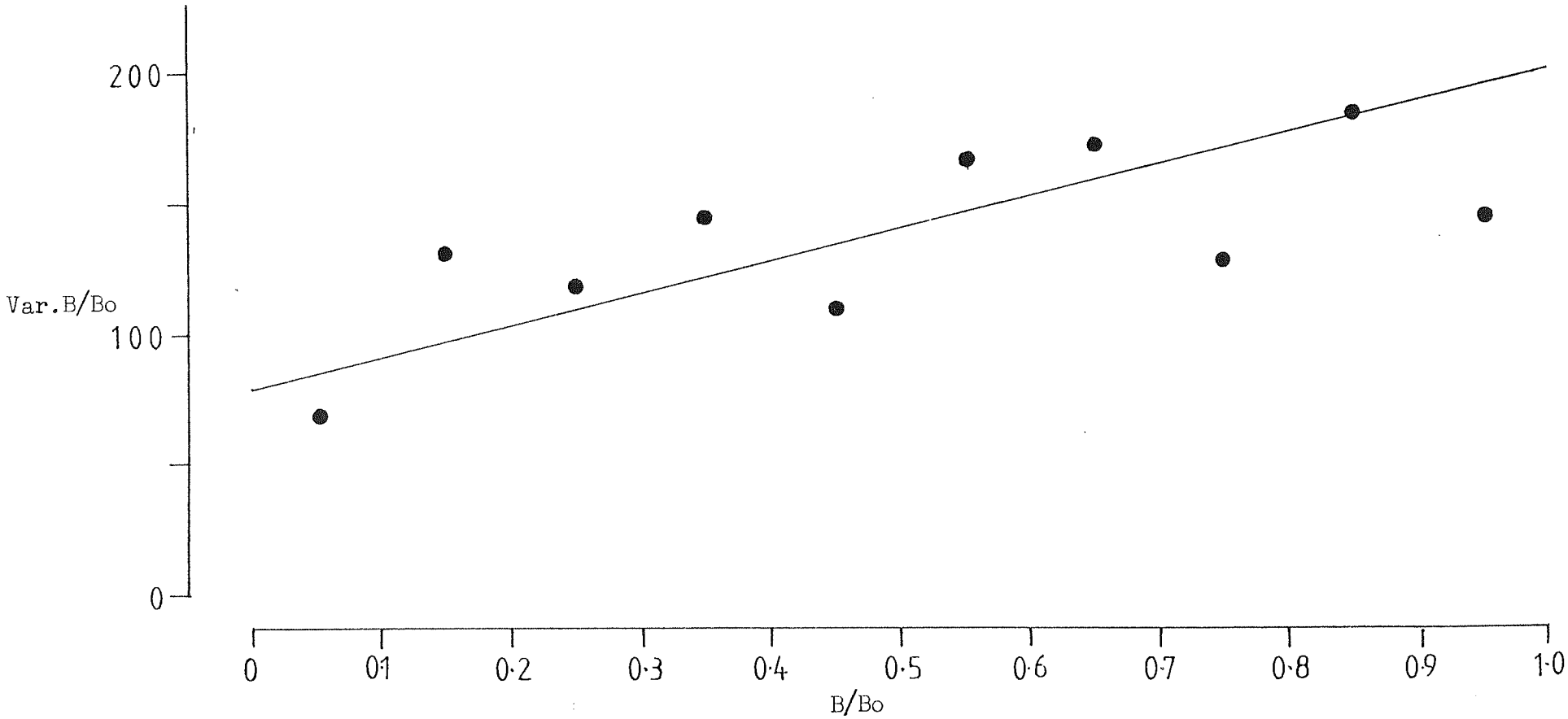


Figure 54 Quality control chart for 5 glucagon assays. Data derived from computer analysis.

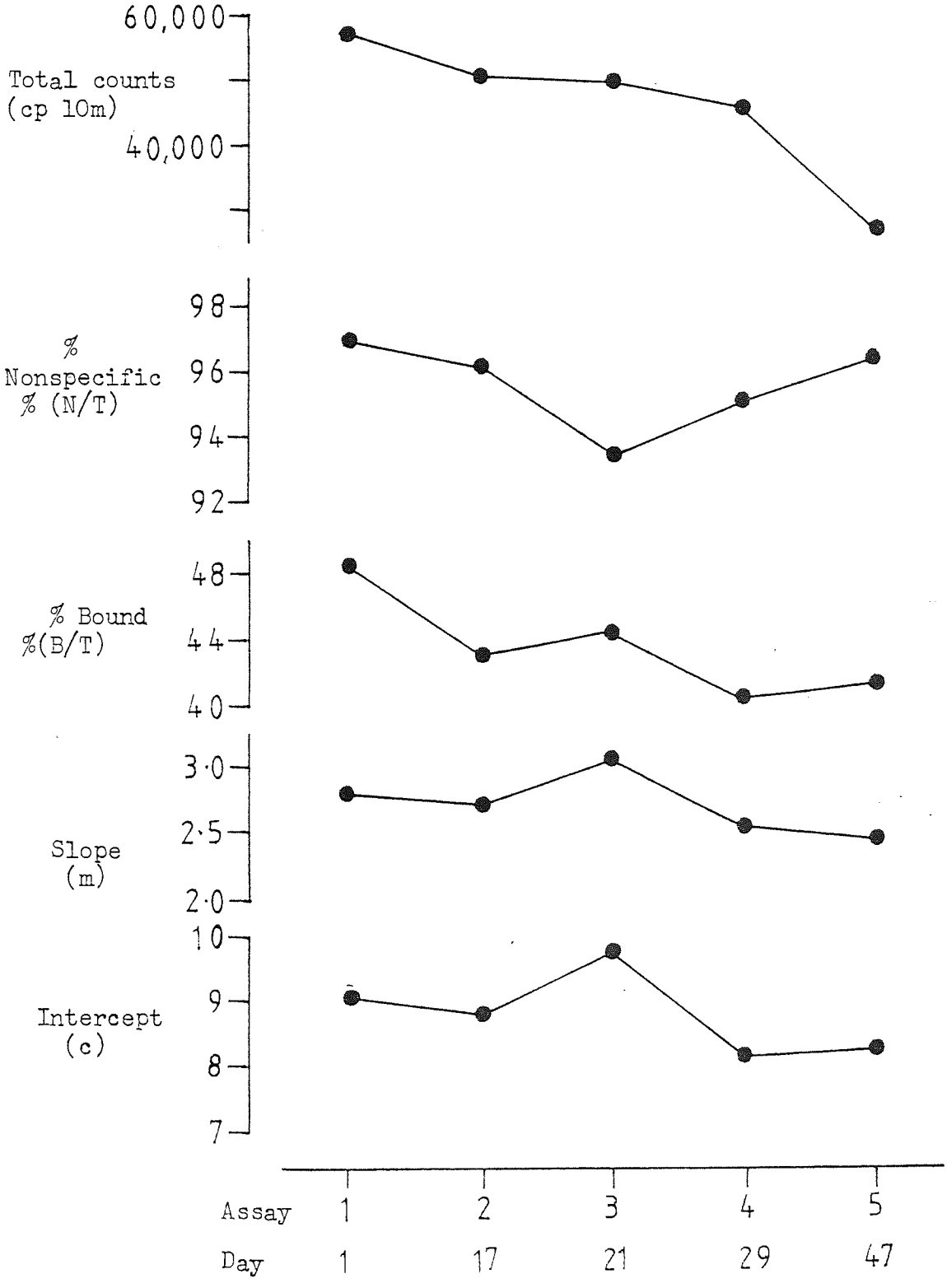


FIGURE 55

COMPUTER ANALYSIS OF RADIOIMMUNOASSAY

DATA PROCESSING: PROGRAMME LISTINGS

```
 0 'BEGIN'  'INTEGER' A, J, M, SC, EX;  'REAL' DEV, SD, SX, SXQ, U;
 1                    SELECT INPUT(5);  SELECT OUTPUT(0);
 2 'COMMENT' CHANNELS-RATIO CALCULATION;
 3 COPYTEXT('('*')');  M:=45-(3*READ);  A:=READ;  A:=READ;
 4 'FOR' J:=1 'STEP' 1 'UNTIL' A 'DO' DEV:=READ;
 5 'FOR' J:=1 'STEP' 1 'UNTIL' A 'DO' M:=M + READ;
 6 'FOR' J:=1 'STEP' 1 'UNTIL' 7 'DO' DEV:=READ;
 7 WRITETEXT('('('('3C')'RATIOS%OF%CHANNELS%1%AND%2%IN%DATA%OF%
 8       ABOVE%FILE;%%%N=%')');  PRINT(M,3,0);  NEWLINE(2);
 9 'BEGIN'  'ARRAY' NI[1:M];  SX:=SXQ:=0;
10       'FOR' J:=1 'STEP' 1 'UNTIL' M 'DO''BEGIN'
11             'FOR' A:=1 'STEP' 1 'UNTIL' 5 'DO' DEV:=READ;
12             U:=READ;  NI[J]:=DEV/U;  PRINT(NI[J],1,5);
13             SXQ:=SXQ + NI[J]↑2;  SX:=SX + NI[J] 'END';
14       U:=SX/M;  SD:=SQRT(SXQ/M - U↑2);  A:=EX:=0;
15       WRITETEXT('('('2C')'MEAN%=%')');  PRINT(U,1,5);
16       WRITETEXT('('%%S.D.%=%')');  PRINT(SD,1,7);
17       WRITETEXT('('%%NUMBER%OF%RESULTS%=%')');  PRINT(M,3,0);
18       WRITETEXT('('('2C')'RATIOS%OUTSIDE%MEAN%+/-%2%X%SD:%')');
19       'FOR' J:=1 'STEP' 1 'UNTIL' M 'DO''BEGIN'
20             DEV:=ABS(U - NI[J]);  'IF' DEV > 2*SD 'THEN''BEGIN'
21             PRINT(NI[J],1,5);  A:=A + 1;  NI[A]:=NI[J] 'END';  'END';
22       WRITETEXT('('('2C')'RATIOS%OUTSIDE%MEAN%+/-%3%X%SD:%')');
23       'FOR' J:=1 'STEP' 1 'UNTIL' A 'DO'
24             'BEGIN'  DEV:=ABS(U - NI[J]);  'IF' DEV > 3*SD 'THEN'
25                   'BEGIN'  PRINT(NI[J],1,5);  SX:=SX - NI[J];
26                   SXQ:=SXQ - NI[J]↑2;  EX:=EX+1 'END';  'END';
27       'IF' EX=0 'THEN' WRITETEXT('('%NONE')') 'ELSE''BEGIN'
28       M:=M-EX;  U:=SX/M;  SD:=SQRT(SXQ/M - U↑2);
29       WRITETEXT('('('2C')'RECALCULATED%PARAMETERS%OMITTING%+/-%3
30       %X%S.D. OUTLIERS:'('C')'MEAN%(CARRIED%FORWARD%TO%LATER%
31       CALCULATIONS)%=%')');  PRINT(U,1,5);
32       WRITETEXT('('%%S.D.%=%')');  PRINT(SD,1,7);
33       WRITETEXT('('%%NUMBER%OF%RESULTS%=%')');  PRINT(M,3,0) 'END';
```

```
                    'END' OF CHANNELS-RATIO CALCULATION;
                    SELECT INPUT(0); SELECT OUTPUT(4);
COPYTEXT('('*')'); SC:=READ; SXQ:=READ; EX:=READ;
'BEGIN' 'PROCEDURE' COLLECT (I, N); 'INTEGER' I, N;
      'BEGIN' 'COMMENT' READ RAW DATA; N:=N * 2; H:=0;
      'FOR' I:=I 'STEP' -1 'UNTIL' -1 'DO' 'BEGIN'
            'FOR' J:=1 'STEP' 1 'UNTIL' 4 'DO' 'BEGIN'
                  NI[J]:=READ; PRINT(NI[J],3,0) 'END';
            'FOR' J:=5,6 'DO' 'BEGIN' NI[J]:=READ; PRINT(NI[J],6,0)'END';
            NI[6]:=NI[6]*0; PRINT(NI[6],6,0);
            'FOR' J:=5,6 'DO' 'BEGIN' NI[J]:=(NI[J]/NI[4]) * 100;
                  PRINT(NI[J],5,2); H:=H + 1; NI[6+H]:=NI[J];
                  'IF' H=2 'AND' N=2 'THEN' 'BEGIN' 'COMMENT' UNKNOWNS;
                        MEAN:=(NI[7] + NI[8])/2; PRINT(MEAN,5,2);
                        'GOTO' NONSTOP 'END'; 'COMMENT' STANDARDS;
                  'IF' H=6 'AND' N=6 'THEN' 'FOR' A:=1 'STEP' 1 'UNTIL' 6
                        'DO' NI[13-K-SC-SXQ)*6 + A + 2]:=NI[6+A];
                  'IF' H=N 'THEN' 'BEGIN' A:=N;
                        LOOP: DUD:=MEAN:=SD:=SX:=0;
                        'FOR' H:=1 'STEP' 1 'UNTIL' A 'DO' 'BEGIN'
                              SX:=SX + NI[6+H]; SD:=SD + NI[6+H]12 'END';
                        MEAN:=SX/A;
                        SD:=SQRT(SD/(A-1) - ((SX * MEAN)/(A-1)));
                        PRINT(MEAN,5,2); PRINT(SD,4,2);
                        'IF' (SD*100)/MEAN > 1 'THEN'
                        'FOR' H:=1 'STEP' 1 'UNTIL' A 'DO' 'BEGIN'
                              INSPECT: DEV:=ABS(NI[6+H] - MEAN);
                              'IF' DEV/SD > 1.5 'THEN' 'BEGIN'
                                    PRINT(NI[6+H],5,2); DUD:=DUD + 1;
                                    'IF' H < A 'THEN' 'BEGIN'
                                          NI[6+H]:=NI[6+A];
                                          A:=A-1; 'GOTO' INSPECT 'END';
                                    A:=A - 1 'END'; 'END';
                        'IF' DUD 'NE' 0 'THEN' 'BEGIN' WRITETEXT
                              ('('('('C58S')'RECALCULATION%%')');
                              'GOTO' LOOP 'END'; NONSTOP:
```

```
                                H:=0;  'IF' C < 3 'THEN''BEGIN'
                                       'COMMENT' STANDARDS;  SYQ:=K+SC+SXQ;
                                       'IF' SYQ=11 'THEN' SY:=SD;
                                       'IF' SYQ=10 'THEN' S[1]:=(SY*SXY*100) /
                                          (V * (XB-MEAN));
                                       'IF' SYQ<11 'AND' K>-1 'THEN' S[12-SYQ]:=
                                          (SD*SXY*XB*100) / (MEAN*V*(XB-MEAN));
                                       'IF' C=1 'THEN' 'BEGIN' PRINT(SXY,22,0);
                                       XL:=MEAN 'END''ELSE' S[K]:=MEAN;
                                       'IF' K=0 'THEN' WRITETEXT('('''('19S')'
                                       ZERO')');  'IF' K=-1 'THEN' WRITETEXT
                                       ('('''('20S')'BNS')');  'IF' K=-2 'THEN'
                                       WRITETEXT('('''('21S')'TC')');  K:=K-1;
                                       'IF'K>0'THEN'SXY:=SXY/2; NEWLINE(1) 'END'
                                'ELSE'  'COMMENT' UNKNOWNS;
                                       'IF' MEAN > S[0] 'AND' MEAN < S[-1] 'THEN'
                                       'BEGIN' YB:=(S[-1] - MEAN)/(S[-1] - S[0]);
                                       SX:=(LN(YB/(1-YB)) - SY)/XB;
                                       SX:=EXP(SX * 2.30259);  PRINT(SX,5,2);
                                       SX:=SX * DIL[C-2]; PRINT(SX,6,2);
                                       'END''ELSE' WRITETEXT('('%%%%.....')');
                           'END' OF H=0;
              'END' OF J=5,6;
              NEWLINE(1);          'END' OF I=1;     C:=C + 1;
              NEWLINE(1)           'END' OF COLLECT;
'PROCEDURE' GHLIMITS(XMIN, XMAX, YMIN, YMAX); 'VALUE' XMIN, XMAX,
              YMIN, YMAX;  'REAL' XMIN, XMAX, YMIN, YMAX;  'EXTERNAL';
'PROCEDURE' GHREGION(XMIN, XMAX, YMIN, YMAX);  'VALUE' XMIN, XMAX,
              YMIN, YMAX;  'REAL' XMIN, XMAX, YMIN, YMAX;  'EXTERNAL';
'PROCEDURE' GHPTPLOT(XV, YV, M, N, C);  'VALUE' M, N, C;  'REAL''ARRAY'
              XV, YV;  'INTEGER' M, N, C;  'EXTERNAL';
'PROCEDURE' GHAXESSI(X, Y);  'VALUE' X, Y;  'REAL' X, Y;  'EXTERNAL';
'PROCEDURE' GHPOINT(X, Y);  'VALUE' X, Y;  'REAL' X, Y;  'EXTERNAL';
'PROCEDURE' GHJOIN (X, Y);  'VALUE' X, Y;  'REAL' X, Y;  'EXTERNAL';
'PROCEDURE' GHCRSET(N);  'VALUE' N;  'INTEGER' N;  'EXTERNAL';
'PROCEDURE' GHCRSIZE(H);  'VALUE' H;  'REAL' H;  'EXTERNAL';
```

```
106 'PROCEDURE' GHPLOTAS(X, Y, P); 'VALUE' X, Y; 'REAL' X, Y;
107         'STRING' P; 'EXTERNAL';
108 'PROCEDURE' GHROTATE(X, Y, A); 'VALUE' X, Y, A; 'REAL' X, Y, A;
109         'EXTERNAL';
110 'PROCEDURE' GHUNROT; 'EXTERNAL'; 'PROCEDURE' GHBORDER; 'EXTERNAL';
111 'PROCEDURE' GHGREND; 'EXTERNAL';
112 'ARRAY' NI[1:(80 - ((6*(SC+SXQ)))], S[-2:12];
113 'INTEGER''ARRAY' DIL, REP [1:6]; 'INTEGER' C, H, IT, K;
114 'REAL' AI, AQ, CT, DUD, MEAN, MW, SY, SYQ, SXY, T, V, XB, YB;
115 'FOR' J:=1 'STEP' 1 'UNTIL' 6 'DO' DIL[J]:=READ;
116 'FOR' J:=1 'STEP' 1 'UNTIL' 6 'DO' REP[J]:=READ;
117 CT:=READ; V:=READ; MW:=READ; T:=READ; IT:=READ; AQ:=READ; AI:=READ;
118 NEWLINE(2); WRITETEXT('('LOW%STANDARDS%OMITTED%FROM%ASSAY:')');
119 PRINT(SC,1,0); WRITETEXT('('%%%%LOW%STANDARDS%IGNORED%IN%CALCULATION:
120 ')'); PRINT(SXQ,1,0); WRITETEXT('('('2C')'RATIO%OF%CHANNEL%1%
121 :%CHANNEL%2%(CR)%=%')'); PRINT(U,1,5);
122 WRITETEXT('('('2C')'STANDARDS'('2C')'%MODE%%%%%%%%TUBE%%TIME%%CHANNEL
123         %%CHANNEL%%CHANNEL%%COUNTS%PER%MINUTE(CHANNEL%2%X%CR)%%%%%%%
124         OUTLIERS%OUTSIDE%%%%CONCN.'('C5S')'CASSETTE%%%MIN%X%0.01%%1%%%%%
125         %%%2%%%%%2%X%CR.%%CHANNEL%1%%CHANNEL%2%%%%MEAN%%%%%%S.D.%%MEAN
126         %+/-%1.5%X%%SD%%%PG/ML.'('2C')'')');
127 K:=11-SC-SXQ; C:=1; SXY:=20000;
128 I:=3; COLLECT (3*K, I);
129 'FOR' J:=1 'STEP' 1 'UNTIL' (18*SXQ) 'DO' SYQ:=READ;
130 M:=4; COLLECT (12, M); K:=11-SC-SXQ;
131 WRITETEXT('('VALUES%OF%TC%AND%ENS%RESPECTIVELY%ARE')');
132 PRINT(S[-2],5,2); PRINT(S[-1],5,2);
133 WRITETEXT('('%%DIFFERENCE%=%')'); PRINT((S[-2] - S[-1]),4,2);
134 WRITETEXT('('%%RATIO%=%')'); PRINT((S[-1] / S[-2]), 1,4);
135 WRITETEXT('('%E0%=')'); SX:=S[-1] - S[0]; PRINT(SX,5,2);
136 SXQ:=S[-2] * NI[4]/100; SYQ:=SD * NI[4]/100;
137 WRITETEXT('('('2C')'MEAN%TOTAL%COUNT%=%')'); PRINT(SXQ,5,2);
138 WRITETEXT('('%S.D.%=%')'); PRINT(SYQ,3,3);
139 WRITETEXT('('%%%PIPETTING%ERROR%=%')');
140 'IF' SYQ↑2 > SXQ 'THEN' PRINT(100 * SQRT((SYQ↑2 - SXQ)/SXQ),3,3)
141         'ELSE' WRITETEXT('('NEGLIGIBLE')');
```

```
142 WRITETEXT('('('('7S')'MEAN%BO/T%=%')');  PRINT(SX/S[-2],1,5);
143             'COMMENT' VARIANCE &C. CALCULATIONS;   SXY:=20000;
144 WRITETEXT('('('('3CS')'CONCN.%%%%%%B%%%%%%MEAN%B%%(MEAN%B)/T%%%%B/BO%%%%
145       %MEAN%B/BO%%%%%VARIANCE%%%%%%%C.OF%V.'('19S')'ASSAY%%%**'('C76S')'
146       (SD/MEAN%X%100)'('12S')'PRECISION%(PG/ML)'('2C')'')');
147 'FOR' J:=1 'STEP' 1 'UNTIL' K 'DO''BEGIN'
148       YB:=S[J];    SX:=SYQ:=XB:=0;  PRINT(SXY,5,0);
149       'FOR' A:=1 'STEP' 1 'UNTIL' 6 'DO''BEGIN'
150             SY:=S[-1] - P[6 + 6*J + A];  PRINT(SY,5,2); XB:=XB+SY;
151             SY:=SY/SX;  'IF' A < 6 'THEN' 'BEGIN'
152                   PRINT(SY,23,3); WRITETEXT('('''('C8S')''')') 'END';
153             SXQ:=SXQ * SY;  SYQ:=SYQ + SY↑2  'END';
154       XB:=XB/6;  PRINT(XB,4,2);  S[J]:=XB/S[-2];  PRINT(S[J],1,5);
155       PRINT(SY,3,3);  SYQ:=SYQ/5 - SXQ↑2/30;  NI[11+J]:=SXQ/6;
156       PRINT(NI[11+J],3,5);  SPACE(3);  PRINT(SYQ,0,3);  NI[J]:=SXY;
157       SXY:=SXY/2;  PRINT(SQRT(SYQ)*100/NI[11+J],5,3);
158       'IF' YB < 500 'THEN' PRINT(ABS(YB),23,2) 'ELSE' WRITETEXT
159       ('('''('21S')'>%500')'); NEWLINE(2) 'END' OF VARIANCE CALCULATION;
160 WRITETEXT('('''('70S')'AT%ZERO%CONCENTRATION%(=%SENSITIVITY):%')');
161 PRINT(S[K+1],2,3);  WRITETEXT('('''('2C6S')'***%(NOTE:%"ASSAY%PRECISION"%
162 BASED%ON%C.V.%OF%BAM%COUNT%RATES;%%THE%TWO%HIGHEST%CONCENTRATIONS
163 %USE%THE%SAME%SLOPE)'('3C')'FINAL%VOLUME')');  PRINT(V,3,0);
164 WRITETEXT('('UL%%%%%%%%MOLECULAR%WEIGHT')');    PRINT(MW,4,0);
165 WRITETEXT('('%%%%%TRACER%CONCENTRATION')');     PRINT(CT,3,0);
166 WRITETEXT('('PG/ML'('2C')'SCATCHARD%PLOT;%%VALUE%OF%P*%=%')');
167 V:=V * MW * 1 & -5;  CT:=CT/V;  PRINT(CT,3,3);
168 SX:=SY:=SXY:=SXQ:=SYQ:=0;
169 'FOR' J:=1 'STEP' 1 'UNTIL' K 'DO''BEGIN'
170       NI[22+J]:=XB:=S[J] * (CT + (NI[J]/V));  SX:=SX+XB;
171       SXQ:=SXQ + XB↑2;  NI[33+JJ:=YB:=S[J]/(1-S[J]);  SY:=SY+YB;
172       SYQ:=SYQ + YB↑2;  SXY:=SXY + XB*YB  'END';
173 WRITETEXT('('('('C')'B/T(P*%+%P):%%%')');
174 'FOR' J:=1 'STEP' 1 'UNTIL' K 'DO' PRINT(NI[22+J],3,2);
175 WRITETEXT('('''('C')'B/T/(1-B/T):%%%')');
176 'FOR' J:=1 'STEP' 1 'UNTIL' K 'DO' PRINT(NI[33+J],3,2);
177 XB:=SX/K;  YB:=SY/K;  MEAN:=(SXY -(XB * SY)) / (SXQ -(XB * SX));
```

```
178 WRITETEXT('('!'('2C5S!')'SLOPE%M%=%')');    PRINT(MEAN,1,4);
179 SD:=YB - (MEAN * XB);
180 WRITETEXT('('%%INTERCEPT%C%=%')');  PRINT(SD,1,4);
181 DEV:=((SXY/K)-(XB*YB)) / SQRT(((SXQ/K)-(XB↑2))*((SYQ/K)-(YB↑2)));
182 WRITETEXT('('%%COEFFICIENT%R%=%')');    PRINT(DEV,1,4);
183 MW:=SXQ-(XB*SX);  DEV:=SYQ-(YB*SY) - (SXY - XB*SY)↑2/MW;
184 WRITETEXT('('!'('CSS')'T-VALUE%%=%')');
185 PRINT(ABS(MEAN/SQRT((DEV/(K-2))/MW)),2,4);
186 PRINT(K-2,4,0);  WRITETEXT('('DEGREES%OF%FREEDOM'('C')'
187 INVERSE%OF%SLOPE%(L/MOLE)%=%')');  PRINT(1/MEAN,2,3);
188 WRITETEXT('('%%-INTERCEPT%(MOLE/L)%=%')');  PRINT((0-SD)/MEAN,2,3);
189 WRITETEXT('('%%FREE%ENERGY%CHANGE%(J/MOLE)%=%')');
190 PRINT(8.314 * T * LN(55.5 & 12 / ABS(MEAN)),5,1);
191 WRITETEXT('('!'('2C')'OPTIMISATION:%%P*OPT%=%')');  PRINT(-4/MEAN,3,4);
192 WRITETEXT('('%%%%%%Q-OPT%=%')');  PRINT(-3/MEAN,3,4);
193 WRITETEXT('('%%%%%%P*-OPT/P*%=%')');  PRINT((-4/MEAN)/CT,1,4);
194 WRITETEXT('('%%%%%%Q-OPT/Q%=%')');  PRINT(3/SD,1,4);
195                         'COMMENT' B/T VS ARITH-DOSE GRAPH;
196 WRITETEXT('('!'('2C')'CONCENTRATION:%')');
197 'FOR' J:=1 'STEP' 1 'UNTIL' K 'DO' PRINT(NI[J],6,0);
198 WRITETEXT('('!'('C')'B/T%VALUES%%%%%')');
199 'FOR'J:=1 'STEP' 1 'UNTIL' K 'DO''BEGIN'
200         PRINT(S[J],2,3);  S[J]:=S[J]*10  'END';
201 SY:=ENTIER(S[K]) +'1';
202 GHLIMITS(1.7, 10.7, 2.5, 8);  GHREGION(0, 20000, 0, SY);
203 GHCRSET(1);                   GHPTPLOT(NI, S, 1, K, -1);
204 GHPTPLOT(NI, S, 1, K, 62);    GHREGION(0, 20, 0, SY/10);
205 GHAXESSI(2, 0.05);
206 GHLIMITS(0.5, 10.5, 1.5, 8);  GHREGION(-2, 20, -1, SY);
207 GHCRSIZE(0.25);   GHPLOTAS(10, SY-0.3, '('B/T%%VS%%CONCENTRATION')');
208 GHCRSIZE(0.20);  GHPLOTAS(6, -0.7, '('CONCENTPATION%(%NG%/ML%)')');
209 GHROTATE(-1.5, 1.0, 1.5708);  GHPLOTAS(-1.5, 1.0, '('MEAN-B%/%T')');
210 GHUNROT;          GHLIMITS(0, 11.75, 1.2, 9.45);        GHBORDER;
211                   'COMMENT' B/BO VS ARITH-DOSE GRAPH;
212 WRITETEXT('('!'('2C')'B/BO%VALUES:%%%%')');
213 'FOR' J:=1 'STEP' 1 'UNTIL' K 'DO''BEGIN'
```

```
214         S[J]:=NI[11+J];  PRINT(S[J],1,4) 'END';
215 GHLIMITS(1.6, 10.6, 11.5, 17);    SY:=ENTIER(S[K]) + 1;
216 GHREGION(0, 20000, 0, SY);         GHPTPLOT(NI, S, 1, K, -1);
217 GHPTPLOT(NI, S, 1, K, 62);         GHREGION(0, 20, 0, SY);
218 GHAXESSI(2, SY/10);                GHCRSIZE(0.05);
219 GHLIMITS(0.6, 10.6, 10.5, 17);    GHREGION(-2, 20, -0.1, SY);
220 GHPLOTAS(10, SY-0.05, '('B/BO%%VS%%ARITH.DOSE%')');
221 GHCRSIZE(0.04);  GHPLOTAS(6, -0.04, '('CONCENTRATION%(%NG/ML%)')');
222 GHROTATE(-1, 0.2, 1.5708);  GHPLOTAS(-1, 0.2, '('B%/%BO')');  GHUNROT;
223 GHLIMITS(0, 11.75, 10.2, 18.45);  GHBORDER;
224               'COMMENT' B/BO VS. LOG-DOSE GRAPH;
225 WRITETEXT('('('2C')'LOG%CONCN.:%%%%%')');
226 'FOR' J:=1 'STEP' 1 'UNTIL' K 'DO' 'BEGIN'
227       NI[J]:=LN(NI[J])/2.30259;    PRINT(NI[J],1,4)  'END';
228 GHLIMITS(1.7, 10.7, 20.5, 25.5);   GHREGION(0, 5, 0, SY);
229 GHPTPLOT(NI, S, 1, K, 62);
230 GHPTPLOT(NI, S, 1, K, -1);         GHAXESSI(1, SY/10);
231 GHLIMITS(0.7, 10.7, 19.5, 26.5);   GHREGION(-1, 5, -0.1, SY);
232 GHCRSIZE(0.05);  GHPLOTAS(2.8, 0.9, '('B/BO%%VS%%LOG.DOSE')');
233 GHCRSIZE(0.04);  GHPLOTAS(1, -0.04, '('LOG%CONCENTRATION%(%PG/ML%)')');
234 GHROTATE(-0.75, 0.2, 1.5708);  GHPLOTAS(-0.75, 0.2, '('B%/%BO')');
235 GHUNROT;  GHLIMITS(0, 11.75, 19.1, 27.35);  GHBORDER;
236           'COMMENT' LOGIT VS. LOG-DOSE GRAPH & REGRESSION;
237 'FOR' J:=1 'STEP' 1 'UNTIL' K 'DO' 'BEGIN'  NI[22+J]:=1;
238       'IF' NI[11+J] < 0 'OR' NI[11+J] > 1 'THEN'  S[J]:=NI[J]:=0
239       'ELSE'  S[J]:=LN(NI[11+J]/(1 - NI[11+J])) 'END';
240 CT:=NN:=0;
241 CIRCULAR;       WRITETEXT('('('C')'LOGIT%TERM:%%%%%')');
242 V:=SX:=SY:=SXY:=SXQ:=SYQ:=0;
243 'FOR' J:=1 'STEP' 1 'UNTIL' K 'DO'
244       'IF' NI[J] 'NE' 0 'THEN' 'BEGIN'  PRINT(S[J],1,4);
245       SX:=SX + NI[J]*NI[22+J];  SY:=SY + S[J]*NI[22+J];
246       SXY:=SXY + NI[J]*S[J]*NI[22+J];  SXQ:=SXQ + (NI[J]↑2)*NI[22+J];
247       SYQ:=SYQ + (S[J]↑2)*NI[22+J];  V:=V+NI[22+J]  'END'
248       'ELSE' WRITETEXT('('%.....%%')');
249 XB:=SX/V;  YB:=SY/V;  MEAN:=(SXY -(XB * SY)) / (SXQ -(XB * SX));
```

```
250 WRITETEXT('('!('2C5S')'SLOPE%M%=%')');  PRINT(MEAN,2,4);
251 SD:=YB - (MEAN * XB);
252 WRITETEXT('('%%INTERCEPT%C%=%')');  PRINT(SD,2,4);
253 DEV:=((SXY/V)-(XB*YB)) / SQRT(((SXQ/V)-(XB↑2))*((SYQ/V)-(YB↑2)));
254 WRITETEXT('('%%COEFFICIENT%R%=%')');  PRINT(DEV,1,4);
255 WRITETEXT('('%%%%SUM%OF%WEIGHTS%=')');  PRINT(V,4,4);
256       'IF' MV 'NE' IT 'AND' MV 'NE' 0 'THEN''GOTO' SHORTCUT;
257 WRITETEXT('('!('2C')'COORDINATES%OF%FIT'('12S')'VALUES%OF%SX,%SY,%SXY,%SXQ,
258       %SYQ:')');  PRINT(SX,4,2);  PRINT(SY,4,2);
259       PRINT(SXY,4,2);  PRINT(SXQ,4,2);  PRINT(SYQ,4,2);
260 WRITETEXT('('!('C')'S.S.D.(FROM%CORRELATION%COEFFICIENT)%=%')');
261 DUD:=SYQ-(YB*SY);  DEV:=(1-DEV↑2)*DUD;  PRINT(DEV,1,4);
262 WRITETEXT('('!(FROM%SLOPE)%=%')');  T:=SXQ-(XB*SX);
263 DEV:=DUD - ((SXY-(XB*SY))↑2) / T;  PRINT(DEV,1,4);
264 WRITETEXT('('%%M.SQ.DEV.%FROMYREGRESSION%=%')');  DEV:=DEV/(K-2);
265 PRINT(DEV,1,4);
266 WRITETEXT('('!('C')'S.S.D.FROM%REGRESSION%=%')');  PRINT(SQRT(DEV),1,4);
267 WRITETEXT('('%%S.S.D.OF%REGRESSION%COEFFICIENT%=%')');
268 DEV:=SQRT(DEV/T);  PRINT(DEV,1,4);  WRITETEXT('('%%T-STATISTIC%=%')');
269 DEV:=MEAN/DEV;  PRINT(DEV,1,4);  PRINT(K-2,2,0);  WRITETEXT('('D.F.')');
270 SXY:=SYQ:=0;
271 'FOR' J:=1 'STEP' 1 'UNTIL' K 'DO''IF' N[J] > 0 'THEN''BEGIN'
272       'IF' S[J] > SXY 'THEN' SXY:=S[J];
273       'IF' S[J] < SYQ 'THEN' SYQ:=S[J]  'END';
274 SYQ:=ENTIER(SYQ);  SXY:=ENTIER(SXY) + 1;
275 'IF' SXY - SYQ < 7 'THEN' SYQ:=SXY - 7;
276 GHREGION(0, 5, SYQ, SXY);  GHLIMITS(14.5, 24, 1.5*CT, 7.5*CT);
277 GHPTPLOT(N, S, 1, K, 62);  GHAXESSI(1, 1);
278 'IF' SD < SXY 'THEN' GHPOINT(0, SD) 'ELSE' GHPOINT((SXY-SD)/MEAN, SXY);
279 'IF' (5*MEAN + SD) < SYQ 'THEN' GHJOIN((SYQ-SD)/MEAN, SYQ) 'ELSE'
280       GHJOIN(5, 5*MEAN + SD);
281 GHREGION(-0.5, 5.5, SYQ-0.5, SXY+0.5);
282 GHLIMITS(13.5, 24.75, 1.5*CT, 8*CT);
283 GHCRSIZE(0.3);  GHPLOTAS(3, SXY, '('IMMUNOASSAY%STANDARD%GRAPH')');
284 'IF' CT=0 'THEN' GHPLOTAS(3.7, SXY-0.5, '('UNWEIGHTED')') 'ELSE'
285       GHPLOTAS(3.8, SXY-0.5, '('WEIGHTED')');  GHCRSIZE(0.25);
```

```
286 GHPLOTAS(0.5, -1, '('LOG%CONCENTRATION%OF%INSULIN%(%PG/ML%)')');
287 GHROTATE(-0.3, 0, 1.5708); GHPLOTAS(-0.3, 0, '('LOGIT%TERM')');
288 GHUNROT; GHLIMITS(13, 24.75, 0.8+CT, 9.05+CT); GHBORDER;
289 'IF' MW=0 'THEN''BEGIN' WRITETEXT('('''('2C')'ITERATIONS%REQUIRED:')');
290       PRINT(IT,2,0); 'IF' IT 'NE' 0 'THEN''BEGIN' WRITETEXT
291       ('('%%%%A0%=%')'); PRINT(A0,2,6); WRITETEXT('('%%%%A1%=%')');
292       PRINT(A1,2,6) 'END'; 'END'; CT:=9;
293 SHORTCUT; 'IF' IT=0 'THEN''GOTO' RUNTHROUGH;
294                 'COMMENT' ITERATION CIRCUIT;
295 MW:=MW+1; WRITETEXT('('''('2C')'ITERATION')'); PRINT(MW,2,0);
296 WRITETEXT('('''('2C')'LOG%CONCN.%%%%%%YHI%%%%%EXP(YHI)%%%%%%YH%%%%%%
297 YH(1-YH)%%%WKG.LOGIT%%%WEIGHT%%%%%THIS%Y%%%%%NEXT%Y'('2C')''')');
298 'FOR' J:=1 'STEP' 1 'UNTIL' K 'DO''IF' NI[J] 'NE' 0 'THEN''BEGIN'
299       S[J]:=MEAN * NI[J] + SD; NI[22+J]:=EXP(S[J]);
300             PRINT(NI[J],3,4); PRINT(S[J],3,4); PRINT(NI[22+J],3,4);
301       NI[33+J]:=NI[22+J] / (1 + NI[22+J]);
302       NI[22+J]:=(1 - NI[33+J]) * NI[33+J];
303             PRINT(NI[33+J],3,4); PRINT(NI[22+J],3,4);
304       S[J]:=(NI[11+J] - NI[33+J]) / NI[22+J] + S[J];
305       NI[22+J]:=NI[22+J]+2 / (A0 + (A1 * NI[33+J]));
306             PRINT(S[J],3,4); PRINT(NI[22+J],3,4);
307             PRINT(NI[11+J],3,4); PRINT(NI[33+J],3,4);
308       NEWLINE(1); NI[11+J]:=NI[33+J] 'END';
309 'GOTO' CIRCULAR;
310 RUNTHROUGH: GHGREND;
311 XB:=MEAN; SY:=SD; WRITETEXT('('''('P')'UNKNOWNS'('2C')''')');
312 'FOR' IT:=1 'STEP' 1 'UNTIL' EX 'DO''BEGIN'
313       WRITETEXT('('DILUTION%=')'); PRINT(DIL[IT],3,2);
314       WRITETEXT('('%%NUMBER%=')'); PRINT(REP[IT],4,0);
315 WRITETEXT('('''('2C')'%MODE%%%%%%%%TUBE%%TIME%%CHANNEL%%CHANNEL%%
316             CHANNEL%%COUNTS%PER%MINUTE(CHANNEL%2%X%CR)%%CONCN.%%%%
317             FINAL%CONC.'('CPS')'CASSETTE%%%MIN%X%0.01%%1
318             %%%%%%%%2%%%%%2%X%CR.%%CHANNEL%1%%CHANNEL%2%%%%%
319             MEAN%%%%%%PG/ML.%%%%%PG/ML.'('2C')''')');
320       M:=1; COLLECT (REP[IT], M) 'END';
321 'END'; 'END'
```

Figure 56a Simplified flowchart of radioimmunoassay data processing programme.

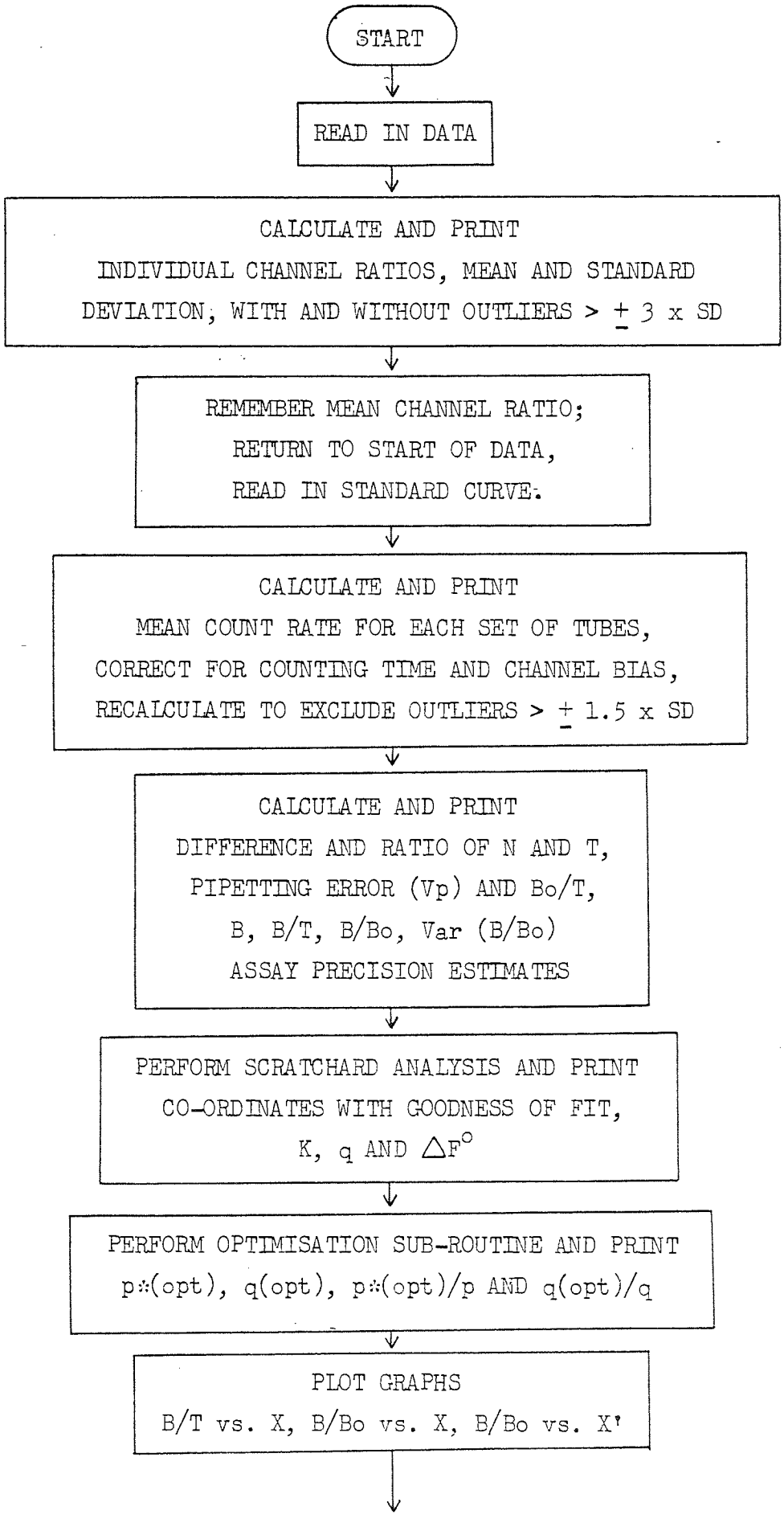


Figure 56b Simplified flowchart of radioimmunoassay data processing programme.

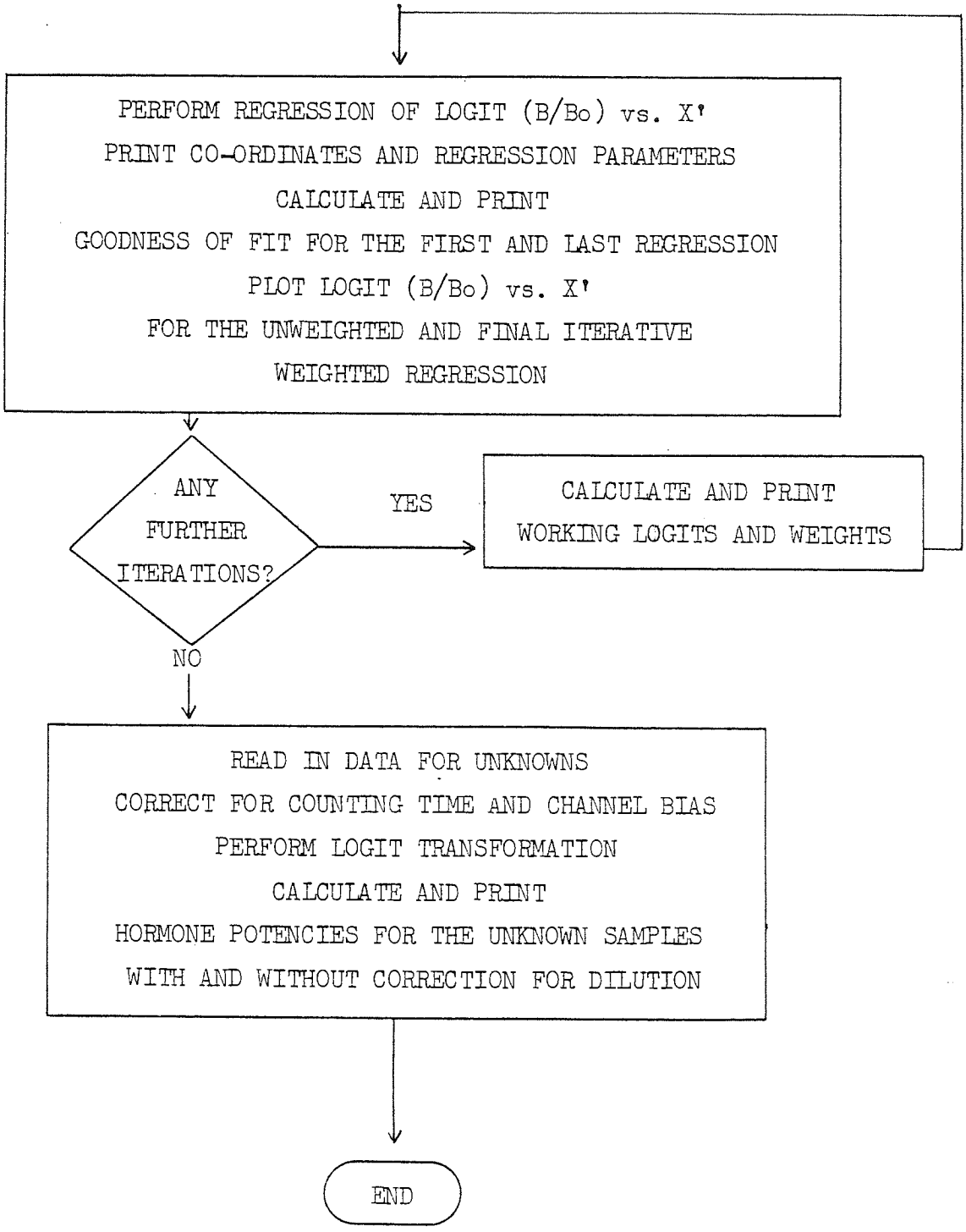


FIGURE 57

SAMPLE COMPUTER OUTPUT FOR
CHANNEL RATIOS IN THE ANALYSIS
OF GLUCAGON RADIOIMMUNOASSAY DATA

DATA INPUT 6,5,77
GLI RIA
FILENAME PIRG6

RATIOS OF CHANNELS 1 AND 2 IN DATA OF ABOVE FILE; M = 354

0,94561	0,94773	0,94815	0,94638	0,94637	0,94588	0,94874	0,94711	0,94706	0,94774	0,94602	0,94482
0,94711	0,95021	0,94837	0,94570	0,94575	0,94985	0,94921	0,94678	0,94931	0,94791	0,94561	0,94668
0,94861	0,94943	0,95093	0,94381	0,94349	0,94928	0,94577	0,94764	0,94827	0,95373	0,94380	0,94412
0,95328	0,94889	0,94618	0,94963	0,94683	0,94741	0,94956	0,94254	0,94562	0,95415	0,94808	0,94713
0,95013	0,95214	0,94757	0,94722	0,94825	0,95032	0,94727	0,94878	0,94897	0,94587	0,94986	0,94925
0,94810	0,94614	0,94969	0,95090	0,94878	0,94690	0,95002	0,95094	0,94941	0,95052	0,95065	0,94669
0,94697	0,94590	0,94978	0,94845	0,94890	0,94635	0,95114	0,94783	0,94555	0,94897	0,94575	0,94907
0,94860	0,94719	0,94761	0,94747	0,94817	0,94739	0,94711	0,95171	0,94824	0,94949	0,94982	0,94745
0,94569	0,95120	0,94915	0,94947	0,94630	0,94542	0,94917	0,94833	0,94634	0,94818	0,94940	0,94957
0,94865	0,94982	0,95016	0,94805	0,94992	0,94604	0,94374	0,95140	0,95282	0,95064	0,94935	0,94626
0,94281	0,94824	0,94908	0,94610	0,94733	0,94808	0,94982	0,94853	0,94648	0,94361	0,94757	0,94677
0,94538	0,94635	0,94767	0,94977	0,94979	0,94482	0,94843	0,94575	0,94592	0,94656	0,94517	0,94611
0,94720	0,94695	0,94838	0,94381	0,95022	0,94547	0,94635	0,94863	0,94527	0,95038	0,94553	0,94406
0,94510	0,94231	0,94803	0,94410	0,94768	0,94564	0,94665	0,94552	0,94438	0,94573	0,94706	0,94558
0,94747	0,94532	0,94633	0,94597	0,94912	0,94469	0,94969	0,94755	0,94874	0,94670	0,94711	0,94810
0,94910	0,94774	0,94762	0,94575	0,94246	0,94627	0,94685	0,95092	0,95130	0,95070	0,94885	0,94964
0,94306	0,94650	0,94435	0,94685	0,94509	0,94802	0,94303	0,94609	0,94949	0,94536	0,94597	0,94556
0,94718	0,94611	0,94770	0,94802	0,94811	0,94784	0,94312	0,94796	0,94462	0,94615	0,94493	0,94543
0,94627	0,94621	0,94423	0,94561	0,94882	0,94519	0,94646	0,94492	0,94620	0,94342	0,94380	0,94280
0,94505	0,94706	0,94336	0,94485	0,94872	0,94663	0,94457	0,94604	0,94668	0,94639	0,94992	0,94697
0,95044	0,94837	0,94824	0,94927	0,94711	0,94904	0,94998	0,94744	0,94890	0,94968	0,94348	0,94828
0,94684	0,94661	0,94806	0,94503	0,95051	0,94882	0,94825	0,94914	0,95011	0,95181	0,94692	0,94120
0,94881	0,94659	0,94891	0,94404	0,95028	0,95085	0,94538	0,95073	0,95051	0,94874	0,94966	0,94765
0,94812	0,95154	0,94891	0,94800	0,94612	0,95092	0,94690	0,94959	0,94743	0,94620	0,94351	0,94865
0,94821	0,94869	0,94685	0,94784	0,94671	0,94978	0,94793	0,94644	0,94802	0,94829	0,94688	0,94601
0,94566	0,94139	0,94677	0,94670	0,94826	0,94270	0,94707	0,94220	0,94469	0,94764	0,95006	0,94975
0,94692	0,94730	0,94975	0,94786	0,95092	0,94721	0,94747	0,94381	0,94465	0,94551	0,94599	0,94808
0,94746	0,94676	0,94738	0,95000	0,94483	0,95000	0,94644	0,94575	0,94831	0,94679	0,94519	0,94510
0,94415	0,94445	0,94589	0,94567	0,94558	0,94569	0,94422	0,94403	0,94815	0,94319	0,94557	0,94523
0,94794	0,95020	0,94956	0,94663	1,01362	0,96684						

MEAN = 0,94759 S.D. = 0,0042766 NUMBER OF RESULTS = 354

RATIOS OUTSIDE MEAN +/= 2 X SD: 1,01362 0,96684

RATIOS OUTSIDE MEAN +/= 3 X SD: 1,01362 0,96684

RECALCULATED PARAMETERS OMITTING +/= 3 X S.D.OUTLIERS;
MEAN (CARRIED FORWARD TO LATER CALCULATIONS) = 0,94735 S.D. = 0,0022125 NUMBER OF RESULTS = 352

NUMBER OF PAGES 2

CHAPTER 5

APPLICATION OF RADIOIMMUNOLOGICAL TECHNIQUES TO THE STUDY OF THE OBESE HYPERGLYCAEMIC SYNDROME IN MICE (GENOTYPE ob/ob)

IMPACT OF RADIOIMMUNOLOGICAL TECHNIQUES ON DIABETES RESEARCH

The discovery by von Mering and Minkowski (1890) that total pancreatectomy resulted in the development of a syndrome in the dog that was similar to that of diabetes mellitus in man, together with the demonstration by Banting and Best that the acute manifestations of the diabetic syndrome of the dog and man were reversed by the administration of insulin isolated from the pancreas (Banting & Best, 1922; Banting et al., 1922a), led to the conclusion that human diabetes mellitus was due to an inherited or acquired insufficiency of the pancreas (Macleod, 1924). Nevertheless, the absence of marked structural changes in the islets of Langerhans of diabetic patients examined at autopsy raised some uncertainties about the role of islet pathology in the aetiology and course of the human disease state (Warres & Root, 1925; Stansfield & Warren, 1928). With the demonstration that ablation of either the adenohypophysis or the adrenal glands ameliorated the severity of the metabolic derangements of the depancreatized animal (Houssay & Biasotti, 1931; Long & Lukens, 1936) came hypotheses that the insulin-antagonistic products of these endocrine glands may be involved in the pathogenesis of diabetes in man (Houssay, 1936; Long et al., 1940; Young, 1940, 1951). Using bioassay techniques which exploited the enhanced tissue sensitivity of surgically prepared alloxan diabetic rodents, Bornstein and Lawrence (1951 a, b) were unable to detect insulin in the plasma of thin ketotics but reported almost normal amounts in that of obese nonketotic diabetics. Subsequent studies conducted by Moloney and Coval (1955) confirmed that the production of a specific insulin deficiency evoked by the injection of an insulin antiserum into rats was associated with the development of a diabetic syndrome. However, the precise nature of the insulin secretory defect in human maturity onset diabetes mellitus was not established until the introduction of the radioimmunoassay for insulin by Yalow and Berson (1959). This technique, which represented the seminal principle for the

development of specific competitive protein binding radioassays for the quantification of virtually all substances of biomedical interest, has led to a more fundamental understanding of both hormone-hormone and hormone-substrate interrelationships. Indeed, most of our present knowledge concerning the mechanisms involved in the regulation of endocrine pancreatic function in both normal and pathogenic states has been a direct result of the application of radioimmunological techniques to the study of diabetes (Buchanan, 1975; Cerasi & Luft, 1976; Turner & Holman, 1976; Unger, 1976a; Yalow, 1976; Cahill, 1977; Unger & Orci, 1977).

ROLE OF ANIMALS IN DIABETES RESEARCH

Experimentation on different species of animals has long been recognised as a valuable approach to the understanding of human physiology. Aristotle drew attention to this during the fourth century B.C. and William Harvey re-emphasised the sentiment almost two thousand years later when he gathered evidence on the circulation of blood in some forty different animal species. Animal experimentation clearly featured prominently in the pioneering investigations of carbohydrate metabolism. Using rabbits, Claude Bernard (1857) studied the regulation of blood sugar and Paul Langerhans (1869) described the pancreatic islets. Von Mering and Minkowski (1890) used dogs to identify an association between the pancreas and diabetes, as did Banting and Best (1922) to demonstrate that insulin extracted from the pancreas could ameliorate the symptoms of diabetes. Recognition of the importance of using animals rather than patients in the early stages of an investigation, together with the value of the development of micromethods which permitted frequent estimation of blood sugar, undoubtedly represented the turning point in diabetes research (Young, 1960; Leibowitz, 1972). Indeed, comparative research has continued to make a substantial contribution to the advance-

ment in this branch of medicine, not only to elaborate the mechanisms involved in the regulation of intermediary metabolism, but also to elucidate the aetiology and pathogenesis of disease states, and to evaluate the preventive and therapeutic means of recourse. To this cause a host of naturally occuring and experimentally induced animal models of human diabetes mellitus has served and continues to serve as one of medicine's most instructive tools (see Renold & Dulin, 1967; Renold et al., 1970; Renold, 1974).

ANIMAL MODELS OF OBESITY AND DIABETES

The spontaneous occurence of obesity and diabetes mellitus has been observed in numerous animal species, particularly those living in close association with man such as domestic pets, farm livestock, animals maintained in zoos and laboratory animals (Wilkinson, 1957, 1958; Meier, 1960; Bray & York, 1971; Karl, 1975). Diabetes mellitus has been described in dogs, cats, pigs, horses, cattle, sheep and goats (Heiss, 1859; Biester, 1925; Baker et al., 1931; Christensen & Schambye, 1950; Schotthauer & Millar, 1951; Ricketts et al., 1953), in addition to foxes (Fox, 1923), monkeys (Sokoloverova, 1960; Hamilton & Brobeck, 1963; Howard, 1972), tree shrews (Rabb et al., 1966), dolphins (Schweisheimer, 1966), hippopotami (Hayashi, 1967), carp (Yokote, 1970) and a wide variety of laboratory rodents including rats and mice (Renold, 1968; Hunt et al., 1976). This may reflect the convenience and frequency of investigations among such species, since equally thorough studies of wild populations have not been reported. However, there is convincing evidence that by removing the forces of natural selection, human intervention has increased the incidence of metabolic disorders in these animals (Renold & Burr, 1970; Renold et al., 1971). For example, selective breeding, nutritional abundance, limited exercise and increased longevity have all been shown to precipitate obesity and diabetes

(Schmidt-Neilsen et al., 1964; Gonet et al., 1965; Rabb et al., 1966; Wise et al., 1969). In addition to the spontaneous syndromes, several experimentally induced animal models have been produced (Delafresnaye & Smith, 1954; Mayer, 1955; Wright, 1960). These methods include pancreatectomy (von Mering & Minkowski, 1890; Banting & Best, 1922; Shapiro & Pincus, 1936), endocrine-induced imbalance (Houssay et al., 1932; Young, 1937; Ingle, 1941; Moloney & Coval, 1955), surgical or chemical lesion of the hypothalamus (Brechner & Waxler, 1949; Mayer et al., 1955), chemical destruction of the islet β cells with alloxan (Luckens, 1948) or streptozotocin (Rakieten et al., 1963) and impairment of β cell function by viral infections (Craighead & McLane, 1968). Since each animal syndrome represents a potential model of a disease state which shares certain features in common with human diabetes mellitus, it is germane to create conditions allowing for the careful and intelligent study of all the major types of animal hyperglycaemic syndromes (Renold et al., 1971; Karl, 1975; Herberg & Coleman, 1977). If the intelligence, means and diligence applied are sufficient, it is most likely that the investigation of each syndrome will provide knowledge of one or perhaps several anomalies representing possible mechanisms of the aetiology and pathogenesis of diabetes mellitus.

Animal models in general offer the opportunity to investigate new hypotheses, to test new approaches and to perform detrimental or terminal procedures not possible in human subjects. Genetic variation between individual animals can be minimised by inbreeding, and environmental influences can be specifically controlled and manipulated. The most extensively studied models of obesity and diabetes are the spontaneous syndromes in laboratory rodents, of which more than a dozen have now been described (see Hunt et al., 1976). A number of reviews on the metabolic anomalies of the various animal models and the relationships between these features and those of human diabetics have demonstrated

both the interest in, and the importance of these species of animals (Renold, 1968; Renold & Burr, 1970; Bray & York, 1971; Cameron et al., 1972; Herberg & Coleman, 1977). Like the human diseases, these syndromes are dependent upon an inherited component which is subsequently modified in expression by the influence of both genetic factors and environmental factors incurred throughout life (Stauffacher et al., 1971; Renold et al., 1972). Such models are therefore appropriate to examine the interaction of genetic and environmental moieties under specified conditions, and enable comparisons to be made with animals of the same strain that are not genetically predisposed to the syndrome (see Renold & Dulin, 1967; Renold et al., 1970; Renold, 1974).

THE OBESE HYPERGLYCAEMIC MUTATION IN MICE

Since the original description of a naturally occuring mutation in the house mouse by Ingalls, Dickie and Snell (1950), the obese hyperglycaemic mouse has become the most widely adopted and thoroughly investigated animal model of obesity and diabetes. The syndrome which provides an excellent model for the study of the aetiology and pathogenesis of human maturity-onset diabetes mellitus associated with obesity has been the subject of numerous reviews (Mayer et al., 1953a; Hausberger & Hausberger, 1960; Mayer, 1960; Christophe, 1965; Hellman, 1965, 1966; Renold, 1968; Westman, 1968; Herberg & Coleman, 1977). The subsequent sections of this thesis are devoted to general considerations of this animal model with special emphasis on the application of radioimmunological techniques to the study of the Birmingham obese hyperglycaemic mouse. The experimental sections represent selected studies conducted during the course of a more extensive investigation designed to elucidate the role of the pancreatic islets in the aetiology and pathogenesis of the obese hyperglycaemic syndrome in mice. Aspects of this work have been reported elsewhere (Atkins et al., 1975a, b; Bailey et al., 1977a, b, c, d; Best et al., 1977; Flatt et al., 1977 a, b).

GENERAL CONSIDERATIONS OF THE OBESE HYPERGLYCAEMIC SYNDROME

GENETIC DEVELOPMENT

The obese hyperglycaemic syndrome arose in the summer of 1949 as a mutation within the V strain of mice at the Jackson Memorial Laboratory, Bar Harbor, Maine (Ingalls et al., 1950). The syndrome is transmitted by a single autosomal recessive gene, obese (gene symbol *ob*, in chromosome 6, linkage group XI) and mice that are homozygous for this gene (*ob*/*ob*) exhibit pronounced obesity and severe disturbances of carbohydrate and lipid metabolism characteristic of certain types of human obesity and maturity-onset diabetes (Mayer et al., 1951, 1953a; Bleisch et al., 1952). The *ob* mutation has been subsequently transferred into the C57BL/6J and the C57BL/KsJ strains at the Jackson Laboratories (Coleman & Hummel, 1973). Studies involving these two inbred congenic strains have demonstrated that many of the metabolic abnormalities of obese mice, like those of diabetes (*db*/*db*) mice, do not solely result from the presence of the mutant gene, but from its interaction with the background genome (Hummel et al., 1972; Coleman & Hummel, 1973; Boquist et al., 1974, 1976). The severity and developmental pattern of the metabolic disturbances of obese mice therefore vary according to the genetic background in which the mutation is maintained (Coleman, 1977a; Herberg & Coleman, 1977). Direct comparison of investigations on this model, maintained in local colonies, either noninbred, inbred, or on the standard C57BL/6J strain, must therefore be made with caution (Westman, 1968).

Several colonies of obese mice are maintained in which the obese mutation is carried on different genetic backgrounds (Coleman & Hummel, 1973). The Swedish colony was established with breeding stock obtained from the Jackson Laboratory colony prior to the transfer of the *ob* gene into the C57BL/6J strain (Hellman, 1965, 1966). Although similar to the BL/6J obese mouse in many respects (Westman, 1968), this noninbred colony

is characterised by large hyperplastic islets which have been used extensively in studies involving islet metabolism (Hellman, 1970; Lernmark, 1971). Another stock of noninbred mice, also derived from the original V strain and characterised by a more severe form of diabetes is maintained at Orléans, France (Lemonnier et al., 1971). The Birmingham colony of obese mice maintained as a noninbred stock at the University of Aston, is descended from breeding pairs (C57BL/6J - ob/+) obtained from the Jackson Laboratory which were outcrossed for higher litter size and faster growth rate in Edinburgh (Atkins et al., 1975a). Unlike inbred C57BL/6J obese mice, this colony has a severe form of diabetes (Bailey et al., 1977a, b).

AETIOLOGY

Although it is established that the obese hyperglycaemic syndrome is transmitted by an autosomal recessive gene (Ingalls et al., 1950), the precise nature of the genetic defect responsible for the syndrome and therefore the primary cause of the disease state await elucidation. Differences between lean and obese mice have been observed prior to weaning (Joosten & van der Kroon, 1974a; Kaplan & Leveille, 1974; Trayhurn et al., 1977) and many of the characteristic features of the syndrome become apparent immediately following the ingestion of solid food (Danielsson et al., 1968; Westman, 1968; Chlouverakis et al., 1970; Genuth et al., 1971; Dubuc, 1976c). Attempts to identify the sequence of onset of the various metabolic abnormalities have been hampered by both the size of the animal and the rapid manifestation of the syndrome. Studies in suckling mice have been thwarted by the exiguity of the animals and their unsuitability for terminal procedures prior to identification of mice bearing the ob/ob genotype. However, considerable evidence, both indirect and circumstantial is now available and several theories have been proposed concerning the primary metabolic disturbance in obese mice (see reviews by Christophe, 1965; Hellman, 1965;

Renold, 1968; Bray & York, 1971; Mahler, 1974a; Herberg & Coleman, 1977). These theories implicate a congenital defect of the hypothalamus, pancreatic islets, skeletal muscle or adipose tissue.

1. HYPOTHALAMUS

Various abnormalities of obese mouse physiology indicate a general malfunction of the hypothalamus. These include hyperphagia (Mayer et al., 1951), impaired temperature regulation (Davis & Mayer, 1954), evidence that the pattern of food intake and metabolic adjustments to dietary manipulation are similar to those observed in animals with hypothalamic lesions (Parson et al., 1954; Fuller & Jacoby, 1955; Anlinker & Mayer, 1956), and more recently altered thyroid and reproductive function (Joosten & van der Kroon, 1974b; Edwards & Hough, 1975; Sinha et al., 1975; Swerdloff et al., 1976). Furthermore, obese mice treated with gold thioglucose developed singificantly smaller ventromedial hypothalamic lesions than similarly treated lean mice, indicating an impairment of glucose utilisation and insulin sensitivity in the hypothalamus of obese mice (Baile et al., 1970). Thus it has been suggested that the primary defect may reside within the obese mouse hypothalamus (Bray & York, 1971; Mahler, 1974a). This defect might represent an incompetence of the central mechanism controlling satiety and hunger, resulting in hyperphagiá and the consequent development of obesity and diabetes (Baile et al., 1970). However, available evidence suggests that the satiety centre of the obese mouse is functional (Chlouverakis et al., 1973; Herberg & Coleman, 1977). Indeed, when lean mice were parabiosed with their obese littermates, the rate of weight gain of the obese partner was reduced, indicating that a satiety factor was transmitted from the lean mouse during parabiosis (Hausberger, 1958a; Haessler & Crawford, 1965; Chlouverakis, 1972a). This observation has been confirmed by an elegant series of experiments conducted by Coleman (1973), who concluded that a defective satiety factor was involved in

the pathogenesis of the obese hyperglycaemic syndrome. Although the islet transplantation studies reported by Strautz (1968, 1970) implicate an islet factor, possibly pancreatic polypeptide (Gates & Lazarus, 1977), in this process, further speculation on these lines must await the provision of additional information. Nevertheless, experimental lesions of the hypothalamus do not produce all of the symptoms of the obese syndrome (Mayer, 1960; Stauffacher et al., 1967; Malaisse et al., 1968) and dietary restriction does not produce metabolic normality (Fuller & Jacoby, 1955; Petersson & Hellman, 1962; Chlouverakis, 1970; Marliss et al., 1975; Dubuc, 1976b). Therefore, although hyperphagia may contribute to the obesity of these mice, one or more additional or alternative primary disturbances are indicated.

2. PANCREATIC ISLETS

Hyperinsulinaemia is an early manifestation of the obese syndrome (Westman, 1968; Chlouverakis et al., 1970; Genuth et al., 1971; Dubuc, 1976c), and it has been proposed that the pancreatic islets of obese mice are genetically predisposed to enhanced insulin secretion (Genuth, 1969; Mahler, 1974 a, b). Numerous pathways exist which could lead to the hyperinsulinaemic state, involving congenital defects of either the hypothalamus (Mahler, 1974a; Herberg & Coleman, 1977), gut (Polak et al., 1975b; Best et al., 1977), pituitary (Beloff-Chain et al., 1975a, 1977b)or the pancreatic islet itself (Atkins & Matty, 1971; Laube et al., 1974; Mahler et al., 1976). Currently available data suggest that the hyperinsulinaemia develops concomitantly with insulin insensitivity (Westman, 1968; Genuth et al., 1971; Flatt et al., 1977b), and it has been suggested that the former may be responsible for the latter (Soll et al., 1975a, b). Insulin insensitivity produced in this way might impair the function of the hypothalamic insulin receptor resulting in hyperphagia and unrestrained hepatic glucose production (Baile et al.,

1970; Szabo & Szabo, 1972, 1975; Bloom et al., 1974). Excess insulin per se is one candidate for the production of insulin insensitivity and there are extensive data to indicate that sustained hyperinsulinaemia reduces both the concentration and the absolute number of insulin receptors in target tissue membranes (Freychet, 1976; Olefsky, 1976). This may be a direct regulatory effect of insulin on its own receptor (Gavin et al., 1974; Roth et al., 1975; Soll et al., 1975c; Freychet et al., 1976), although it is conceivable that other factors may be involved in the mediation of this effect (Flier et al., 1976; Czech, 1977; Flatt et al., 1977b; Le Marchand et al., 1977). Despite convincing evidence to suggest that both hyperinsulinaemia and insulin insensitivity can be abolished by either caloric restriction (Batt & Mialhe, 1966; Chlouverakis & White, 1969; Abraham & Beloff-Chain, 1971) or pre-treatment with β cytotoxic agents (Mahler & Szabo, 1971; Solomon et al., 1974; Cuendet et al., 1976), the hypothesis that hyperinsulinaemia is essential in the generation of insulin insensitivity in obese mice has been challenged by demonstration that it is possible to reduce insulin levels while maintaining insulin insensitivity and obesity (Batchelor et al., 1975; Boozer & Mayer, 1976).

In addition to the possible genetic predisposition of obese mouse islets towards increased insulin secretion, other congenital defects of the islets may exist. Mahler (1974 a, b) has suggested that if elevated levels of insulin are not solely responsible for the development of insulin insensitivity and obesity in the syndrome, then the islets of obese mice may secrete inappropriate amounts of an insulin antagonist. This would correspond with islet hypertrophy and hyperplasia, possibly reflecting the absence of an islet inhibitory factor (Strautz, 1970). The type of antagonist envisaged could be an 'abnormal' insulin which retains insulin-like immunoreactivity but possesses structural differences to render it biologically less effective. Insulin insensitivity would

presumably then result from competition between the abnormal insulin and the biologically active insulin at target tissue receptors. A precursor molecule such as proinsulin exhibits several of the properties attributed to an abnormal insulin acting as an insulin antagonist. Circulating levels of proinsulin in obese mice have not yet been reported but it seems unlikely that the levels are raised sufficiently to produce the severe insulin insensitivity observed in these animals because the proportion of proinsulin and insulin in obese mouse islets is only moderately raised (Atkins, 1972) as might be expected under conditions of rapid synthesis and release (Berne, 1975). The concept of insulin antagonism by the excessive secretion of an abnormal insulin is not in agreement with the work of Stauffacher and colleagues (1967) who demonstrated that the proportion of immunologically and biologically active insulin in the serum was similar in the two main phenotypes. Indeed, Strautz (1968, 1970) has shown that although transplantation of islets from lean mice into obese mice improved insulin sensitivity and reduced weight gain, blood glucose and insulin concentrations, lean mice transplanted with obese mouse islets were not affected. These observations have been interpreted as an indication that instead of producing an excess of insulin antagonist, the islets of obese mice are deficient in one or more factors that normally inhibit β cell hyperfunction and are necessary for the maintenance of normal carbohydrate and lipid metabolism (Strautz, 1970). This is consistent with the report of Coleman (1973) that parabiosis of lean and obese mice suppressed the weight gain and both the blood glucose and plasma insulin concentrations of the obese parabiont. Histological and ultrastructural examinations of the islets have revealed a number of distinct cell types (de Hoyos-Guevara, 1969; Lacy & Greider, 1972), and more recent studies employing immunological techniques have demonstrated the presence of a wide variety of peptide containing cells within these structures (Larsson et al., 1975; Polak et al., 1975a;

Fujita, 1976; Bloom et al., 1977; Dockray et al., 1977). However, the identity of the hypothetical factor and the role of these peptides in physiological processes await further elucidation. The presence of an inhibitor of insulin secretion derived from the α_1 cells of the islets has been demonstrated by Hellman and Lernmark (1969 a, b). Although the precise nature of the inhibitor remained uncertain for many years, it is now evident that somatostatin containing cells distributed at the periphery of the islets can exert a local inhibitory effect on β cell function (Baetens et al., 1976; Galey et al., 1976; Unger & Orci, 1977a). Nevertheless, in view of these morphological observations and the report by Patel and colleagues (1976) of only marginally decreased somatostatin content of the obese mouse pancreas, it seems unlikely that this peptide is of primary importance in the aetiology of the syndrome.

Thus, although the obese hyperglycaemic syndrome is characterised by marked alterations of islet structure and function, including the early manifestation of hyperinsulinaemia, it is uncertain whether a congenital defect of the islet exists or whether such a defect could be a primary cause of the syndrome. In the light of present knowledge it seems unlikely that a congenital defect could be a single primary cause of the syndrome because an increased proportion of body fat is detectable before a significant alteration of islet function (Chlouverakis et al., 1970; Joosten & van der Kroon, 1974a). The issue of insulin insensitivity is also difficult to rationalise solely on the basis of an islet defect because insulin insensitivity in obese mice is associated with a differential insulin resistance of skeletal muscle and adipose tissue, the former being very much more resistant than the latter (Chlouverakis & White, 1969; Stauffacher & Renold, 1969). To account for this it is necessary to postulate either that the factor producing insulin insensitivity is capable of exerting a differential

influence on the insulin responsiveness of different tissues or that the tissues themselves possess congenital defects that affect their responsiveness to insulin.

3. MUSCLE

Since it is possible to detect a resistance of skeletal muscle to insulin during the early manifestation of the obese syndrome, the possibility has been broached that this represents a primary cause of the syndrome (Stauffacher et al., 1965, 1967). Hyperinsulinaemia might then be explained as an adaptation to the increased peripheral demand for insulin, and the excessive deposition of fat would result because adipose tissue is more responsive to insulin than muscle (Chlouverakis & White, 1969; Genuth et al., 1971). A greater response of adipose tissue to insulin than of muscle to insulin may well contribute to the excessive accumulation of fat in obese mice (Stauffacher et al., 1967; Cuendet et al., 1976), but the theory of muscle insulin resistance as the cause of hyperinsulinaemia is difficult to reconcile for two reasons. Firstly, a congenital defect of muscle insulin resistance would require a feedback mechanism to enable islet recognition of the increased requirement for insulin. Hyperinsulinaemia, insulin insensitivity and obesity precede a significant elevation of blood glucose levels, indicating that the initiation of hyperinsulinaemia is not an adaptation to hyperglycaemia (Westman, 1968; Dubuc, 1976c). Therefore an unidentified feedback mechanism between muscle and islets must be postulated. Secondly, insulin sensitivity is restored by prolonged dietary restriction (Batt & Mialhe, 1966; Chlouverakis & White, 1969) suggesting that muscle insulin resistance is modified following changes in food consumption and is not entirely intrinsic. Whether or not muscle is capable of any intrinsic modulation of the effects of agents causing its insensitivity to insulin is unknown. These reasons, plus

evidence that excessive fat accumulation precedes a demonstrable insensitivity to insulin (Westman, 1968; Chlouverakis et al., 1970; Dubuc, 1976c) indicate that a congenital defect of muscle might contribute to the manifestation of the obese hyperglycaemic syndrome but is unlikely to represent a single primary causitive factor.

4. ADIPOSE TISSUE

The most obvious manifestation of the obese syndrome is an excessive accumulation of adipose tissue. This feature is also one of the earliest known distinguishing characteristics of obese mice (Chlouverakis et al., 1970; Joosten & van der Kroon, 1974a; Dubuc, 1976c) and it is not therefore unreasonable that considerable attention should be focused on the adipose tissue as a possible site of primary metabolic disturbance (Hellman, 1965). The deposition of fat in obese mice is associated mainly with hypertrophy of the subcutaneous and abdominal depots and there are conflicting reports that hyperplasia of these depots may also exist (Hausberger, 1965; Hellman, 1965; Johnson & Hirsch, 1972; Abraham, 1973). Numerous metabolic defects of the adipose cells have been noted in adult obese mice (see Hellman, 1965; Christophe et al., 1967; Bray & York, 1971; York, 1975; Herberg & Coleman, 1977), and strong support for the location of a primary metabolic fault in the adipose tissue comes from the observation that the proportion of body fat remains elevated in obese mice despite the maintenance of normal body weight by caloric restriction (Alonso et al., 1955; Chlouverakis, 1972b; Marliss et al., 1975). However, abnormalities of adipose tissue metabolism in weaning mice have not been established. It is therefore unclear whether the abnormalities of fat metabolism observed in adult obese mice represent intrinsic defects or merely secondary manifestations of the expression of the ob gene (see Loten et al., 1976; Thenen & Mayer, 1976). Insulin administration will mimic most of the abnormalities

seen in obese mouse adipose tissue, providing evidence against a primary defect at this locus. Indeed, recent studies have demonstrated that the internal environment is of paramount importance and the inherent properties of minor importance in determining obesity in genetically predisposed mice (Ashwell et al., 1977). An additional problem associated with a possible primary defect of adipose metabolism is the need to postulate a feedback system from adipose tissue to the islets. Several groups (Salans et al., 1968; Stern & Hirsch, 1972) have proposed the existence of such a system in other forms of obesity, but there is little experimental evidence to justify this view (see Mahler, 1974a). The early manifestation of inordinate fat deposition provides a strong basis for implicating the adipose tissue as a primary location of metabolic imbalance, however, further studies are clearly required to substantiate this idea. As previously noted, the single primary fault hypothesis does not seem realistic, and if a primary defect of either the hypothalamus, islet, muscle or adipose tissue does become established, it seems likely that this will not be the only fault responsible for the syndrome, but one of many faults in different locations that are all part of a diverse multifactorial origin of the syndrome. Accordingly, in addition to the possible primary causes of the obese hyperglycaemic syndrome already discussed, several other potential disturbances of metabolism have been explored, for example, those hormones which exert hyperglycaemic and insulin antagonistic effects.

5. GLUCAGON

Hypersecretion of glucagon as a causative factor of the hyperglycaemia in obese mice was postulated by Mayer and colleagues (Mayer et al., 1953b; Clarke et al., 1956). Support for this hypothesis was derived from observations that the absolute number of α_2 cells in the pancreas of adult obese mice is greater than in lean mice, despite a dramatic increase in the relative proportion of β cells in the islets

of these animals (Gepts et al., 1960; Hellman, 1961). Consonant with this, it has been shown that the total pancreatic GLI content of obese mice is significantly higher than in lean mice (Findlay et al., 1973). However, considerable divergence exists between the reported circulating glucagon concentration in these animals (Lavine et al., 1975; Herberg et al., 1976; Mahler et al., 1976; Beloff-Chain et al., 1977a), and no studies have been performed to examine the functional activity of the α_2 cells in the mice, despite evidence to suggest that glucagon represents a potent stimulus for both hepatic glucose production and insulin secretion in the mutant (Westman, 1970). In contrast to recent studies (Beloff-Chain et al., 1977a), Lavine and colleagues (1975) reported that plasma C-GLI levels are greater in young fed obese mice than in their lean counterparts, although the reverse was observed following a 48 hour fast. After 16 hours of fasting, a higher plasma C-GLI concentration was found in young obese mice, but the converse was observed in older mice fasted for the same period of time (Mahler et al., 1976). These observations may be considered in the context of <u>in vitro</u> perfusion studies which demonstrated that the obese mouse pancreas secretes singificantly more GLI than the lean mouse pancreas, and whereas high glucose levels suppress GLI secretion in lean mice, the obese mice exhibit a paradoxical increase of secretory activity (Laube et al., 1974). Such a response is consistent with the increase in plasma C-GLI reported in young fed obese mice (Lavine et al., 1975), but it does not explain why the reverse should occur after prolonged fasting. Furthermore, and ironically, the <u>in vitro</u> studies of Laube and colleagues (1974) were conducted on older mice of equivalent age to those used by Mahler and coworkers (1976) in their demonstration of reduced plasma C-GLI concentration in obese mice. Thus, the role of glucagon in the obese hyperglycaemic syndrome is far from clear and it would appear that the extent of changes that accompany different nutritional states may alter with age.

6. GROWTH HORMONE

Growth hormone is known to exert an insulin antagonistic action in obese mice (Lostroh & Krahl, 1974, 1976), and chronic administration of this hormone will produce a compensating increase in insulin secretion together with hypertrophy and hyperplasia of the pancreatic islets where potential for this response exists (Mahler, 1974a). However, three studies have noted that growth hormone levels are normal or even slightly reduced in obese mice (Roos et al., 1974; Sinha et al., 1975; Larson et al., 1976). Furthermore, the obese hyperglycaemic syndrome develops in obese mice that are also homozygous for the dwarf gene and have no circulating growth hormone (Joosten & van der Kroon, 1975). These studies indicate that growth hormone is not only unlikely to be of aetiological significance in the syndrome, but is also unlikely to play any significant role exacerbating the metabolic abnormalities of the disease state.

7. GLUCOCORTICOIDS

The demonstration that implantation of ACTH secreting tumours in lean mice produces many characteristics of the obese hyperglycaemic syndrome including hyperglycaemia (Shull et al., 1956), augmented lipogenesis (Zomzely & Mayer, 1956), obesity, pancreatic β cell hyperplasia, and hyperphagia (Hausberger, 1958b) has led to the suggestion that elevated glucocorticoids are responsible for the hyperglycaemia and insulin insensitivity of the obese mouse. Consonant with this, is the observation of adrenal cortical hypertrophy (Hellerström et al., 1962), increased glucocorticoid production *in vitro* (Carstensen et al., 1961), and a reduction of body weight, blood glucose, serum insulin, together with an improvement of both glucose tolerance and insulin sensitivity in bilaterally adrenalectomised obese mice (Naeser, 1973; Solomon & Mayer, 1973). Indeed, numerous studies have confirmed the

presence of elevated levels of circulating glucocorticoids in adult obese mice (Naeser, 1974a; Dubuc et al., 1975; Edwardson & Hough, 1975; Herberg & Kley, 1975). However, these and other studies on younger animals (Naeser, 1974b; Coleman & Burkart, 1976; Dubuc, 1976a) have concluded that although adrenal cortical dysfunction may well contribute to the magnitude of the insulin resistance and obesity, elevated glucocorticoid levels are unlikely to be of aetiological significance due to the time-sequence of changes in obese hyperglycaemic mice.

THE BIRMINGHAM OBESE HYPERGLYCAEMIC MOUSE

GENETIC BACKGROUND

The colony of genetically obese hyperglycaemic mice (Mus musculus, genotype ob) maintained at the University of Aston in Birmingham is derived from original C57BL/6J-ob/+ breeding pairs obtained by Professor D. S. Falconer at The Institute of Animal Genetics, University of Edinburgh in 1966. These mice had been backcrossed 12 or more times to the C57BL/6J strain at the Jackson Laboratory, Bar Harbor, Maine and could therefore be considered over 99% with respect to the C57BL/6J genome (Coleman, 1977b). Heterozygous mice were subsequently outcrossed at Edinburgh to Bateman's high lactation strain and a third generation strain derived from pair-matings of MacArthur's and Goodale's large body weight strains (Falconer, 1973). A breeding pair of this noninbred stock was transferred to Birmingham where the syndrome has been maintained in a closed noninbred colony for approximately 40 generations. Comparison of data presented in this thesis with previously published reports support the view that the phenotype of the Birmingham strain is intermediate with respect to the severity of diabetes and lifespan as compared to the typical clinical development of the ob/ob syndrome on either the C57BL/KsJ or C57BL/6J background (see Coleman & Hummel, 1973; Herberg & Coleman, 1977).

THE ASTON COLONY

All animals were bred and accommodated within the animal house at The University of Aston in Birmingham. Litters derived from known heterozygous (ob/+) monogamous pairs consisting of obese (ob/ob), lean heterozygous (ob/+) and lean homozygous (+/+) mice in an approximate ratio of 1:2:1 were housed individually, prior to separation of the visually obese littermates at approximately 3 weeks of age. Lean homozygous mice (+/+) were bred from established homozygous (+/+) parents. Groups of animals were housed in plastic cages (48 x 30 x 15 cm), and the bedding of wood shavings and straw was changed twice weekly. The room was air-conditioned at 22 ± 2°C with a regular lighting schedule of 9.5 hours light (08.00-17.30 hours) and 14.5 hours dark. Tap water and a standard 3/8" pellet diet (Heygates Breeding Diet, L. A. Pilsbury, Birmingham) were supplied ad libitum.

CHARACTERISTICS OF THE GENOTYPES

The obese hyperglycaemic syndrome in mice is transmitted as an autosomal recessive trait which is characterised in the homozygous (ob/ob) condition by obesity, hyperglycaemia and hyperinsulinaemia. Studies employing lean littermate mice as 'normal' controls have invariably used a mixture of the genotypes ob/+ and +/+ in the approximate proportion 2:1. A selective breeding programme within the Aston colony has provided established ob/+ and +/+ genotypes, thereby permitting characterisation of the essential features of each genotype.

1. METHODOLOGICAL APPROACHES

Animals

Groups of six identically reared 20 weeks old male mice of each genotype were used in these studies. Mice heterozygous for the ob gene were selected on the basis of breeding experiments with established heterozygous females. The animals were fed ad libitum or fasted for either 6 or 24 hours prior to an experiment as indicated in the appropriate sections.

Administration of test substances

Glucose (2g/kg/5ml sterile water) was administered either by intraperitoneal injection or by gastric intubation. Arginine hydrochloride (1.5g/kg/5ml saline) and monocomponent porcine insulin (0.25 U/kg/5ml saline or 100 U/kg/5 ml saline) were administered by the intraperitoneal route.

Blood sampling

Blood samples (approximately 0.1 ml) were obtained from conscious mice by the tail-tip amputation method (Grice, 1964). Plasma samples were stored in tightly capped tubes at -20°C prior to analysis.

Analytical techniques

Plasma glucose was determined by the glucose oxidase method using a Beckman analyser (Stevens, 1971) and plasma insulin was determined by radioimmunoassay as previously described in detail.

Statistical method

Where appropriate, the mean (m) and the standard error of the mean (sem) of a group of data were calculated. Different groups of data were compared by Student's t test, and a statistically significant difference was accepted for probability levels of $p < 0.05$.

2. BODY WEIGHT AND FOOD CONSUMPTION

Mean body weight and food intake data are shown in figure 58. Heterozygous (ob/+) mice were approximately 2 g heavier than age matched homozygous (+/+) mice and consumed significantly ($p < 0.05$) more food. At this stage in the syndrome, the heterozygous (ob/+) animals consumed slightly more food than obese mice, although the food intake of the obese animals continued to exceed that of the lean homozygous (+/+) mice.

3. PLASMA GLUCOSE AND INSULIN CONCENTRATIONS

Plasma glucose and insulin concentrations of freely fed and 24 hour fasted mice are illustrated in figure 59. The heterozygous (ob/+) animals

exhibited significantly elevated levels of plasma glucose ($p < 0.01$) and plasma insulin ($p < 0.05$) than freely fed lean homozygous (+/+) mice. Following a 24 hour fast, the plasma concentration of both the substrate and the hormone were significantly reduced ($p < 0.001$ & $p < 0.01$, respectively) in the two groups of lean mice. Although both the plasma glucose and insulin concentrations of heterozygous (ob/+) mice continued to exceed those of the homozygous (+/+) lean animals, a relatively greater fall in the circulating insulin level of ob/+ mice rendered the difference between the hormone concentrations insignificant.

Obese mice were characteristically hyperglycaemic and hyperinsulinaemic regardless of nutritional status, and the plasma concentrations of glucose and insulin observed in these animals were grossly elevated ($0.01 > p < 0.001$) when compared with either of the lean phenotypes. Both the plasma glucose and insulin concentration of obese mice declined significantly ($p < 0.01$ & $p < 0.001$, respectively) following a 24 hour fast. However, the effect of food restriction on the circulating insulin concentration was particularly marked in these animals due to the extent of the hyperinsulinaemia in the fully fed state.

The relationship between plasma glucose and insulin concentrations and the nutritional status of obese mice is further illustrated in figure 60. Obese mice fed ad libitum displayed distinctive diurnal variations in food consumption, plasma glucose and plasma insulin concentration, and peak insulin levels in the region of 150 ng/ml coincided with the height of the feeding period. The role of oral feeding in the maintenance of the hyperinsulinaemia in fully fed obese mice is exemplified by the rapid-onset of marked changes in circulating insulin concentration which accompanied both the withdrawal of food, and the re-introduction of the standard pellet diet to a group of 24 hour fasted obese mice. Indeed, in the absence of a significant decline in plasma glucose concentration, the circulating plasma insulin concentration of obese mice displayed a dramatic reduction ($p < 0.01$) following 6 hours of food restriction outside of the peak feeding period.

4. GLUCOSE TOLERANCE

The intraperitoneal glucose tolerance curves of freely fed mice are illustrated in figure 61. Heterozygous (ob/+) mice showed a significant impairment of glucose tolerance as indicated by the persistence of elevated plasma glucose concentrations ($p < 0.01$). The plasma insulin concentrations of the heterozygous (ob/+) group continued to exceed those of the lean homozygous (+/+) mice during the test, but unlike the +/+ animals, only a small non-significant increment in the plasma insulin concentration was detected despite the presence of sustained hyperglycaemia. These features were clearly exaggerated in the homozygous (ob/ob) condition, and fully fed obese mice exhibited a gross impairment of glucose tolerance together with a complete lack of an insulin secretory response to a glucose challenge.

As illustrated in figure 62, the β cell response to intraperitoneal glucose could be partially restored by fasting the obese animals for a period of 24 hours prior to the test. However, despite a transient two fold ($p < 0.05$) increment in the plasma insulin concentration, glucose tolerance became more severely impaired ($p < 0.001$), presumably reflecting the difference in the magnitude of the hyperinsulinaemia in the two nutritional states. Nevertheless, administration of glucose by gastric intubation resulted in a dramatic improvement in the glucose tolerance curves of fully fed obese mice (figure 63). Plasma glucose concentrations were significantly lower ($p < 0.001$) throughout the test period, and a marked decline ($p < 0.05$) was observed 60 minutes after administration of glucose by the gastric route. These changes were accompanied by a sharp increase ($p < 0.01$) in the circulating plasma insulin concentration at 30 minutes. However, the insulin secretory response was transient, and plasma insulin subsequently returned to pre-stimulatory levels despite the persistence of significantly elevated ($p < 0.01$) plasma glucose concentrations. In contrast to the marked differences in the response

of obese mice to intraperitoneal and intragastric glucose, lean homozygous (+/+) mice exhibited a slight improvement in glucose tolerance together with a small increment in the plasma insulin response to glucose administered by the gastric route.

5. ARGININE STIMULATION

The plasma glucose and insulin responses of 24 hour fasted mice to intraperitoneal administration of arginine hydrochloride are illustrated in figure 64. Heterozygous (ob/+) mice showed a rapid 7-fold ($p < 0.001$) increase in plasma insulin concentration which was associated with a significant reduction ($p < 0.01$) in the plasma glucose concentration. A similar, though less marked, trend was observed in lean homozygous (+/+) animals despite a comparatively modest increment ($p < 0.01$) in circulating insulin levels. Obese mice however, exhibited an extensive rise in the circulating level of insulin which remained significantly elevated ($p < 0.001$) throughout the entire test period. The plasma glucose concentrations of these animals were paradoxically increased at 15 minutes, but subsequently declined, though not significantly, in the presence of sustained hyperinsulinaemia. As illustrated in figure 65, a marked insulin secretory response was also observed in fully fed obese mice together with a marked increase in plasma glucose concentration.

6. INSULIN SENSITIVITY

The intraperitoneal low dose (0.25 U/kg) insulin sensitivity curves of 24 hour fasted mice are shown in figure 66. Administration of mono-component porcine insulin (Actrapid, Novo Industi A/S) resulted in a rapid reduction ($p < 0.05$) in the plasma glucose concentration of both lean heterozygous (ob/+) and lean homozygous (+/+) mice. Despite a small decrease in the rate of glucose disappearance in heterozygous (ob/+) mice, the insulin sensitivity of this group of animals was not

significantly impaired. The pattern of changes in the plasma insulin concentration was also similar in the two groups of lean mice, although the rate of insulin disappearance was approximately 25% greater in lean heterozygous (ob/+) mice. Obese mice however, exhibited marked insulin insensitivity, and plasma glucose concentrations were unaffected despite a significant increase ($p < 0.01$) in the circulating insulin concentration. The increment in the plasma insulin level was transient in obese mice, illustrating a rapid rate of insulin disappearance in the ob/ob animals.

The severity of the insulin insensitivity of obese mice is further illustrated in figure 67. Administration of a massive dose (100 U/kg) of insulin to fed, 6 hour fasted and 24 hour fasted obese mice resulted in a significant reduction ($p < 0.001$) in plasma glucose concentration regardless of the nutritional status of the animals. However, the decline in plasma glucose concentration of fully fed obese mice was significantly impaired during the latter stages of the test period despite the presence of severe hyperinsulinaemia corresponding to a plasma insulin concentration of approximately 3 ug/ml. This phenomenon was abolished following a 24 hour period of food restriction and indeed could not be detected 6 hours after initiation of a fast, thereby indicating a rapid improvement in insulin sensitivity in the absence of severe hyperinsulinaemia.

7. CONCLUSIONS

The Birmingham obese hyperglycaemic syndrome is characterised in the homozygous (ob/ob) condition by gross obesity and numerous abnormalities of glucose homeostasis which become markedly less severe in adult mice heterozygous with respect to the ob gene. Nevertheless, in the present studies, lean heterozygous (ob/+) mice displayed significant abnormalities which although less marked than in the presence of an additional ob gene, were characteristic of the obese littermates.

Homozygous obese (ob/ob) mice were typically hyperphagic, hyperglycaemic and hyperinsulinaemic; glucose tolerance was grossly impaired and an insulin response to intraperitoneal glucose was not observed in freely fed animals. Arginine evoked a dramatic rise in the circulating level of insulin and produced a delayed reduction in the hyperglycaemia of 24 hour fasted mice. During insulin hypoglycaemia tests obese mice showed marked insulin insensitivity and a rapid rate of insulin disappearance.

Heterozygous (ob/+) animals consumed more food than lean homozygous (+/+) mice and exhibited increased body weight, together with higher circulating levels of glucose and insulin in both the freely fed and 24 hour fasted states. Glucose tolerance was impaired in ob/+ mice although plasma insulin levels continued to exceed those of +/+ mice during the test. The heterozygous (ob/+) animals also displayed higher plasma insulin levels after administration of arginine, concomitant with lower circulating levels of glucose. The rate of glucose disappearance during insulin sensitivity tests was slightly impaired in ob/+ mice, and the rate of insulin disappearance was marginally enhanced.

These data demonstrate a small but significant effect of the ob gene on metabolic status in the heterozygous (ob/+) condition, thereby illustrating that the ob allele is incompletely dominant.

A number of studies have suggested a gene dosage effect in lean heterozygous (ob/+) mice. Animals of this genotype have been shown to display intermediate levels of adipose tissue oxidation (Yen et al., 1968), plasma corticosterone (Herberg & Kley, 1975), release of a hitherto uncharacterised insulin secretogogue from the pituitary

(Beloff-Chain et al., 1975b), and other differences including oxygen consumption (Kaplan & Leveille, 1974), epididymal fat cell size (Joosten & van der Kroon, 1974a), pituitary ACTH (Edwardson & Hough, 1975), carcass fat and serum insulin (Dubuc, 1976c) have been implied. Thus, on the basis of available information, the metabolism of lean heterozygous (ob/+) mice can be classified as partially intermediate between that of the +/+ and ob/ob genotypes. Accordingly, it is now eminently apparent that the results of metabolic studies on obese hyperglycaemic mice should only be discussed in comparison to lean homozygous (+/+) animals of the same strain. The genetically uncharacterised lean littermate mice may be carriers of the mutant gene and therefore different to metabolically intact animals.

Although the partial penetrance of the ob gene necessitates the use of lean homozygous (+/+) animals as controls in metabolic studies, the lean heterozygous (ob/+) mice provide an excellent opportunity to investigate the genetic determinants of diabetes mellitus without interference from the multiple metabolic abnormalities resulting from the severity of expression of the ob gene in the homozygous (ob/ob) condition. Indeed, the application of radioimmunological techniques to the study of the minor metabolic anomalies of the lean heterozygous (ob/+) mice exemplifies the practical advantages and enormous potential of sensitive and precise microanalytical techniques in diabetes research.

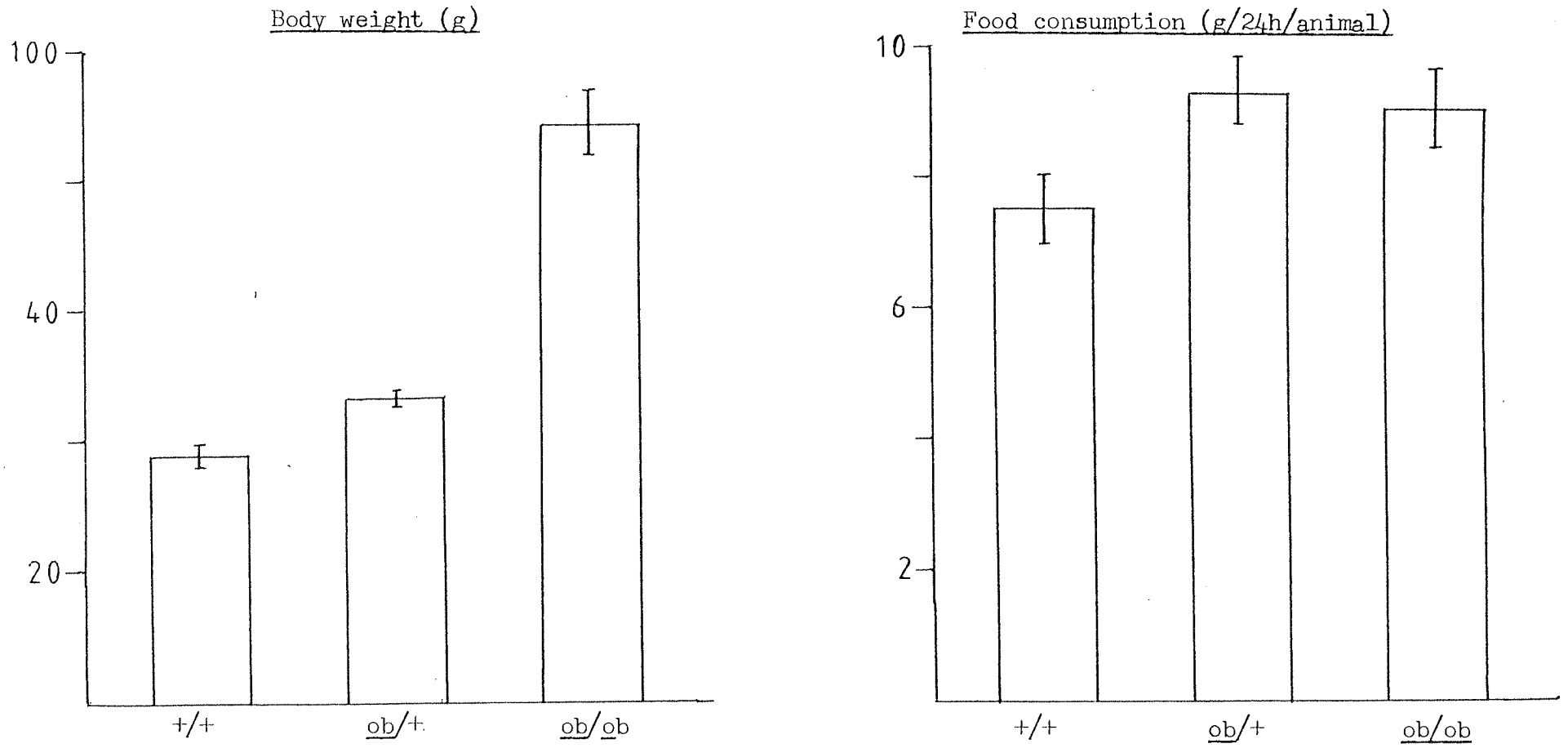
At 20 weeks of age, male heterozygous (ob/+) mice exhibited hyperphagia, hyperglycaemia, hyperinsulinaemia, impaired glucose tolerance, diminished insulin secretory activity following intraperitoneal glucose, and an excessive β cell response to arginine. However, in contrast to obese mice, the heterozygous (ob/+) animals showed little weight gain and no signs of insulin insensitivity. Thus, although both obesity and insulin insensitivity contribute significantly to the later manifestations of the ob/ob syndrome, they appear to be of little importance in the

aetiology of the disease state, and possibly arise as a consequence of the prolonged period of extreme hyperphagia and the subsequent development of marked hypertrophy and hyperplasia of the islets of Langerhans of young obese mice. The existence of hyperglycaemia concomitant with hyperinsulinaemia and normal insulin sensitivity in the heterozygous (ob/+) mice implies an enhanced rate of hepatic glucose output mediated by a paradoxical release of glucagon or by an incompetence of a hypothalamic mechanism. Indeed, the most plausible explanation for the development of the metabolic disturbances observed in the present studies concerns hyperactivity of a humoral and/or neural mechanism which is normally activated during the ingestion of food substances, and results in the production of a marked insulin secretory response. Support for this hypothesis is derived from fasting and refeeding experiments which have clearly demonstrated an intimate relationship between nutritional status and the extent of the hyperinsulinaemia in both heterozygous (ob/+) and homozygous (ob/ob) mice. Since the insulin secretory response to intraperitoneal glucose was defective in both genotypes, the massive secretory response which accompanied intragastric administration of glucose to obese mice affords further evidence to support this viewpoint.

In conclusion, the hypothesis is presented that the over-ingestion of specific food substances (essentially glucose), as a result of the absence of a normal satiety factor, represents the primary stimulus for the development of a hyperactive hormonal-hypothalamic insulin secretion mechanism leading to the production of hyperinsulinaemia and, in genetically predisposed animals, hypertrophy and hyperplasia of the pancreatic islets. The development of islet hypertrophy co-existent with marked hyperphagia and mild obesity leads to the appearance of peripheral insulin insensitivity and relative hyperglucagonaemia resulting in the production of hyperglycaemia. Preferential impairment

of insulin enhanced glucose utilisation by muscle due to the absence of a normal satiety factor, results in the excessive accumulation of body fat. As a result of the persistent insulin stimulation via the hormonal-hypothalamic mechanism, the islets develop structural lesions, and the β cells gradually become unresponsive to a glucose stimulus, resulting in a greater impairment of glucose tolerance and a progressive increase in the hyperglycaemia.

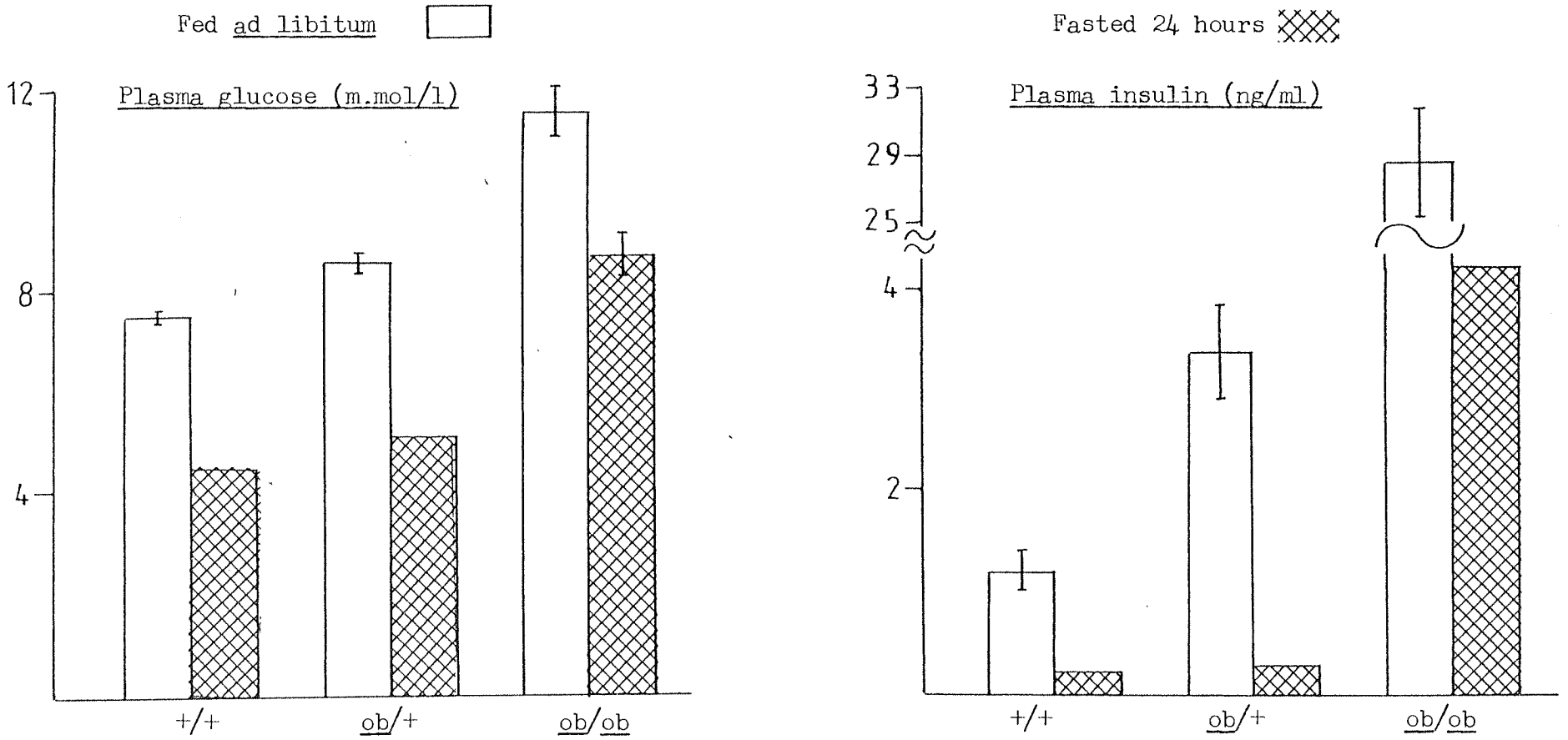
Figure 58 Body weight and food consumption in +/+, ob/+ and ob/ob mice (m ± sem, n = 6)



Statistical analysis Body weights: ob/ob > ob/+, +/+ at $p < 0.001$.

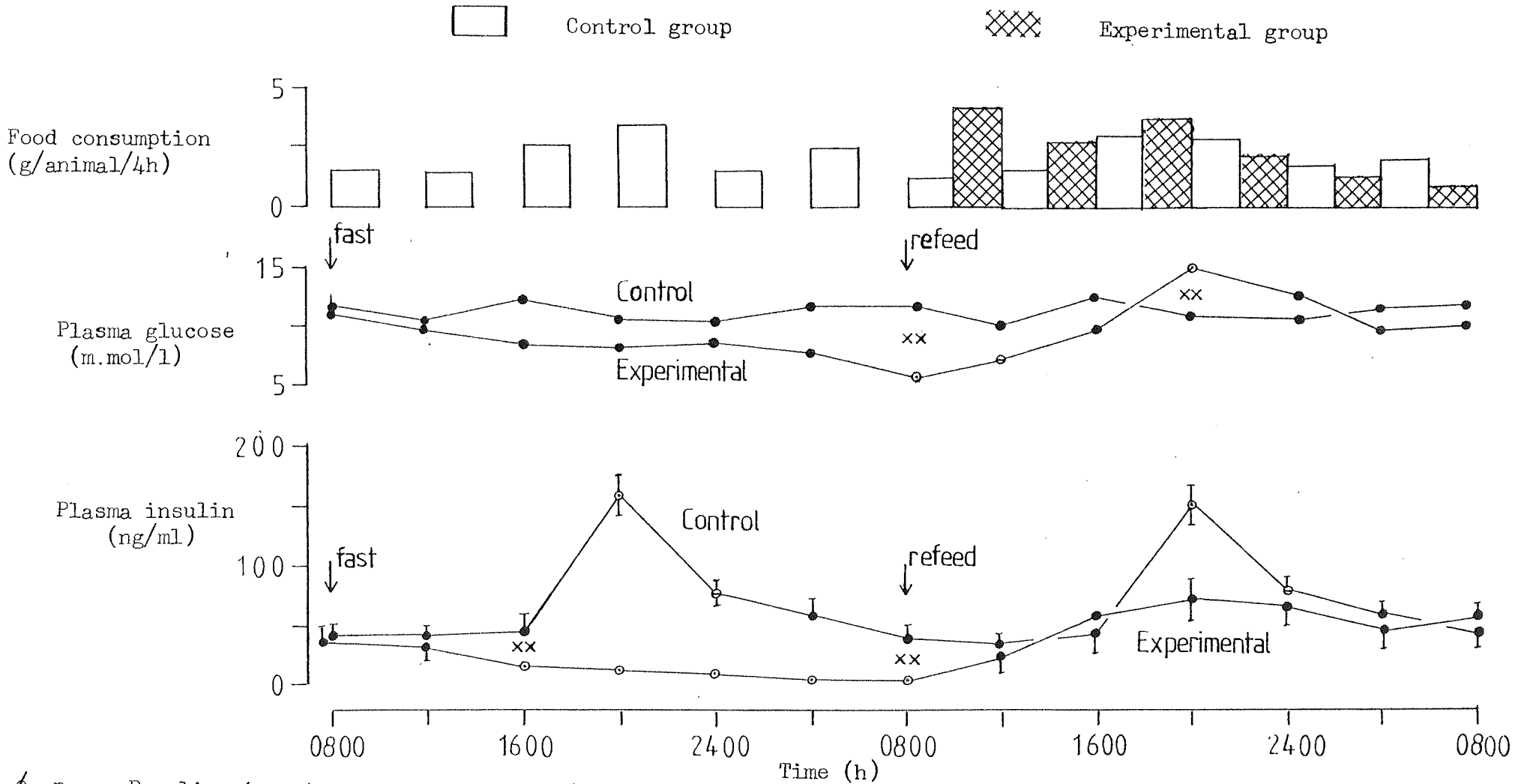
Food consumption: ob/+ > +/+ at $p < 0.05$.

Figure 59 Plasma glucose and insulin concentrations in fed and 24 hour fasted +/+, ob/+ and ob/ob mice (m ± sem, n = 6).



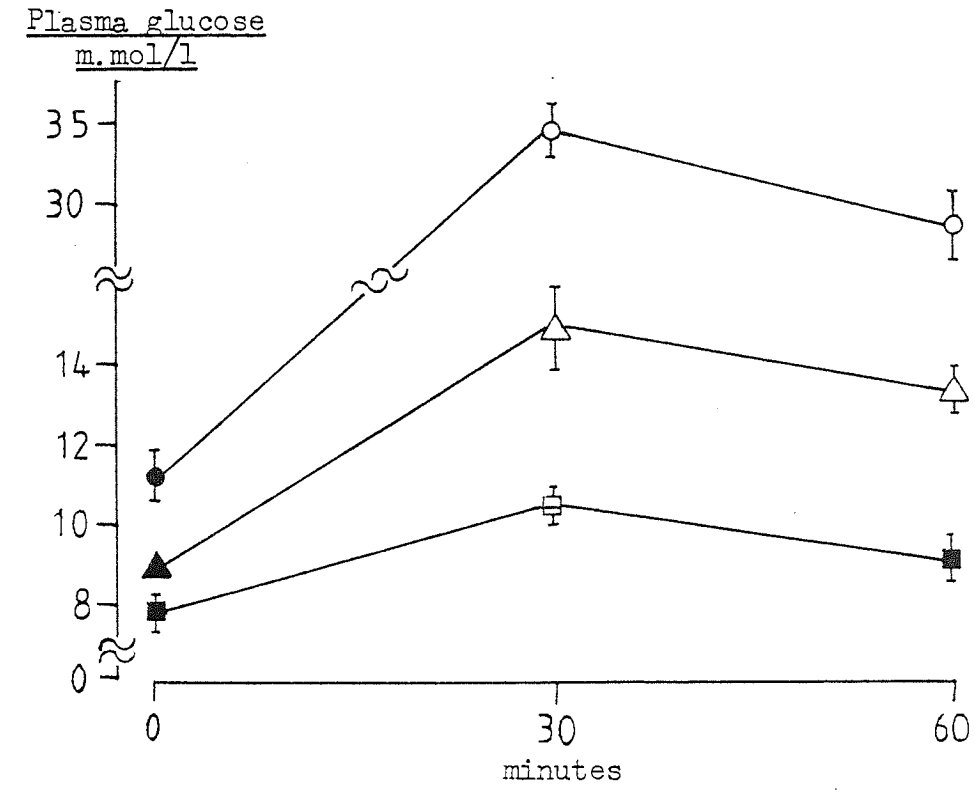
Statistical analysis: Plasma glucose: fed ob/ob > ob/+, +/+ at $p < 0.001$, ob/+ > +/+ at $p < 0.01$; fasted ob/ob > ob/+ > +/+ at $p < 0.001$; fed > fasted ob/ob at $p < 0.01$, ob/+, +/+ at $p < 0.001$. Plasma insulin: fed ob/ob > ob/+, +/+ at $p < 0.001$, ob/+ > +/+ at $p < 0.05$; fasted ob/ob > ob/+, +/+ at $p < 0.001$; fed > fasted ob/ob at $p < 0.001$, ob/+, +/+ at $p < 0.01$.

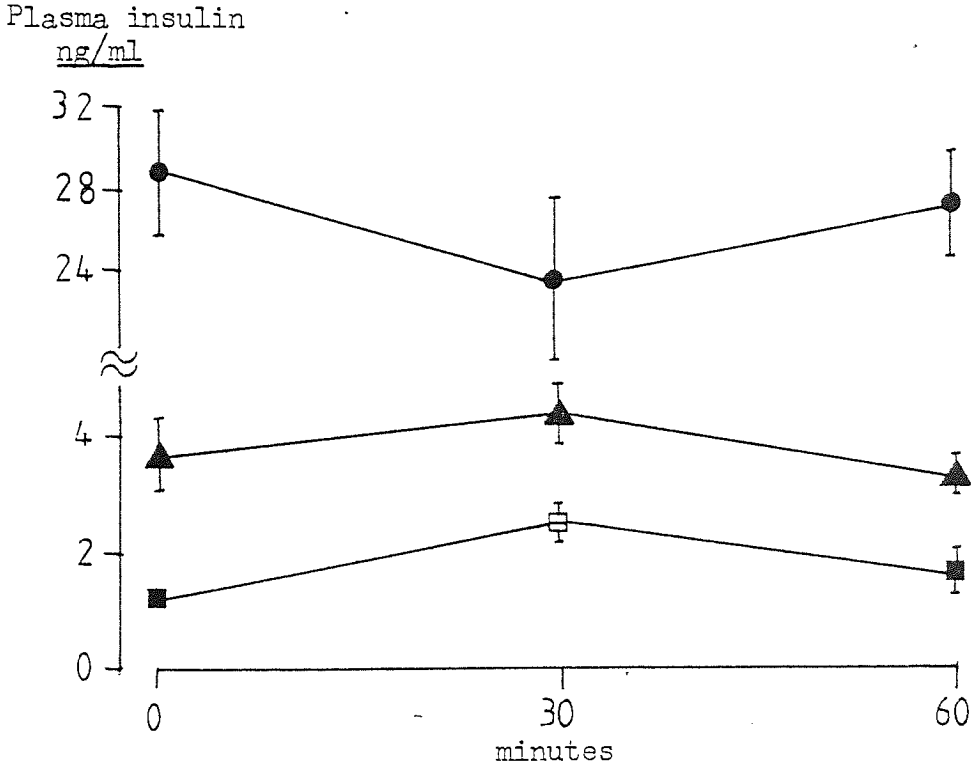
Figure 60 Diurnal variations in food consumption, plasma glucose and insulin concentrations in fasting and feeding ob/ob mice (m ± sem, n = 6)ϕ.



ϕ p vs. Baseline (0800) ϴ = p < 0.05; ⊙ = p < 0.01; ⊙ = p < 0.001.
Differences to controls x = p < 0.05; xx = p < 0.01; xxx = p < 0.001.

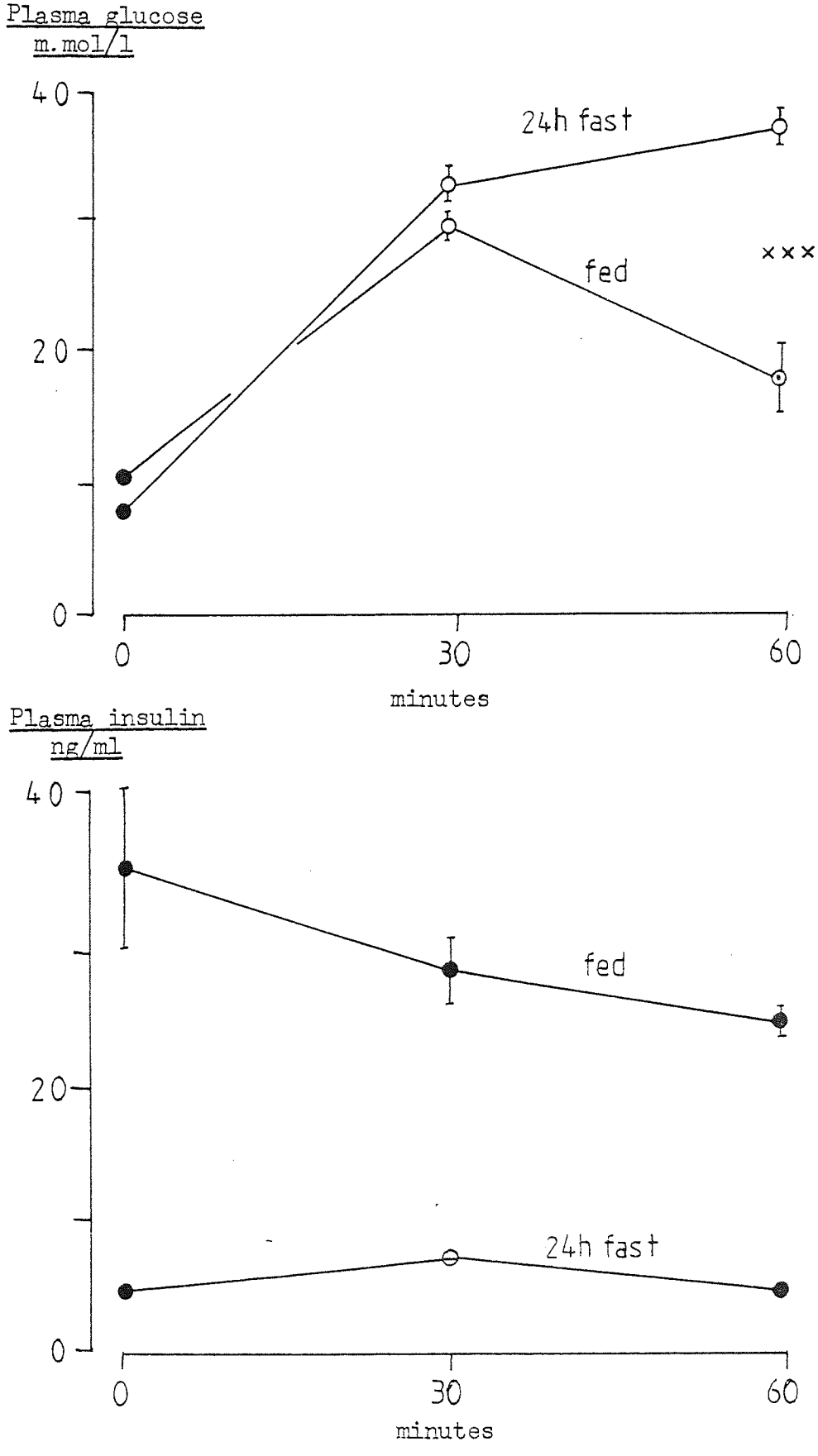
Figure 61 Glucose tolerance in fed +/+ (■——■), ob/+ (▲——▲) and ob/ob (●—●) mice (m ± sem, n = 6).





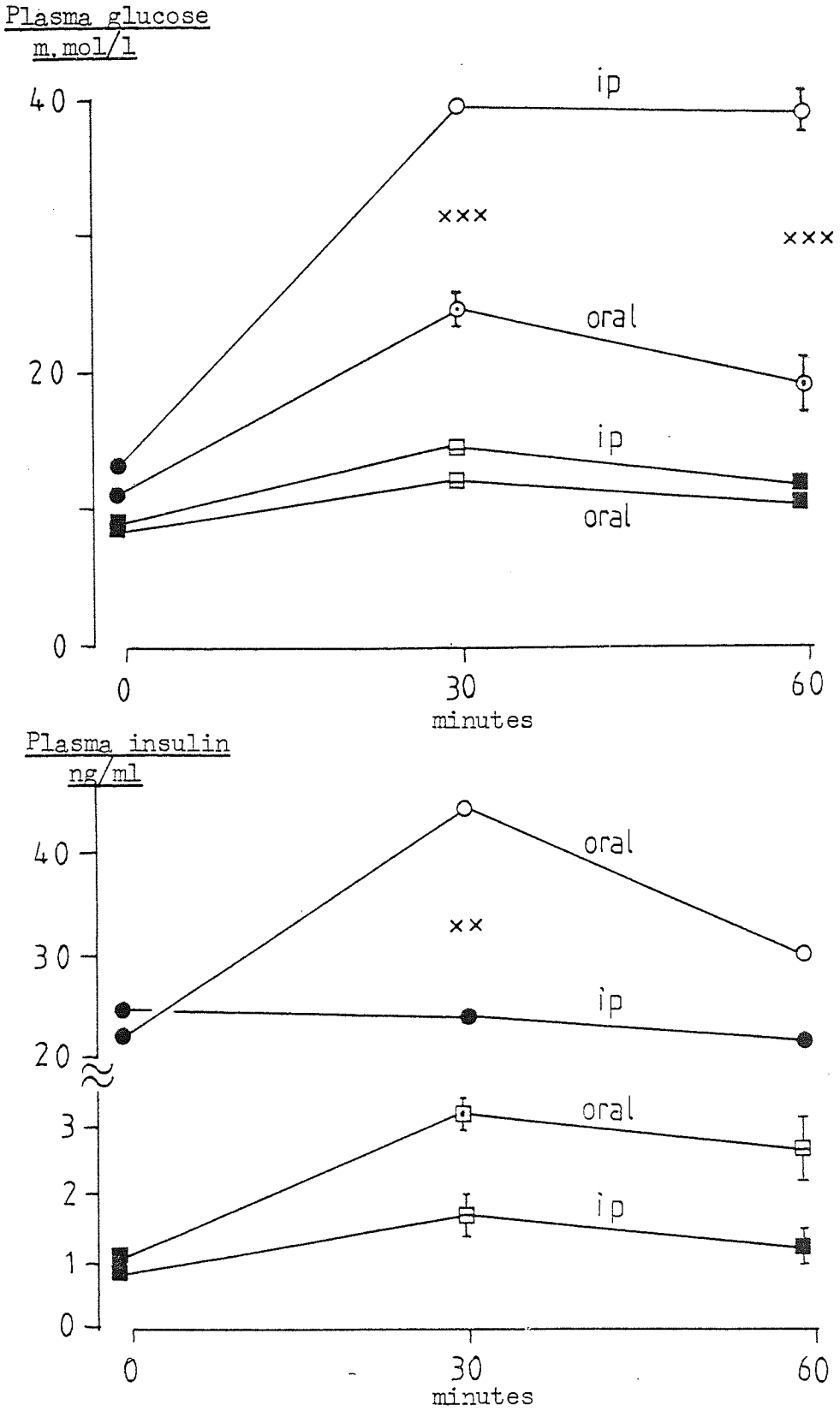
p vs. Baseline ⊟△⊖ = $p < 0.05$; □△○ = $p < 0.001$.

Figure 62 Glucose tolerance in fed and 24 hour fasted obese mice (m ± sem, n = 6).$^{\phi}$



$^{\phi}$p vs. Baseline Θ = p < 0.05; ⊙ = p < 0.01; O = p < 0.001.

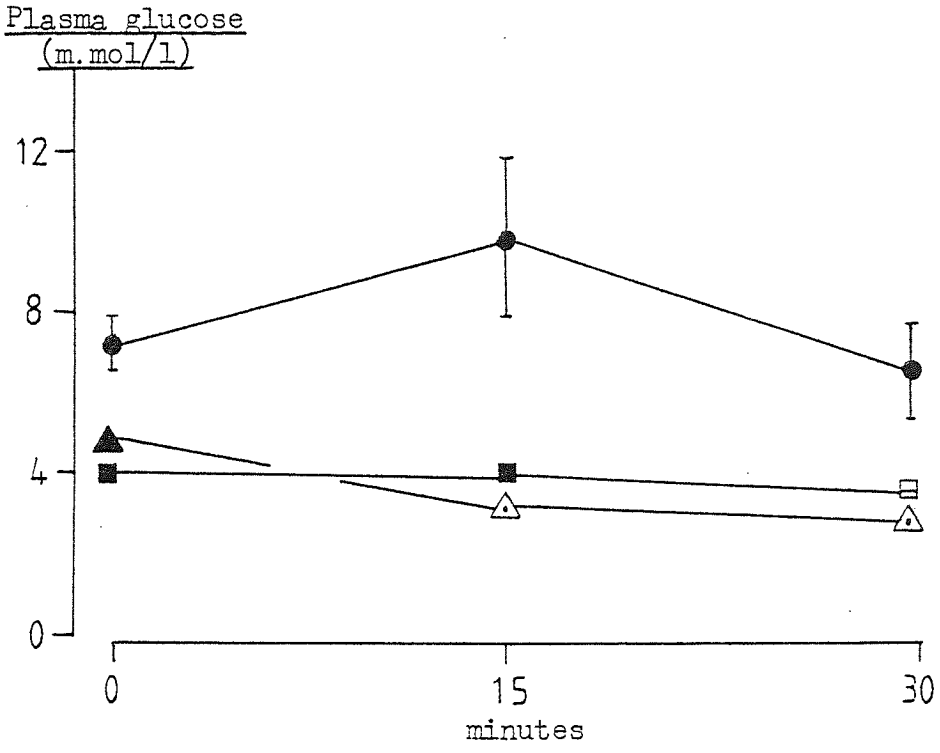
Figure 63 Oral and intraperitoneal glucose tolerance in fed +/+ (■——■) and ob/ob (●—●) mice (m ± sem, n = 6)

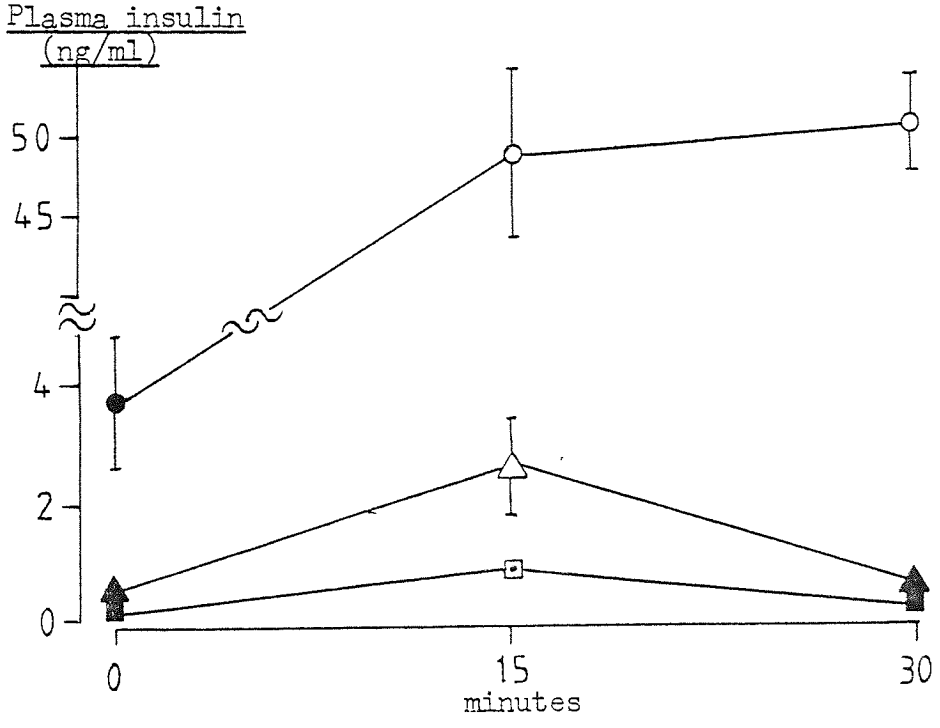


p vs. Baseline ⊟ ⊖ = $p < 0.05$; ⊡ ⊙ = $p < 0.01$; □ ○ = $p < 0.001$.

Comparison with genotypes x oral > i.p. at $p < 0.05$; xx oral > i.p. at $p < 0.01$; xxx oral i.p. at $p < 0.001$.

Figure 64 Arginine stimulation in 24 hour fasted +/+ (■——■), ob/+ (▲——▲) and ob/ob (●—●) mice (m ± sem, n = 6).

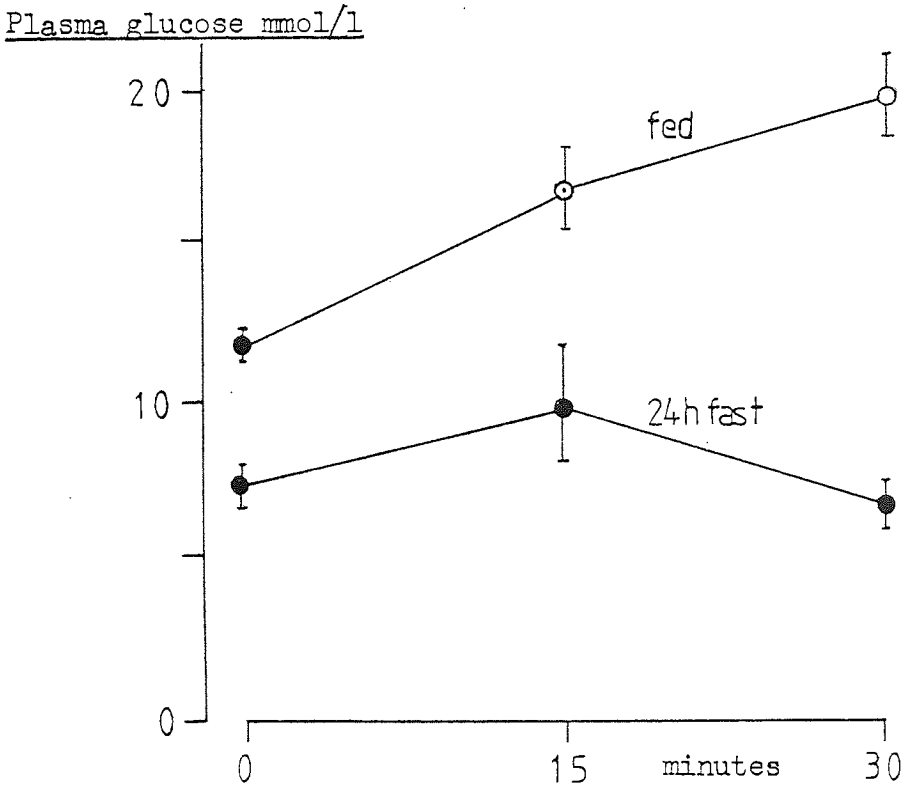


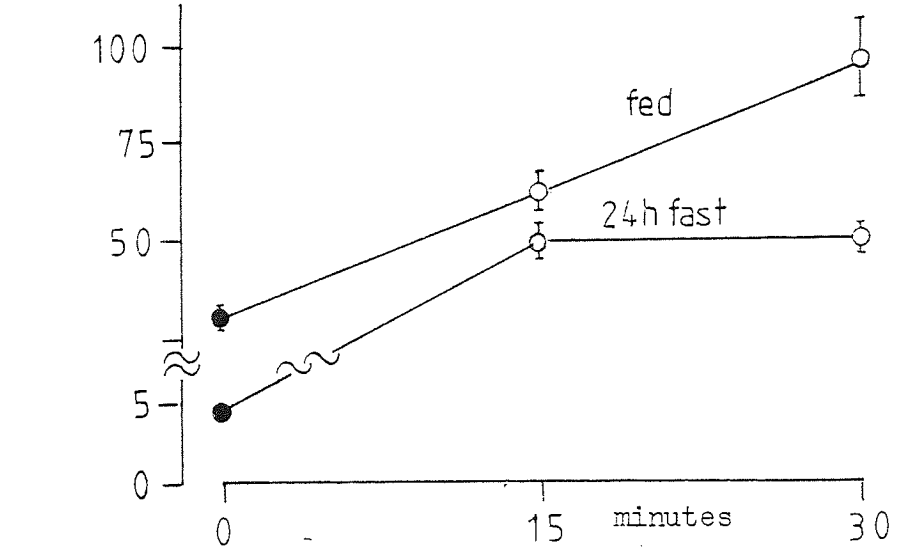


p vs. Baseline ⊟△⊖ = < 0.05; ⊡△⊙ = < 0.01;

□△○ = p < 0.001

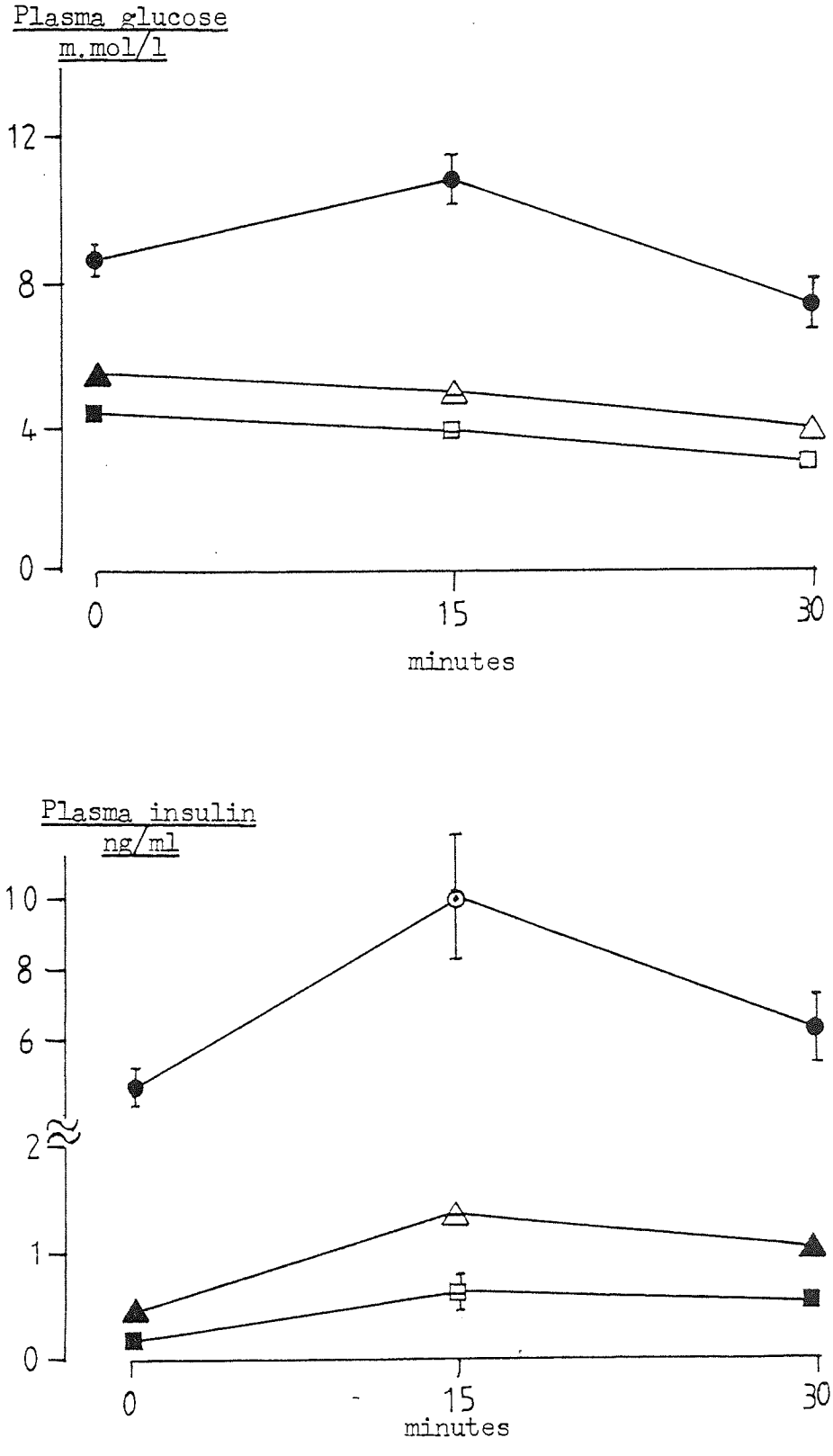
Figure 65 Arginine stimulation in fed and 24 hour fasted ob/ob mice (m ± sem, n = 6)$^{\phi}$.





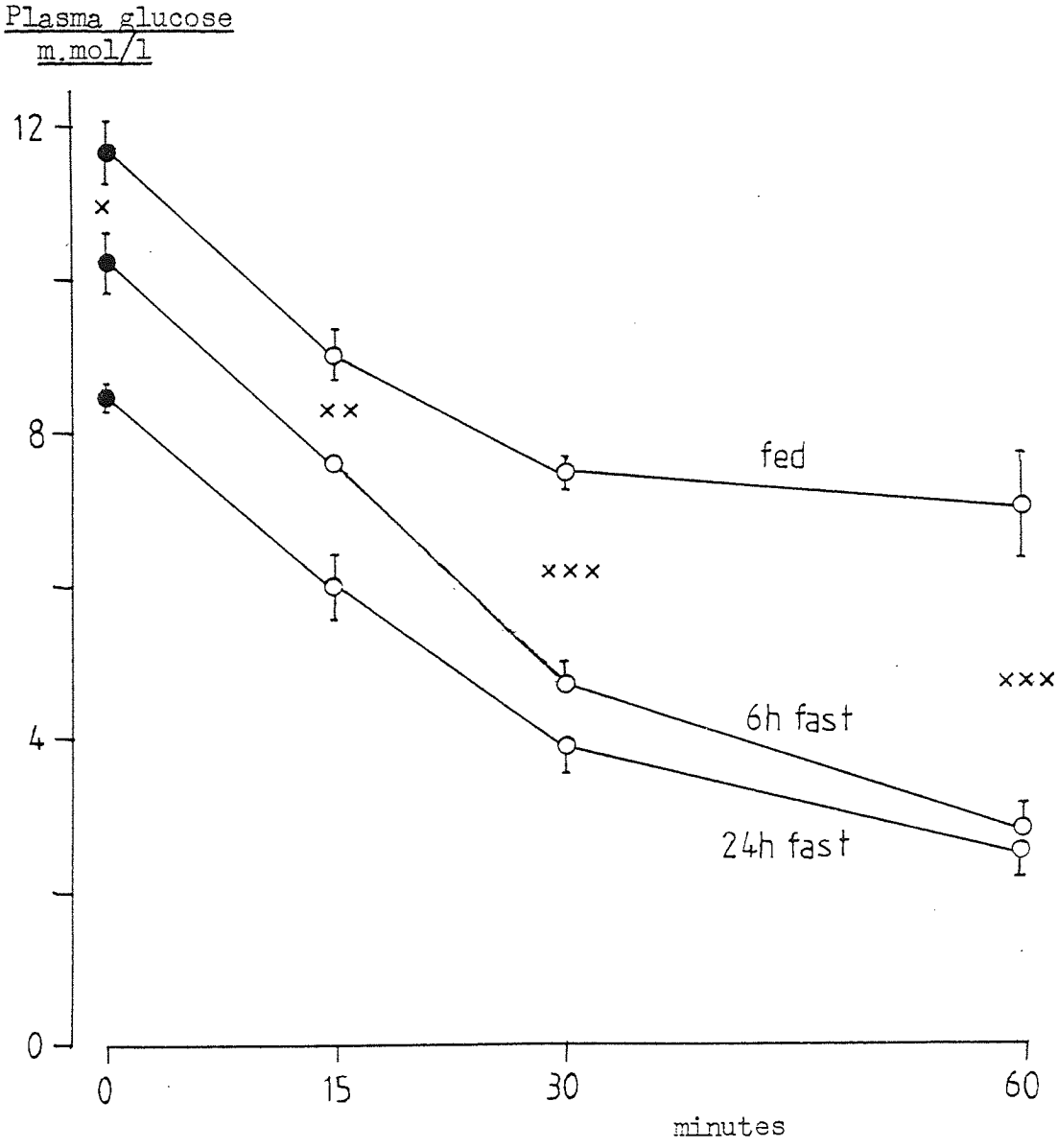
$^{\phi}$p vs. Baseline ⊙ = p < 0.01; O = p < 0.001.

Figure 66 Insulin sensitivity (0.25 U/kg) in 24 hour fasted +/+ (■——■), ob/+ (▲——▲) and ob/ob (●—●) mice (m ± sem, n = 6).



p vs. Baseline ⊟ △ ⊖ = $p < 0.05$; ⊡ △ ⊙ = $p < 0.01$; □ △ ○ = $p < 0.001$.

Figure 67 Insulin hypoglycaemia (100 U/kg) in fed, 6 hour fasted and 24 hour fasted ob/ob mice (m ± sem, n = 6).



p vs. Baseline 0 = p < 0.001

Comparison between fed and 6 hr.fasted mice x = p < 0.05; xx = p < 0.01; xxx = p < 0.001.

CHAPTER 6

THE REGULATION OF PANCREATIC ALPHA CELL FUNCTION AND THE ROLE OF GLUCAGON IN THE PATHOGENESIS OF THE OBESE HYPERGLYCAEMIC SYNDROME IN MICE (GENOTYPE ob/ob)

INTRODUCTION

The role of insulin in normal physiology and in the pathogenesis of diabetes mellitus has been appreciated since the studies of von Mering and Minkowski (1890) in pancreatectomised dogs and the epoch-making discovery of Banting and Best (1922) over 50 years ago. In recent years, considerable attention has focused on the contribution of glucagon to the regulation of metabolism in both health and human disease states (see Lefebvre & Unger, 1972; Marks, 1972; Bloom, 1974a; Unger, 1974 a, b, 1976 a, b, c; Felig et al., 1976a; Unger & Orci, 1976, 1977 a, b; Buchanan, 1977). A pathophysiological role for glucagon in human diabetes has been inferred from observations that in diabetics, plasma glucagon levels are inappropriately elevated for the degree of hyperglycaemia (Aguilar-Parada et al., 1969b; Heding & Rasmussen, 1972; Gerich et al., 1973; Weir et al., 1973; Alford et al., 1977) and are not suppressible after carbohydrate feeding (Müller et al., 1970; Unger et al., 1970) or oral glucose (Buchanan & McCarroll, 1972; Day & Anderson, 1973). Strong support for glucagon's importance in diabetes has been provided by investigations with somatostatin (Gerich et al., 1974; Dobbs et al., 1975), and further evidence has been derived from animal studies which have demonstrated that many forms of diabetic hyperglycaemia including alloxan diabetes in dogs and rats (Müller et al., 1971; Meier et al., 1972), streptozotocin diabetes in rats (Katsilambros et al., 1970; Amherdt et al., 1974), diazoxide and mannoheptulose induced diabetes in dogs (Müller et al., 1971), anti-insulin serum diabetes in rats (Müller et al., 1971), surgically induced diabetes in dogs and rats (Vranic et al., 1974; Matsuyama & Foà, 1974; Wider et al., 1976), and spontaneous diabetes in rodents (Lavine et al., 1975; Herberg et al., 1976) are accompanied by a relative or absolute hyperglucagonaemia.

Since recent studies have demonstrated a definite physiological role for glucagon in the regulation of basal and insulin-inhibited splanchnic glucose production (Altszuler et al., 1976; Cherrington et al., 1976; Felig et al., 1976b; Bomboy et al., 1977), the possibility arises that diabetic hyperglycaemia is a consequence of a relative hyperglucagonaemia. Indeed, Unger and Orci (1975, 1977b) support the hypothesis that diabetes is a bihormonal disease, dependent on a relative or absolute insulin lack for glucose underutilisation, and an absolute or relative glucagon excess for glucose overproduction. However, conflicting evidence has emerged as to whether abnormal alpha cell function is a direct consequence of insulin deficiency or is due to an intrinsic defect of the alpha cell itself (Unger et al., 1970; Müller et al., 1971; Samols et al., 1972; Buchanan & Mawhinney, 1973 a, b; Gerich et al., 1973, 1975, 1976a; Raskin et al., 1975; Warne et al., 1977). The importance of the relative hyperglucagonaemia in the pathogenesis of diabetes also remains controversial (Unger & Orci, 1975; Barnes & Bloom, 1976; Levine, 1976; Sherwin et al., 1976; Barnes et al., 1977 a, b; Buchanan, 1977), as does the role of glucagon in the development of insulin insensitivity of diabetes (Eaton, 1975).

Although considerable interest and controversy have developed in recent years concerning the possible significance of abnormally high circulating glucagon levels encountered in diabetes and the relevance of this hormone to hyperglycaemia, very little attention has been given to the role of glucagon in the aetiology and pathogenesis of the obese hyperglycaemic syndrome in mice. Mayer and colleagues (Mayer et al., 1953b; Clarke et al., 1956) originally proposed that excessive glucagon secretion may contribute to the hyperglycaemia of obese mice, and support for this hypothesis has been derived from histological investigations (Gepts et al., 1960; Hellman, 1961; Findlay et al., 1973) and functional studies conducted on a variety of different strains of genetically obese

and diabetic rodents (Laube et al., 1973, 1974; Frenkel et al., 1974; Lavine et al., 1975; Ziegler et al., 1975; Herberg et al., 1976; Mahler et al., 1976; Beloff-Chain et al., 1977a).

In the present investigation, a sensitive and reliable glucagon radioimmunoassay was used to examine the regulation of alpha cell function and to assess the role of glucagon in both the normal physiology and in the pathogenesis of the obese hyperglycaemic syndrome in mice.

MATERIALS AND METHODS

ANIMALS

Genetically obese hyperglycaemic mice (genotype ob/ob) and lean homozygous mice (genotype +/+) from the Aston colony were used for this study. Groups of 6-12 animals were fed ad libitum or fasted for 24 hours prior to an experiment as indicated in the appropriate sections. The mice were allowed free access to tap water and were used at 18-22 weeks of age.

CHEMICALS

Adrenaline, arginine hydrochloride, 2-deoxy-D-glucose, noradrenaline bitartrate, pilocarpine hydrochloride and porcine glucagon were purchased from Sigma Chemical Company Ltd., London. Heparin was obtained from BDH Chemicals Ltd., Poole, and monocomponent porcine insulin (Actrapid) was purchased from Novo Industri A/S., Copenhagen. All other chemicals were of analytical grade. The source of reagents used in the radioimmunoassay procedures are given in the previous chapters.

ADMINISTRATION OF TEST SUBSTANCES

All test substances were dissolved immediately prior to use in either warm distilled water (glucose only) or saline (0.9% w/v, NaCl), and administered by intraperitoneal injection at a dose corresponding to 5 ml/kg body weight.

BLOOD SAMPLING

In the following experiments, blood samples for the determination of plasma glucose, immunoreactive insulin (IRI), immunoreactive glucagon (C-GLI) and non-esterified free fatty acids (FFA) were obtained from conscious mice by the tail-tip amputation method described by Grice (1964). Blood was collected in small heparinised polystyrene tubes immersed in an ice bath. The samples were centrifuged at 4°C and an aliquot of each plasma was taken for the various analytical techniques. Plasma for the glucagon assay was extracted as previously described using a final ethanol concentration of 60%. All samples were stored at -20°C in tightly capped tubes prior to analysis.

ANALYTICAL TECHNIQUES

Plasma glucose was determined by the glucose oxidase method using a Beckman autoanalyser (Stevens, 1971), and FFA were measured by the colorimetric method described by Duncombe (1964). Plasma IRI and C-GLI were determined by radioimmunoassay as previously described.

STATISTICAL METHOD

Statistical analyses were performed with the aid of a desk computer (Olivetti Programma 101, British Olivetti Ltd., London). Mean values $\pm$ standard error of the mean (sem) were calculated for groups of data, and where appropriate the significance of difference between these groups evaluated by unpaired Student's t-test (Snedecor, 1967). Probability levels of $p < 0.05$ were taken as statistically significant.

EXPERIMENTAL PROCEDURES

Although the plasma glucose IRI, C-GLI and FFA concentrations reported in this chapter were from individual mice, serial sampling was not routinely performed due to the relatively small plasma volume of these animals (Abraham et al., 1971). Thus in the majority of experiments, each animal was bled only once after treatment, and a rest period of

approximately 10 days was allowed before a further study was commenced. The preinjection levels were established in each series of experiments by bleeding randomly selected untreated animals.

The following studies were performed between 09.00-14.00 using mice that had been previously accustomed to the manipulative procedures.

1. BASAL STUDIES

To investigate the relationship between circulating glucagon and plasma glucose and insulin concentrations, basal levels of glucose, IRI and C-GLI were determined in both the fed state and in the 24 hour fasted state.

2. ROLE OF CIRCULATING GLUCAGON IN GLUCOSE HOMEOSTASIS

In order to evaluate the role of circulating glucagon in the regulation of glucose homeostasis, groups of fully fed mice were treated with either porcine glucagon or C-terminal reactive antibovine glucagon antiserum. Serial blood samples were taken for the analysis of plasma glucose and IRI concentrations.

Glucagon

The effect of exogenous glucagon was examined in mice given a single intraperitoneal injection of porcine glucagon (1 mg/kg). Glucagon was dissolved in a small volume of 0.01M sodium hydroxide and diluted to a final concentration of 200 µg/ml with saline. Blood samples were taken immediately prior to, and 10, 30 and 60 minutes following administration of the test substance.

Glucagon antiserum

The physiological role of endogenous glucagon in the maintenance of plasma glucose and insulin concentrations was examined using groups of mice given a single intraperitoneal injection of undiluted C-terminal reactive rabbit glucagon antiserum (ROG2/1, 1.5 ml/100g). Control animals received an equivalent volume of normal rabbit serum. Blood

samples were taken from fed mice and then 180 and 260 minutes after treatment.

3. CONTROL STUDIES

In view of the importance of glucagon in stress situations (Bloom, 1973; Bloom et al., 1973; Lindsey et al., 1974), control studies were considered an essential prerequisite to further investigations on the regulation of glucagon secretion in conscious mice. Groups of mice fed ad libitum or previously fasted for a period of 24 hours were given an intraperitoneal injection of saline (0.5ml/100g). Blood samples for analysis of plasma glucose, IRI and C-GLI were withdrawn at either 15, 30 or 60 minutes after administration of the control injection.

4. STIMULATION/SUPPRESSION STUDIES

In order to examine the pancreatic α cell function of obese hyperglycaemic mice and to evaluate the role of glucagon in the pathogenesis of the ob/ob syndrome, plasma glucose, IRI, C-GLI and in one experiment FFA responses to known stimulators and suppressors of glucagon secretion were studied in both freely fed and 24 hour fasted mice.

Arginine

Arginine stimulation tests were performed on 24 hour fasted mice. Arginine hydrochloride (1.5g/kg) was administered by intraperitoneal injection and blood samples were withdrawn after a period of either 15 or 30 minutes.

Glucose

The suppressive effect of glucose on glucagon secretion was examined during the course of glucose tolerance tests. Glucose (2g/kg) was administered by intraperitoneal injection to freely fed and 24 hour fasted mice. Blood samples were then taken at either 30 or 60 minutes.

Insulin

The inhibitory effect of insulin on pancreatic α cell function was studied in 24 hour fasted mice. Porcine insulin (0.25 U/kg) was administered by the intraperitoneal route to both lean homozygous (+/+) mice and obese (ob/ob) mice. To evaluate the role of glucagon in the manifestation of insulin insensitivity in obese mice, changes in plasma glucose and C-GLI concentrations were also examined following a high dose (100 U/kg) insulin hypoglycaemia test in ob/ob mice. Plasma samples were prepared from blood collected at either 15 or 30 minutes after the injection of insulin.

Insulin antiserum

The effect of insulin antiserum on glucagon secretion was determined in groups of mice allowed free access to food. Guinea pig insulin antiserum (GPA2/3-4, binding capacity approx. 15 U/ml) was administered by intraperitoneal injection at a dose of 1 ml/100g and 2 ml/100g to lean and obese mice respectively. Control animals received an equivalent volume of normal guinea pig serum. Blood samples were taken 180 and 360 minutes after treatment.

2-Deoxy-D-glucose

The effect of a non-metabolisable analogue of glucose, 2-deoxy-D-glucose, on pancreatic α cell function was examined using freely fed mice. 2-Deoxy-D-glucose (750 mg/kg) was administered by intraperitoneal injection and blood was withdrawn after a period of either 30, 60 or 180 minutes.

Heparin

The inhibitory effect of free fatty acids on glucagon secretion was examined by heparin-induced elevation of plasma FFA levels in 24 hour fasted mice. Heparin was administered by two intraperitoneal injections (0.25 ml/100g at 0 and 20 minutes, corresponding to a total dose of 200 U/kg). Blood samples for the determination of plasma glucose,

IRI, C-GLI and FFA were collected at either 30 or 60 minutes after the initial injection. The preinjection levels were established by bleeding randomly selected untreated mice.

5. NEURAL STUDIES

In order to evaluate the possible participation of the autonomic innervation and circulating catecholamines in the regulation of glucagon secretion, plasma glucose, IRI and C-GLI responses to both parasympathomimetic and sympathomimetic agents were examined in freely fed and 24 hour fasted mice.

Pilocarpine

The effect of the parasympathomimetic agent, pilocarpine, was determined in 24 hour fasted mice. Pilocarpine hydrochloride (35 mg/kg) was administered by intraperitoneal injection and blood samples were collected at 15 and 30 minutes after treatment.

Noradrenaline

The effect of the sympathomimetic agent, noradrenaline, was examined in groups of freely fed mice. Noradrenaline bitartrate (1.84 mg/kg) was administered by the intraperitoneal route. Blood was withdrawn after a period of either 30 or 60 minutes.

Adrenaline

The effect of adrenaline was examined using mice allowed free access to food. Adrenaline (1 mg/kg) was administered by intraperitoneal injection and blood samples were collected at either 30 or 60 minutes.

RESULTS

1. BASAL STUDIES

Basal plasma glucose, IRI and C-GLI concentrations of fully fed and 24 hour fasted mice, together with the IRI/C-GLI molar ratios are illustrated in figure 68. The IRI/C-GLI molar ratios were calculated assuming molecular weights of 6000 and 3500 respectively. Both groups

of mice exhibited characteristic changes in circulating glucose and IRI following the withdrawal of food. Plasma glucose and insulin concentrations of obese mice were significantly greater (all $p < 0.001$) than those observed in lean homozygous (+/+) mice regardless of nutritional status. Circulating C-GLI concentrations were also elevated in obese mice under these conditions, although the difference did not achieve statistical significance. However, the IRI/C-GLI molar ratios of the obese animals were grossly elevated ($p < 0.001$) in both the fed and 24 hour fasted states, indicative of a gross abnormality of α cell function. The plasma C-GLI concentrations and the IRI/C-GLI molar ratios of both lean and obese groups were significantly reduced following a 24 hour period of food restriction.

2. ROLE OF CIRCULATING GLUCAGON IN GLUCOSE HOMEOSTASIS

Glucagon

Plasma glucose and IRI responses to glucagon are shown in figure 69. Both lean and obese groups exhibited a marked rise ($p < 0.001$) in plasma glucose and IRI concentrations immediately following administration of the test substance. In lean homozygous (+/+) mice, the effect of glucagon was transient. However, the obese animals displayed both an exaggerated and prolonged response to glucagon stimulation. Plasma glucose concentrations remained significantly elevated ($p < 0.01$) in obese mice throughout the test, but plasma insulin concentrations gradually declined to prestimulation levels towards the end of the observation period.

Glucagon antiserum

The effects of C-terminal reactive glucagon antiserum on plasma glucose and IRI levels of lean and obese mice are presented in figure 70. The data are illustrated following correction for sham treatment. Administration of glucagon antiserum resulted in a significant reduction

($p < 0.001$) in plasma glucose concentrations of both groups of mice. The plasma glucose levels of obese mice continued to fall throughout the test, reaching values comparable to those observed in lean animals. In contrast to these marked changes, relatively small alterations in plasma insulin values were observed following sequestration of circulating C-GLI. The plasma insulin levels of lean homozygous (+/+) mice were not significantly depressed at 180 minutes. The circulating insulin concentrations of obese mice, however, remained significantly depressed ($p < 0.05$) throughout the test, despite a trend towards the return to baseline values during the latter stages of the observation period.

3. CONTROL STUDIES

The responses of fully fed and 24 hour fasted mice to control injections of saline are illustrated in figures 71 and 72, respectively. Plasma glucose and insulin concentrations remained stable throughout the test period. However, the administration of control injections to both lean and obese mice was accompanied by a gradual increase in plasma C-GLI concentrations, reaching significantly elevated values ($p < 0.001$, all groups) after a period of 60 minutes. Obese mice were particularly sensitive to this form of treatment as illustrated by both the time course and the severity of the changes in C-GLI concentrations.

Since these data indicate a significant stimulatory effect of control injections on circulating C-GLI concentrations of both lean homozygous (+/+) and obese mice, C-GLI responses in subsequent studies are presented following subtraction of that component of the response attributable to sham stimulation.

4. STIMULATION/SUPPRESSION STUDIES

Arginine

The effects of arginine on plasma glucose, IRI & C-GLI concentrations of lean and obese mice are shown in figure 73. Administration of arginine

resulted in significant increases of circulating IRI and C-GLI in both groups of mice. In lean homozygous (+/+) animals, the effect was transient in nature, and although plasma C-GLI concentrations remained significantly elevated ($p < 0.01$) during the latter stages of the test period, a significant reduction ($p < 0.01$) in plasma glucose concentration was observed. Obese mice displayed exaggerated and prolonged secretory responses, together with a slight increase in the circulating glucose concentration. Plasma C-GLI attained values significantly greater ($p < 0.001$) than those recorded in lean mice during the test period, indicative of abnormal α cell function.

Glucose

Plasma glucose, IRI and C-GLI responses of fully fed and 24 hour fasted mice to glucose are illustrated in figures 74 and 75, respectively. The changes in plasma glucose and insulin concentrations of lean and obese mice followed a characteristic time course. Lean mice exhibited transient elevations ($p < 0.05$) of both glucose and insulin concentrations following a glucose challenge. Obese mice displayed severe glucose intolerance which was further aggravated following a 24 hour fast despite the restoration of a weak insulin secretory response. In the fed state, plasma C-GLI concentrations of both groups of mice declined steadily to reach significantly reduced ($p < 0.001$) values upon termination of the observation period. The withdrawal of food prior to the test resulted in a delayed reduction in C-GLI concentration of lean mice, and a complete lack of suppression of circulating C-GLI in the obese group. Indeed, obese mice displayed a paradoxical increase ($p < 0.05$) in C-GLI concentration following a glucose challenge which coincided with a small increment in the fasting insulin concentration. These data provide further evidence for an abnormal α cell function in obese mice and suggest that the suppression of glucagon secretion by glucose is closely related to the prevailing insulin concentration.

Insulin

The responses of lean and obese mice to a low dose (0.25 U/kg) of insulin are shown in figure 76. In lean homozygous (+/+) mice, this dose of insulin resulted in a prompt rise ($p < 0.05$) in the circulating insulin level which was accompanied by a progressive fall ($p < 0.01$) in both plasma glucose and C-GLI concentrations. However, the obese animals failed to respond to the low dose of insulin, and both plasma glucose and glucagon concentrations remained elevated despite a significant increase ($p < 0.01$) in the circulating insulin level.

In contrast, obese mice treated with a high dose (100 U/kg) of insulin exhibited both a marked and progressive decline ($p < 0.001$) in plasma glucose concentrations which were associated with a significant decrease ($p < 0.001$) in circulating C-GLI (figure 77). Thus suppression of endogenous plasma C-GLI in obese mice requires vastly elevated concentrations of circulating insulin.

Insulin antiserum

Plasma C-GLI responses of groups of mice to insulin antiserum and normal control serum are illustrated in figure 78. Administration of insulin antiserum resulted in significant increments in plasma C-GLI concentrations in both lean and obese mice when compared with their respective controls. Circulating C-GLI continued to increase throughout the period of observation in both experimental groups, but the final concentration was significantly greater ($p < 0.05$) in obese mice. These data indicate that in the fed state, the prevailing insulin level exerts a significant effect on glucagon secretion in both lean homozygous (+/+) and obese mice.

2-Deoxy-D-glucose

The effects of the non-metabolisable analogue of glucose, 2-deoxy-D-glucose, on plasma glucose, IRI and C-GLI are illustrated in figure 79. Administration of 2-deoxy-D-glucose resulted in marked elevations in the plasma glucose and C-GLI concentrations of both groups of mice. Despite the severity of the hyperglycaemia and hyperglucagonaemia in these animals, no significant changes in circulating insulin were observed. The effects of intracellular glucopenia were particularly pronounced in the obese group, and the circulating C-GLI concentrations of obese mice attained significantly greater values ($p < 0.001$) than those recorded in lean mice during the early stages of the test. Since the primary metabolic block produced by 2-deoxy-D-glucose concerns the competitive inhibition of phosphohexoseisomerase (Wick et al., 1957), these observations suggest that glucose metabolism plays an important role in the regulation of glucagon secretion in these animals.

Heparin

Plasma glucose, IRI, C-GLI and FFA responses to heparin are shown in figure 80. Heparin evoked considerable elevations ($p < 0.001$) in the FFA concentrations of both lean and obese mice. The extent of the hyperlipidacidaemia was most prominent in the obese group, and the FFA concentrations of obese mice reached significantly greater ($p < 0.05$) peak values despite the existence of comparable prestimulation concentrations to those observed in lean homozygous (+/+) mice. Although a tendency towards decreased circulating levels of glucose and insulin was observed in both groups during the latter stages of the test period, no significant changes in either the plasma glucose or plasma IRI concentrations were recorded. However, the circulating C-GLI concentrations of both lean and obese mice were significantly ($p < 0.05$ and $p < 0.001$, respectively) reduced following heparin administration. In obese mice, the plasma C-GLI concentration declined rapidly to attain

values significantly lower ($p < 0.05$) than those of lean homozygous (+/+) mice despite the presence of mild hyperinsulinaemia. These data preclude a generalised abnormality of α cell function in obese mice to suppression by metabolic substrates, and provide additional evidence that the α cells of the obese animal may be selectively insensitive to modulation by circulating glucose.

5. NEURAL STUDIES

Pilocarpine

The effects of pilocarpine on plasma glucose, IRI and C-GLI are illustrated in figure 81. Administration of pilocarpine resulted in marked elevations ($p < 0.01$) of plasma glucose and C-GLI concentrations, together with a rapid increase in the circulating insulin levels of both lean and obese mice ($p < 0.05$ and $p < 0.01$, respectively). The magnitude and pattern of the changes in plasma glucose concentrations were similar in the two groups, but the obese animals exhibited an exaggerated increase in circulating insulin which subsequently declined despite the persistence of significantly elevated ($p < 0.01$) plasma glucose levels. The plasma C-GLI response to pilocarpine was most prominent in lean homozygous (+/+) mice, and the plasma C-GLI concentrations of this group attained significantly higher values ($p < 0.05$) than those observed in their obese counterparts.

Noradrenaline

The responses of fully fed lean and obese mice to noradrenaline are shown in figure 82. Both groups of mice exhibited significantly elevated ($p < 0.001$) circulating concentrations of glucose and C-GLI following the administration of noradrenaline. In lean mice, the plasma insulin concentrations declined steadily throughout the test period. However, no significant changes in circulating IRI were recorded in the obese animals and a tendency towards increased insulin concentrations was observed in these mice. The plasma C-GLI concentration of the obese

group was significantly greater ($p < 0.01$) than that observed in lean mice during the early stages of the test but rapidly declined to base-line values thereafter.

Adrenaline

Plasma glucose, IRI and C-GLI responses to adrenaline are illustrated in figure 83. Administration of adrenaline resulted in significant elevations of plasma glucose and C-GLI in both groups of mice ($p < 0.001$ and $p < 0.01$, respectively). The circulating glucose levels continued to rise during the test, but the plasma C-GLI concentrations of both lean and obese mice declined during the latter stages to essentially prestimulatory values. Plasma insulin concentrations of both groups of mice were significantly reduced by adrenaline, despite the presence of hyperglycaemia and hyperinsulinaemia.

DISCUSSION

Recently, attention has been focused on the contribution of glucagon to the maintenance of hyperglycaemia in human juvenile-onset diabetics (Gerich et al., 1974b; Eaton, 1975; Unger & Orci, 1975; Felig et al., 1976; Sherwin et al., 1976) and in the experimentally induced diabetes of animals (Müller et al., 1971; Vranic et al., 1976). Numerous studies suggest that the elevated systemic glucose concentrations associated with these forms of diabetes mellitus arise from the combination of reduced pancreatic β cell secretory capacity and persistent glucagon production of pancreatic or gastrointestinal origin (Unger, 1976b; Unger & Orci, 1976, 1977b). However, the hyperglycaemia that occurs in some forms of adult-onset diabetes and in animals with genetically based diabetes and obesity is more complex due to the simultaneous presence of hyperglycaemia, relative or absolute hyperglucagonaemia, and either moderate or severe hyperinsulinaemia (Unger et al., 1970; Herberg et al., 1976; Mahler et al., 1976; Alford et al., 1977).

Accordingly, two popular hypotheses have emerged to account for the pancreatic α cell dysfunction in diabetes mellitus. The first suggests that insulin lack per se is responsible for the abnormal glucagon metabolism in diabetes (Buchanan & McCarroll, 1972; Marks, 1972; Warne et al., 1977). This is supported by observations that every known insulin-deficient state is associated with either a relative or absolute excess of circulating glucagon (see Unger & Orci, 1975). Moreover, insulin deprivation in human diabetics and alloxan diabetic dogs results in hyperglucagonaemia that can be reversed by insulin administration (Müller et al., 1971; Gerich et al., 1975; Warne et al., 1977). The second hypothesis proposes that diabetes mellitus is a bihormonal disease in which abnormal glucagon secretion results from a primary pancreatic α cell insensitivity to glucose (Unger et al., 1972; Gerich et al., 1973, 1976; Unger & Orci, 1976, 1977b). Evidence in support of this postulate is derived from observations that the diabetic α cell responds inappropriately to both hyper- and hypoglycaemia (Unger et al., 1972; Gerich et al., 1973), and that diabetic hyperglucagonaemia is often found in the presence of normal or elevated insulin levels (Unger et al., 1970; Gerich et al., 1976a). In the present study, the regulation of pancreatic α cell function together with the role of glucagon in the pathogenesis of genetic diabetes has been examined in the Birmingham strain of obese hyperglycaemic mice (genotype ob/ob) using sensitive and reliable radioimmunoassay techniques. As previously illustrated, this animal model displays hyperglycaemia concomitant with marked hyperinsulinaemia and severe insulin resistance.

Although the precise nature of the mechanism of glucagon secretion is unknown (Gerich, 1976; Gerich et al., 1976b; Porte et al., 1976; Unger & Orci, 1976), it is generally considered that splanchnic glucose concentrations represent the major regulatory signals for the release

of pancreatic glucagon which is subject to local modulation by the secretory activity of the adjacent β cells (Unger & Orci, 1976; Buchanan, 1977). The results of several previous studies (Lavine et al., 1975; Mahler et al., 1976; Beloff-Chain et al., 1977), and the data presented here demonstrate that obese mice maintain basal hyperglucagonaemia despite vastly elevated circulating levels of glucose and IRI. Clearly, a deficit in either a glucose or insulin-dependent glucose mechanism that inhibits glucagon secretion could account for the hyperglucagonaemia of these animals. However, in view of the extreme hyperinsulinaemia of obese mice, it appears that the diabetic α cell is markedly insensitive to the normal inhibitory action of insulin, thereby resulting in the secretion of inappropriate amounts of glucagon. Indeed, consideration of the IRI:C-GLI molar ratio indicates the existence of a gross abnormality of α cell function in obese mice. The IRI:C-GLI ratio was significantly elevated in the obese animals regardless of nutritional status. However, in agreement with classical theory developed by Unger and associates (Unger, 1974a, b; 1975, 1976), the molar ratio was significantly reduced in both groups of mice following a 24 hour period of food restriction, thereby favouring hepatic glucose production. Such changes were nevertheless manifested by alterations in the plasma IRI levels, and C-GLI concentrations declined in both groups of mice following a 24 hour fast. Food deprivation in many species results in compensatory responses of glucose mobilisation that are mediated by transient increases in circulating glucagon concentrations and progressive reductions in insulin secretion (Unger, 1974a; Gerich, 1976). However, recent studies (Fischer et al., 1976) have demonstrated that fasting hyperglucagonaemia predominantly arises as a result of decreased metabolic clearance rather than hypersecretion of glucagon. This phenomenon challenges the importance of enhanced glucagon secretion, and emphasises the role of the insulin:glucagon molar ratio and direct neural influences in the

regulation of hepatic metabolism during fasting (Shimazu & Amakawa, 1968; Parrila et al., 1974; Cherrington et al., 1976). Thus, in situations where enhanced glucagon secretion occurs during fasting, it is possible that the necessary stimulus for the metabolically intact α cell is secondary to changes in plasma glucose and β cell secretory activity (see Lavine et al., 1975; Eaton et al., 1976). However, the results of the present study support the contention (Felig et al., 1976) that one of the primary physiological roles of glucagon is to prevent the hypoglycaemia that would otherwise accompany food-mediated insulin secretion. Thus compared with fasted mice, obese animals in particular, exhibited grossly elevated circulating IRI levels combined with marked elevations in the plasma C-GLI concentrations. As previously illustrated, the dramatic increase in β cell secretory activity that occurred following oral or intragastric feeding was not dependent on the prevailing plasma glucose concentration, thereby implicating an uncharacterised mechanism of gastrointestinal origin. It is attractive to suggest that such a pathway also contributes to the elevated plasma C-GLI concentrations of fully fed mice.

The intimate local interrelationship between adjacent α and β cells within the pancreatic islets modulates the hormonal output of each functional endocrine unit to various physiological influences (Orci & Unger, 1975; Weir et al., 1976; Unger & Orci, 1977 a, b), and thereby plays a major role in determining the insulin:glucagon molar ratio and consequently the metabolic activity of the liver (Unger & Orci, 1976, 1977 a, b). Although the precise nature of hormone-receptor interactions are complex (Czech et al., 1977), studies using purified plasma membranes of liver have shown that although the insulin binding was severely reduced by 70-80% in obese mice, the glucagon binding was only decreased by 20-30% when compared with liver membranes prepared from lean littermate mice (Freychet, 1976). Accordingly, it is possible that under such conditions,

minor alterations in the circulating C-GLI concentrations observed in obese mice can exert significant effects on hepatic glucose production despite the relative magnitude of the IRI:C-GLI molar ratio. Indeed, the present study demonstrates that the sensitivity of the liver to exogenous glucagon is unimpaired in obese mice, and that plasma glucose concentrations of fully fed mice rose rapidly following a glucagon stimulus. The circulating IRI levels were also dramatically elevated during the test, suggesting that endogenous glucagon of either pancreatic or gastrointestinal origin might contribute to the hyperinsulinaemia as well as to the hyperglycaemia and insulin resistance of obese mice. To investigate these first two possibilities plasma glucose and IRI responses to a selective glucagon deficiency evoked by administration of a C-terminal reactive glucagon antiserum were examined in fully fed mice. Sequestration of circulating C-GLI had only a small effect on the plasma IRI concentrations of lean homozygous mice, indicating that the contribution of endogenous C-GLI to the maintenance of circulating IRI levels was negligible in these animals. However, endogenous C-GLI contributed significantly to the hyperinsulinaemia in fed obese mice as illustrated by a 20% fall in the plasma IRI concentration. Nevertheless, since the circulating IRI concentration remained grossly elevated compared to that observed in lean mice, it appears that circulating C-GLI is of relatively minor importance in both the aetiology and maintenance of the hyperinsulinaemia of obese mice. However, these data do not preclude the participation of local glucagon release in the modulation of insulin secretion, although recent kinetic evidence suggests that such a mechanism is unlikely to be of physiological significance in these circumstances (Pek et al., 1976). In sharp contrast to the relatively minor role of C-GLI in the maintenance of circulating IRI levels, the induction of a specific glucagon deficient state resulted in the production of marked hypoglycaemia in both groups

of mice. The plasma glucose concentrations of lean homozygous (+/+) mice and obese mice fell by approximately 59 and 70% respectively, indicating that circulating C-GLI plays a primary role in the maintenance of plasma glucose levels in the fed state. However, the response of lean homozygous (+/+) mice during the latter stages of the test suggests that an additional mechanism, possibly mediated via the autonomic innervation (Shimazu & Amakawa, 1968) may become functional during hypoglycaemia. The apparent lack of this plateau response in obese mice together with the restoration of normoglycaemia suggest that enhanced hepatic glucose production rather than decreased peripheral glucose utilisation is the major cause of the hyperglycaemia in the obese animals, and that this condition is a direct consequence of a relative hyperglucagonaemic state. Thus, it is envisaged that following sequestration of circulating C-GLI, the IRI:C-GLI molar ratio was considerably elevated in obese mice, thereby favouring a rapid increase in hepatic glucose uptake and an almost total suppression of hepatic glucose production. Previous studies (Szabo & Szabo, 1972, 1972a) have also suggested that the hyperglycaemia in obese mice is a consequence of unrestrained hepatic glucose production. However, Olga and Andrew Szabo (1975) have concluded that diabetic hyperglycaemia may result from a defective insulin sensitive hypothalamic receptor. The present data do not preclude such a defect in obese mice, but suggest that the hyperglycaemia results directly from the relative hyperglucagonaemia displayed by the obese animals. Accordingly, these observations provide strong evidence that pancreatic α cell dysfunction contributes significantly to the maintenance of diabetic hyperglycaemia in obese mice.

As previously indicated obese mice maintain basal hyperglucagonaemia despite vastly elevated levels of both glucose and insulin, suggesting a deficit in either a glucose or insulin-dependent glucose mechanism that

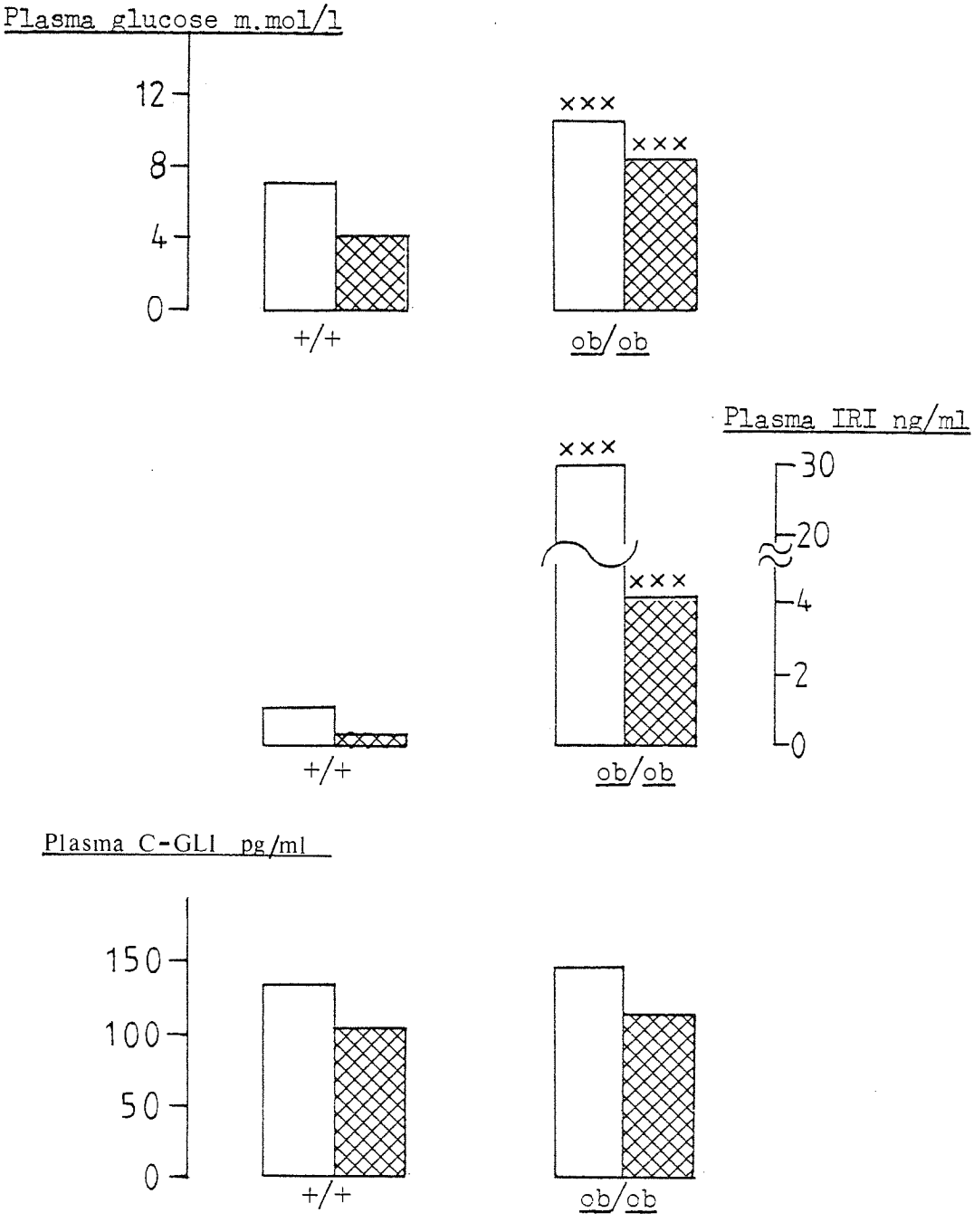
normally inhibits glucagon secretion. To further investigate the α cell function of obese mice and to evaluate the precise nature of the secretory defect, the action of known stimulators and suppressors of glucagon secretion were examined in conscious mice. The use of anaesthetics in this series of experiments was rejected since anaesthesia was associated with profound effects on plasma C-GLI and IRI metabolism of the mouse (Bailey & Flatt, unpublished). Furthermore, there is convincing evidence to support an important role of the autonomic nervous system in the regulation of endocrine pancreatic function (Watari, 1968; Kobayashi & Fujita, 1969; Orci et al., 1970; Bloom, 1974a; Woods & Porte, 1975; Porte et al., 1975, 1976; Smith & Porte, 1976). However, for exactly this reason, it was important to evaluate the participation of stress-induced changes in the overall response to the administration of test substances. Administration of control injections to either fully fed or 24 hour fasted mice resulted in non-significant changes in the plasma glucose and IRI concentrations. However, consistent with an important physiological role as a stress hormone (Bloom, 1973; Lindsey et al., 1974; Frohman & Nagai, 1976; Gerich et al., 1976c; Benson et al., 1977), C-GLI concentrations increased progressively during the test period to attain significantly elevated values after sixty minutes. This effect was slightly more pronounced in obese mice, suggesting the existence of a functionally intact neural mechanism. Indeed complimentary studies designed to evaluate the possible contribution of the autonomic nervous system to the overall regulation of endocrine pancreatic function in these animals, demonstrated that both parasympathomimetic and sympathomimetic agents were capable of exerting a fine control on the secretory activity of the pancreatic islets. Thus, in view of the well defined trend towards increased plasma C-GLI concentrations during the latter stages of the test period, it was necessary for validation of subsequent experiments to correct all poststimulation

C-GLI concentrations for that component of the response attributable to sham stimulation. The changes which accompanied this mild form of stress were however minor when compared with the gross alterations in circulating C-GLI which generally accompanied the administration of established stimulators and suppressors of glucagon secretion. Thus, arginine evoked massive increments in the circulating C-GLI concentrations of both lean homozygous (+/+) mice and obese mice. Consistent with a previous report (Laube et al., 1974) employing the isolated perfused ob/ob pancreas, and clinical observations in human diabetics (Unger et al., 1970), the obese animals displayed a marked hyperresponsiveness to arginine stimulation which undoubtedly contributed to the magnitude of the changes in both the plasma glucose and IRI concentrations. Demonstration that the obese animals have a high glucagon secretory capacity in vivo provides evidence against the contention (Lavine et al., 1975) that glucagon secretion is maintained at a maximal rate in obese mice. Indeed, since the exaggerated glucagon response to arginine stimulation in human juvenile-onset diabetics can be corrected by infusion of insulin (Gerich et al., 1975; Raskin et al., 1976), these data suggest that α cell insensitivity to endogenous insulin may contribute to the hyperglucagonaemia of obese mice. Further evidence of abnormal α cell function in the obese animals is derived from the lack of suppression of circulating C-GLI following the administration of glucose to fasted mice. Thus, in the freely fed state when the hyperinsulinaemia was severe, glucose elicited a prompt suppression in plasma C-GLI which was accompanied by a slight fall in circulating IRI and an impaired glucose tolerance curve. However, when the prevailing IRI concentration in obese mice was reduced by a 24 hour period of food restriction, administration of glucose resulted in a paradoxical increase in the circulating C-GLI concentration which might also have been responsible for the transient elevation in plasma IRI and the

deterioration in glucose tolerance. The dependence of the suppressive effect of glucose on the prevailing IRI concentration is consistent with reports (Unger et al., 1972; Gerich et al., 1976a) that simultaneous infusion of insulin and glucose partially restores normal α cell responsiveness to hyperglycaemia in human diabetics, and suggests that the impaired ability of glucose to suppress α cell function in obese mice results from an insensitivity to the normal inhibitory action of insulin. To investigate this possibility and to clarify the nature of the relationship between circulating insulin and α cell dysfunction in obese mice, plasma C-GLI responses were examined following the administration of exogenous insulin and the induction of a selective insulin deficient state with insulin antiserum. In contrast to lean homozygous (+/+) mice, the obese animals exhibited a total lack of suppression of plasma C-GLI and glucose concentrations following administration of a low dose of insulin, thereby indicating that the mechanism responsible for the insulin-induced suppression of α cell function is defective in obese mice. However, by increasing the insulin dose by a factor of 400, a marked reduction in plasma C-GLI concomitant with a prolonged hypoglycaemic response was observed in the obese animals. These observations demonstrate a gross insensitivity of the diabetic α cell to exogenous insulin and, in view of the essential role of circulating C-GLI in the maintenance of basal hyperglycaemia, strongly suggest that lack of suppression of α cell function by endogenous insulin contributes significantly to the manifestation of severe insulin resistance in these animals. The ability of exogenous insulin to reverse the hyperglucagonaemia in obese mice is consistent with clinical observations in juvenile-onset diabetics (Gerich et al., 1975; Warne et al., 1977). Nevertheless, the α cell defect in obese mice is not a consequence of insulin lack since administration of insulin antiserum demonstrated a significant suppressive effect of high concentrations of endogenous insulin on α

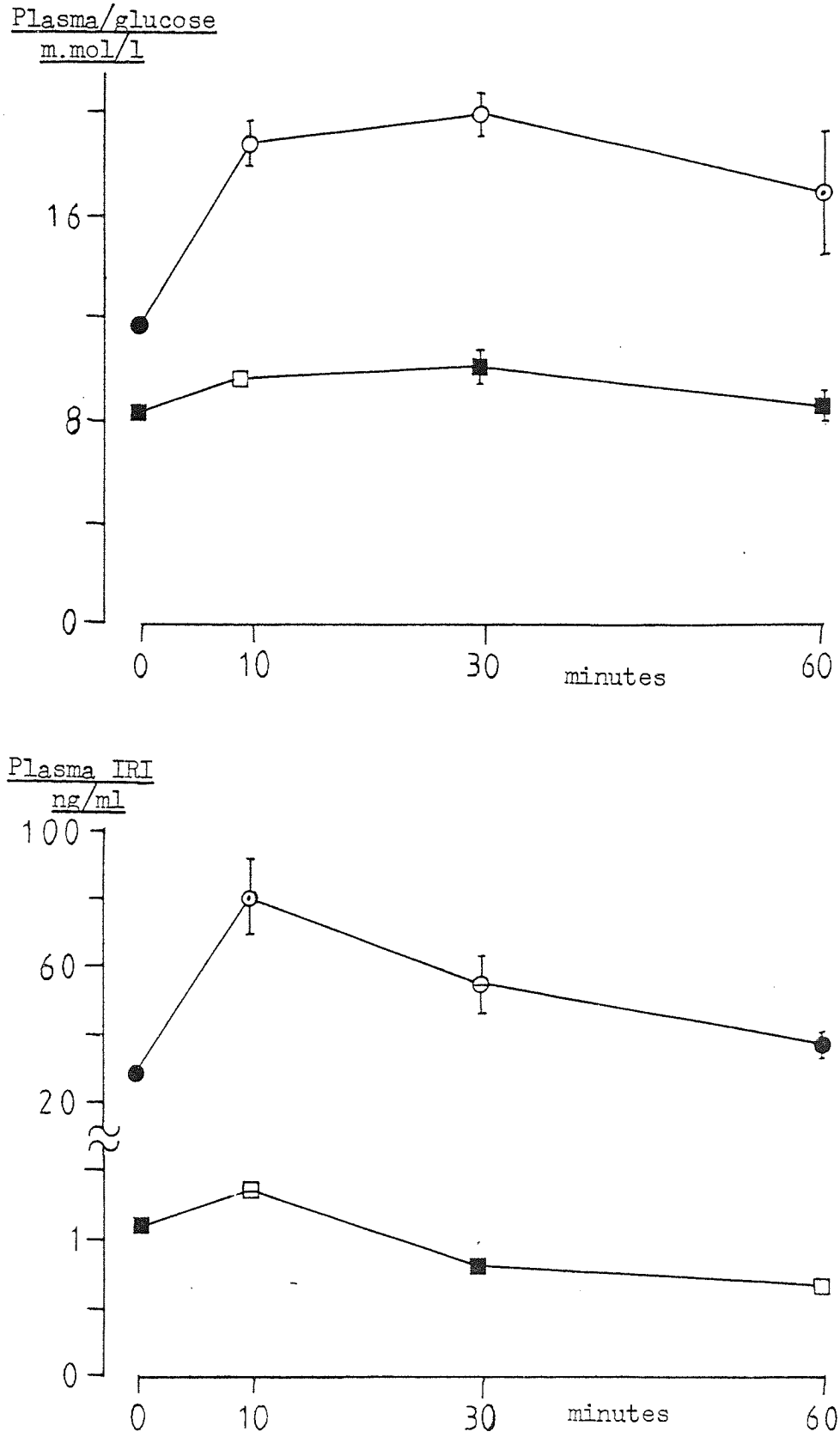
cell function in fully fed obese mice. Indeed, the present data provide convincing evidence that the pancreatic α cell function of obese mice is grossly insensitive to modulations in the endogenous circulating insulin concentration and that the defective response to glucose is mediated by this mechanism. Thus, it is envisaged that the normal inhibitory action of glucose on α cell function which is mediated by an insulin sensitive glucoreceptor or glucose sensing mechanism (see Unger & Orci, 1976; Weir et al., 1976) is defective in obese mice. To further characterise the nature of the α cell defect in these animals and to establish the role of glucose in the regulation of α cell function, the effects of intracellular glucopenia and heparin-induced hyperlipidacidaemia were examined in groups of mice. Consonant with previous studies (Müller et al., 1971), 2-deoxy-D-glucose evoked a marked increase in circulating C-GLI concentrations concomitant with the production of severe hyperglycaemia. Since the primary metabolic block produced by this anologue concerns the competitive inhibition of phosphohexoseisomerase (Wick et al., 1957), it appears that glucose must be phosphorylated and further metabolised before it can inhibit glucagon secretion. However, in agreement with the clinical studies of Gerich and colleagues (1976a), heparin-induced elevation of plasma free fatty acids resulted in a comparable decline in the circulating C-GLI concentration of both lean and obese mice, thereby precluding a generalised defect of the α cell to suppression by metabolic substrates in obese mice. Edwards and Taylor (1970) have suggested that the suppression of glucagon secretion is an energy-dependent process. The results of the present study are in agreement with this hypothesis and suggest that a gross insensitivity of an insulin-dependent metabolic step in either the glycolytic or pentose phosphate pathways is responsible for the α cell dysfunction of obese hyperglycaemic mice.

Figure 68 Plasma glucose, IRI and C-GLI concentrations of fed (▭) and 24 hour fasted (▨) +/+ and ob/ob mice (m ± sem, n = 8)$^{\phi}$.



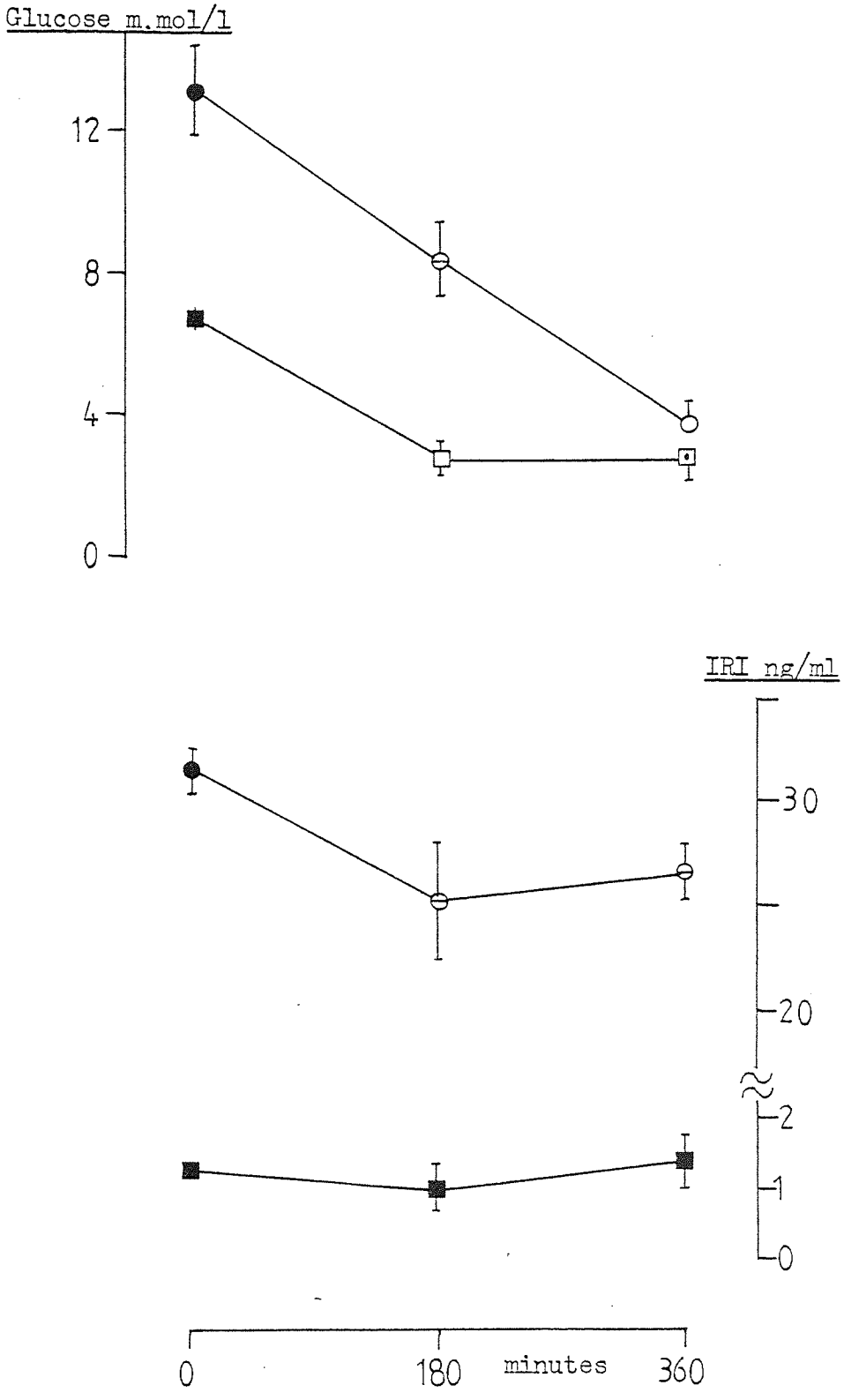
$^{\phi}$Comparison between genotypes x = $p < 0.05$; xx = $p < 0.01$;

Figure 69 Effect of glucagon on plasma glucose and IRI concentrations in fed +/+ (■——■) and ob/ob (●——●) mice (m ± sem, n = 6)$^{\phi}$.



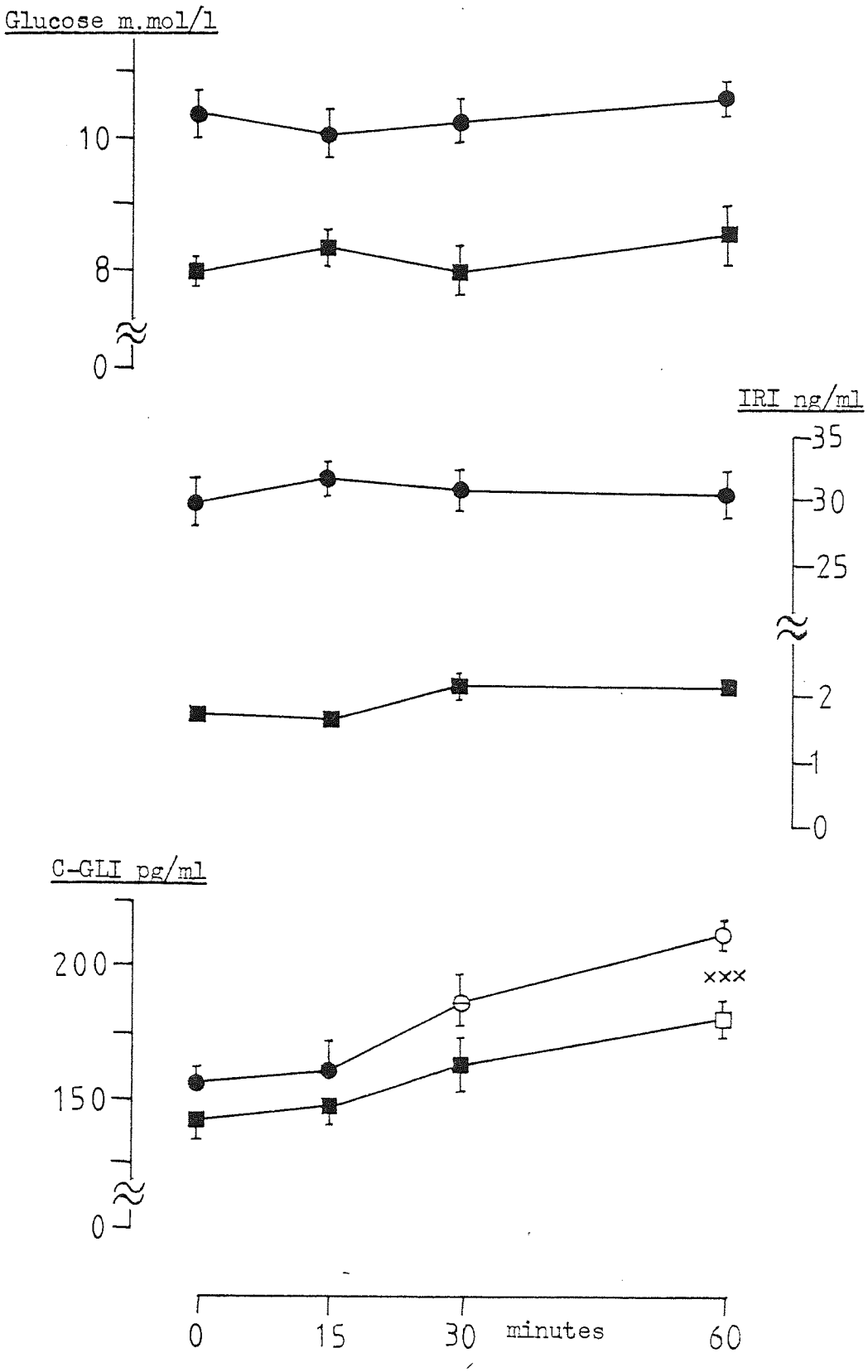
$^{\phi}$p vs. Baseline ⊟ ⊖ = p < 0.05; ⊡ ⊙ = p < 0.01; □ ○ = p < 0.001.

Figure 70 Effect of C-terminal reactive glucagon antiserum on plasma glucose and IRI concentrations in fed +/+ (■——■) and ob/ob (●——●) mice (m ± sem, n = 6)$^{\phi}$.



$^{\phi}$p vs. Baseline ⊟⊖ = p < 0.05; ⊡⊙ = p < 0.01; □○ = p < 0.001.

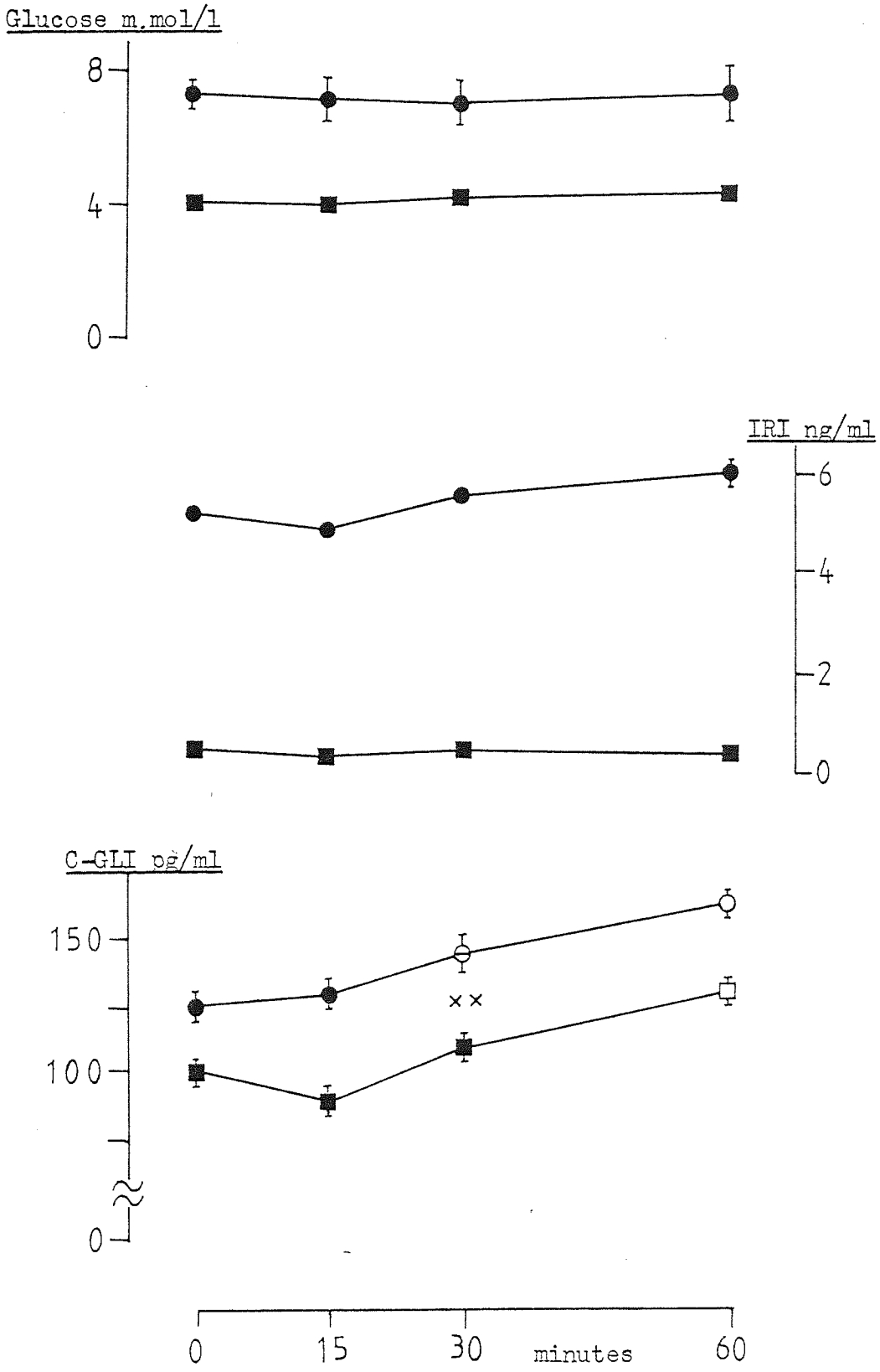
Figure 71 Effect of saline on plasma glucose, IRI and C-GLI concentrations in fed +/+ (■——■) and ob/ob (●——●) mice (m ± sem, n = 6)$^{\phi}$.



$^{\phi}$p vs. Baseline ⊟ ⊖ = p < 0.05; □ ○ = p < 0.001.

Comparison between genotypes xxx = p < 0.001.

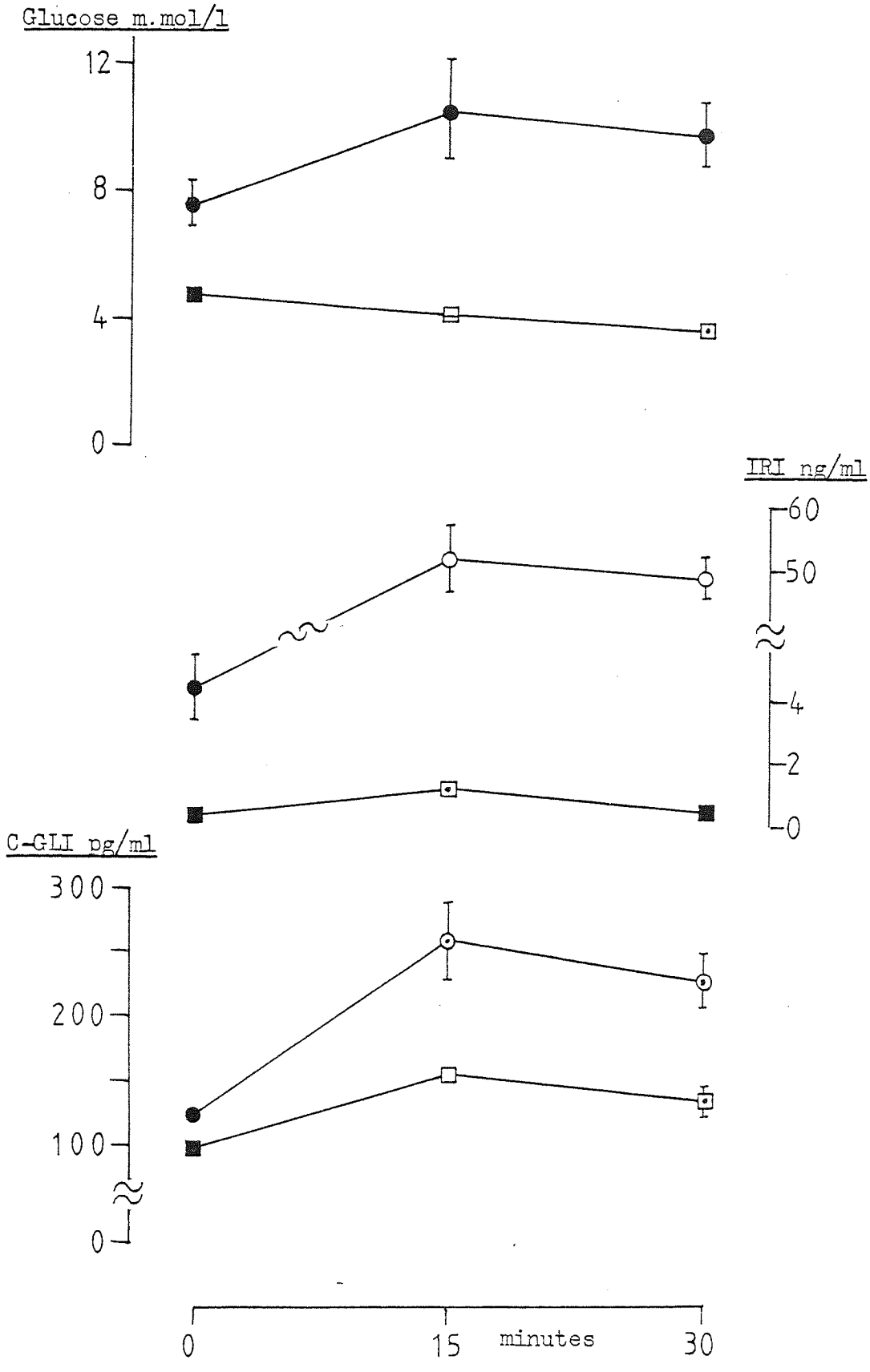
Figure 72 Effect of saline on plasma glucose, IRI and C-GLI concentrations of fasted +/+ (■——■) and ob/ob (●——●) mice (m ± sem, n = 6)$^{\phi}$.



$^{\phi}$p vs. Baseline ⊟ ⊖ = p < 0.05; □ ○ = p < 0.001.

Comparison between genotypes xx = p < 0.01.

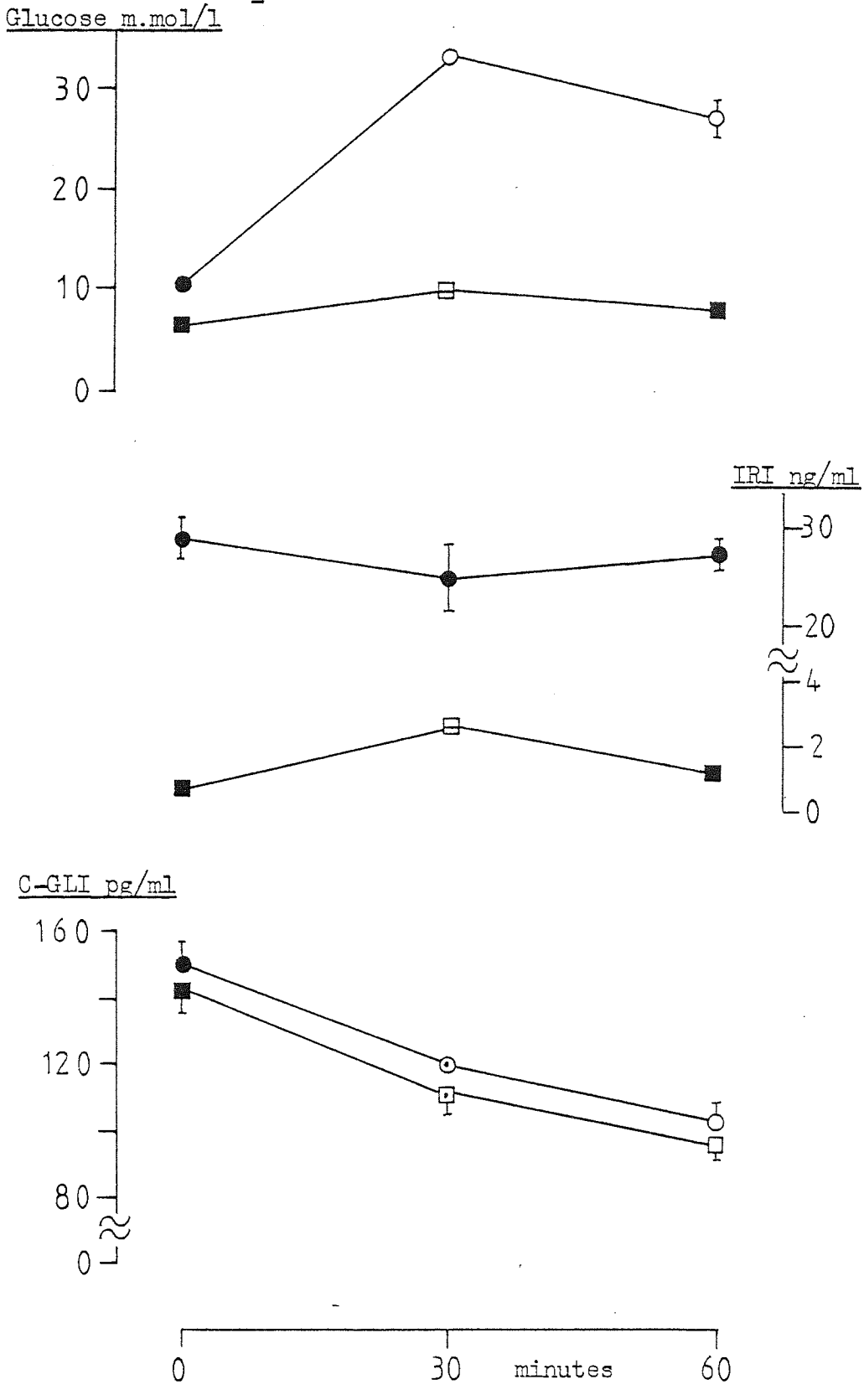
Figure 73 Effect of arginine on plasma glucose, IRI and C-GLI in fasted +/+ (■—■) and ob/ob (●—●) mice (m ± sem, n = 6)$^{\phi}$.



$^{\phi}$p vs. Baseline ⊟⊖ = p < 0.05; ⊡⊙ = p < 0.01; □○ = p < 0.001.

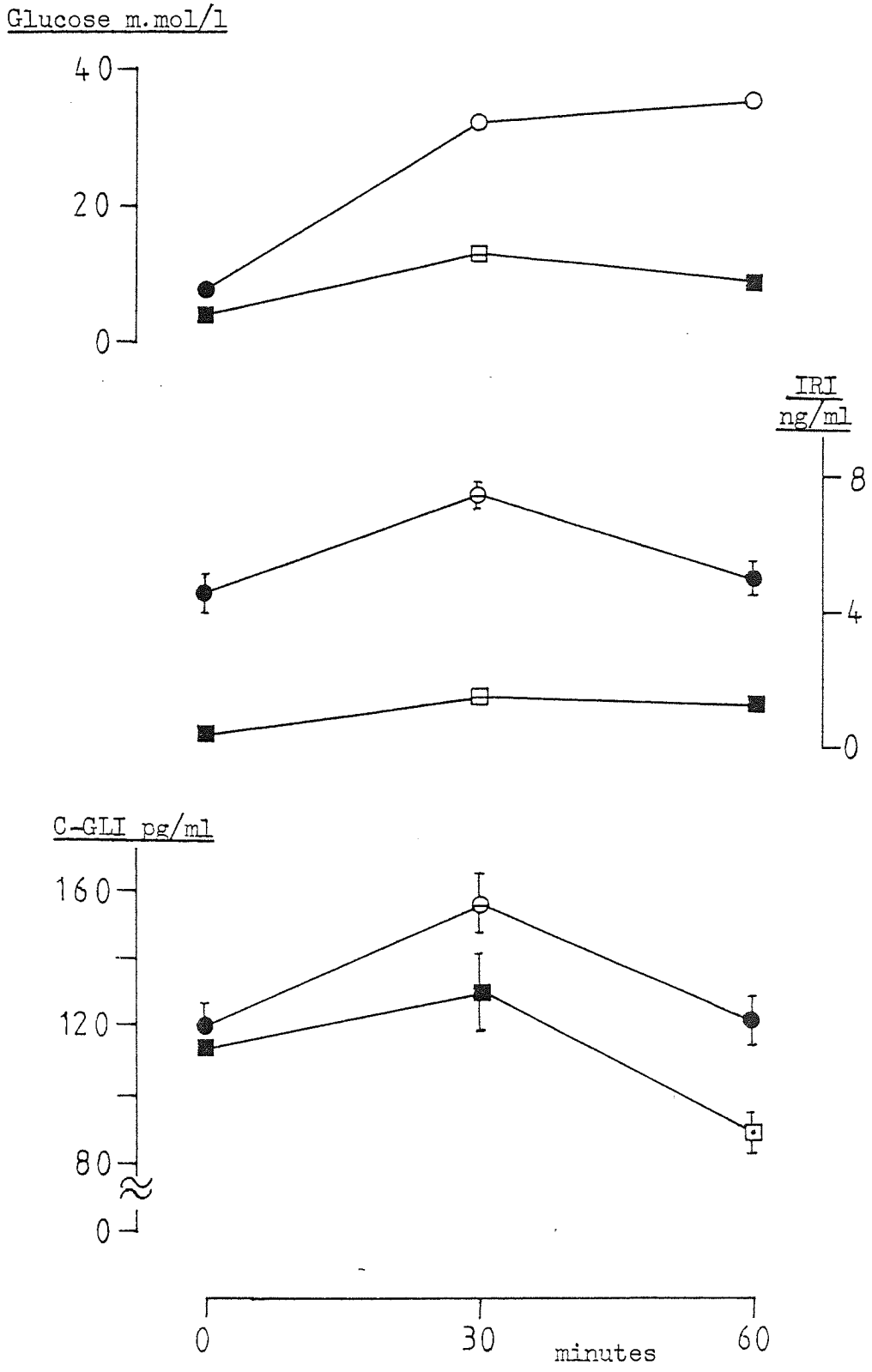
Comparison between genotypes xx = p < 0.01; xxx = p < 0.001.

Figure 74 Effect of glucose on plasma glucose, IRI and C-GLI concentrations in fed +/+ (■—■) and ob/ob (●—●) mice (m ± sem, n = 6)$^{\phi}$.



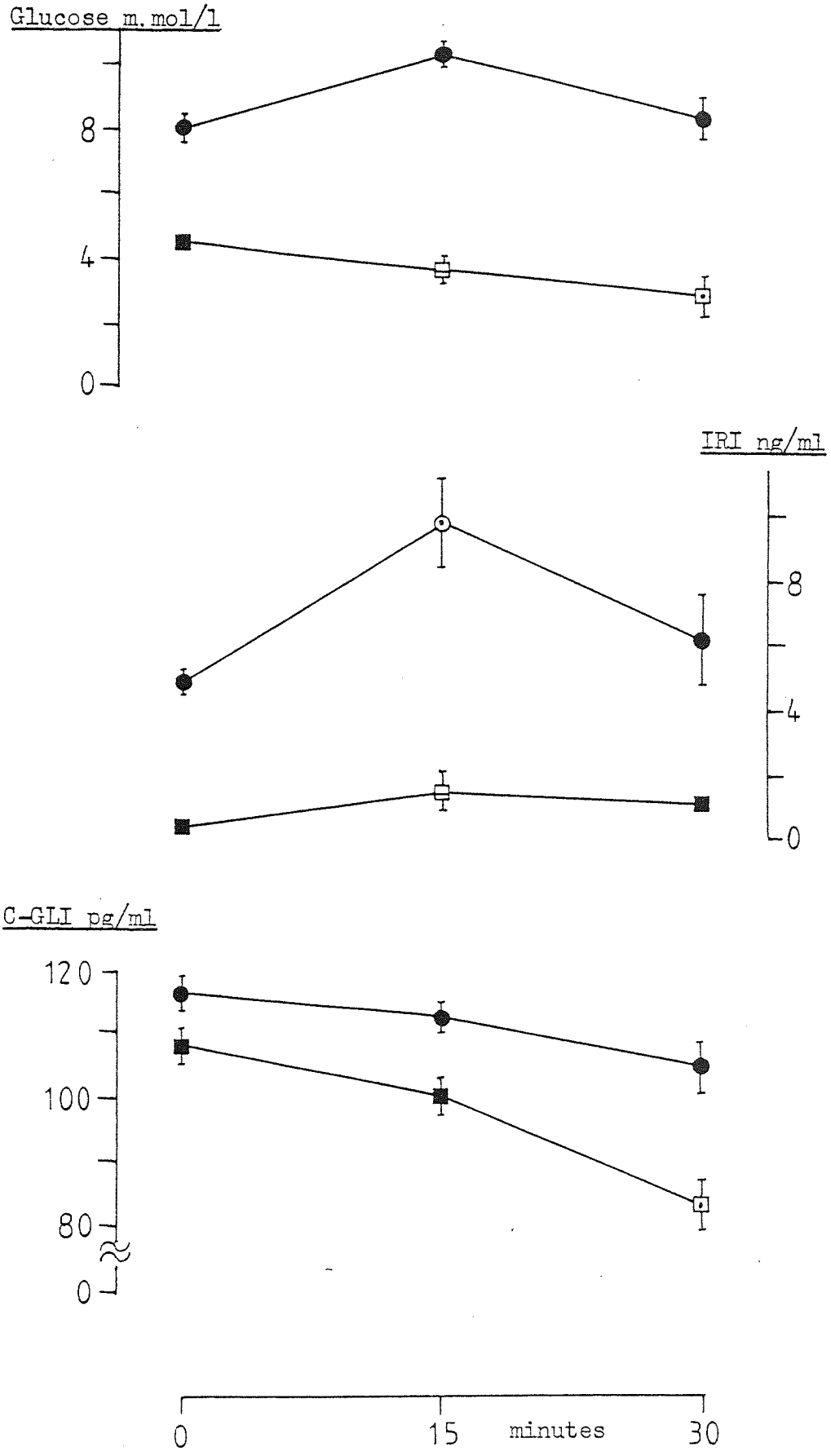
$^{\phi}$p vs. Baseline ⊟ ⊖ = p < 0.05; ⊡ ⊙ = p < 0.01; □ ○ = p < 0.001.

Figure 75 Effect of glucose on plasma glucose, IRI and C-GLI concentrations of fasted +/+ (■——■) and ob/ob (●——●) mice (m ± sem, n = 6)$^{\phi}$.



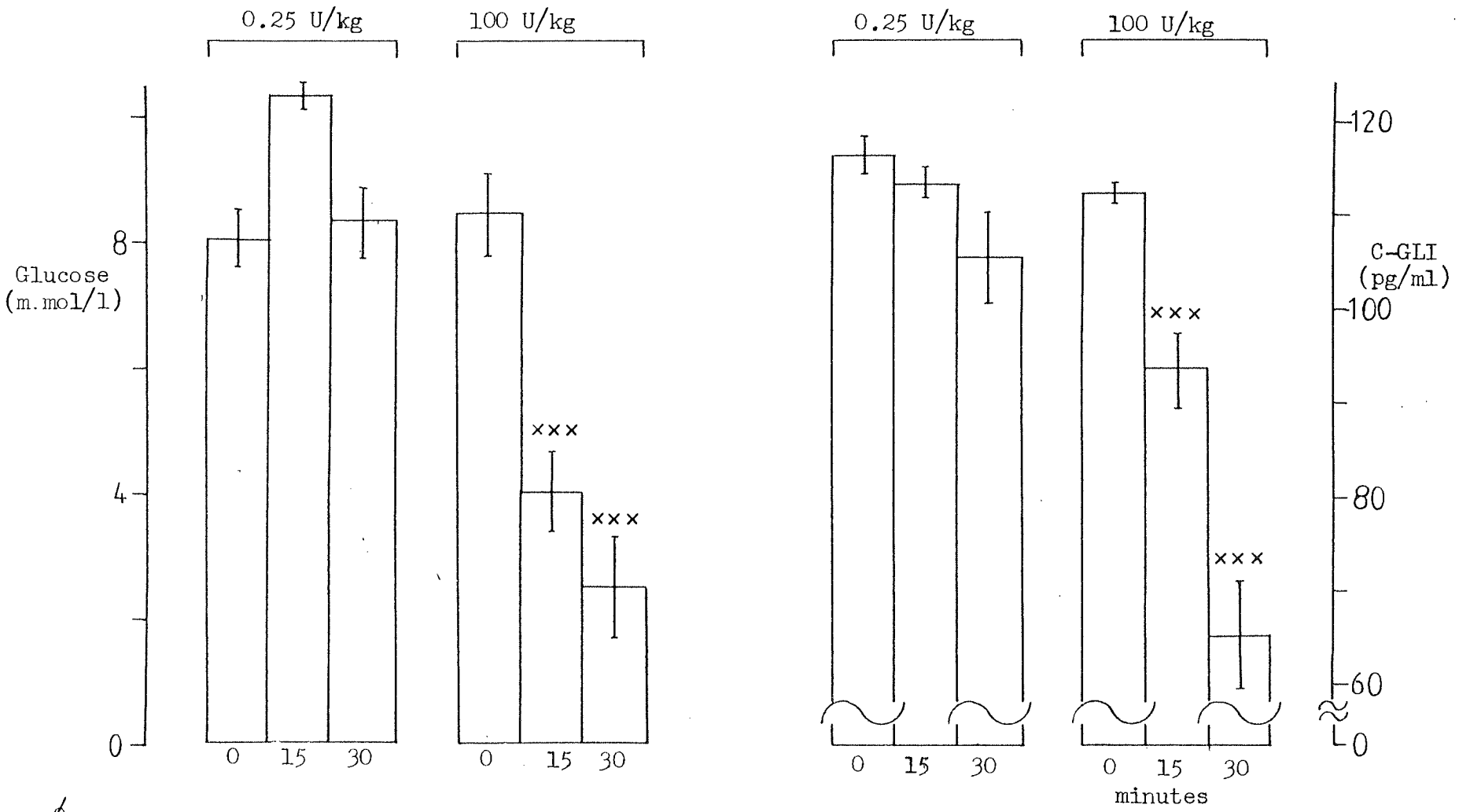
$^{\phi}$p vs. Baseline ⊟ ⊖ = p < 0.05; ⊡ ⊙ = p < 0.01; □ ○ = p < 0.001.

Figure 76 Effect of insulin (0.25 U/kg) on plasma glucose, IRI and C-GLI concentrations of fasted +/+ (■—■) and ob/ob (●—●) mice (m ± sem, n = 6)$^{\phi}$.



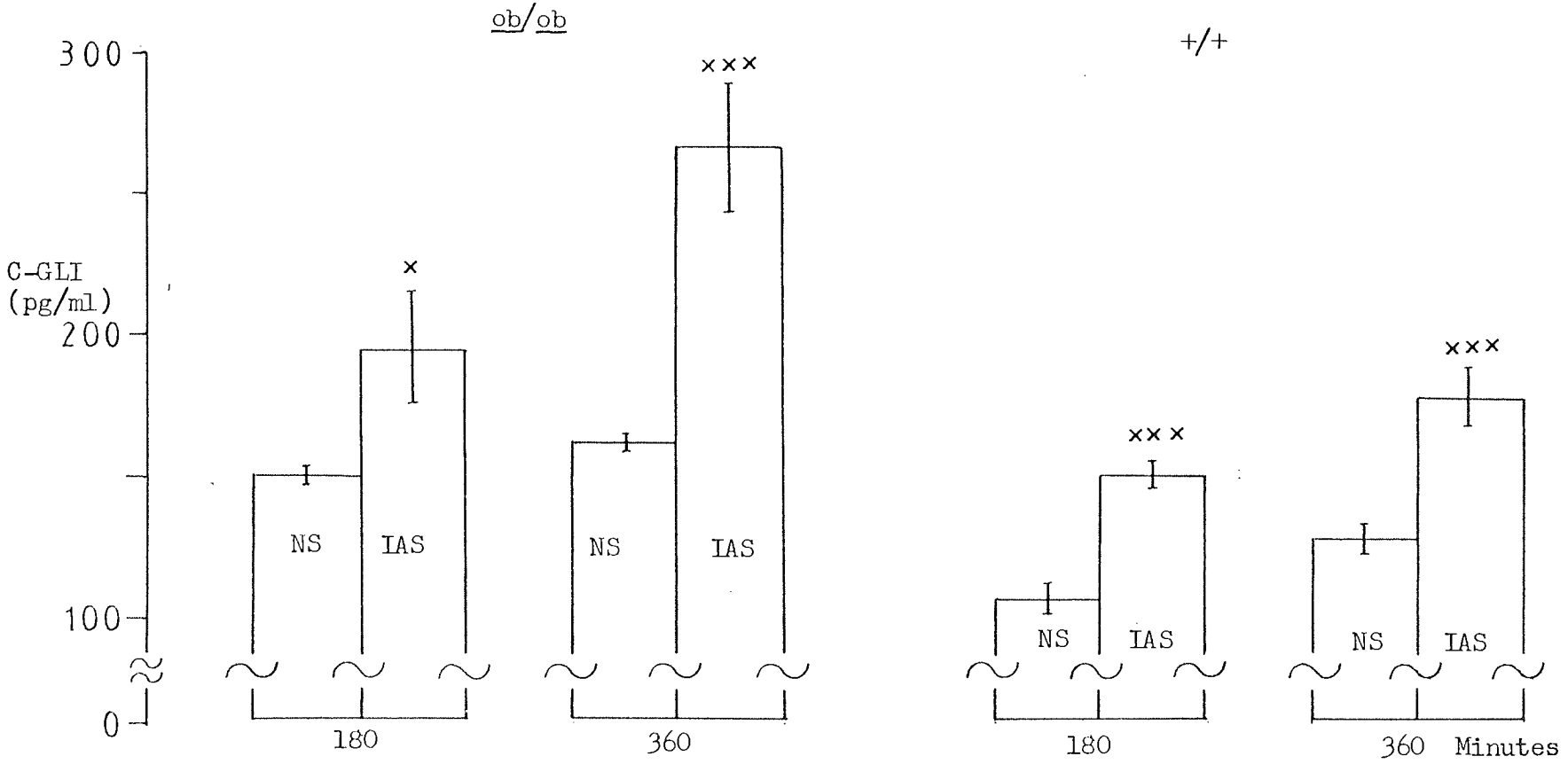
$^{\phi}$p vs. Baseline ⊟ ⊖ = p < 0.05; ⊡ ⊙ = p < 0.01; □ ○ = p < 0.001

Figure 77 Effect of low dose (0.25 U/kg) and high dose (100 U/kg) insulin on plasma glucose and C-GLI concentrations in fasted obese mice (m ± sem, n = 6)ϕ.



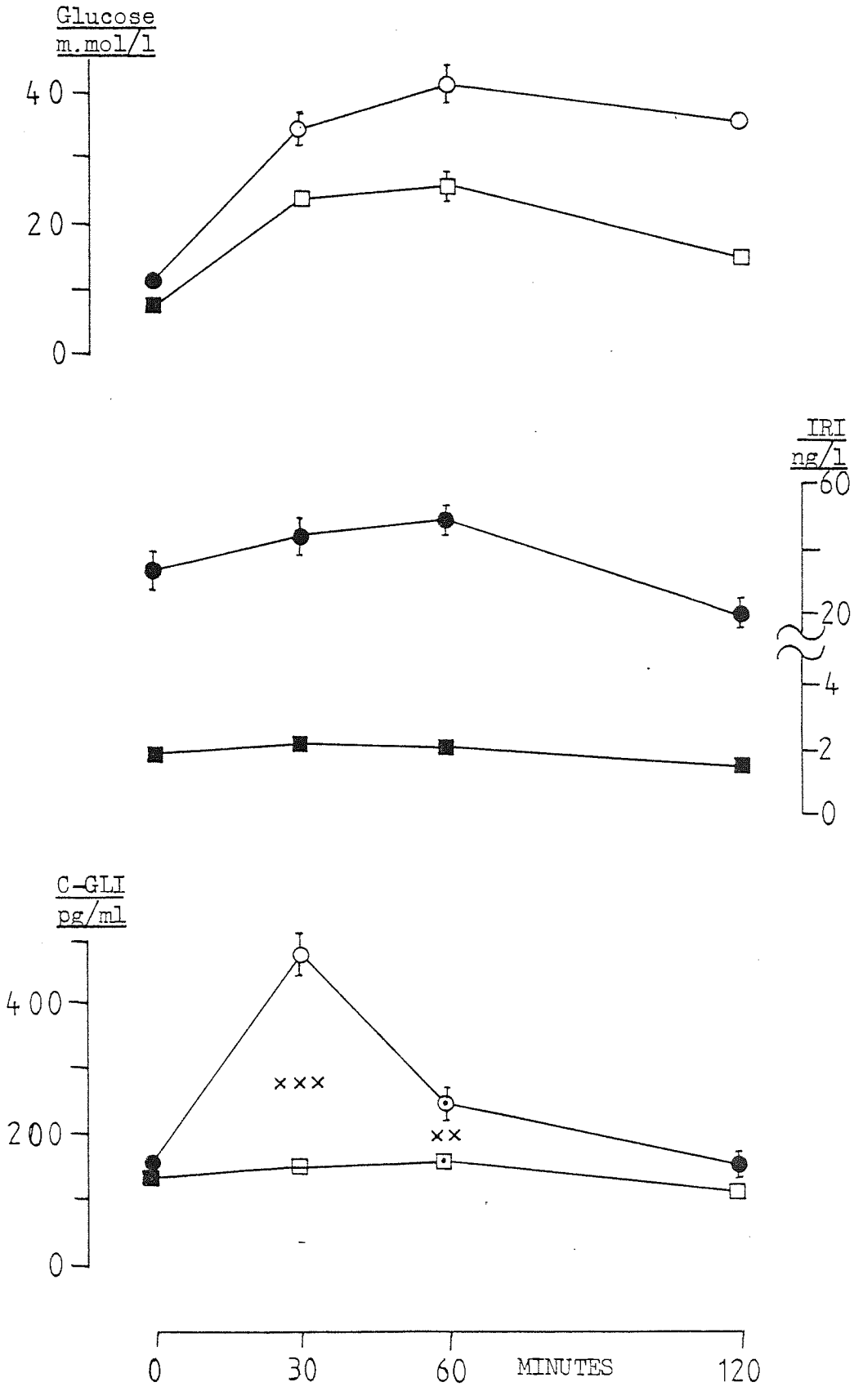
ϕ p vs. Baseline xxx = p < 0.001

Figure 78 Effect of insulin antiserum (IAS) and normal serum (NS) on plasma C-GLI concentrations of +/+ and ob/ob mice (m ± sem, n = 6)$^{\phi}$.



$^{\phi}$p vs. Controls x = p < 0.05; xxx = p < 0.001.

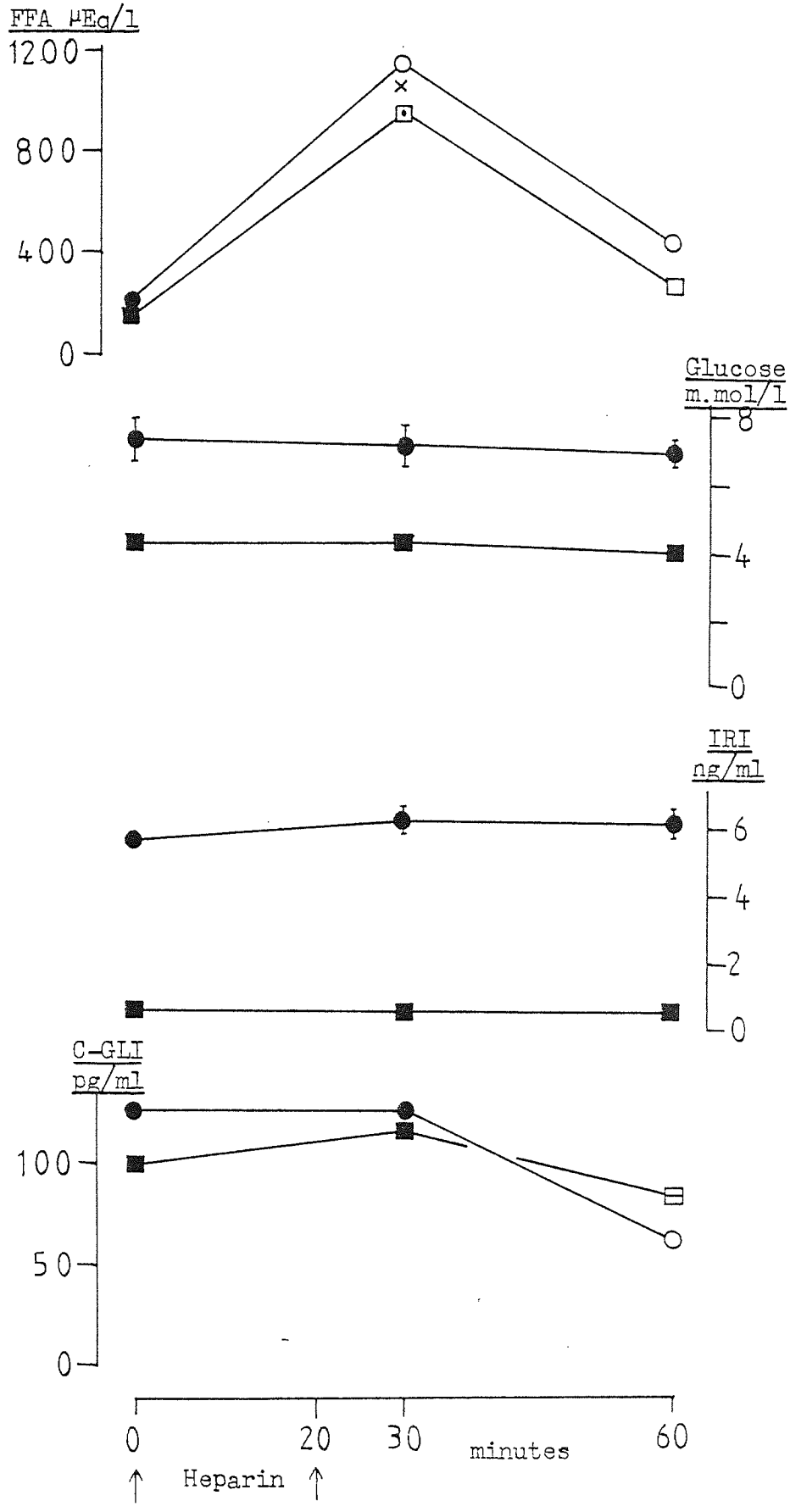
Figure 79 Effect of 2-deoxy-D-glucose on plasma glucose, IRI and C-GLI concentrations of fed +/+ (■——■) and ob/ob (●——●) mice (m ± sem, n = 6)$^{\phi}$.



$^{\phi}$p vs. Baseline ⊟ ⊖ = p < 0.05; □ ○ = p < 0.01; □ ○ = p < 0.001.

Comparison between genotypes xx = p < 0.01; xxx = p < 0.001.

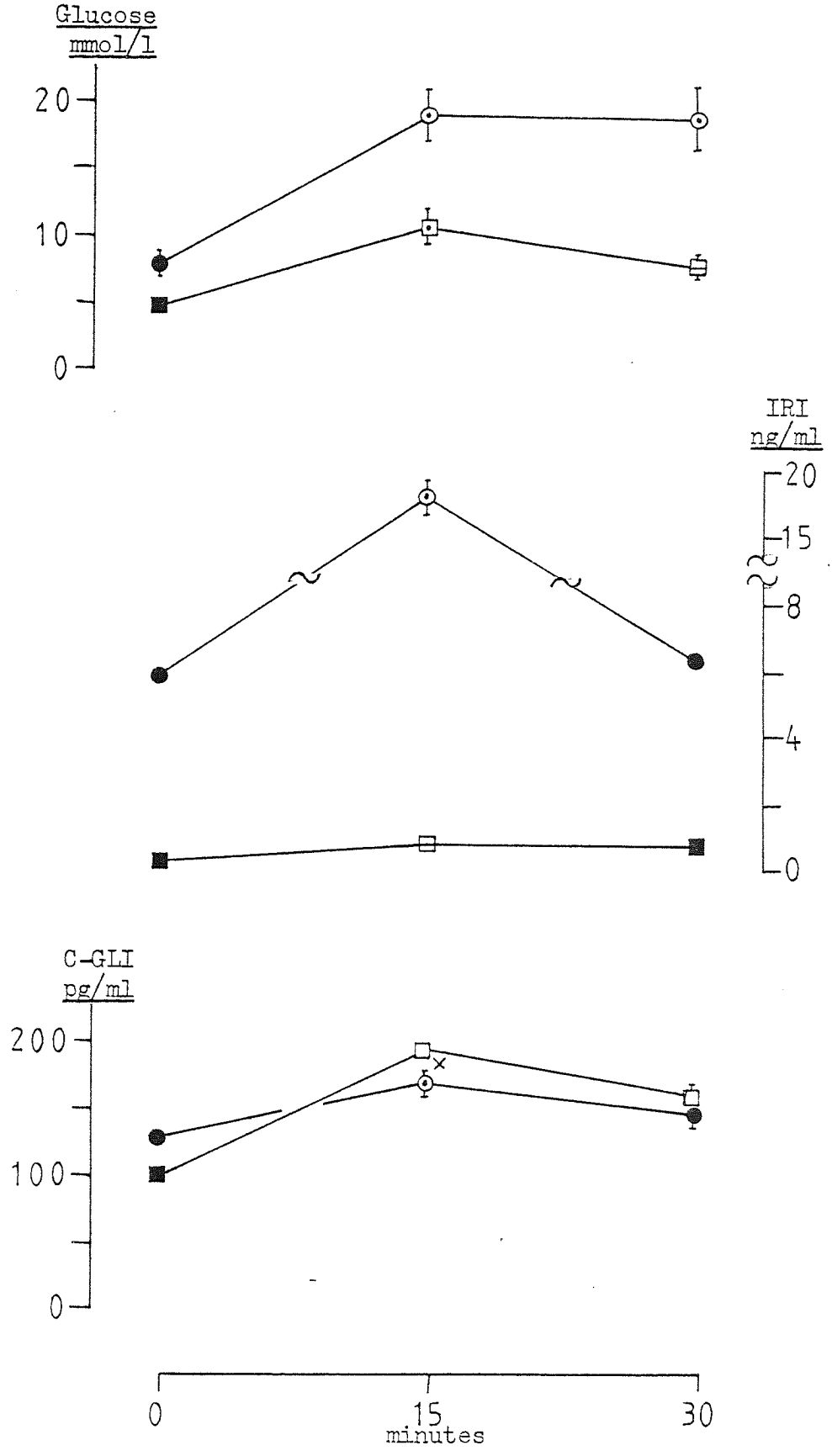
Figure 80 Effect of heparin on plasma FFA, glucose, IRI and C-GLI concentrations in fasted +/+ (■—■) and ob/ob (●—●) mice (m ± sem, n = 6)$^{\phi}$.



$^{\phi}$p vs. Baseline ⊟ ⊖ = p < 0.05; ⊡ ⊙ = p < 0.01; □ ○ = p < 0.001.

Comparison of genotypes x = p < 0.05.

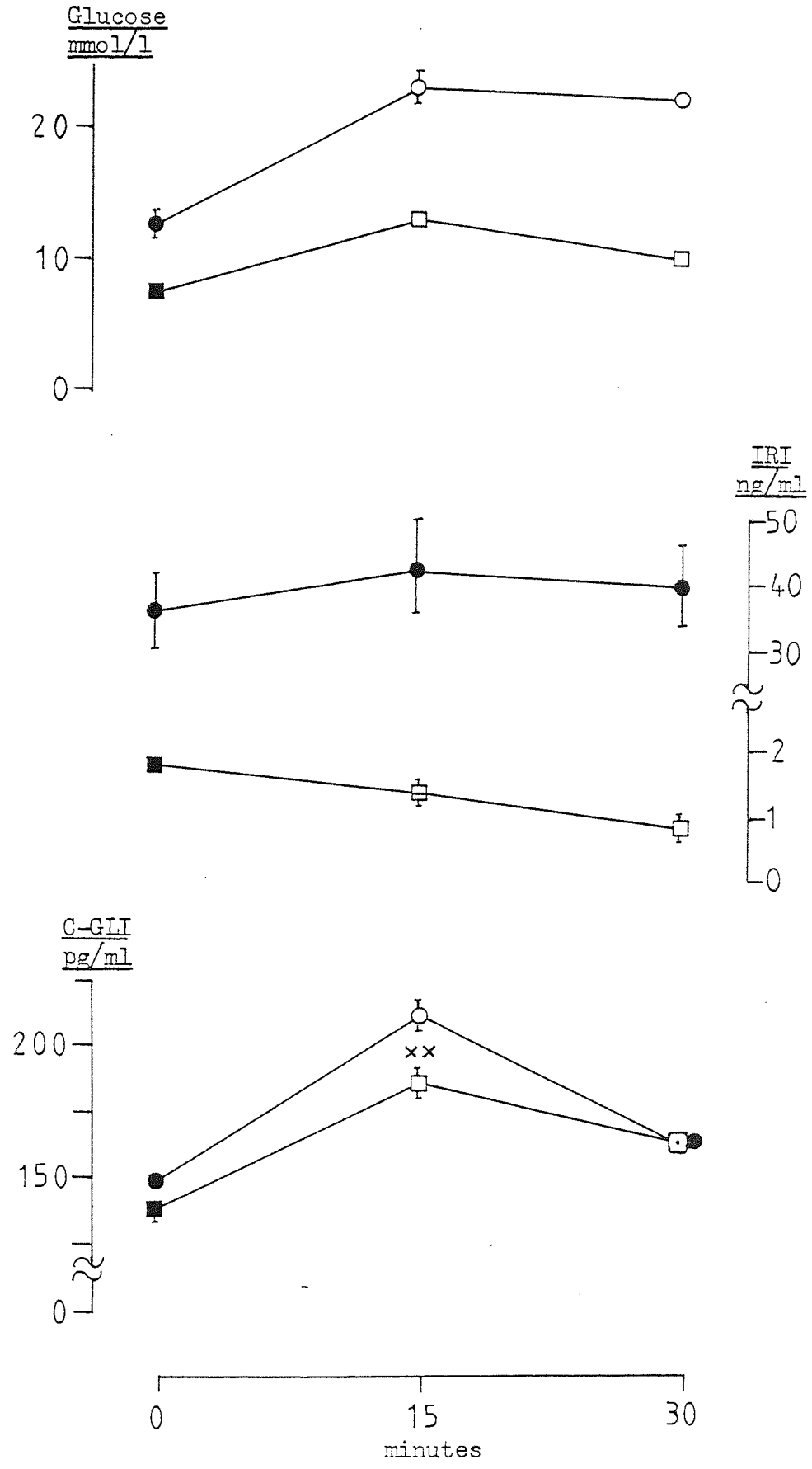
Figure 81 Effect of pilocarpine on plasma glucose, IRI and C-GLI concentrations of fasted +/+ (■——■) and ob/ob (●——●) mice (m ± sem, n = 6)$^{\phi}$.



$^{\phi}$p vs. Baseline ⊟,⊖ = p < 0.05; ⊡,⊙ = p < 0.01; □,○ = p < 0.001

Comparison of genotypes x = p < 0.05

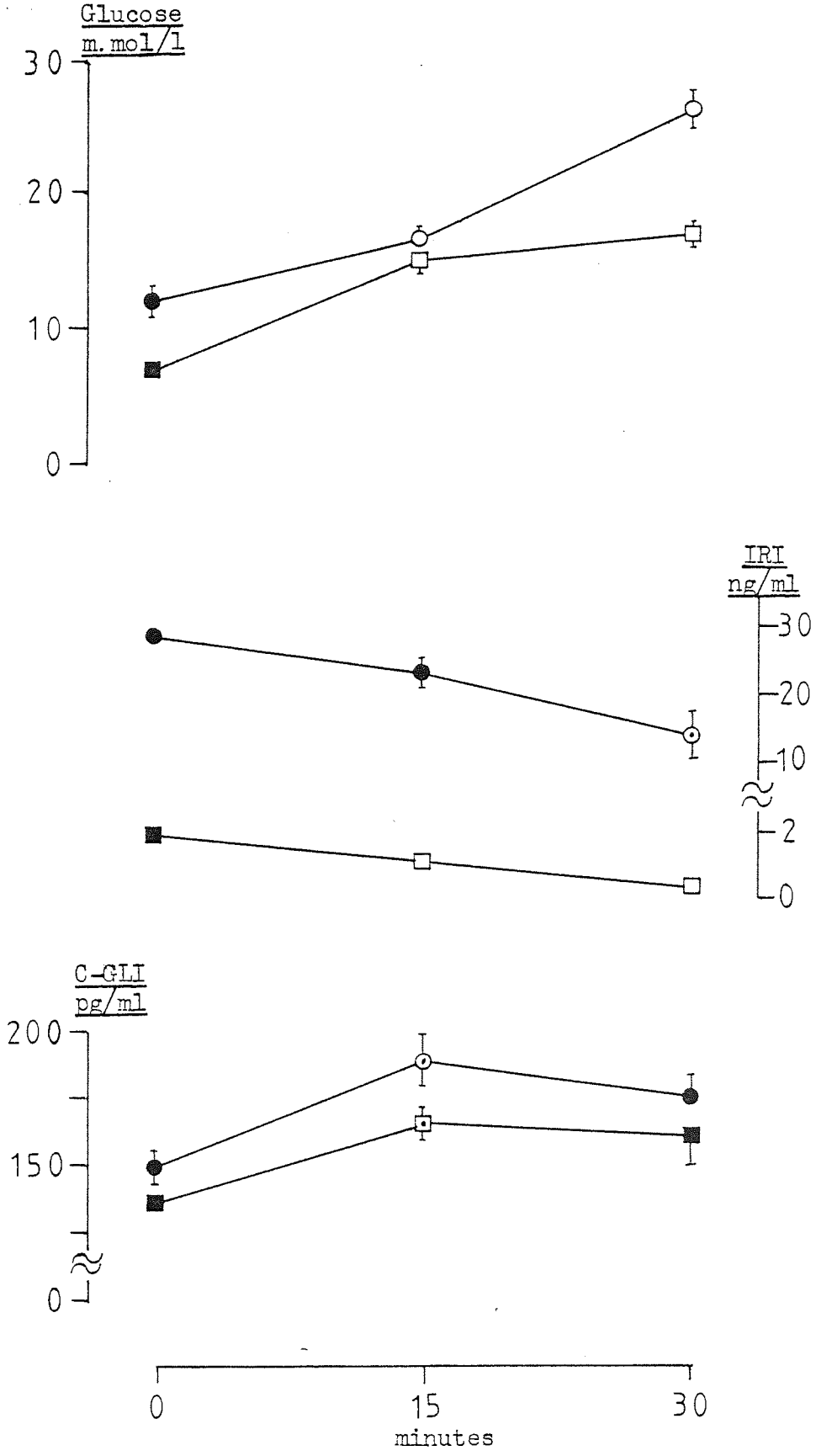
Figure 82 Effect of noradrenaline on plasma glucose, IRI and C-GLI concentrations of fed +/+ (■——■) and ob/ob (●——●) mice (m ± sem, n = 6)$^{\phi}$.



$^{\phi}$p vs. Baseline ⊟ ⊖ = p < 0.05; ⊡ ⊙ = p < 0.01; □ ○ = p < 0.001.

Comparison of genotypes xx = p < 0.01

Figure 83 Effect of adrenaline on plasma glucose, IRI and C-GLI concentrations of fed +/+ (■—■) and ob/ob (●—●) mice (m ± sem, n = 6)$^{\phi}$.



$^{\phi}$p vs. Baseline ⊟ ⊖ = p < 0.05; ⊡ ⊙ = p < 0.01; □ ○ = p < 0.001

CHAPTER 7

THE OBESE HYPERGLYCAEMIC SYNDROME IN MICE (GENOTYPE ob/ob):- GENERAL CONCLUSIONS

The work described in this thesis concerns the establishment of sensitive and precise radioimmunoassays for insulin and glucagon and their application to the study of the obese hyperglycaemic syndrome in mice. The data presented here, together with previously published reports (Atkins et al., 1975 a, b; Bailey et al., 1977 a, b, c, d; Best et al., 1977; Flatt et al., 1977 a, b) permit the following conclusions to be made concerning the general characteristics, endocrine pancreatic function, aetiology and pathogenesis of the obese hyperglycaemic syndrome in the Birmingham strain of mice:

GENERAL CHARACTERISTICS

GENETIC DEVELOPMENT

The obese hyperglycaemic syndrome is transmitted by a single autosomal gene, obese (gene symbol ob) with partial penetrance.

OBESE HYPERGLYCAEMIC (ob/ob) MICE

Adult homozygous (ob/ob) mice exhibited pronounced obesity and numerous abnormalities of glucose homeostasis characteristic of certain types of human obesity and maturity-onset diabetes mellitus. Obese mice were typically hyperphagic, hyperglucagonaemic, hyperglycaemic, hyperinsulinaemic and were characterised by marked islet hypertrophy coexistent with β-cell hypertrophy and hyperplasia. Glucose tolerance was grossly impaired and neither an insulin nor a glucagon response to intraperitoneal glucose was observed in freely fed animals. Arginine evoked a dramatic rise in the circulating levels of insulin and glucagon and produced a delayed reduction in the hyperglycaemia of 24 hour fasted mice. During insulin hypoglycaemia tests obese mice showed marked insulin insensitivity, impaired suppression of circulating glucagon and a rapid rate of insulin disappearance.

LEAN HETEROZYGOUS (ob/+) MICE

Adult heterozygous (ob/+) mice displayed mild abnormalities of glucose homeostasis and therefore provide a suitable model to investigate the genetic determinants of diabetes mellitus without interference from the multiple metabolic abnormalities resulting from the severity of expression of the ob gene in the homozygous (ob/ob) condition. Lean heterozygous (ob/+) mice exhibited hyperphagia, hyperglycaemia, hyperinsulinaemia, impaired glucose tolerance, diminished insulin secretory activity following intraperitoneal glucose, and an excessive β cell response to arginine. Notably, heterozygous (ob/+) mice showed little weight gain and no signs of insulin insensitivity.

ENDOCRINE PANCREATIC FUNCTION

INSULIN SECRETION

The insulin secretory mechanisms of the pancreatic islets in obese hyperglycaemic mice were exquisitely sensitive to both the stimulatory and inhibitory action of hormones, amino acids and drugs. This phenomenon was partially related to a direct modulation by glucagon secreted by the adjacent α cells, but also reflected both the increased β cell mass and the predisposition of the β cells to a rapid rate of synthesis and release. However in marked contrast, the insulin secretory response to intraperitoneal glucose was defective in adult obese mice suggesting a loss of ability of the β cells to recognise and respond to variations in the endogenous circulating glucose concentration.

GLUCAGON SECRETION

Obese mice exhibited pancreatic α cell dysfunction favouring the production of a hyperglucagonaemic state. Thus, while arginine and other secretogogues evoked exaggerated glucagon secretory responses, the suppressive actions of both glucose and insulin were defective.

Investigation of these defects revealed a marked insensitivity of the diabetic α cell to the inhibitory effect of insulin, thereby suggesting that both the excessive secretory responses and the lack of suppression of α cell function by glucose result from insensitivity to local regulation by endogenous insulin. Further examination suggested that a gross insensitivity of an insulin-dependent metabolic step in either the glycolytic or pentose phosphate pathways may be responsible for the α cell dysfunction in obese hyperglycaemic mice.

AETIOLOGY AND PATHOGENESIS

Although the studies reported in this thesis were conducted using 18-22 weeks old mice, complimentary studies have been performed during the developmental phase of the syndrome. These investigations have demonstrated the following age-related sequence of changes in the Birmingham strain of obese hyperglycaemic mice:

> Obesity was well established at 5 weeks of age and increased until approximately 30 weeks after which the body weight stabilised and gradually declined. Freely fed mice showed a marked hyperinsulinaemia upon weaning which peaked at 10-12 weeks and slowly receded beyond this age. A relative hyperglucagonaemia was apparent at 5 weeks of age and plasma glucagon concentrations remained slightly elevated thereafter. Plasma glucose concentrations became significantly raised at 7-8 weeks, reached a maximum at 10-12 weeks and fell progressively beyond this age. Insulin insensitivity was manifest shortly after weaning and continued to worsen until 25-30 weeks. The development of hyperinsulinaemia corresponded with a progressive blunting of the acute plasma insulin response in freely fed animals and a

positive secretory response was not observed between 8-30 weeks of age. The insulinotrophic effects of arginine and glucagon were essentially unchanged during this period.

Histological examination of the pancreatic islets of obese mice revealed the development of islet hypertrophy co-existent with β cell hypertrophy and hyperplasia. The process increased until 20-25 weeks of age after which further enlargement of the islets was not apparent. Small intracellular vacuoles became evident within the islets at about 10 weeks, coinciding with the redistribution of α cells from the periphery to the central portions of the islets. The vacuoles increased in size and number throughout the animals' life span and appeared to replace β cells. Although the rate of β cell hypertrophy and hyperplasia was much greater than the rate of vacuolation at 10-20 weeks, in the absence of further islet enlargement after about 25 weeks, the increasing vacuolation was associated with a reduction in the number of β cells.

These studies indicate that hyperactivity of the pancreatic β cells is one of the earliest detectable abnormalities in genetically predisposed ob/ob mice of the Birmingham strain. Indeed, impaired α cell function was also apparent at 5 weeks of age, corresponding to the youngest mice examined for plasma C-GLI.

Although it seems unlikely that either insulin or glucagon are of primary aetiological significance in the obese hyperglycaemic syndrome, it is eminently apparent that alterations in the endocrine pancreatic function of young ob/ob mice play a major role in the development of abnormalities of glucose homeostasis. The data reported in this thesis demonstrate an important role for glucagon in the pathogenesis of the

syndrome. Thus, although circulating glucagon was of minor importance in the maintenance of hyperinsulinaemia, further implicating an insulin-secreting mechanism of gastrointestinal origin, lack of suppression of α cell function contributed significantly to both the insulin insensitivity and the hyperglycaemia in obese mice.

HYPOTHESIS

On the basis of presently available information it is suggested that over-ingestion of specific food substances represents the primary stimulus for the development of hyperactive hormonal-hypothalamic-islet stimulating mechanism leading to the production of hyperinsulinaemia and, in genetically predisposed animals, hypertrophy and hyperplasia of the pancreatic islets. The development of islet hypertrophy co-existent with marked hyperphagia and obesity leads to the appearance of insulin insensitivity and α cell dysfunction, resulting in the production of hyperglucagonaemia, hyperglycaemia and severe insulin insensitivity. Preferential impairment of insulin enhanced glucose utilisation by muscle results in the excessive accumulation of body fat. As a result of persistent stimulation, the islets develop structural lesions, and the β cells gradually become unresponsive to a glucose stimulus, resulting in a decrease in the insulin:glucagon molar ratio, a further deterioration of insulin sensitivity and a greater impairment of glucose tolerance.

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