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ASPECTS OF THE OBESE HYPERGLYCEMIC SYNDROME IN MICE

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SUMMARY

The obese hyperglycemic mouse colony was set up and established at this institution. Since the radioimmunoassay of insulin was still a recent method, it was fraught with many inherent difficulties. It was thus investigated and aligned with our special requirements.

The sequential development of the inherent obese hyperglycemic syndrome has been studied in these animals by investigating the relationship between the growth rate, blood sugar and serum insulin changes with regard to age. A close relationship was observed in these three parameters. There were primarily three phases in the development of the syndrome:

(a) the dynamic phase until about week 24 characterized by an increased growth, hyperglycemia and increased serum insulin.

(b) a static phase when weight gain was minimal and the weights of the animals were stable. Hyperglycemia too was fairly stable within its own limits and serum insulin concentrations were reduced with time.

(c) this third phase was characterized by a reduction in all three parameters. However this apparent amelioration of the disease terminated in ketosis.

The results of the investigations strongly suggest that obesity is probably a secondary manifestation of tissue resistance to insulin. This "abnormality" is present to an exaggerated degree in the obese animals thus resulting in hyperglycemia, excessive insulin production, and accretion of adipose tissue. This state of affairs is brought to a conclusion when an insufficiency of insulin production results and the animals succumb to diabetes *per se*.

This tissue resistance was made even more evident during the course of studies involving primarily potassium and glucose uptake "*in vivo*". The investigations were carried out in animals between 16 and 20 weeks of age using the conventional glucose tolerance test under conditions of potassium surplus and deficiency. The importance of potassium was stressed in that further impairment in glucose tolerance was observed in animals maintained on a potassium deficient diet, and an improvement was apparent in animals fed on high potassium diet. This is partially explained by an increased serum insulin and therefore presumably an increased secretion of insulin. Sodium and potassium were measured in tissue, serum and urine. The obese and normal animals on the control 41B diet did not show much variation in these parameters. The glycogen content of liver and pancreas was much higher and that of muscle was much lower in the obese. The various changes of the parameters on the experimental diets have been discussed in the text. Polyuria is obviously not a

characteristic in this animal at this stage of development in the syndrome. Blood glucose, acetoacetate and weight relationships too were studied and discussed. As explained, there is no simple or direct relationship between blood sugar and acetoacetate.

In the studies involving alloxan and caffeine, it was found that the obese animals were not as insensitive to alloxan or as vuncrable to caffeine as was supposed originally.

From these studies it appears that the changes and differences observed and reported in the obese are in all probability more in degree than in kind, i.e. none of the metabolic variants in this syndrome have been proven to be unequivocally and absolutely different from that of the normal.

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Diabetes is a wonderful affection, not very frequent among men, being a melting down of the flesh and limbs into urine. Its cause is of a cold and humid nature, as in dropsy. The course is the common one, namely, the kidneys and the bladder; for the patients never stop making water, but the flow is incessant, as if from the opening of aqueducts. The nature of the disease is then chronic and it takes a long period to form; but the patient is short-lived, if the constitution of the disease be completely established; for the melting is rapid, the death speedy. Moreover, life is disgusting and painful; thirst unquenchable; excessive drinking which, however, is disproportionate to the large quantity of urine, for more urine is passed; and one cannot stop them either from drinking or making water. Or if for a time they abstain from drinking, their mouths become parched and their bodies dry; the viscera seem as if scorched up; they are affected by nausea, restlessness, and a burning thirst; and at no distant term they expire.

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CHAPTER I

GENERAL INTRODUCTION

A. Historical Review

Diabetes mellitus is a disease of antiquity with a written record dating back possibly to 3000 B.C. - The Papyrus Ebers. However it is only in the last century or so that its metabolic features began to be adequately understood. It is probably the most common hereditary endocrine disease. There is a general agreement that this often represents a genetic disease, many of whose manifestations are deleterious to the individual with the disease and some of which impair reproductive fitness. The endocrine defect irrespective of the mode of inheritance apparently results in the absolute or relative deficiency of insulin. This does not necessarily imply defective or inadequate insulin production from the pancreas. It may possibly involve merely a lack of effectiveness of insulin on the target tissue. It has become increasingly obvious that diabetes is a generalised, complex disease exerting its effects on a great number of aspects of metabolism. From the time of its discovery it has been characterized by the excretion of glucose in the urine and hence its name, which means "to pass through". The disease was first attributed to a weakness of the kidney because of the polyuria. It should not however be confused with another not uncommon condition - nondiabetic mellituria which is characterized by glucosuria in the presence of normal glycemia.

The term Diabetes in actual fact now covers a group of conditions with the common symptom - hyperglycemia. Until recently hyperglycemia was generally the only reliable diagonistic criterion of Diabetes mellitus.

The discovery of insulin by Banting and Best and its ability to reverse the metabolic abnormalities produced has been a major step forward in the understanding of this syndrome. The endocrine role of the pancreas was first recognised by Minkowski and von Mering (1886) who first produced diabetes in the dog by total pancreatectomy. Somebody once said that "Diabetes is like a drama, in which the individual's genetic makeup is the stage, his ability to resist the onset of the disease, the hero; and the life stresses e.g. obesity, pregnancy, infection, surgery, aging, serious illness or certain endocrinopathies, the villains." Thus diabetes is a life long disease which begins to run its course at birth.

As a result of wider understanding, the mortality from the disease has been falling but its incidence is unfortunately on the increase. It is possible for the syndrome to be detected in a matter of weeks as in the juvenile (or youth-onset, Ketosis prone) type. Here the essential abnormalities are associated with absolute insulin deficiency. In the maturity-onset variety the onset of the disease can take years to develop and even then a number of patients are completely unaware of any clinically disabling symptoms. In fact very often the discovery of the disease is accidental. Maturity-onset diabetes is frequently associated with a delayed release of endogenous insulin in relation to carbohydrate challenge. However some

do have or develop a subnormal capacity for insulin synthesis and storage. Warren et al. (1966) aptly described the situation associated with this syndrome when he said "Few diseases definitely associated with any one function, metabolic or otherwise, show as wide a range of concomitant pathological change as does diabetes or as frequent absence of demonstrable pathological change".

In all experimental forms of diabetes whether by pancreatectomy or by induction by chemical or hormonal agents the common denominator has been beta-cell damage. Although it is certainly true that glucose infusion, certain chemical and hormonal induced biochemical changes in the animal results in its being ultimately deprived of islet function, the question of how this is brought about merits further investigation. It is possible that this common end result merely masks different mechanisms which result first in relative and finally in absolute deficits of insulin. These induced forms of diabetes,which are commonly caused and recognized by their insulin lack, could well differ in any number of ways from the naturally occurring inherent type of diabetes.

In contrast to the experimental forms of diabetes in animals, islet changes are reported to be present only in a minority of human diabetics, (Hartcroft, 1956, Warren and co-workers, 1966, Editorial 1954) and there is a considerable overlap in the weight and islet size of normal and obese diabetic tissue (MacLean and Ogilvie, 1955; Ogilvie, 1964), i.e. all types of diabetic islet lesions do occur in non-diabetics although they are less common.

This is especially so with regard to the hydropic changes which are the consequence of glycogen infiltration (Toreson 1951.) These changes are reversible in its earlier stages. In man they are reversed partially by insulin therapy and they are rapidly obscured by post mortem changes. Nevertheless there is histopathological evidence in humans based on islet counts and measurements (Haist et.al. 1949),insulin extracted from the pancreas (Scott and Fisher 1938, Wrenshall et al 1952; Hartcroft and Wrenshall 1955) and the assay of insulin in urine (Mirsky et al 1948) that absolute deficits of insulin do occur.

Diabetes and obesity are intimately linked particularly in the adult. However the actual relationship is not clear. Obesity is characterized by hyperinsulinism, insulin resistance or insensitivity and impaired glucose tolerance. Diabetes on the other hand is often associated with the lack or insufficiency of insulin with impaired glucose utilization. Does obesity cause diabetes or vice versa or are they simply a coincidental association?

The easiest and poorest definition of obesity is based on weight alone. It is then easily recognisable only if a person is grossly overweight. Obesity may perhaps be regarded as a body type. It has been the experience of many obese people who have lost weight after an extremely rigid dietetic regime that they regain weight very quickly with only a slight relaxation. On the other hand many underweight people too have struggled to put on weight for years in vain. Thus it seems that the natural weight of a person or animal must be controlled by genetic factors. Obesity may perhaps be more suitably defined as a condition

involving an excessive generalised deposition and storage of fat. Obese hyperglycemic animals on a restricted food intake still contain more body fat than the lean animals. Extreme obesity then is the manifestation of an aberration in the physiological complexity regulating food intake. Hyperphagia is an essential feature of obesity which may be said basically to represent the imbalance between energy output and energy intake.

Until recent years obesity has been looked upon favourably as indicative of prosperity and good health. However the relationship of increased mortality and excessive weight and the practical problems associated with it have been increasingly recognised during the last few decades. Opinion has reversed and today the market is filled with all varieties of low-calorie foods, slimming gadgets etc. for those who wish to attain a more stream-lined figure.

The medical profession on its part too has only recently interested itself with the practical problems of obesity. This awakening was no doubt brought about by the increasing awareness of the general association of obesity with diabetes mellitus, hypertension, arteriosclerosis and all their attendant complications. The exact causal relationship between obesity and these major killing diseases has still to be understood.

A disease of affluence like diabetes may be an expression of genotype which manifests itself deleteriously under one set of environmental conditions, but under another alternative type of environment, e.g. under conditions of food scarcity, it

could confer a certain survival advantage compared with the non-diabetic genotype. In prehistoric times when man and animals were dependent on the caprices of nature and had to withstand long periods of starvation alternating with periods of abundance this gene mutation would have ensured conservation of calories in the form of fat for the times of need. However under the present conditions of civilization, where food is plentiful, this efficient mode of caloric conservation has led to obesity which is further enhanced by a culture which increasingly favours a sedentary way of life. Thus the mutation which prolongs or confers life in one environment, becomes a stress and a cause of mortality under another.

Aging obesity is common to all mammalian species when the body progressively contains a greater proportion of fat without necessarily showing an increase in weight, as the mature animal grows older (Mayer J. 1949). The average fat content of women is appreciably greater than in men of the same age in young adulthood (Brožek 1961 and Keys and Brožek 1953).

The multiple etiology of obesity is perhaps best demonstrated by the fact that although obesity is the common manifestation, obesity itself has several different causes. Thus the herditary obese hyperglycemic mouse is an example of one form of metabolic obesity, and gold-thioglucose obesity is an example of regulatory obesity. Hyperphagia or its development during obesity is often partly dependent on the changes observed in tissue metabolism, rather than the cause of obesity, however it is undoubtedly an important feature in all cases irrespective of

the form or cause of obesity.

There is a parallelism between the metabolic changes seen in human obesity and those seen in certain experimental forms of obesity in animals. Himsworth (1949) and Butterfield (1964) observed that obese individuals exhibited a diminished sensitivity to the hypoglycemic action of insulin which apparently is associated with increased levels of circulating non-esterified fatty acids. Beck et al (1964), Gordon (1960), Karam et al (1963) demonstrated that the rise of plasma insulin in obese individuals is much greater and more prolonged in comparison to normal people and the fasting levels of plasma insulin may be elevated. Enlargement of the beta cells has been reported in non-diabetic obese subjects. Ogilvie (1933). Christophe et.al. (1959) reported elevated plasma insulin levels in obese mice and the enlargement of beta cells.

The work of Hausberger (1958) on parabiosis of hereditary obese mice with non-obese littermates suggests that the pancreas of the normal animals possesses or secretes some factor which is functionally lipostatic even in the obese animals.

The observation that obesity in humans is very often associated with impaired glucose metabolism has focussed attention on laboratory animals, particularly those that develop spontaneous obesity and hyperglycemia e.g. the obese hyperglycemic mouse which is the earliest known form of spontaneous diabetes in rodents (Ingalls et al 1950) This animal has since become an extremely popular research tool. Diabetic hyperosmolar non-ketotic coma too is being recognised with increasing

frequency since it was described in 1957 (de Graeff and Lips 1957; Sament and Schwartz 1957). This particular mutant is comparable to the maturity-onset, ketosis-resistant variety of the syndrome.

There are at least thirteen known mutations among rodents which exhibit a tendency towards development of diabetes. (Renold and Dulin 1967). It has also been reported in a number of other species including cattle, horses, pigs, sheep, cats and dogs (Mier 1960).

The interaction of multiple genetic and environmental factors makes it difficult to study the aetiology of the syndrome in humans. Thus the animal model, whose genetic and physical environment can be easily controlled, is very helpful in the study of diabetes - obesity.

Since all the work for this thesis has been carried out primarily on the obese hyperglycemic mouse (ob/ob) a brief general review is now given.

B. THE OBESE HYPERGLYCEMIC MOUSE

Origin and development

A new mutation called obese and designated by the symbol ob occurred in the V stock in Jackson Laboratories in the summer of 1949. They originated from the crossing of the off-spring of the V Stock males and C57BL/6 females with the V Stock males (Mayer et al. 1951a). These animals become first recognisable between the ages of four to six weeks when the body appears slightly shorter and squarer with expansive hind quarters. From this stage weight gain is rapid until the animals are about three months old after which the weight gain continues but at a comparatively slower rate. Ultimately the obese animals are about four times the weight of their normal littermates (Ingalls et al. 1950). In this mutation diabetes and obesity are believed to be caused by a simple autosomal recessive mode of inheritance. "This mutation has since been maintained by repeated back-crosses of transplanted ob/ob ovaries to the C57BL/6J inbred strain".. (Johnson F.N. 1967 - Personal communication).

Reproduction

A common experience encountered by numerous investigators under normal conditions of breeding is that the obese homozygote irrespective of sex is sterile. Thus it has inevitably become common practice to mate heterozygote animals (ob/+) to obtain the homozygote (ob/ob). This form of crossing produces obese animals in the ratio 1:3 non-obese.

Although the sterility in this type of animal is attributed to a recessive gene it has become increasingly evident that the sterility irrespective of sex is merely a secondary effect produced possibly by pituitary insufficiency or malfunction or hormone inactivation. The sterility is also very much more simply overcome in the male animal than in the female.

Lane and Dickie (1954) found that the male animal was not totally infertile and was quite capable of breeding provided he was kept on a restricted diet and not allowed to exceed a weight between twenty-five and thirty grams. The authors presented no evidence to indicate that sterility in the female obese mouse could either be corrected or prevented by diet restriction. However, they demonstrated that the ova of the obese female were viable by the very complex and delicate technique of transferring ova from the obese female to the non-obese female. Since ovulation apparently cannot occur spontaneously in the female it had to be induced first.

Drasher et al. (1955) found that the uteri of the obese animals regressed far more rapidly after oestrogen stimulation than their sibling controls. The fact that there was only half the number of normal cells in comparison to their sibling controls was indicated in that obese mice contain only half the quantity of DNA found in the control animals. The uteri of both types respond to the same extent on a short term treatment, but the uteri of the obese type underwent pronounced regression after six weeks. The fact that the obese gene is responsible for this alteration in pattern in some way is obvious because

the uteri of the C57BL/6 strain which forms the genetic background of these animals is characterised by a delayed regression after oestrogen treatment. The obese animal in spite of its inherent sterility and unstimulated uteri is capable of response to exogenous oestrogen. The uterine weights of castrated, untreated animals of both types are the same.

Runner (1954) induced ovulation in prepuberal mice by administration of an extract of pituitary gland hormogenate. Non-obese female recipients of the ovaries from obese females had successful pregnancies resulting in off-spring. Runner and Gates (1954) induced conception in sterile obese females by administration of gonadotrophic hormones. Implantation of the resulting blastocysts was produced by giving progesterone and pregnancy maintained by larger amounts of this hormone. Parturition was brought about by stopping of progesterone and administering relaxin or by Caesarian section. Lactation followed automatically. However in the obese female the reproductive organs are immature, development having apparently ceased with the onset of obesity between four to five weeks of age. The requirement for this delicate balance between oestrogen and progesterone in the obese female is further demonstrated by the work of Smithberg and Runner (1957). They induced ovulation, conception, maintained pregnancies, induced parturition and obtained the first 100% phenotypically obese litter from a pair of obese parents and a 50% obese litter from an obese mother (ob/ob) and a heterozygote father (ob/+

The obese female is comparable to the non-obese prepuberal female in that corpora lutea are absent and there is an absence of progesterone. Mammary glands are well developed only in pregnant animals and the interpubic ligaments elongated only after treatment with relaxin. It was also found that the obese males on a restricted diet showed a low frequency of matings. A large number of unsuccessful pregnancies with merely reabsorption sites were obtained too.

Hellman et al (1963) found that obese males were characterized not only by smaller testes and hypoplastic seminal vesicles but by atrophic Leydig cells, the total volume of these cells being only half that in the normal male. Despite these very marked regressive changes in the interstitial cell system, all the stages of normal spermatogenesis was present with only a slight decrease of spermatozoa. Steroid - 3βol dehydrogenese, essential for the formation of nonbenzenoid steroid hormones, was detected in the Leydig cells. The activity of this enzyme is subject to stimulation by gonadotrophics and thus the authors feel that the abnormalities of the male animal too are due to the deficiency of gonadotrophic secretion.

Morphology and Metabolism

Bleish et al (1952) made an attempt to study in general the morphological abnormalities of these animals. Portions of literally every organ, gland and tissue were fixed and stained. Although insulin-resistance, diabetes, centripetal

obesity and skin-changes were suggestive of pituitary-adrenal hyperfunction, no significant histological changes were found in this particular study to support this possibility. They were not able to demonstrate any significant structural changes in any of the other glands or organs examined with the exception of the islets of Langerhans. The obese animals had much larger and more numerous islets. The non-obese siblings and parent animals too showed an increase in islet number and size in comparison to the Swiss controls studied, but a decrease in comparison to the obese type. The obese also had comparably less amounts of hepatic glycogen and the presence of fatty lives in these animals was not unusual. In spite of the high glucosuria (3%) in these animals glycogen nephrosis was not evident. Parent and non-obese siblings had more glycogen than the obese animals but less than the Swiss controls. Cutaneous ulceration and atrophy were common, particularly at the sites of pressure, e.g. flank, posterior portions of thighs and legs, the gluteal and shoulder regions Alopecia was frequent in these animals while in the non-obese normal animals the skin and coat were luxuriant and healthy. The centripetal development of obesity gave rise to an increase of about 50% of the abdominal circumference, 25% of the thoracic girth and 20% of the cervical circumference. The facies and extremities remained normal. The testes as observed by Lane and

Dickie (1954) often did not descend into the scrotum even after the application of abdominal pressure. Normal active spermatogenesis was evident.

Mayer et al (1953b)during their further preliminary investigations into the aetiology of the syndrome of these fat mice, confirmed insulin resistance of up to 20 IU insulin and that the syndrome is the result of a recessive mendelian genetic lesion. They postulated that this lesion causes the partial blockage of the oxidation of C_2 fragments, prevents lipolysis, and promotes the synthesis of fatty acids from pyruvate. During their investigation the supersensitivity of the obese animals to thyroxine in comparison to their non-obese controls was demonstrated very clearly. After treatment the obese animals experienced an average weight loss of 5·7 grams/week, a decrease in food intake by one-third, a drop in blood glucose levels by almost 50%, and increased basal metabolic rate of 62% and a mortality of 50%. Thus although thyroxine undoubtedly facilitates lipolysis, it has exceedingly unfavourable physiological effects. In contrast the normal animals increased the food intake by about 20% and basal metabolic rate by 8%, experienced a slight weight gain and a slight reduction in blood sugar levels and no animals died after thyroxine administration.

Diethylstilbestrol, a pituitary depressant and an alpha cytotoxic agent had no significant effect on either group by itself but increased the toxicity of thyroxine greatly in the obese animal. Non-obese

animals seemed to experience a greater increase in weight.

The effects of propylthiouracil and thiouracil were tested on the non-obese variety only. Basal metabolic rates and food consumption were reduced but body weight remained stable.

Prolactin produced an increase in basal metabolic rate but not in body weights, and did not reduce the blood sugar levels in the obese. It left the non-obese completely unaffected on all scores.

Mayer et al.(1953) showed that the diabetogenic activity of growth hormone, which is completely ineffective on the non-obese animals, induces the onset of hyperglycemia in the young obese and increases the blood glucose levels by 200% in the older animals. ACTH treatment and even partial pancreatomy proved absolutely lethal to these over-weight creatures while their lean controls were able to withstand the above treatments much better and evidenced a longer survival rate. Both types however survived adrenalectomy for at least six weeks (on saline) with very little ill effects.

Mayer et al. also looked at the problem from the nutritional aspect. They found that the obese animals experienced weight loss and disappearance of glucosuria on high fat and high vitamin diets but there was a deterioration of the general condition of the animals. Obese animals on a high fat diet still retained their insulin insensitivity. A high protein-carbohydrate

diet checked weight gain and glucosuria but had no adverse effects on the general condition. However, a glucose-free diet containing fructose checked glucosuria but not weight increase. Lean animals were not put on this glucose free diet but remained in apparently good health on all the other diets. Onset of hyperglycemia normally occurred between the ninth and tenth weeks and insulin resistance was detectable in both the fed and fasted states.

From this work Mayer et al (1953) postulated a second block, at the hexokinase stage in that glucose ingestion not fructose induced hyperglycemia. The authors felt that the existence of the C_2 blockage i.e. incomplete oxidation of acetate fragments in these animals was a pre-requisite condition for the hexokinase block. They attributed this to the reduced availability of high energy phosphate bonds as the result of the incomplete oxidation of acetate. They also felt that the sensitivity of the obese animals to growth hormone supported this in that growth hormone causes insulin resistance, or rather enhances it, probably because growth hormone mobilizes the fatty tissue resulting in the release of free fatty acids in the system, which in turn compete with glucose in the tissues. Thus glucose uptake is inhibited or suppressed resulting in hyperglycemia. The non-obese animals are however not effected by growth hormone administration. ACTH has similar anti-insulin effect i.e. tissue resistance to insulin plasma antagonism and increased gluconeogensis and hepatic glucogensis. Guggenheim and Mayer (1952) showed that pyruvate oxidation too was depressed

in the obese animal.

Carstenson et al. (1961), and Hellerström et al. (1962) found evidence of the hyperactivity of the adrenal cortex in that the adrenal glands in the obese animal were at least twice as large and probably produced larger quantities of corticosterone in animals that were allowed to feed ad. lib. The excess volume of the glands was attributed to the adrenal cortex whose hyperplasia was caused by an enlargement of all the layers. This enlargement was not evident in the glands of animals maintained on a restricted diet. The authors found a linear relationship between body weight and adreno-cortical volume both in the obese and the non-obese animal. Corticosterone was found to be the principal glucocorticoid produced by mouse adrenals. Hellerström et al. (1962) also found that the boundary between adrenal cortex and medulla was more indistinct in the obese animal on an ad. lib. diet. There was a pronounced lipid depletion of particularly the inner layers of the cortex. There was no detectable difference in the medullary part. These findings are in contrast to Bleish et al. (1952), who found that the adrenal could be restored to normal size by diet restriction. Thus although this hyperadrenocortism may be partially responsible for insulin resistance, obesity and hyperglycemia, it may nevertheless be considered a secondary phenomenon.

Mayer et al. (1951a) found that when the animals

were allowed a free choice of diet, the obese animals had an obvious preference for a high fat, low carbohydrate and low protein diet while the normal animal preferred a more normal distribution of food, i.e. they derived about 43% of their calories from carbohydrates, 26% from protein and 29% from fat. The obese animals obtained 52% from fat, 20% from protein and 28% from carbohydrates. The obese animal also had a calorie intake which was 25% higher than their lean littermates.

Following on from these results Mayer et al (1951b) further intestigated the carbohydrate metabolism of these animals and established that they were hereditarily diabetic as well as obese. This conclusion was drawn primarily on blood sugar studies of both obese and non-obese animals in the fed and fasted state and on studies of their insulin resistance or tolerance. Fed obese animals had blood sugar levels of 200 mg% which dropped 50-60% after a four hour fast. The blood sugar levels were also unaffected by the massive doses of insulin. The normal animals had a blood sugar level of about 110 mg%, were not as sensitive to fasting and were extremely sensitive to insulin. Both types were responsive to adrenaline. The sugar levels soared to 500 mg% after a dose of 1 mg/kg. Obese animals had a glucosuria of 3%. The liver of obese animals also had an extremely low glycogen content in comparison to the normal animal.

Anliker and Mayer (1956) studied the feeding patterns in both the normal and obese animals. They found that in

the normal animal there was a twenty-four hour cycle with an initial phase of rapid feeding followed by a second phase of slower feeding. This cycle was absent in the obese animal. However, when the weight levels out the cycle appears and becomes more distinct.

Lane and Dickie (1958) found that the mean life span of the obese animal could be increased by nearly a third if their food intake was restricted. Obese animals when allowed a free access to food had a mean life span of 457 days, animals on a restricted diet 795 days and normal animals 747 days. Two of the restricted female obese were allowed to feed ad. lib. after 800 days and survived on average up to 1020 days. Thus on a limited food intake the obese animal on average has a longer life span than the ordinary normal animal.

Mayer (1954) observed that decreased activity preceded obesity in this strain of mice and the young obese animals studied increased their weight at the rate of 16 gm/month. Mayer also observed that in the obese animals which also carried the waltzing gene there was a restriction in weight gain of about 50%. A cessation of weight increase can also be brought about when the obese mouse is pair-fed with its normal littermate. This has been attributed to the fact that the activity in these animals increases with total or partial fasting. Pair-feeding also imposes a certain amount of restriction on food intake. These factors inclusive of the heavier weight of these animals brings about weight stabilization.

Alonso and Maren (1955) found that obese animals allowed

free access to food had the same amount of body protein but more fat than the normal control animals, even in severely underfed animals when body weight was less than 30% of the lean littermates. However, if maintained on a restricted diet, they still had more fat but less body protein than the lean animals.

Thus a decrease in physical activity in adults of less than 2% of the normal activity is an essential contributing factor for the development of obesity (Mayer 1953). Thus it could be of considerable interest to learn more about the factor, or factors, which bring about this decrease in activity before the actual onset of frank obesity, Mayer et al (1954) have further shown that enforced activity in these mutant mice brought about a considerable loss of weight in spite of the increase in food intake.

Mayer et al.(1957) found that obese animals on average gained less weight than their controls when exercised, while the lean animals maintained the same weight in both the exercised and non-exercised group. There is no significant difference in the serum cholesterol levels between the exercised and the non-exercised animals under these experimental conditions. Total duration of exercise was one hour daily at a speed of 0.435 km/hr.

The work of Baile et al (1970) suggested the presence of ventromedial hypothalamic lesions of varying degree in the obese hyperglycemic mouse. The extent

of these lesions and degree of hyperphagia is minimal under the conditions of reduced glucose utilization of the Glucoreceptors of this area and high blood glucose concentration. The obese animals developed smaller lesions than their lean littermates. Thus there was still some restriction of food intake. It would be interesting to know if this is inherent or merely the secondary result of reduced glucose utilization, insulin sensitivity and hyperglycemia.

Lochaya et al (1961) found that a high fat, low carbohydrate diet had insignificant effects on the metabolism of the adipose tissue of obese mice. In contrast the normal mouse adapted itself by decreasing the rate of its glucose metabolism to the various metabolites of glycogen, carbon dioxide and fatty acids and incorporating its carbon into the glyceride-glycerol instead. Glucose uptake too in animals maintained on this diet appeared to be inhibited after the administration of insulin, which seems to have an indifferent effect on lipogenic activity or glucose incorporation into glyceride-glycerol. These effects were found both in saturated and unsaturated fat diets. Glucose carbon recovery after stimulation with adrenaline was diminished. This indicates a compensatory mechanism in the absence of dietary carbohydrate and the importance of regulating fatty acid metabolism with emphasis on the re-esterification of fatty acids resulting from lipolysis. The obese animals however appear to have no need of or to lack any compensatory mechanism.

Christophe et al (1961 a and b) felt that the obesity

in this mutant was the result of some metabolic anomaly of its adipose tissue. They found a depression of glucose metabolism and to a lesser extent pyruvate metabolism during their investigations of the epididymal and mesenteric adipose tissue of the obese mice in terms of unit nitrogen or wet weight. The severity of these defects became more pronounced with age and that of glucose metabolism was detectable in the young animal just at the onset of obesity. They also found that fatty acid synthesis from the acetate carbon was greatly increased. This incorporation of acetate into fatty acids in the obese tissue in the absence of glucose was comparable to the response of non-obese tissue in the presence of glucose. This accelerated lipogenesis occurred maximally at the onset of obesity i.e. in the youngest, least hyperglycemic and least obese animal. This apparent greater independence of lipogenesis from glucose permits lipogenesis under conditions of fasting or conditions where normally lipolysis is encouraged in the obese animal. Thus hyperphagia in these animals is probably one of the results of the metabolic anomalies present and this increased lipogenesis is responsible for this type of metabolic obesity. They also found that although the basal activity in the obese mouse was about one-third that of the lean animal its relative response in terms of the basal activity was similar to the lean mouse. The epididymal tissue of the obese accounted for 4 to 6% of the total body weight as opposed to the 1·6 to 1·8% in the non-obese littermate. The increased accumulation

of tissue lipids was accompanied by increasing amounts of nitrogen. Mice were approximately 3, 4, 5 and 12 months old.

Leboeuf et al (1961) found a depressed glucose metabolism which was more pronounced by its diminished response to insulin in comparison to the non-obese. The impaired lipolysis too is made evident by the diminished ability of the tissue to prevent free fatty acid release after the addition of insulin and the diminished stimulation of free fatty acids by adrenaline. Although basal release of free fatty acid is similar in both animals, the glyceride-glycerol synthesis is only half or less in the obese. Thus this tissue is unable to utilize the free glycerol resulting from lipolysis and thus glycerophosphate a metabolite of glucose becomes essential to esterify the fatty acids released by lipolysis.

The earlier investigations of Bates et al (1955 a.b.c.d.) Mayer et al (1955) and Marshall et al. (1959) were agreed with the above that hyperphagia in the obese hyperglycemic mouse is the result of an inbalance in fat metabolism. This form of metabolic obesity is characterized by both excessive lipogenesis and decreased lipolysis resulting in a positive energy balance which is further increased by the hyperphagia. The authors for the first time distinguished between metabolic obesity and regulatory obesity. They found that under the conditions of a twenty-four hour fast rate of peripheral lipogenesis in the obese animal was still 140% of the controls and hepatic lipogensis was at least twice the control value while lipolysis was only 50 to 60%.

There were significant changes in the size of certain organs e.g. liver, kidney and heart which are associated with non-specific hyperphagia. However, the enlargement of the pancreas, thymus and the decrease in the size of the brain were associated with this particular form of obesity. Hypogonadism and gonadal atrophy were also observed. Further indications of pituitary hypofunction was found in that the pituitary of the young obese animals was smaller than the controls. The fat content of the young obese was ten times that of the mature obese and twenty times that of their respective controls. Some of the excess weight in this animal can be attributed to the slightly increased water content in terms of weight. However the water content in terms of the percentage body weight is about one-third that of the control animals.

Hellman et al. (1962 a.b.c.d.) Hellman (1963) do not entirely agree with the theory of excessive lipogenesis in the obese animal in terms of fat cell number. They found a reduction in the number of fat cells per unit tissue weight, but there was no reduction of nitrogen in comparison to the non-obese. However there was an increased number of mast cells (non-fat cells). About 50 per 100 epididymal fat cells in the obese as opposed to the 3 per 100 in the non-obese. The presence of this increased number of mast cells in believed to be probably related more to the state of obesity than its fundamental cause. These mast cells contain heparin which was believed essential to the enzyme system concerned with triglyceride breakdown. (Hellman et al. 1963).

Zomzely and Mayer (1959) found the acetate pools in fed obese and non-obese animals were the same but twice as great in obese animals after fasting for eighteen hours. The obese animals synthesized cholesterol three times faster than the non-obese.

Anderson and Hollifield (1966) further confirmed the excessive lipogenesis in adipose tissue under conditions of prolonged fasting, finding that there was no fall in the activity of the enzyme glucose-6-phosphate dehydrogenase. The non-obese and other forms of regulatory obesity showed the expected fall. Thus this attempt at gluconeogenetic adaptation to starvation is another unique feature in the metabolism of the animal.

Hughes and Tolbert (1958) too found that the oxidation of acetate and excretion of CO_2 from this acetate was considerably reduced in the obese and glucose metabolism was depressed very markedly in the male animal.

Treble & Mayer (1963) found that the glycerokinase activity in the obese mouse to be forty times that of the control lean littermates.

Stein et al. (1967) found an increased proportion of linoleic acid an essential fatty acid in fasted obese animals in comparison to increased oleic acid in the lean animals. The authors felt that this selective retention and mobilization of fatty acid reflected a lower monoglyceride lipase activity. They felt that this indicated the existence of a monoglyceride pathway which enabled the re-esterfication of free fatty acids without invol-

ving

the L-glycerophosphate and this adaptation further enhanced the impaired fatty acid mobilization particularly during starvation in these fat mice.

Mayer et al. (1951) found that oxygen consumption in the obese on the basis of body weight was less than 50% and their basal metabolic rate on the basis of body surface was less than 40% of the non-obese. They also felt that hypothyroidism was indicated in that the obese animals are hypersensitive to thyroxine. On administration of this hormone they decreased their food intake and were able to catabolize their fat. The hypercholesteremia in these animals too they felt added support to this theory. However they lacked morphological evidence.
Goldberg and Mayer (1952) in their studies on this strain, used the anoxia method and iodine uptake to verify the theory of hypothyroidism and the lower basal metabolic rates in these animals. Radioactive iodine uptake was normal and the dependence of oxygen tension was the same in both types.

However Mclintock and Lifson (1957 a. and b.) disagreed with Mayer's views and works on metabolism and energy balance in these animals on the basis mainly of methodology. These authors found that total CO_2 output of young adult obese mice was significantly higher than the non-obese. This higher total energy expenditure was accounted for by a/ increased basal metabolism, b/greater energy expenditure with regard to feeding and c/larger energy output required for activity although voluntary activity was

reduced which perhaps is indicative of a reduced efficiency in these animals. However, further work requires to be done in view of the discrepancies.

Mayer and Silides (1953) felt that the hyperglycemia in these animals was the result of the hyperfunction of the alpha cells of the pancreas resulting in the excessive production of a hormone other than insulin, i.e. glucagon. It has been suggested by Mayer et al. (1953a) that growth hormone is the trophic hormone for glucagon. Non-obese as other strains of mice were totally unresponsive to prolonged and massive treatment with growth hormone. However, the obese mice are hypersensitive to the hormone in that a single dose of 0.1 mg. of growth hormone was sufficient to precipitate hyperglycemia. In fact growth hormone actually reduced the blood sugar levels slightly in some of the lean animals. Diethyldithiocarbamate had the effect of normalizing weight slightly, blood glucose levels, growth hormone response and insulin resistance and prevented the hyperglycemic effect of cobaltous chloride in the obese animal. This is further supported by morphological evidence. In the normal animal temporary hyperglycemia and a small weight loss followed by a weight gain was observed. Treatment with a pancreatic hormone preparation containing insulin caused lethal convulsions in non-obese animals in contrast to a hyperglycemic effect on the obese. Some of the obese animals had normal postprandial blood glucose levels. Onset of hyperglycemia was observed between the twelfth and eighteenth weeks.

Shull and Mayer (1956a)found that hyperglycemia in obese animals was elicited by cortisone as well but not quite to the same extent and ACTH was ineffective, while the non-obese were unresponsive. This is in contradiction to Mayer et al. (1953). The authors found that the pattern response of both types to growth hormone was different as well. Fasting also abolished this hyperglycemic response in the obese, and response was diminished when the animals were maintained on a carbohydrate free diet. The authors felt that growth hormone inhibited the peripheral uptake of glucose.

Silides and Mayer (1956) found that growth hormone administration reduced lipogenesis from acetate in the obese while it increased lipogenesis in the non-obese. Glucagon behaved in the same way and they thus felt that this further supported the view that growth hormone is trophic to glucagon. However, when glucagon was administered to fasted non-obese animals, the lipogenesis was similar to that of untreated fasted obese animals. Lipogenesis from acetate is reduced in both types when maintained on a high fat diet and this decrease is more pronouncedin the obese than in non-obese. Fain et al (1965) and Fain (1967) found that the addition of growth hormone in the presence of glucocorticoids to free white-fat cells increased lipolysis and this lipolysis was abolished by inhibitors of protein synthesis e.g. cycloheximide, puromycin and actinomycin. Excessive amounts of corticosterone were found in the obese. (Hellerström et al. (1962) Zomzely et al. (1959)

in their studies using the hereditary obese hyperglycemic mouse and the goldthioglucose obese mouse, found that obese animals retained proportionately more steriod hormones (progesterone and testosterone) than the non-obese.

Christophe and Mayer (1959. a and b) studied the action of one of the earliest known sulfonylureas carbutamide (BZ-55) on the obese hyperglycemic mouse and its lean littermates. Although chronic oral treatment over a period of seven weeks had no effect on weight increase or food intake on either variety of mouse, it failed to improve the diabetic condition of this animal and besides produced a paradiabetic condition in the lean littermates after four weeks. These lean animals also responded like the obese to glucagon and growth hormone administration. The acute administration of carbutamide did elicit a hypoglycemic reaction from the non-obese fasted animals while it appeared ineffective with obese fasted mice. Two month old and four month old mice were used. Body fatty acid content and the rate of lipogensis were not affected in both varieties of mice. Serum and hepatic cholesterol levels in the obese were elevated. Although hepatic cholesterogensis in the non-obese was increased, the hepatic and body cholesterol levels were not. Mayer and Jones (1953) found the level of cholesterol in the obese mouse to be double that of the lean animal between four to five months of age. The levels were further elevated by fasting, growth hormone and a fortnight's regimen on a high carbohydrate and high protein diet. Levels were decreased by thyroxine and unaffected by ACTH. In the lean variety, ACTH, and growth hormones increased the

levels while throxine, high protein and high carbohydrate diets reduce the levels. Zomzely and Mayer (1958) confirm some of the above work and also the fact that hypercholesterogenesis is characteristic of metabolic obesity even when weights are similar to control animals.

Mayer and Yanoni (1956) found an increased rate of glucose absorption in obese including the hereditary variety which they believed could be an adaptation to prolonged hyperphagia or of a prolonged consumption of a high carbohydrate diet. Binder et al. (1966) found the small intestine in the obese to be heavier than that of the non-obese, but in proportion to their increased body weight. They found no differences in the histochemistry or impairment of <u>in vitro</u> transport of methionine and glucose.

Kornacker and Lowenstein (1964) found that specific activity of the citrate cleavage enzyme was 3·3 times greater in the livers of obese mice even during starvation, while the activity of the acetate activating enzyme was the same in both types.

Shull and Mayer (1956b) found the total turnover of liver glycogen measured in terms of the incorporation of uniformly labelled C-14 glucose to be greater in the obese animals. The levels of liver glycogen per g. of tissue were comparable in both obese and controls. However, due to the greater liver mass, the absolute values in the obese were twice that of the controls. The muscle glycogen in obese too was significantly higher. Shull et al. (1956) also found the liver phosphorylase activity to be significantly higher than the non-obese. The liver glucose-6- phosphatase activity in terms of

unit weight were similar. Hellman et al. (1961) too found that glucose uptake, CO_2 formation and glucose incorporation into lipid and insoluble residue both in liver and diaphragm for both groups of animals in terms of unit weight was the same. In liver the glucose carbon was also incorporated into glutamine and alanine, and glutamine, alanine and glutamic acid in the diaphragm. However in the obese animal glutamine formation was four to five times greater. There was no difference in activity between glutamic-oxalacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) in both varieties of mice. Westman (1968b) found that obese epididymal adipose tissue had an eightfold higher insulin degrading activity than that of the lean littermates but there was no differences in the liver samples of these animals.

Wrenshall et al. (1955) found that the islets of obese animals particularly on a high protein diet contained more insulin, as well as being hyperplastic and degranulated and supported the postulate that the hypersecretion of insulin was only secondary to that of glucagon. Clarke et al. (1956) found that there was a rise of blood glucose and hyperglycemic-glycogenolytic factor and a decrease in pancreatic insulin in the obese animal within twenty-four hours of growth hormone treatment, this further supported the above theory.

Gepts. et al (1960) found that four month old obese mice had six times as much islet tissue as the normal animals but hypertrophy was due to an increase in size

of the islets. They had six times as many beta cells and three times as many alpha cells. The obese pancreatic islets are large and contain more than 90% beta cells. These alpha cells too showed signs of hypertrophy and degranulation but not to the same extent as the beta cells.

Hellman et al. (1961) found the total islet volume of these hereditary obese mice to be ten times that of their normal littermates with a regular arrangement of the islets. Hellman and Petersson(1966) associated the increase in nuclear and nucleolar size in the beta cells with the increased activity.

Westman (1968c) found the presence of A1 and A2 cells in these animals including the lean animals, however the frequency of the A1 cells was lower in the obese and the authors attributed this to the function of age and obesity. Glucose-6-Phosphate splitting enzyme activity too decreased with age. These twenty month old obese animals still had an increased pancreatic islet volume in comparison to the lean animals and showed no sign of degeneration.

The high activity of Glucose-6-Phosphatase and the important role it may play in the metabolic release of insulin was discussed by Hellman and Hellerström (1961.) Täljedal (1969;) and Hellman et al. (1969.) These last authors felt that this role could be attributed to ATP instead. Thus the present trend of investigations tends to indicate that the hypersecretory activities of the islet were an adaptation to hyperglycemia and thus the likely villain

in this syndrome could well be part of the impaired peripheral glucose metabolism.

Batt and Miahle (1966) found that the insulin resistance of these animals could be attributed to the excessive food intake in that obese with a restricted body weight responded to insulin administration as did their lean littermates.

Christophe and Mayer (1959) found high levels of circulating insulin-like activity together with hyperglycemia. The aging animals (fourteen months) too showed a tendency towards insulin depletion. Serum insulin-like activity in obese animals was greater than 1000 μU/ml and in the lean animals 33 μUnits/ml. Gershoff et al. (1966) too demonstrated the diminishing glucose utilization in aging obese animals (eight/nine months) and the possibility of the existence of the circulating insulin in "bound" form. In the old obese animals the "free" insulin measured was 178 ± 23 μUnits/ml. and in the control animals it was 21 ± 4 μUnits/ml. The Bioassay technique used by Christophe et al.(1959) was able to estimate both forms of the hormone.

The paradoxical effects of alloxan and caffeine on the obese animal and the antagonism between alloxan and caffeine were studied by Solomon and Mayer (1962), Kuftinec and Mayer (1964) and Mayer (1966). This work will be extensively discussed later in this thesis.

Morse et al. (1956) did some preliminary electrolyte measurements on obese mice. They found intracellular and

and extracellular dehydration but the intracellular concentration of potassium was the same. Mayer and Hagman (1953) found that although 12% of the excess weight in these animals was due to water, the total body water and its percentage weight was similar in both types of animals of the same weight. Blood volume was not increased in the obese.

The inability of these animals to draw upon their adipose tissue or fat reserves even in an emergency was demonstrated by Mayer and Barnett (1953) and Davis and Mayer (1954). They found that the animals were extremely sensitive to cold and died of exposure within a few hours. Administration of throxine, thyrotrophic hormones or dinitrophenol prolonged life for a while but ACTH and cortisone enhanced this sensitivity. It was also observed that although the respiratory rates were increased the oxygen consumption was not. This inability to adapt was not the result of obesity or the decreased thickness of its pelage. These findings further support that these animals are not able to oxidise the acetate adequately. The normal animals show remarkable resistance even under conditions of fasting and exercising.

Besides their obvious value in diabetic research, these animals also have other uses, they also proved susceptible to "decompression illness" because of their large amount of adipose tissue. Nitrogen released in the bloodstream after rapid decompression from high atmospheric pressure accumulates in various tissue including bone.

The amount and rate of nitrogen released is in proportion to the amount of body fat. Nitrogen is five times more soluble in fat. Antopol et al. (1964).

Jehl et al. (1955) found the survival rate of these animals when infected with Ehrlich mouse ascite carcinoma cells was greater than non-obese animals. This was partly explained by the fact that although these tumour cells require glucose, their growth is inhibited by large concentrations. Thus the hyperglycemia existent in these animals had a protective effect.

<u>The Raison d'etre.</u>

As may be observed from the foregoing literature review that although certain changes may be suspected, development of this sydrome in the obese animals, has never been studied. Although obesity and diabetes are evidently associated, the exact relationship of this association needs to be investigated.

It might be concluded that the best approach would be to study the development by measuring parameters of body weight, blood sugar levels and serum insulin levels with time at very frequent and constant intervals. As the work described in this thesis will show, this has been done.

Although the relationship between carbohydrate metabolism and the potassium ion has been established for a long time, there is a limited amount of information concerning its uptake and regulation or its effects on

obesity and diabetes or vice versa in terms of carbohydrate regulation and there is no information available in mice. Thus it was felt that the investigations should involve the measurement of the cations sodium and potassium in serum and tissue under different experimental conditions as explained in Chapter V and its effects on carbohydrate metabolism were studied primarily by measuring blood glucose and serum insulin levels and estimating the glycogen content of tissue.

Alloxan and caffeine too have been shown to have some very interesting effects on the obese animals in contrast to the normal lean animals. Alloxan which is normally diabetogenic in its action reduced the levels of hyperglycemia in the obese animals while caffeine had the reverse effects. (Solomon and Mayer 1962; Kuftinec and Mayer 1964; Mayer 1966). These authors have offered no physiological explanation for this unusual and paradoxical effects in the obese animal. Therefore, an attempt was made to investigate this phenomenon and to explain its effects in the light of other investigations Chapter VI.

Also in this research we had first of all to establish the colony of obese hyperglycemic mice and to investigate the insulin immunoassay. At the time of this investigation the insulin immunoassay was a new method with many inherent difficulties and had not been explored to any great extent. It had never been used on mice and therefore it was essential to investigate this method fully and to align it with our needs.

CHAPTER II

METHODS AND MATERIALS

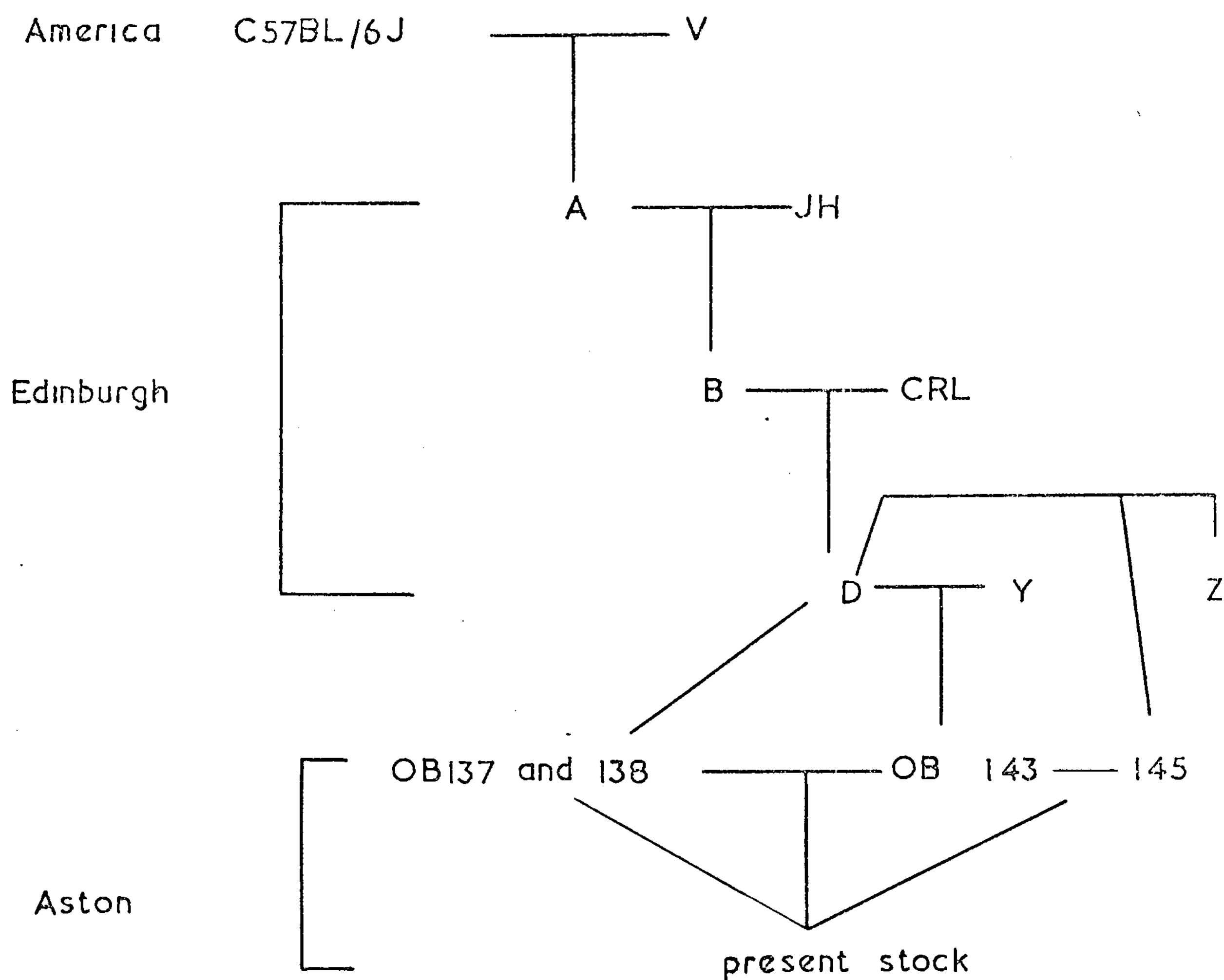
Plate A 30 week old, male, obese hyperglycemic mouse (weight 105 g) and it's lean littermate (weight 35g)

A. BREEDING HISTORY OF THE ASTON OBESE HYPERGLYCAEMIC MOUSE

The Aston obese mouse as it is now designated was first set up at this establishment in 1966 and may be described as "a non-inbred strain of mixed origin". (Dr. D.S. Falconer personal communication 1968). The original two heterozygote pairs and a number of unproven male and female mice were obtained from the Institute of Animal Genetics, Edinburgh in 1966. They in turn had obtained heterozygotes from Bar Harbour in 1957. The animals at Edinburgh were outcrossed to a non-inbred strain (JH) selected for its high litter size. This was maintained as a closed stock for five generations till 1960 and then outcrossed again to a non-inbred strain (CRL) for high growth rate and maintained as a closed stock for ten generations till February 1966. The mice of matings OB/137 and OB/138 were obtained from this last outcross to the CRL strain. The above closed stock resulting from the outcross to the CRL strain was then further outcrossed to two inbred strains of local origin. The other mice of matings OB/143 and OB/145 were the immediate progeny of the last outcrossing. These ancestries have been further mixed by our interbreeding them in this laboratory (see Fig.1 below). We had the assurance of the Institute of Animal Genetics that the only gene causing pathological obesity in the mice we were given was the recessive gene obob.

Setting up of Stock:

Since the recessive mutant gene results in the inability of both sexes to breed it became necessary to breed the obese



Key

A offspring of C57BL/6J x V strains

B " " " A x JH strains

D " " " B x CRL strains

Z & Y 2 unknown inbred strains of local origin

OB 137 & 138 mice obtained prior to matings (D x Z) & (D x Y)

OB 143 & 145 " " after matings (D x Y) & (D x Z)

Fig 1

Breeding history of the Aston obese-hyperglycemic mouse

homozygote animals (obob) from the normal phenotypically heterozygote animals (ob/+). (Fig.2).

Theoretically from the above one would obtain 25% obese homozygotes, 25% of the normal homozygotes and 50% of the heterozygotes. The problem here lies in that the heterozygotes and wild type homozygotes are identical in appearance and if by chance a pair of the normal homozygotes were mated, no obese mice would be forthcoming. These two genotypes can be distinguished only by breeding.

This was done in our case by breeding from a pair of animals, of which one was a proven heterozygote and the other was of an unknown genotype. If the unknown turns out to be a normal homozygote there will be no obese animals. However, by using the above method one stands a 66.6% chance of success. (Fig.3)

As our numbers of proven heterozygotes was limited we also adopted the random mating of animals, i.e. any two normal phenotypes were paired. In this way we had a 50% chance of success since each animal had the 66.6% chance of being the desired genotype.

We increased and maintained stocks by the above two methods using the new proven heterozygotes in the same way. However, we allowed each pair to have two litters before we discarded them from the breeding unit. Every effort was made to prevent inbreeding by avoiding the mating of siblings.

Obese males were capable of reproducing up to 12 weeks. The female animals were infertile. However one female animal who had been left in the same cage with a heterozygote male quite

Key

gene +	normal allele
gene ob	recessive allele
+/+	Homozygote (double dominant wild type) } normal
ob/+	Heterozygote } normal
obob	Homozygote (double recessive mutant sterile) obese

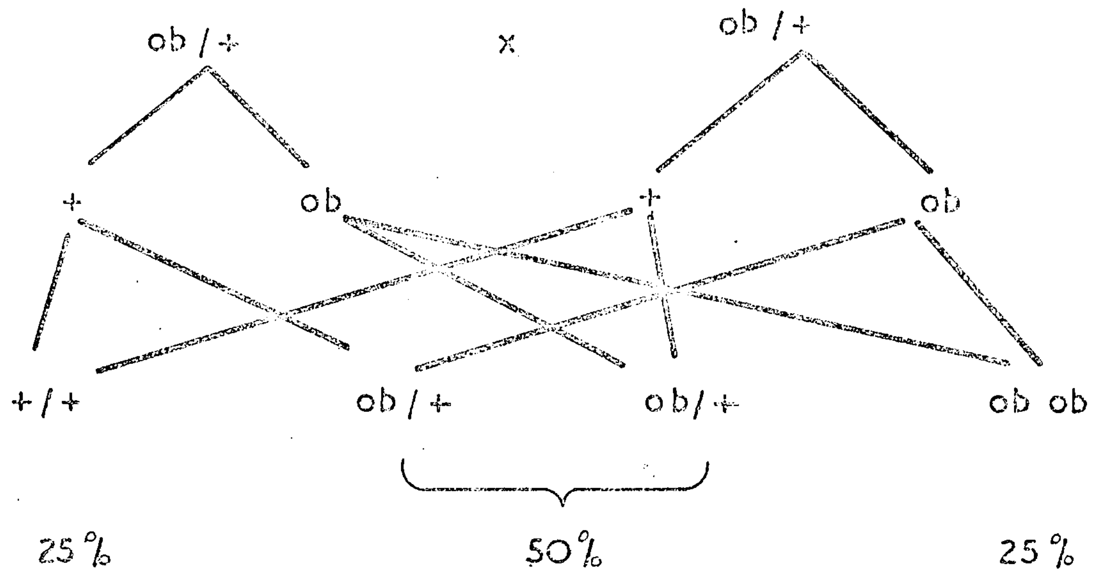


Fig 2

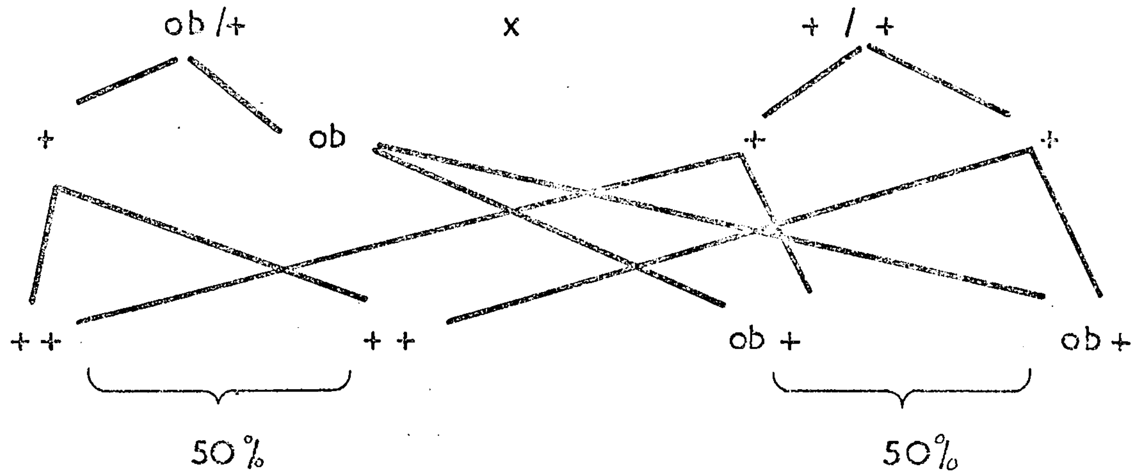


Fig 3

Inheritance of the obese gene

accidently provided us with 50% obese animals. Previous deliberate attempts on our part had failed even in weight restricted female animals. This confirms the recessive gene theory (Runner, 1954).

The young were weaned and sexed when they were three weeks of age and mated when they were six to eight weeks. Full records were kept of dates of matings, birth, litter size and parentage. The average litter size was about thirteen. At five weeks the obese were separated from their lean littermates.

The spontaneous death rate among the young animals up to about six months of age was infrequent and a few old mice have been known to survive up to about eighteen months. The general state of the colony was excellent. No widespread infections or epidemics were observed.

An attempt was made to breed American obese animals from heterozygotes obtained directly from Bar Harbour in March, 1967. However, it was not as successful because they did not breed as easily or as prolifically as our own stock. The animals including the obese were very small and excitable in comparison to our animals and their pelage was jet black. The pelage of our animals was extremely varied from albino to all shades of brown, gold and grey with "piebald spotting" in some.

Characteristics of the Obese Animals:

The obese animals were rather placid in comparison to their non-obese litter mates, although we did have the odd highly-strung animal. Some of the males when maintained in solitude became vicious when they had to share a cage. At one point a

male animal apparently ate two of its companions alive! Generally the obese animals are less sensitive to a change of environment. They are extremely slow, lack curiosity and they do not mind being handled, i.e. they make little or no attempt to escape or even to bite the handler. They can be placed on the bench for a few minutes and often will merely sit there or crawl a few paces at the very most. They remain comparatively placid in comparison to the normal animal even when they are dropped. On the whole one had the impression that the above characteristics seemed more pronounced with increasing body weight. They probably found it too much of an effort even to move themselves unnecessarily. This is very reminiscent of the "obese personality" associated with human beings. Thus their conduct is very different from the normal animals whose behaviour is the complete opposite and who tend to be very fidgety even under restraint. Their inactivity, refusal to mate even when weight was restricted, the hyperphagia, selection of food, all contribute to this picture. It will be interesting to clarify this behaviour further as to whether it was the cause or the effect of the syndrome or merely a part of it. The comparative inactivity is noticeable even when the animals are still young at the onset of frank obesity. The animals can be often recognised just before onset with a little experience by the squaring of the hindquarters and abdominal girth, i.e. between 3 to 4 weeks. They may also be recognised by a reddening of the skin in the anogenital region. This reddening is probably caused by the stretching of the skin. Most obese animals remained in good conditions even when they

were about a year old. Comparatively few at this age developed alopecia, ulceration of the skin and shedding of the pelage resulting in bald patches.

Handling and maintenance of the Animals:

All the animals used were housed in polythene cages. The floor of the cages was covered with sawdust and wood shavings. The young were additionally supplied with straw or cotton wool. The cages were cleaned out twice a week and the water supply was renewed daily. The rooms in which the animals were housed were regulated at an average temperature of 20°C (Range 18°C - 22°C). The experimental animals were kept in groups of not more than five in the small cages (30 x 15 x 11.5 cm) and not more than ten in the large cages (40 x 28 x 15 cm). They were fed and watered *ad lib.* except in the experiments where it is stated otherwise. Animals that were fasted overnight or over a longer period of time were always put into a clean cage with fresh sawdust. Water was supplied *ad lib.* at all times.

Before the animals were actually used in an experiment they were handled for a few days previously to get them accustomed to the experimental procedure. This also involved transit from the animal house to the laboratory and being put into the plastic tubes for the blood sampling procedure. All this was carried out in order to try to eliminate the effect of emotion on blood sugar in the animals which had been injured or were under stress. Transitory glycosuria too can occasionally be found.

Some preliminary experiments on obese animals and normal animals showed that although the p value is not very significant

it was felt that these preliminary precautions were worth taking (see below, Table I and the experiment on stress).

The animals used in the metabolic studies were housed individually in the metabolic cages, (to be described later,) in a separate part of the Animal House to which access was limited to only the investigators. This had the advantage of being quieter and therefore some stress was avoided. Room temperature was maintained at $22^{o}C \pm 3^{o}C$ and it had artificial lighting between 7.0 a.m. and 7.0 p.m.

Blood glucose levels as affected by stress:

The effects of stress were investigated by first examining the blood glucose of animals in the animal house. Stress was induced by comparatively rough handling, a rough and noisy journey to the laboratory when the blood sugars were determined immediately afterwards. The sugars were tested for the third time an hour later when the animals had calmed down. The results showed an increase in blood glucose concentration of 12% in the obese and 14% in the non-obese resulting from the stress, returning to pre-stress levels an hour later, $p < 0.1$ in the non-obese animals. This experiment also confirms the observation that normal animals are more sensitive to experimental conditions.

Choice of animals:

The animals used were all male animals between sixteen and twenty weeks of age unless stated otherwise. Obesity and hyperglycemia were established and the animals at this stage seemed to be at their prime and able to withstand experimental

TABLE 1. EFFECT OF STRESS ON THE BLOOD GLUCOSE CONCENTRATION (mg/100 ml.)

	Obese animals			Normal animals		
Animal*	Before stress	Immediately after	One hr. later	Before stress	Immediately after	One hr. later
R	190	240	200	98	100	95
Bl	133	156	140	75	93	80
G	167	173	160	100	126	93
B	188	199	185	86	90	82
–	208	234	195	103	115	100
RR	145	150	150	91	105	100
Mean	171.8	192.0	171.1	92.1	104.8	91.6
S.E.M.	± 11.7	±15.7	±10.1	± 4.2	± 5.5	±3.5

* Animal distinguished by coloured markings on the tail

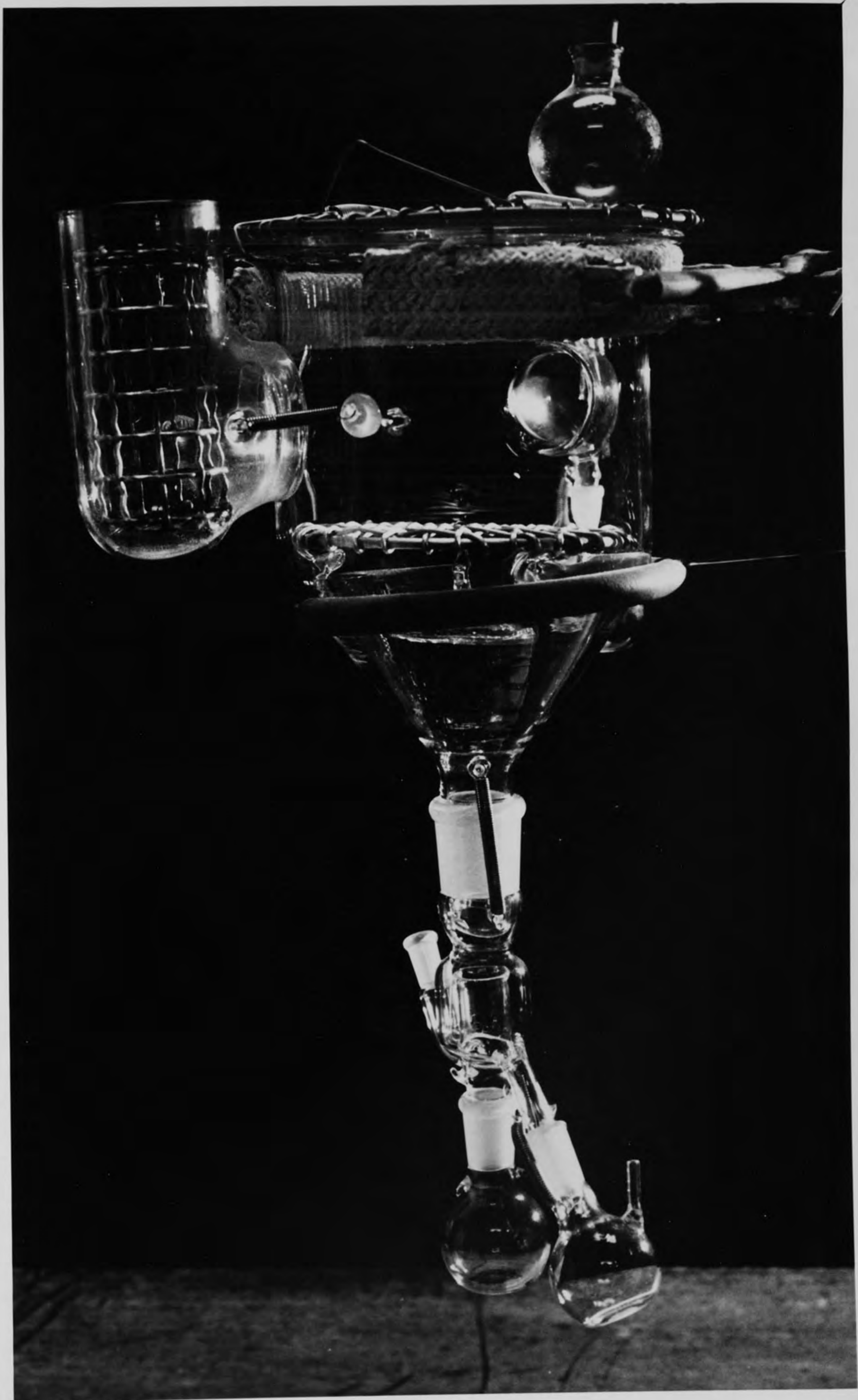


Plate B Metabolic cage

procedures better. They were also capable of maintaining a fairly constant body weight. Males were used because they are not subject to the cyclic hormonal behaviour of the females.

The obese animals were bred in the animal house in a separate breeding unit. Albino TO animals were obtained commercially between six to eight weeks prior to the experiments.

B. THE METABOLIC CAGES

The "minor metabowl" 4 in. (Jencons Ltd. Cat.No. H89/3) was used (Plate B). These consist of a central chamber 4" in diameter and are supplied with a glass lid. The cage has a stainless steel grid base to support the animal to which is attached a little conical shaped piece of glass which acts as a urine deflector. The unit includes a separator of faeces and urine which is easily taken to pieces for cleaning. The separator chamber is designed with an angled base to ensure speedy run-away of urine and also avoids small stagnant pools collecting in the lower channel of the chamber. Attached to the control chamber are the food and water holders. All attachments are of standard ground glass joints provided with hooks, held together with suitably sized springs or rubber bands.

These cages were designed for complete basal metabolic studies and were modified as follows:

Modifications

The glass lids were replaced by ones made from wire mesh grids and held in position by a system of lid-retaining springs.

The floor grid had to have more wire mesh threaded through so that the holes of the grid were reduced to a quarter of the

original size. This added to the animals' comfort and prevented large pieces of food pellet falling through and clogging up the separator.

Valves for the drinking bottles were made to prevent air bubbles forming making it impossible for the animals to drink. The animals also developed the habit of urinating into the flask which collected the drips from the water bottle. To prevent this a piece of perspex was glued as shown in Fig.4. to reduce the width of the inlet so that although the animal was able to drink it was prevented from sitting inside.

Another problem was the food column. The original design was far too narrow to take the pellets of the 41B diet. The powdered variety of the diet was tried but the animals only scattered it all over the cage. The food column was cut away completely and the food unit was redesigned (Fig.5). A circular wire mesh basket with a diameter to hold four pellets was made and a glass container was made to support the wire basket so as to allow the dust particles to collect at the bottom. It was necessary to have the depth and slope so that the animal was unable to drag anything out. The food unit was fitted on to the side of the cage and was retained by hooks and springs.

The joint by which the separator was fitted on to the cage and the separator and accessories were also made a size larger to prevent clogging and contamination.

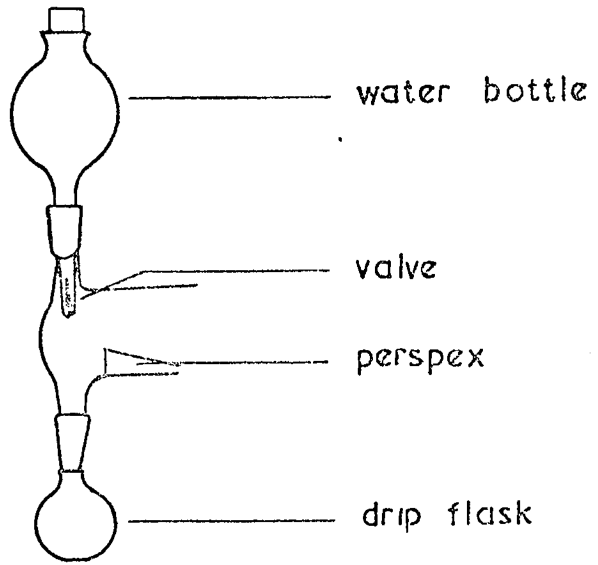


Fig. 4

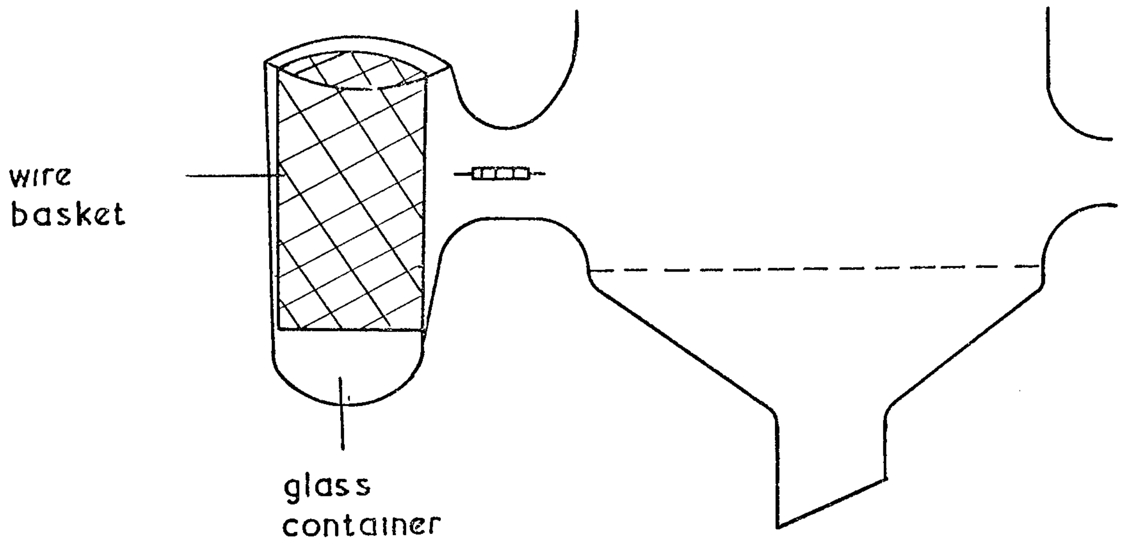


Fig 5

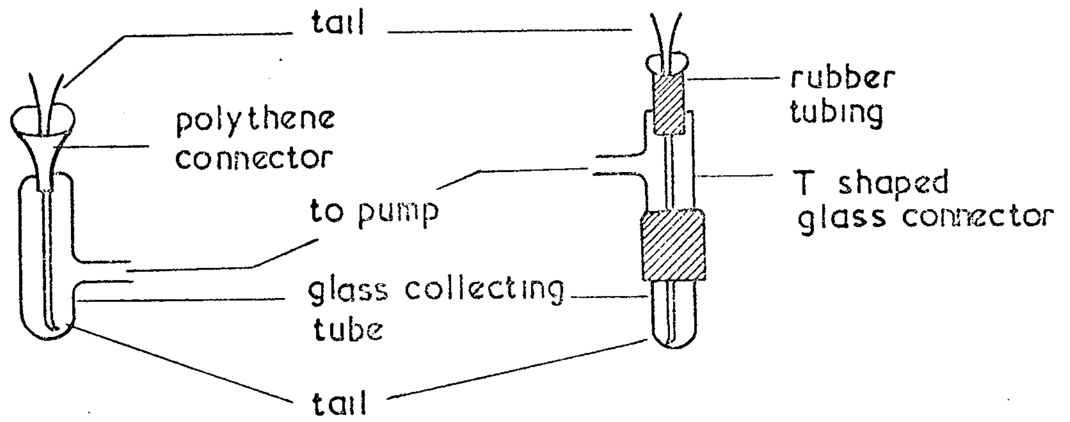


Fig. 6a

Fig. 6b

Plate C. Appartus for caudal blood sample collection

C. BLOOD SAMPLING TECHNIQUE

Several methods were tried but the one described below was found to be the most suitable. The piece of apparatus described here was developed to provide a convenient, inexpensive and easily cleaned piece of equipment which would restrain a mouse without causing too much trauma (see Plate C).

The blood sample was obtained by cutting off the extreme tip of the mouse's tail with a sharp pair of scissors. The animal was restrained in a polythene centrifuge tube, with small ventilation holes in the closed end. The animal was persuaded to enter the tube and if this proved impossible it was gently forced in. The tail was passed through the stem of a polythene funnel which fitted snugly over the mouth of the centrifuge tube, thus keeping the animal in position. If the animal was too small it almost invariably kept moving up and down and made blood collection tedious. To prevent this a small light rubber bung was placed in the tube in front of the animal. This also served to occupy the animal happily while blood was being obtained. The tube was clamped firmly to a retort stand. The tail was then passed through a suitable polythene connector into a specially made T-shaped collecting tube which was attached to a suction pump (see Fig.6(a) below).

Alternatively, if a suitably-sized polythene connector was unavailable, the tail was passed through a suitable piece of rubber tubing, a T-shaped glass piece was fitted over it and connected to the suction pump. Another small suitable piece

of rubber tubing was fitted over the bottom piece of glass tubing and a little collecting tube attached to it (see Fig.6(b) below).

Care had to be taken all the time to ensure that the suction exerted by the vacuum on the tail was optimal (100 - 200 mm Hg) Care was also taken so that the rubber, glass tubing or polythene connector did not fit too tightly over the tail. These factors if neglected seem to be some of the major causes of making blood flow extremely sluggish and difficult.

At the beginning the tails were warmed in warm water as well. Before placing the tail in the collecting T-tube it was wiped dry with Kleenex tissue paper. If necessary a drop of heparin was placed on the tail tip as soon as it was cut. A couple of grains of heparin were also placed in the bottom of the collecting tube. However, this procedure of warming the tail was found to be generally unnecessary and was resorted to only in case of extreme difficulty.

The blood was pipetted out from the T-shaped tube always using the same blood pipette. Between samples it was rinsed out with saline and distilled water and dried by rinsing it out with acetone and drawing air through it.

In the case of the obese animals the centrifuge tube proved to be too small. Therefore 100 ml. polythene bottles with the bottoms cut off were used. The animal was placed in the bottle with its tail emerging through the mouth of the bottle and the animal was kept in by means of a rubber bung with a hole in it.

In the case of the young mice (2 - 6 weeks) shortened centrifuge tubes were used or alternatively 25 ml. polythene sample vials.

This piece of equipment, although extremely crude according to present day standards of equipment, has been found to be extremely successful generally in these laboratories. It enables repeated representative sampling with no contamination of the blood and little suffering by the animal.

The other methods tried were:

(ii) Retro-orbital sinus puncture

The animal was lightly anaesthetized with Fluothane and placed on its side. The eye was held in position in such a way that the animal was held down at the same time. A microcap as required was pushed down at the corner of the eye and given a slight twist when it had reached the ophthalamic venous plexus and withdrawn slightly. It filled by capillary action. The microcap pipette was removed as soon as it was filled and the blood was delivered into the required vessel. The animal's eye was then gently wiped and its eyelid closed. (Dr. B.J. Weir, personal communication).

(iii) Cardiac Puncture

The animal was anaesthetized and restrained in a dorsal recumbent position. The thorax was swabbed with disinfectant and the apex beat palpated with the index finger was located immediately anterior to the xiphoid cartilage. A one-ml. syringe was used. The needle was introduced first straight down and then forward into the area of the heart. Blood was then

withdrawn.

(iv) Jugular venipuncture (Kassel & Levitan, 1953).

The mouse was lightly anaesthetized and held by the skin on the back of the neck such that the head was extended and the thorax and neck exposed. The hair of the neck and upper thorax was removed so that one was able to see the jugulars on both sides and the needle inserted into the distended vessel.

D. COLLECTION OF BLOOD AND PREPARATION OF SERUM FOR INSULIN AND ELECTROLYTE DETERMINATIONS.

The animal was decapitated with a large sharp pair of scissors and the blood was allowed to flow into 25 ml. beakers and poured immediately into polypropylene tubes. The tubes were allowed to stand in the refrigerator for twenty minutes. The clot was loosened with a fine platinum wire or a fine drawn out glass rod and spun at 400 g (on a MSE Bench Centrifuge) for ten minutes. The serum was decanted into small vials. The last drops were collected by fine drawn out Pasteur pipettes by capillary action (sucking with a Pasteur pipette resulted in haemolysis- this was also the experience of Weichselbaum et al. (1940)). Every effort was made to avoid even the slightest haemolysis. The serum was then deep frozen until required. The blood was allowed to stand for only such a limited time because a slow but appreciable transference of potassium from the red blood corpuscles to plasma occurs with time between 4° and $8^{o}C$ (Bernstein, 1953).

E. GLUCOSE TOLERANCE TESTS

The animals were fasted for seventeen hours. A sample of blood was removed from the tail as described previously, and the animal was injected with 100 mg. glucose in 0.4 ml. distilled water intraperitoneally. Blood samples were removed one hour and two hours after the administration of glucose. The urine was tested with Labstix just before the removal of blood each time.

The urine was obtained by applying gentle pressure on the abdomen and each section of the Labstix was lightly brought into contact with clean and clear urine.

Controls were injected with 0.4 mls. of distilled water. The young were injected with 300 mg.%/100 g. body weight of glucose.

F. ADMINISTRATION OF DRUGS

(i) Oral administration of KCl

The animal was held with its head back so that the mouth and oesophagus were in as straight a line as possible. A long piece of polythene tubing which had a syringe containing the required quantity of KCl (1 ml of 1 % KCl) inserted at one end was used. The free end was inserted into the corner of the mouth and guided down into the stomach. One ml. of 1 % KCl was then pumped into the stomach. Controls were given 1 ml. of deionised water.

(ii) Alloxan

5 mg. of alloxan in 0.2 ml. of buffer were administered intra-

peritoneally to each animal. The controls were injected with 0.2 ml. of buffer alone. The buffer was 0.1M citric acid adjusted to pH 3.2 or 4.0 with 0.2M Na_2HPO_4 (Hale, 1965). Alloxan was injected after a forty-eight hour fast.

(iii) Caffeine

2 mg. of caffeine in 0.2 ml. distilled water were administered to each animal after a 48-hour fast. The controls were injected with 0.2 ml. of distilled water.

(iv) Insulin

0.5 units were administered intraperitoneally into the normal TO mice and 2.5 units into the obese animals.

G. DIETS

(i) High Sodium Diet

The mice were maintained on a 41B diet but their drinking water was replaced with a 2% saline solution.

(ii) Low Sodium Diet

Mice were maintained on a special diet obtained from Nutritional Biochemical Corporation, U.S.A.

(iii) High Potassium Diet

The drinking water was replaced with a 1% Potassium chloride solution and the animals fed the 41B diet.

(iv) Low Potassium Diet

Mice were maintained on a special diet obtained from

Nutritional Biochemical Corporation, U.S.A.

In all the above cases animals were put on a forty-eight hour fast before they were put on their experimental diets. They were maintained on the experimental diet for a maximum of two weeks. The detailed analysis of the 41B diet, the sodium deficient diet and low potassium diet are given below.

Control Group.

The control group was put back on the normal 41B diet and normal drinking water after their forty-eight hour fast.

All these animals had their blood sugars measured daily or every other day. At the end of the experimental period of two weeks they were decapitated and the serum free from haemolysis was collected for insulin assay and electrolyte analysis.

In all the experiments as far as possible every effort was made to ensure that the animal was its own control in the light of the greater variation to which obese animals are subject.

DIET 41 B RAT AND MOUSE RESEARCH DIET

as supplied by Pilsbury's Ltd.,Birmingham, England.

INGREDIENTS

BARLEY	DRIED YEAST PRODUCTS
WHEAT	MINERAL SUPPLEMENT
OATS	VITAMIN SUPPLEMENT
DRIED MILK POWDER	ENGLISH WHITE FISH MEALS

CALCULATED ANALYSIS

	%		p.p.m
Crude Protein	17.069	Fe	65
Crude Oil	2.732	Mn	32
Crude Fibre	4.352	Cu	7
Digestible Crude Protein	14.52	I	4.18
Digestible Oil	2.114	Co	0.89
Digestible Fibre	1.723	Zn	8.29
Arginine	0.801		I.U/lb.
Lysine	0.877		
Methionine	0.309	Vitamin A	4641
Cystine	0.261	Vitamin D_3	1160
Tryptophan	0.192		mgm/lb
Histidine	0.326	Vitamin E	8.698
Leucine	1.089	Thiamine	2.809
Tyrosine	0.362	Riboflavine	1.588
Isoleucine	0.710	Niacin	25.004
Phenylalanine	0.674	Pantothenic Acid	6.801
Threonine	0.555	Choline	595
Valine	0.837	Biotin	0.41
Glycine	0.981	Folic Acid	0.38
Ca	1.3	Pyridoxine	2.8
p	0.72	Inositol	Not less than 100
Ca:P	1:0.6		
Na	0.575		mcgm/lb
CI	0.870	Vitamin B12	6.4
Mg	0.154		
Na	0.736%/kg. fat free diet		
K	0.78%/kg. fat free diet.		

SODIUM DEFICIENT TEST DIET

Composition

	%
Sucrose	72.0
Vitamin free casein	18.0
Butter fat (salt free)	5.0
Sodium free salt mixture	5.0

supplemented with Vitamin Diet Fortification mixture as follows:

		gms/100lbs.diet
Vitamin A concentrate	(200,000 units/gm)	4.5
Vitamin D concentrate	(400,000 " ")	0.25
Alpha tocopherol		5.0
Ascorbic acid		45.0
Inositol		5.0
Choline chloride		75.0
Menadione		2.25
p Aminobenzoic acid		5.0
Niacin		4.5
Riboflavin		1.0
Pyridoxine hydrochloride		1.0
Thiamine hydrochloride		1.0
Calcium pantothenate		3.0
Biotin		20 mgms/100 lbs. diet
Folic acid		90 " " "
Vitamin B-12.		1.35" " "

Sodium content (measured in lab. from ashed diet)
less than 0.1%/Kg. fat free diet

Potassium content (measured in lab. from ashed diet)
1.715%/Kg. fat free diet.

Formulated per modification. Itter et al. 1935.

The above diet was obtained from Nutritional Biochemicals Corporation, Cleveland 28, Ohio, U.S.A.

LOW POTASSIUM DIET

Composition

	%
Corn Starch	64.2
Casein	30.0
Butterfat	3.5
Calcium carbonate	1.3
Sodium chloride	1.0
Magnesium	400 mgms/Kg.

Above diet is with added vitamin supplements

Sodium (measured in the lab. from ashed diet)	1.169%/Kg. fat free diet
Potassium (measured in the lab. from ashed diet)	$<$0.01%/Kg. fat free diet.

The above diet was obtained from Nutritional Biochemicals Corporation, Cleveland 28, Ohio, U.S.A.

Water content

Sodium	0.2823 ± 0.03 mEq/L
Potassium	less than 0.1 mEq/L

Total number of samples measured = 10

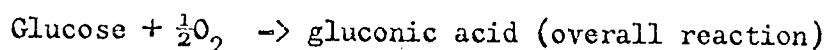
H. CHEMICAL AND ANALYTICAL PROCEDURE

i. Blood Sugar Determinations

Glucose-oxidase method (Lipscomb et al. 1958)

The commercially available kit was used (Boehringer Corporation, (London) Ltd. TC-MI, II or III. All estimations were carried out in duplicate

Blood glucose was determined enzymically and the reactions by which glucose is oxidised by the above enzyme are given below after Foresch and Renold (1956).



Glucose + $\frac{1}{2}O_2$ -> gluconic acid (overall reaction)

1. β glucose + glucose oxidase(FAD) -> gluconolactone-(FAD) + glucose oxidase H_2-($FAD.H_2$)
2. Gluconolactone + H_2O -> gluconic acid
3. Glucose oxidase H_2 ($FAD.H_2$) + 2O -> glucose oxidase FAD+H_2O_2
4. H_2O_2 + peroxidase -> H_2O + O_2

This method is based on the specific aerobic oxidation of glucose by glucose oxidase to gluconolactone which in turn is converted to gluconic acid in an aqueous solution. The resulting hydrogen peroxide which is inhibitory to glucose oxidase is reduced by a suitable chromogenic oxygen acceptor in the presence of peroxidase to produce a reddish brown dye. The glucose concentration measured at a wavelength of 436 nm on the SP 600 is proportional to the intensity of the dye.

Above 350 mg % the calibration curve was no longer linear and the deproteinized supernatant was diluted with water (1:5).

With the small amounts of blood available in certain experiments, e.g. when three week old mice were used, it was

found necessary to scale down the procedure. By halving the quantities the final volume of 2.6 mls. was found to be sufficient provided the height of the cuvette was adjusted for alignment with the light path in the spectrophotometer. It was also necessary to make small corrections for the cuvettes.

ii. The Simultaneous determinations of Acetoacetate and Glucose in whole blood.

These methods have been described by Salaway (1969) and the estimations were made on the Technicon Autoanalyser. A brief description of the methods is given.

To obtain blood for both determinations a solution of 60 I.U/ml. Heparin in 0.1% (w/v) sodium fluoride was used (25 ml. of heparin, 50 ml. of sodium fluoride and 25 ml. of distilled water). The diluent was freshly mixed each day before use. (Heparin was obtained as 'Pularin' heparin injection (25,000 I.U/5 mls) from Evans Medicals Ltd., Speke, Liverpool.) Owing to the small size of the animals only 0.1 ml. of whole blood was pipetted into 0.8 ml. of the above diluent and the volume made up to 1 ml. with distilled water. If the readings were too high 0.2 ml. of this diluted solution was pipetted into a further 0.8 ml. of diluent.

Calculations

Both sugars and acetoacetate concentrations were read off from the respective calibration graph which was always prepared at the same time that the samples were run through. Normally the diluted samples were multiplied by 5. This was

necessary because the manifold had been specially geared to estimate glucose and acetoacetate in 0.2 ml. of whole blood in a total volume of 1 ml. (i.e.a 20% concentration of whole blood). However both parameters in mice were measured in a 10% concentration of blood and when necessary in a 2% concentration.

The Ferricyanide method for glucose Determinations

This well established technique is based on the procedure of Hoffman (1937); yellow potassium ferricyanide in an alkaline solution is reduced to colourless ferrocyanide and the diminution of colour photometrically measured. Much of the non-glucose reducing substances are removed by dialysis so that values closer to the true glucose are obtained (a difference of 10%). This method is described on the 'Technicon' Method Sheet N-2b with the minor modification of capillary blood being diluted five-fold with the heparin fluoride diluent solution described and the sensitivity maintained by changing the size of the pump tubes (Salaway 1969). Potassium cyanide was added to the saline diluent to increase the sensitivity.

The Acetoacetate Determination

This method is described by Salaway (1969). It is a chemical method in which the acetoacetate is coupled to 2.5-dichlorobenzene diazonium chloride (pH 4.5) to produce yellow formazan derivatives which are measured photometrically. The whole reaction takes about 25 minutes to complete. The addition of 2N-sodium hydroxide changes the yellow to various coloured solutions, the actual colour depending upon the

concentration of acetoacetate in the solution. At low concentrations the solution is purple, as the concentrations of acetoacetate get higher the solution progressively takes on a yellow colour and when acetoacetate is present in excess the solution becomes intensely yellow. The solutions were prepared as described by Salaway (1969). The stock solution is believed to be stable for years at -20°C (Salaway, personal communications). The stock solution was 0.8 M.

Reagents

NADH 5mM

This was obtained from Boehringer. The working solution was prepared immediately before use by dissolving 4.7 mg. NADH in 1 ml. of water.

Beta-hydroxybutyrate hydrogenase

This too was obtained from Boehringer as a suspension containing 10 mg /2.0 ml. It should be diluted with 10 mM Phosphate Buffer pH 7.0 to give a final concentration of 1 mg. /ml.

Potassium Phosphate Buffer 0.1M pH 7.0

100 ml. of 0.2 M potassium dihydrogen orthophosphate was mixed with 60 ml. of 0.2M sodium hydroxide. This was diluted to 200 ml. and its pH checked.

Preparation of the Working Standard solutions

Approximately two litres of distilled water were placed in a refrigerator at 4°C to chill overnight. At least 0.2 ml. of

the stock acetoacetate solution was diluted in 800 ml. of the ice-cold distilled water resulting in a solution of 0.2 mM acetoacetate from which the working standard solutions were prepared as below:

Standard Solution (mM)	Volume of 0.2 mM stock (ml)	Volume of water (ml)	Actual conc. of acetoacetate (mM)
0.1	20	180	0.02
0.2	40	160	0.04
0.4	80	120	0.08
0.6	120	80	0.12
0.8	160	40	0.16
1.0	200	-	0.20

These solutions were then dispensed into clean autoanalyser cups, capped, with clean caps and stored at -20°C.

To check the concentration of the standard solution

The following solutions were pipetted into 1 cm. path length silica cuvettes and left for a few minutes at room temperature until the optical density at 340 nm remained stable. This optical density was recorded (E_1)

	No substrate (Blank)	Tests in duplicate	
0.1M phosphate buffer (pH 7.0)	1.0 ml	1.0 ml	1.0 ml
water	1.85 ml	0.85 ml	0.85 ml
NADH 5mM	0.05 ml	0.05 ml	0.05 ml
Acetoacetate (approx 0.2 mM)	-	1.0 ml	1.0 ml

0.1 ml of beta-hydroxybutyrate dehydrogenase was then added to each cuvette and the decrease in optical density followed at 340 nm until the reaction reached completion. This takes between twenty and thirty minutes. The final optical density (E_2) was noted.

The difference between E_1 and E_2 gives the optical density at 340 nm for the blank (E_B) and the test (E_T). The total optical density change is therefore ($E_T - E_B$).

The concentration of acetoacetate is obtained from the formula:

$$\text{acetoacetate concentration (mM)} = \frac{E_T - E_B}{2.07}$$

iii. Labstix Reagent Strips (Ames Co.) for urine analysis

These commercially supplied strips conveniently combine five rapid semi-quantitative simplified tests for pH, protein, glucose, ketones and blood in the urine.

iv. Electrolyte determinations

a. Sodium and Potassium

Urinary, serum and tissue extract sodium and potassium values were determined by means of an "Eel" single channel SP 900 flame photometer. The standard solutions for the measurement of sodium and potassium in urine, serum and tissue extract were prepared specially in each case to correspond with the dilution used. The sodium and potassium stock solutions were prepared from quantities of sodium chloride and potassium chloride which had been dried for 48 hours. An appropriate amount of sodium chloride was added to the potassium stock solution to prevent

the spectral interference by sodium in the potassium estimations. Both test and standard solutions were sprayed in duplicate, deionised water being sprayed between each reading. Between samples an appropriate standard was sprayed as well. This was to ensure that there was no drift in the machine. Clean dust-free containers for the samples being analysed were used to minimise contamination of the flame. The aqueous stock solütions and working standards were stored in clean polythene bottles with non-wetting surfaces and screw caps. This avoided the gradual diffusion of contaminants which is known to occur from the walls of glass containers. Routine precautions in the use of this instrument involved the maintenance of a constant gas and air pressure (10 lbs./sq.in.) and the continual checking of the standard readings and zero settings.

Calculation

Standard curves were prepared from the galvanometer readings of the instrument. The deflection is read off the graph to give the ion concentration in terms of mEq/litre.

b. Chloride

The chloride in the urine samples was measured using the "Eel" chloride meter. Similar precautions were observed during the estimations. The readings were obtained directly from the instrument in terms of mEq/litre.

v.a. Preparation of Tissue for sodium and potassium analyses.

Liver, pancreas, left kidney, left gastronemius and thigh muscle were removed from the animal as quickly as possible. They were cleared of fat, blotted and weighed in previously weighed

10 ml. beakers or similar sized small containers. They were then dried to constant weight at 105°C. This was achieved within forty-eight hours. They were ground up in a mortar with three changes of a mixture of 1:1 petroleum ether (B.P.40 - 60°C) and ether. The resulting powder was dried with hot air using a hair dryer and placed in an oven at 105°C for twelve hours, and stored in a desiccator. 20 mg of fat free dry tissue were weighed into nickel crucibles and ashed at 500°C in a muffle furnace for eighteen to twenty hours until white. The resulting ash was dissolved in 4 ml. of hydrochloric acid (100 ml. of 13.2 N.HCl/L deionised water) and analysed on the Eel SP900 flame photometer for sodium and potassium (MacIntyre and Davidson 1958).

All the standard solutions and blank were made up in the same acid solution so that it was finally of the same concentration in the standard and the blank as in the sample solutions that were sprayed. Thus any variation in readings which might result from the presence of the acid was eliminated.

The mortar was rinsed with acetone, wiped clean and rinsed three times with deionised water and dried with the hair dryer between each sample.

Calculations

The amount of sodium and potassium in the sample was read off from the calibration graph and converted by a simple calculation to mEq/Kg of fat free dry tissue.

b. Total water content

This was calculated as the difference between the tissue wet weight and dry weight before the extraction of fat from the tissue. Dried tissue was cooled in a dessicator before weighing.

c. Urine Analysis

A 1:200 dilution was made with deionised water (0.25 mls. of urine in 50 mls. of water).

d. Serum Analysis

A 1:100 dilution was used.

vi.a.. Estimation of Glycogen in tissue

The method adopted was that of van Handel (1965). The animals were anaesthetized with fluothane (halothane) in a desiccator and pieces of liver, pancreas, right kidney and right leg muscle were removed in that order within one and a half minutes from the time the animal was unconscious on the table. The piece of liver was removed within 30 seconds. The tissue was deep frozen by immersion in liquid nitrogen immediately after removal. This was an attempt to preserve the "in vivo" conditions.

The tissue was weighed and put into 1.5 ml. of 30% potassium hydroxide and heated in a boiling water bath until it had entirely dissolved. The released glycogen was then precipitated with 4 ml. of ethanol and 0.2 ml. of a saturated sodium sulphate solution as a co-precipitant. The precipitate was centrifuged down at 400 g for ten minutes. The supernatant was decanted and the glycogen was hydrolysed by autoclaving with 1 ml. of normal sulphuric acid at 15 lbs/sq.in. pressure for twenty minutes.

The hydrolysate was neutralised and analysed for glucose using the ferricyanide method.

To endeavour to eliminate the effect of diurnal variation in glycogen the tissue was removed from the mice between 10.0 a.m.

and 12.30 p.m. The animal was killed as soon as the required tissue was removed. Other tissues required for electrolyte analyses were then removed.

vii. Packed cell volume of Blood

This was estimated using the Hawksleys Haematocrit Centrifuge The packed cell volume was then read off the scale provided.

Serum Protein Estimation

The total serum proteins were estimated by the method of Reinhold (1958), more commonly known as the Biuret method. The proteins form a coloured complex with the copper ions in alkaline solutions (biuret complex). The intensity of this coloured complex is proportional to the protein concentration, at a wavelength of 555 nm on the SP.600.

I. GENERAL WASHING PROCEDURE

All glassware was firstly rinsed out in tap water. All glassware used for glucose and electrolyte estimations was soaked in pyroneg, rinsed thoroughly in tap water and several times (5 - 6)in distilled water and deionised water separately.

The beakers and any other equipment for iodinated insulin were allowed to stand for at least twenty-four hours in a strong solution of pyroneg or Decon 75 (Diversey Ltd.) rinsed out several times and resoaked in a fresh solution of detergent. This procedure was repeated twice more, rinsed thoroughly in tap water and several times in deionised water. These beakers were specially reserved for iodinated insulin.

All other glassware, plastic ware, inclusive of the

polythene disposable pipette tips for the Eppenderf pipettes (including the new ones) were allowed to soak overnight in a strong solution of detergent and rinsed in distilled water. They were then soaked in distilled water overnight and rinsed out at least three times with deionised water and dried in the oven. The washed pipette tips enabled easier delivery of the solutions.

The nickel crucibles used for the sodium and potassium estimations in tissue were scrubbed and rinsed in hydrochloric acid and then treated as the glassware. Before use, the crucibles were placed in the muffle furnace at 500° overnight.

J. STATISTICAL ANALYSIS OF DATA

The mean, standard deviation, standard error, t values were all calculated by computer. A statistical significant difference between groups was taken as $P < 0.05$. In all the figures the standard error of the mean has been represented by vertical bars.

CHAPTER III

THE INSULIN ASSAY

HISTORICAL BACKGROUND

The methodological procedure by means of which insulin assays are conducted are of two kinds:

A. Biological, either in vivo or in vitro

B. Immunological

In vivo Bioassays

The biological "in vivo" methods are solely of historical interest. The two bio-assays which have been most widely used to compare the relative potency of samples of insulin have been those depending on the ability of the hormone to produce convulsions in mice and hypoglycemia in rabbits! (Marks & Pak, 1936, Hemmingsen, 1939, Fieller, 1940, Burns et al., 1950, Smith, 1950.) Both these procedures have been proved to be too insensitive to detect the minute quantities of insulin present in the body fluids (Lorraine, 1958). They may however be useful for assaying commercial lots of insulin.

More sensitive methods utilized on a limited scale in clinical investigations include tests depending on the fall of blood sugar levels in chemically diabetic animals whose sensitivity to insulin has been increased by the removal of endocrine glands, e.g. alloxan-diabetic adrenodemedullated hypophysectomized rats (Anderson et al. 1947) and in alloxan diabetic hypophysectomized, adrenalectomized rats (Bornstein, 1950). However, the radical surgery and the resulting precarious metabolic state in addition to the extremely short life expectancy and therefore the very

limited number of assays made the "*in vitro*" assays more desirable. The reliability of these methods is discussed by Lorraine,(1958) and Bornstein, (1964).

In vitro Bioassays

The "*in vitro*" methods too may be classified into two groups:

1. The Isolated rat diaphragm
2. The Epididymal fat pad method.

The first method is dependent on the fact that insulin causes an increase in the synthesis of glycogen from glucose in the isolated rat diaphragm (Gemhill, 1941, Groen et al.,1952 Vallence-Owen and Hurlock, 1954, Randle, 1954, Manchester et al. 1959 (modification) . The major disadvantage of this method is its lack of specificity and the fact that a variety of plasma fractions have been shown to contain substances which interfere with the procedure (Randle and Taylor, 1961). Other factors, e.g. adsorption of insulin to inert objects e.g. glass, leather or paper and the degradation of insulin by proteolytic enzymes liberated by the diaphragm (Piazza et al. 1959) are again now recognised. Newerly and Berson in 1957 came to the conclusion that the binding of insulin need not be specifically related to its action. However, both adsorption and degradation of insulin may be inhibited by the addition of proteins such as serum albumin, ACTH, gelatin and prolactin to the incubation medium. Also Randle (1957), Wright (1957), Groen et al. (1958) have demonstrated that when insulin concentrations are determined

in successive dilutions of the same plasma sample differing results are obtained, the values of the diluted samples being generally higher. This finding has been attributed to the presence of inhibitors, the effects of which presumably diminish with dilution.

The epididymal fat pad method depends on the ability of insulin to affect the *in vitro* metabolism of glucose in adipose tissue (Martin et al. 1958). However, the specificity of this test has been questioned by Winegrad et al. (1959). He showed that G.H. prolactin and ACTH interfered with the assay. Cahill et al. (1964) obtained much higher values using the assay as compared with those obtained using the radioimmunological assay. The activity thus measured by bioassays is now designated as "insulin-like activity" (ILA) (Berson and Yalow, 1964).

Bioassay techniques are, therefore, not only difficult to perform but the results thus obtained are neither precise nor easily reproducible. Besides all these methods depend on the use of tissues, and all tissues have an inherent variability. The biological response depended upon in an assay is not always specific for the hormone being assayed. The extreme variability which is the result of the above severely limits the sphere of usefulness of bio-assays.

Immunological Methods

Immunological procedures are again of two main varieties:

A. Reactions depending on haemagglutination or haemolysis inhibition

B. Radioimmunological assays.

The reactions of the first type involve pre-treatment of erythrocytes with bisdiazotised benzidine or tannic acid. These erythrocytes are then coated with insulin and exposed to a fixed or pre-determined quantity of antibody. This antiserum has been preincubated with various standards of known concentration or serum of unknown concentration before the addition of the coated red blood corpuscles. The end point of the response is the inhibition of the agglutination of the erythrocytes which in turn is dependent on the amount of antigen present in either the standard or the unknown samples. In the absence of the complement, the cells are agglutinated by insulin anti-sera, but in the presence of the complement the agglutinated cells undergo lysis with the release of haemoglobin into the solution (Arquilla and Stravitsky, 1956).

The specificity of the above procedure has been questioned by Arquilla et al.(1960) on the grounds that the values obtained are very much higher than those obtained by radioimmunological methods. Berson and Yalow (1964) demonstrated that the sensitivity was relatively low and thus the test is unsuitable for widespread clinical or research application.

The radioimmunological technique was first developed by Yalow & Berson (1960). The use of radioimmunological methods

have revolutionised the technique of assay of protein hormones in the blood. Insulin was the very first hormone to be determined by this method. It probably is the hormone most widely assayed by this means. Radioimmunological procedures are the most popular at present because they are comparatively precise, specific and well suited to the simultaneous determination of large numbers of samples. They also permit the measurement of very low concentrations of insulin in very small volumes of plasma (0.1 μU/100 μl). They do not require complete identity between labelled and unlabelled hormones. They are based on immunological specificity rather than on biological activity. This has also been the most widely studied method for the determination of hormones in the blood and is increasingly used in clinical and research laboratories. Its disadvantages are comparatively few but it demands an extremely high degree of precision and accuracy. The sensitivity of the various methods is compared below:

<u>Sensitivity of some insulin assay methods (Goetz 1965)</u>

Microunits	1 10 100 1000
Milliunits	1 10 100 1000
Units	1 10 100
Radioimmunoassay	
Fat pad	
Diaphragm	
Mouse hypoglycemia	
Mouse convulsion	
Rabbit hypoglycemia	
Source	

PLASMA

PERIPHERAL PANCREATIC

EXTRACTED INSULIN

The method is based on the principle of isotopic dilution in the presence of specific antibodies. This principle depends on the competition between the radioactively labelled hormone and unlabelled hormone, for the specific binding sites of the antibody. Thus when insulin is added to the insulin antibody the insulin is bound by the antibody in a reversible reaction which follows the Law of Mass Action or in other words, after equilibrium has been obtained the amount of insulin bound to antibody depends upon the quantities of free insulin and antibody in the system.

The antibody is of a fixed concentration with the capacity to bind both the labelled and the unlabelled hormone. The labelled hormone (iodinated insulin) reacts with the antibody to form a labelled-hormone-antibody complex. The subsequent addition of unlabelled hormone (which is either the standard of known concentration or the sample of unknown concentration) results in the 'isotopic dilution' or the diminishing of the radioactivity of the antigen-antibody-complex. As the result of the competition between the labelled and the unlabelled hormones for the antibody, the labelled hormone bound to the antibody (bound radioactivity 'B') is inversely proportional to the unlabelled hormone, that is the higher the concentration of the free hormone or insulin measured the smaller will be the radioactive count (Bound insulin). After separating the bound from the free hormone the quantity of labelled hormone to bound hormone can be measured. Standards of pure insulin of varying concentration are determined in every assay and a calibration

graph is prepared. The concentration of insulin in any given sample is directly found by referring to the calibration graph.

In the radioimmunoassay of the hormones in the blood the points given below are customarily assumed:

1. The radioactively labelled and unlabelled hormones behave identically.
2. The circulating hormone is chemically and immunologically identical with the standard hormone preparation.
3. The amount of hormone bound by the antibody is independent of the concentration of the hormone.
4. The different antisera made against a particular antigen will give a similar quantitative response in the immunoassay system.

The labelled hormone of high specific radioactivity is used as it is desirable to keep the concentration of the labelled hormone as small as possible.

The general principles involved in assays of this type are discussed further by Berson and Yalow (1964), Felber (1966), Felber et al. (1966), Wright and Taylor (1967). A good general review "Immunoassay of hormones" by Ciba Foundation Colloquium on Endocrinology (1962) has been published and the kinetics of the antibody-antigen reactions as applicable to this technique is well discussed by Berson and Yalow (1959).

The several forms of radioimmunoassay for insulin described in the literature follow the same principle basically. They differ mainly in the manner in which the free labelled hormone

(free radioactivity) is separated from the bound labelled hormone antibody complex or antibody bound hormone (bound radioactivity) (Yalow and Berson, 1960; Morgan and Lazarow, 1963; Goetz et al., 1963; Hales and Randle, 1963; Grodsky and Forsham, 1960; Meade and Kiltgaard, 1960; Genuth et al., 1965; Herbert et al., 1965; Rosselin et al., 1966.)

The two most widely used methods of radioimmunoassay are Berson and Yalow (1964) where the separation of free insulin and antibody bound insulin is effected by electrophoresis and Hales and Randle (1963) and Morgan and Lazarow (1963) by the precipitation with a second antibody.

<u>Experimental Development of the Radioimmunoassay</u>.

The method adopted in the present investigations is the double antibody method, based on that described by Hales and Randle, 1963. The primary advantage of this method is that the necessary reagents for the assay are commercially available. (Radioimmunoassay Kit 1M39; Technical Bulletin 68/6, Radiochemical Centre, Amersham).

Each kit comprises of a bottle of iodinated insulin - I^{125} (0.1 µg) with a minimum specific activity of 50 µc/µg and an equivalent quantity of insulin binding agent (5 bottles) sufficient for 400 assays. This kit now includes a bottle of standard human insulin.

In this method, the complex of insulin and anti-insulin is rendered insoluble by a second antibody. The precipitate is separated from the free insulin by filtration and its radioactivity is measured. Originally Sköm and Talmage (1958) used this method to separate insulin from the antibody.

Hales and Randle developed this non-equilibrium system to increase the sensitivity of the method. The labelled hormone will be added last, that is after the incubation mixture containing the unlabelled insulin and antiserum has had time to reach equilibrium. In this system the unlabelled hormone has been given priority to bind with the antibody and the labelled hormone will occupy the vacant antibody sites.

Modification of Hales and Randle Procedure - Amersham Kit Method, Technical Bulletin 68/6.

The procedure described in the bulletin was initially followed. However as the result of the great variability obtained between triplicate and often quadruplicate samples it was necessary to modify the procedure for the separation of bound radioactivity from free radioactivity. The radioactivity was counted on a Nuclear Enterprises Gammamatic Counter.

Some of the steps taken to improve the accuracy of the method are given. The height of the Millipore 'Pyrex' micro-analysis filter holder (Catalogue No.XX1002500) recommended on the bulletin was cut down to about 2.5 cm. This was done in an attempt to ensure that the tiny droplets from the Pasteur pipette did not accidentally get left behind during the washing procedure. Great care was taken to deposit the incubation mixture containing the hormone-antibody-complex and free iodinated insulin right in the middle of the membrane filter and with every sample two portions of exactly one ml. of ice-cold buffer C were delivered in exactly the same manner as the contents of the reaction tubes. This precaution was taken to

avoid contamination and therefore possible loss around the lower rims of the filter holder. As there was no convenient refrigerator available, the reaction tubes were always transferred from refrigerator to the laboratory over trays of ice and so left throughout the time it was necessary for the tubes to be left outside.

Unfortunately in spite of every single care taken the results obtained in the first months of the investigation were most unsuccessful as the result of the variability between the samples. This was much later explained by Circular 68/13 which reported that the micropore filters supplied were substandard and other laboratories had suffered similarly. Meanwhile it was decided to abandon the microfiltration procedure and to attempt the separation of bound and free radioactivity by centrifugation based on Morgan and Lazarow's method (1963). After the second incubation of eighteen hours after the addition of iodinated insulin, the tubes were centrifuged in a refrigerated centrifuge (MSE Mistral 4L), precooled to $4^{o}C$ at 1600 g for one hour. The contents of the tube were decanted and drained and 500 μl of ice-cold Buffer C_1 was added. The precipitate at the bottom of the tube was resuspended by shaking in an eccentric mixer ("Whirlimixer" from Fisons Scientific Apparatus Ltd.) The tubes were then recentrifuged for another hour at 1600 g. The contents were decanted, drained and the precipitate resuspended in 500 μl of ice-cold buffer C_1 and centrifuged as before. The precipitate was washed twice in Buffer C_1. After this centrifugation the tubes were decanted and drained, ensuring that no drops of liquid were left at the bottom. The tubes

containing the precipitate were then air-dried and put into vials and counted on the Gammamatic. Empty tubes were included for background activity both at the beginning and the end of each batch.

This method for the separation of bound and free radioactivity has proved to be extremely successful in comparison to the microfiltration procedure. It is very much less tedious and saves a great deal of expense and time. It has also been successfully adopted by other workers in these laboratories. All experiments regardless of number can be very conveniently completed within seventy-two hours. Greater sensitivity was obtained when the first incubation period was extended to 24 hours. The whole washing procedure was further standardised and made independent of possible errors, e.g. the unequal filter quality mentioned before and it was very much less exacting on the operator. Finally it does not require the usual stringent decontamination of vials as the disposable polypropylene tube containing the precipitate is placed in the vial, thus another source of possible error is avoided. Quabbe (1969) adopted the above washing procedure independently. His centrifugation time however was only thirty minutes. This centrifugation method has another great advantage in that a single tube can serve for the incubation of the reactants, isolation of the precipitate and finally the measurement of the radioactivity.

Inter-assay variability was reduced by strictly standardising the whole procedure as much as possible. This contributed towards more reliable comparisons between test

samples:

1. The preparation of serum for the insulin immunoassay.
2. Use of 5.1 mg. albumin/ml. for making up the buffers A_1, B_1 and C_1.
3. Standardisation of incubation periods i.e. 24 hours each for both incubations.
4. Standardisation of centrifugation periods to one hour and the addition of 0.5 ml. of Buffer C_1.
5. Using the immunoassay kit within two weeks of its arrival.

Intra-assay variability was reduced by avoiding temperature changes while the tubes were actually being handled.

The Micro Method

Method 1.

There were two major problems during the insulin studies. One was that the quantity of serum (about 0.4 ml) needed was still far too great and the other was the hyperinsulinemia of the obese animals. Some of the values were extremely high and there is some doubt about the accuracy of the method beyond about 400 μU/ml. Thus it became essential to modify the existing Hales and Randle procedure even further.

The basic concept of this immunoassay consists of the competition between a constant amount of insulin I^{125} (250 pg) and a variable quantity of either standard insulin or serum insulin for available binding sites on a fixed amount of dilute guinea-pig anti-insulin serum (1:16,000 dilution). All the reagents are used in aliquots of 100 μl. It was felt that

if the concentration of each reagent used was reduced by a factor of ten in a total volume of 0.15 ml, the equilibrium would be preserved. Each reagent was diluted in its respective diluent. However, there were serious technical difficulties during the separation of the insulin antibody bound complex from the free insulin. This problem was overcome by the use of Beckman microplastic tubes (PRO 22, conical tip, 45 mm by 4.5 mm inside diameter) with attached stoppers. The separation of the precipitate was achieved by stoppering the tube firmly and inverting it with a sharp flick of the wrist and allowing to stand, to drain thoroughly (if possible). The two halves of the tube were separated with a blade. However this method still left unsolved the second problem of hyperinsulinism. Besides the counting time had to be extended by at least three times and even then the difference between the background count and the highest standard was getting a little too close for comfort.

Method II

While the concentration of antibody and labelled insulin remains the same the concentration of the unlabelled insulin i.e. the standards and unknown sample, vary greatly during an ordinary assay. Thus it was felt that by diluting the serum from the obese animal one would solve both problems as the concentration of serum actually measured would be reduced and would be within the reliable range of the assay and the assay is sensitive enough to measure a minute amount of insulin, i.e. 1 μU/ml .

The serum was diluted 1:10 with Buffer B_1 and the assay was carried out as usual. The results were corrected for the dilution.

Equipment

Eppendorf Marburg Micropipettes with changeable plastic tips.
(V.A.Howe and Co.Ltd., London).
Polypropylene immunoassay test tubes (Hopkins and Williams).
Polypropylene micro test tubes PRO 22 with attached stoppers.
(Beckmans Instruments Ltd., Glenrothes, Fife, Scotland).

Procedure

Insulin assays were performed on diluted obese mouse serum (dilutions 1:2 ; 1:4 ; 1:10 with Buffer B_1) and on increasing concentrations of ordinary mouse serum (0.1 ml. to 0.4 ml. of serum, the volume of standards made up with Buffer B_1. The recovery of varying quantities of added insulin to mouse serum was also studied. The results are presented in Tables 2, 3 and 5.

The insulin assay itself was studied by the performance of the assay over a series of standards ranging from 1 μU to 400 μU/ml. Microassay (Method I), the assay by Ekins et al. where the separation of the precipitate was effected by charcoal was compared with it in a few preliminary standard studies and finally standard mouse insulin was compared with it. Since until recently, i.e. just before the termination of this project, mouse insulin had been unavailable, ox insulin standards had to be used.

All solutions were prepared in double glass distilled deionised water (Elgastat). Ordinary distilled water was of variable quality and has been known to be responsible for the variations in the quantity of insulin bound to the antibody (Hales and Randle, 1963). All the insulin standards generally used were made up separately from the second stock solution (Bulletin 68/6) just before the assay. This was to ensure that errors were not the result of dilution. However the standards between 1μU/ml and 10 μU/ml were made from a 10 μU/ml standard dilution. All assays were done in triplicate. Any set of triplicates which did not agree within 10% was discarded. Washing procedure was considered satisfactory when the control tubes which contained no binding agent contributed to no more than 5% of the total counts. All measurements were made on serum. The reproducibility of the assay and the stability of serum insulin was further tested by assaying several samples of the same serum during an assay, during separate assays on different days and after periods of six, twelve and eighteen months. (Table 4).

The Charcoal Method

This procedure was a personal communication (Drs. R.C. Turner and R. Ekins).

The basic principle of the method is the same as that of the Hales and Randle method, i.e. "on the competition of insulin in the sample to be assayed and of radioactive insulin for reaction with an antibody which is specific to insulin.

The amount of radioactive insulin bound to the antibody therefore varies inversely with the concentration of insulin in the assay sample". One of the main differences is that in the Hales and Randle method the bound precipitated insulin only was counted, whereas in this method both the bound and the free insulin were counted.

In order to do this, cooled charcoal suspension is added to the tubes, each containing unlabelled and labelled insulin and antibody. The excess labelled insulin which is not bound to the antibody is bound by the charcoal. The supernatant is separated from the charcoal and both are counted separately.

It is recommended that when the supernatant and charcoal are counted, both vials should contain approximately equal counts. The amount of radioactive insulin which is bound depends on the amount of free antibody available after the unlabelled sample has been added. The greater the concentration of the antibody the greater the amount of labelled insulin which will be bound. The amount of antibody which will bind approximately 50% of the labelled insulin has been found to be most suitable.

Reagents

Insulin Binding Agent

It was decided to continue the use of this reagent which is supplied in the Kit 1M39. The 1:16,000 dilution of antibody binds approximately 40% of the standard dose of 250 pg insulin.

Iodinated insulin

Each vial contains 0.1 μg of iodinated ox insulin dissolved in one ml. of Buffer A_1. This was obtained from Wellcome Laboratories.

Buffers

Concentrated Phosphate Buffer I

$NaH_2PO_4 . 2 H_2O$	7.8 g
Na_2HPO_4	101.15 g

This was made up to one litre with deionised water.

Buffer IA

Before use this was diluted with deionised water in the ratio 1:5 and 0.3 g. % of crystalline bovine serum albumin was added. pH was adjusted to 7.4.

Buffer II

0.9 g. of sodium chloride was added to Buffer I.A.

Buffer III

0.1 g. % crystalline bovine serum albumin was added to Buffer I.

Charcoal Suspension

One g. of "Norit A" charcoal was added to 20 ml. of Buffer III and stirred for 2 hours at 4°C.

Insulin Free Serum

One g. of "Norit A" charcoal was added to 20 ml. of mouse serum, shaken and stirred for 40 mins. on the modified

blood cell suspension mixer (see below). The mixture was separated at 200,000 g (on the MSE Superspeed 50) for 10 min. This procedure was repeated once more.

An alternative recommended procedure in the event of the non-availability of an ultra-centrifuge was to leave the above serum and charcoal mixture for a week and then to spin at 1600 g. for several hours.

Apparatus

Mixer

A machine for mixing the solutions thoroughly after the addition of charcoal was required. It was recommended that to ensure thorough mixing the samples should be turned through two different planes in a slowly rotating manner.

The blood cell suspension mixer (Matburn Ltd) easily lent itself to modification. The only modification required here was a very much larger rotor plate which would carry several samples at the same time.

The spring clips on the rotor plate were removed and a plywood board of a thickness of 3/16" and of 16 in. in diameter was screwed on. White polythene-coated spring clips No.10 were placed on the outer rim, $\frac{1}{2}$" from the edge with a distance of $\frac{3}{4}$" between the clips (Total No. 64). Another circle of clips was placed at a diameter of 9 in. (total No.38) leaving a distance of 3 in. between the two rings or circles of clips.

All other equipment used, e.g. pipettes, tubes, vials etc. were the same as used for the Hales and Randle method. Insulin standards were made up in Buffer A_1.

Tube	Diluent	Insulin Std. or Sample	Insulin free serum	Anti-serum	I_{125}	Charcoal
Blank tube	500 µl	-	50 µl	100 µl	100 µl	100 µl
Standards	400 µl	100 µl	50 µl	"	"	"
Sample	490 µl	10 µl	40 µl	"	"	"

Procedure

The diluent (Buffer I), insulin standards, insulin free serum and anti-serum were added according to the above table, shaken and then incubated for 24 hours at 4°C. The tracer I_{125} was added, shaken and incubated at 4°C for another 24 hours. Charcoal which had been precooled to 4°C on the previous day was added in 100 µl aliquots, mixed for 1 hour slowly rotating at 4°C. The tubes were then centrifuged at 1,000 g for 20 min. and the supernatant was separated into another tube by means of a Pasteur pipette or by the method described below, which was found to slightly increase the accuracy. The tubes containing the precipitates and supernatants were put into separate vials and counted as usual.

Separation of Supernatant from the Charcoal Precipitate

The investigators from Dr. Ekins' laboratory used the Pasteur pipette taking care to suck up air bubbles together with the supernatant as apparently the delivery of the solution was better. In this laboratory strips of filter paper (Whatmans No. 1) were used. The supernatant was gently decanted into another tube and the sides gently tapped as usual to get the

last drops in as well. One end of the filter paper was put into the lower tube which held the decanted supernatant. The other end was folded into the tube above containing the precipitate but ensuring that it did not come into contact with the precipitate. Thus all the drops as far as possible were absorbed on to the filter paper strip which was then pushed back into the tube containing the supernatant.

This technique although more successful is more difficult. It requires nimble fingers and practice and attention if one is to avoid the charcoal in the tube, particularly from around the rim where the edge of the cap fits into the tube.

Calculation

The Co/Ci ratio was calculated according to Hales and Randle (1963) (Co is the radioactivity of the insulin antibody complex in the absence of insulin - the zero value, the Ci is the radioactivity of this antibody complex when the concentration of insulin is "i". A standard graph was obtained by plotting the standard Co/Ci values against the insulin concentrations of the standards (μU/ml). This Co/Ci ratio has a value of unit when $i = 0$. (Hales and Randle (1963) The Co/Ci ratio of the unknown serum samples were calculated and the corresponding insulin content in μU/ml was obtained from the standard graph. Serum insulin concentrations are expressed as μUnit equivalents of ox insulin/ml. Background counts and blank values were all accounted for before the calculation of the Co/Ci ratios.

Results

Standard Curves

The standard curves obtained with Hales and Randle's method but

with our modification for the separation of the precipitate from the supernatant may be seen on all the graphs. (Figs.7 - 10). By plotting the concentration of insulin against the Co/Ci value a linear graph is obtained up to 400 μUnits/ml for both ox and mouse insulin. The slopes of standard ox insulin (0.9/100) and standard mouse insulin (0.6/100) are significantly different ($p<0.001$). (Fig.9).

The slopes of the micro assay I and the standard assay do not vary significantly. The antibody has been diluted from 1:16,000 to a final dilution of 1:80,000, the labelled insulin from 250 pg to 25 pg and the concentration of the unlabelled insulin too has been reduced proportionally by a factor of ten. All the reagents have been diluted five times before the assay. Since the proportion of the reactants are still the same the equilibrium has been preserved and the assay barely unchanged (Fig.10).

The charcoal immunoassay was quite unsuccessful in our hands (Fig.7). Besides, the insulin-free serum was found to contain 6.0 ±0.33 μUnits insulin/ml.

The effect of dilution and concentration of serum. (Tables 2 and 3).

The results of the insulin immunoassay, using the standard Hales and Randles procedure with our modification, on the diluted serum sample and on the samples where the insulin concentration has been increased proportionally by increasing the volume of serum and the standards showed direct proportionality between the concentration of insulin and the volume of neat serum used.

Reproducibility and stability of the insulin determinations and the insulin in serum. (Table 4).

The reproducibility of the insulin assay is excellent. The mean value for four different experiments and using 24 samples of the same serum was 34.83 μUnits/ml and the standard error of the mean was 0.3931.

The insulin content of serum was stable for at least eighteen months. The stability was not tested beyond this period.

Recovery of ox insulin from mouse serum (Table 5)

As may be observed from the tables, samples of serum were assayed before and after the addition of varying quantities of standard ox insulin. The mean percentage recovery was 101.33±2.47 although the variation between the samples ranged from 92% to 125%.

Discussion

The standard procedure and Hales and Randle (Technical Bulletin 68/6) with our modification of the separation of anti-body-bound insulin complex from the free insulin in the precipitate gave us excellent and reproducible results. Apparently the insulin content of serum has not been affected by deep freeze storage. The non-interference with this immunoassay by other serum components is demonstrated by the proportional insulin concnetrations in different volumes of neat serum used and also by the good recovery of ox insulin from most of the serum samples investigated. This certainty of the stability of the serum samples has one great advantage in that one is able to store ones experimental samples and assay them conveniently

in the distant future. The method is sensitive up to 1 μUnit/ml. However in this region extreme attention and precision is required. The proportionality of the serum insulin concentrations to the increasing volumes of neat serum enable one to use this simpler way of insulin estimation in cases of very low insulin content. The relationship obtained between insulin concentration and serum dilution solves the problem of hyperinsulinemia. Thus one is able to work in the mid-regions of the assay with greater comfort and ease.

The highest error will occur when the counts are the highest, i.e. when the concentration of insulin is very low. However once the radioactivity has been converted to the Co/Ci relationship and the accuracy is dependent on the slope of the line of the calibration graph, the greatest error would be at the other end of the scale, i.e. at the higher insulin concentrations, particularly as the curve after 400 μUnits/insulin is no longer continually linear. The low insulin concentrations may also be immunoassayed with ease and accuracy by using standard insulin as a carrier, if necessary.

The above observations prove the validity of microassay II where the insulin standards are assayed exactly as the standard method but the serum samples are diluted (1:10) with buffer B_1 and the results are corrected for the dilution. The serum samples of ordinary mice were generally assayed straightforwardly. The use of microassay II will enable the investigator to measure serum insulin values "in vivo". To obtain sufficient serum for the standard macroassay one had to cull the animal and even then

it was sometimes a struggle or an impossibility to obtain sufficient clear serum free of haemolysed blood. Haemolysed blood was observed to result in decreased insulin content, the decrease presumably being proportional to the degree of haemolysis. It is not certain about the affinity of the red blood corpuscles for insulin or how much insulin is actually lost or trapped during coagulation during the preparation of serum. This was compensated for by standardizing the procedure of serum preparation. The packed cell volume in mice is 41.92 $\pm$0.69.

The difference in affinity of mouse insulin and ox insulin for the antibody is clearly demonstrated by the two different linear slopes for each of the above "insulin types". The slopes are significantly different ($p<0.001$).

The microassay I was more exacting and there was a greater scatter which may be attributed to handling and/or to the separation of the precipitate from the supernatant. Here the precipitate has not been washed with the albumin rich Buffer C_1 and it is possible that the precipitate has in addition some products which are the result of radiation degradation. This method was given only preliminary study and it was decided to discard it in favour of microassay II in the light of its handling difficulties and that it did not solve the problem of hyperinsulinism.

The charcoal immunoassay too was cursorily studied and discarded. More practice was required to separate the precipitate from the supernatant which like the above was not washed in a protein rich buffer. The variation between the two

graphs (Fig.7) may perhaps be due to the difference in the albumin content of the buffers of each method. The albumin content of Buffer B_1 of the Hales and Randle method contained 5.1 mg/ml while Buffer II of the Charcoal method contained only 3 mg/ml. Thus the binding capacity of the anti-insulin serum may be influenced in some way by the protein content of the reactants. From this it may be surmised that a low protein content results in a lower insulin value.

However this relationship is probably relative and further experiments must be performed to evaluate this point. All our estimations have been carried out with buffers of a protein content of 5.1 mg/ml. Total serum protein in mice as assayed by the Biuret method was 7.35 ± 0.18 mg/ml. Thus it is quite feasible that the actual serum insulin content is higher than the estimated value.

This relationship might also be the result of insulin degradation, or it might effect the charcoal binding of the antibody-insulin complex.

The detection of 6 μUnits/ml in the insulin free serum also suggests that the recommended treatment of serum with Norit A charcoal is insufficient or alternatively Norit A may not be a suitable adsorbant for the preparation of insulin-free serum. This is mainly compensated for by the addition of this supposedly insulin-free serum to all the tubes.

The immunoassay assay of insulin by the Hales and Randle method is so very sensitive that it is not surprising that it can be subject to a number of causes for error. Precision required

during pipetting can never be too strongly stressed. While the reactants are being mixed on the eccentric mixer one must be careful to avoid even minute splashes. The washing procedure by centrifugation which was adopted in this laboratory although comparatively crude compared to ultrafiltration etc. has proved most successful in our hands. The assay tubes were never allowed to stand between centrifugations during the washing stage because of the possibility of the precipitate redissolving in Buffer C_1. Care was taken to avoid temperature changes and all the glass-ware used was rinsed out with the respective ice-cold buffers immediately before use. The syringes and pipettes used were further washed out with the solution used. The same applicator was always used throughout and carefully cleaned as above between solutions. The most dilute solutions were always dispensed first.

Minimum periods of incubation are vitally important but longer periods of pre-incubation up to seventy-two hours did not seem to make much difference. However, any change in incubation periods or other conditions would affect the insulin standards in the same way.

In the charcoal method one must be sure that all the charcoal is removed from the insulin free plasma. Besides the tubes must be handled with attention after the rotary type of mixing. Bits of dried charcoal around the rim of the tube and cap may be lost .

FIG.7. Comparison of the Hales and Randle modified procedure with the procedure involving the separation of the precipitate with charcoal.

FIGS.8 and 9. Standard graph of the Hales and Randle modified procedure (0 - 25 μ units/ml. ox insulin) and the comparison of ox and mouse insulin standards.

FIG.10. Comparison of the modified Hales and Randle macro and microassays.

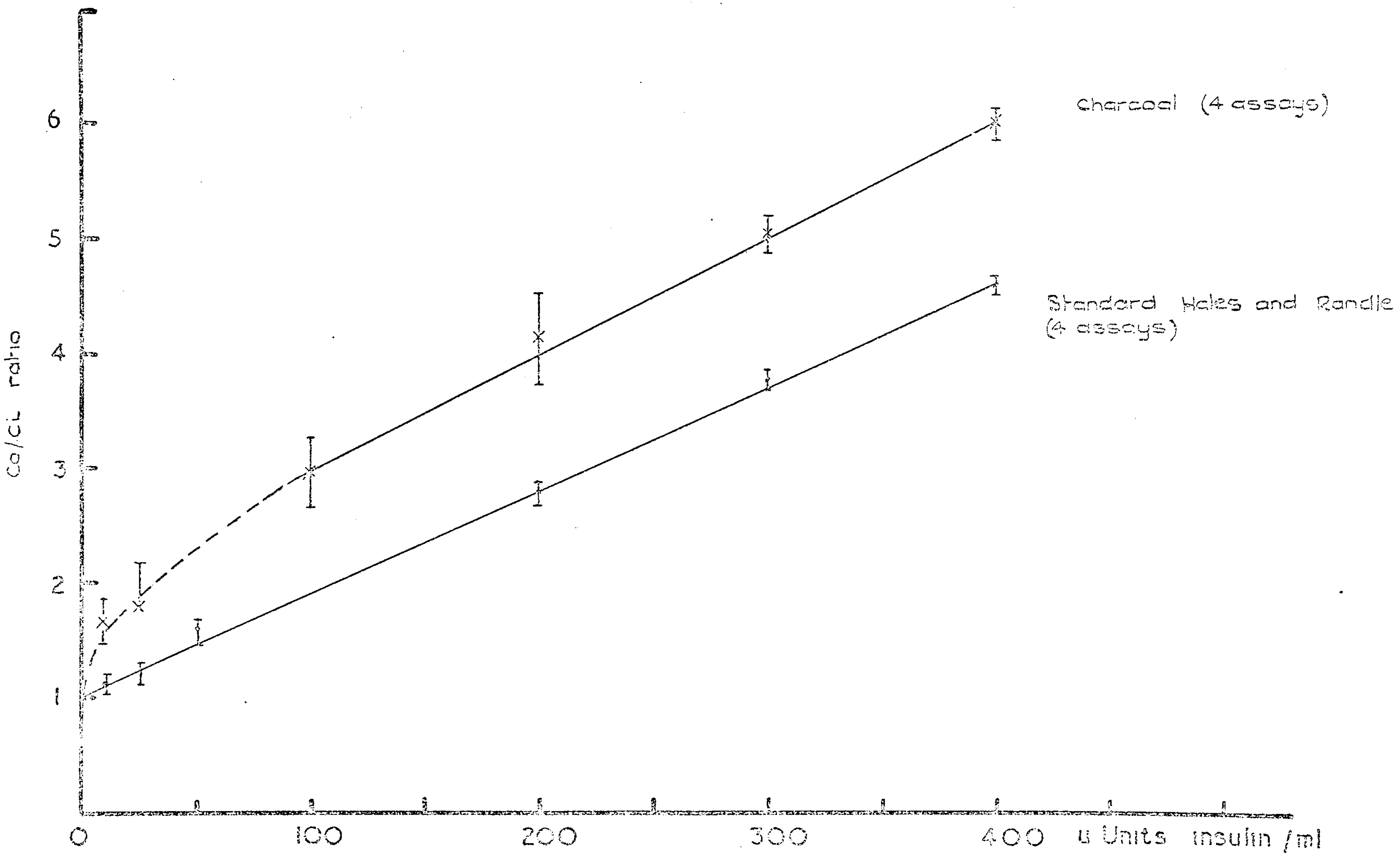


Fig 7 Comparison of the Hales and Randle method with the charcoal method

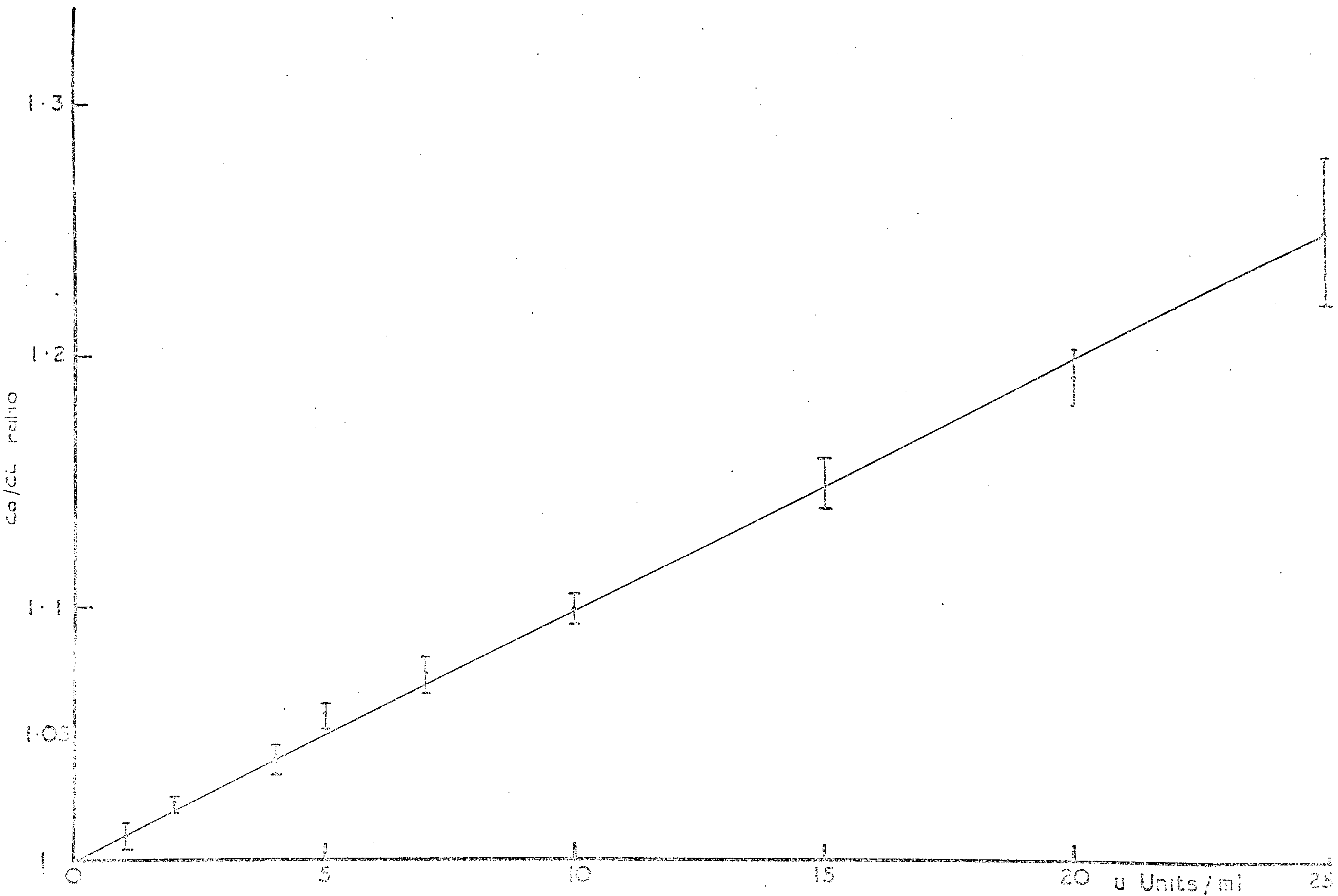


Fig. 8 Insulin standard graph (O u Units - 25 u Units/ml ox insulin) (4) assays

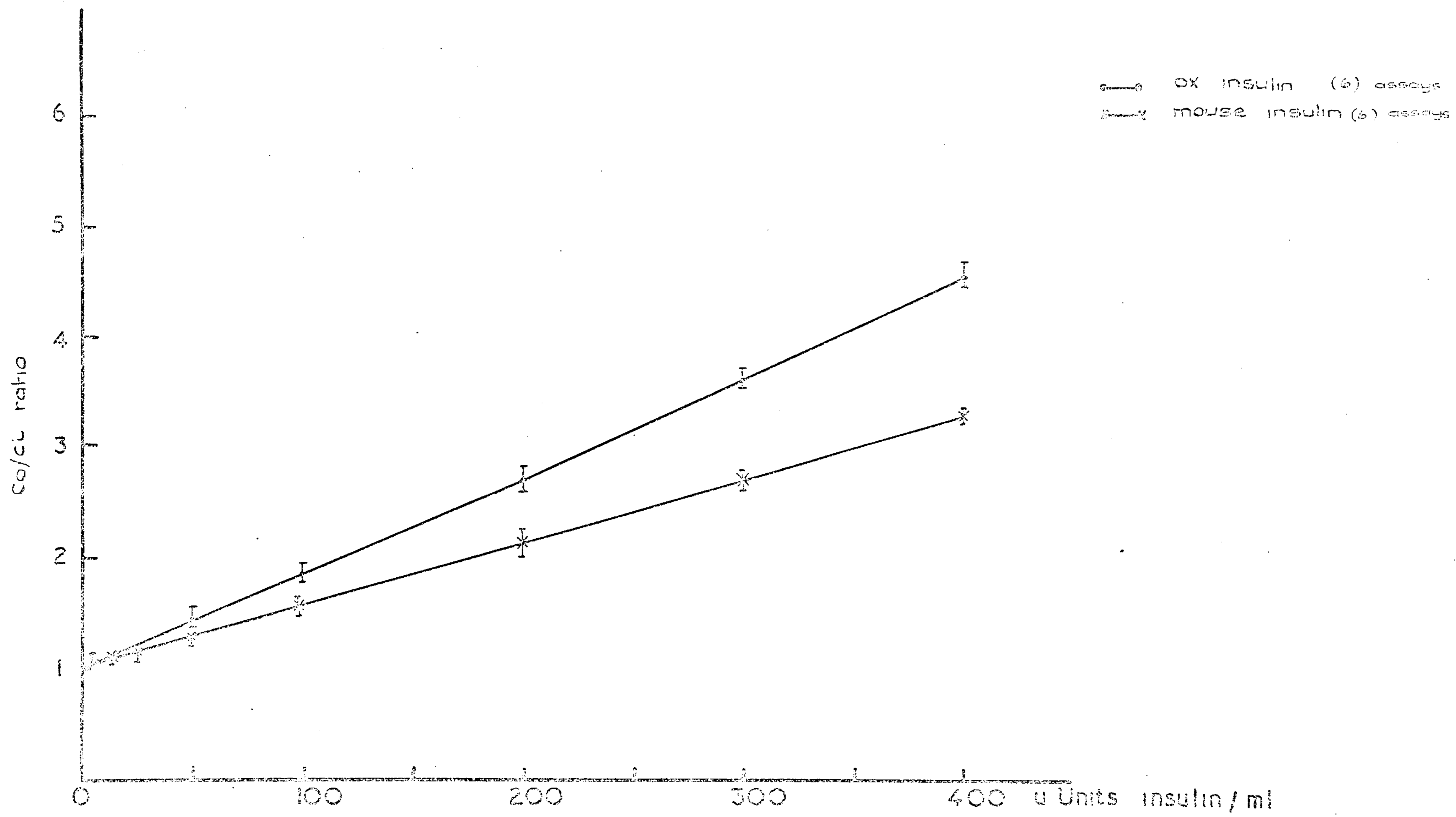


Fig 9 Standard graph of ox and mouse insulin (Hales and Randle)

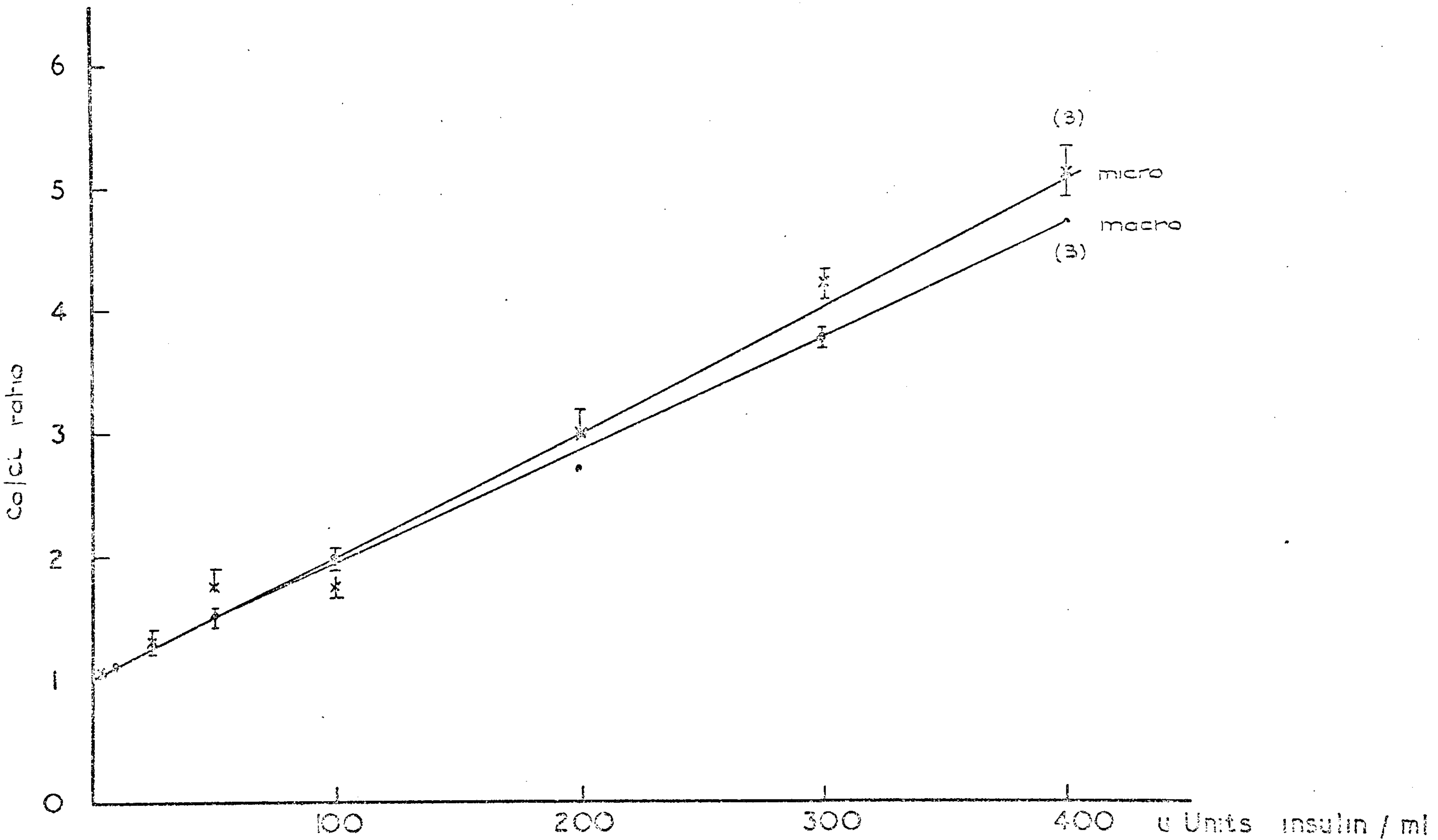


Fig 10 Standard graph of the standard macro assay and micro assay I

TABLES 2 - 5 Analysis of the following parameters during the insulin immunoassay:

1. the effect of serial dilution and concentration of standard ox insulin and mouse insulin
2. the stability of insulin
3. the reproducibility of the assay
4. the recovery of added exogenous ox insulin from the serum sample

TABLE 2. Serial dilution of obese mouse serum - insulin assay

Results have all been corrected for dilution.

Dilution	Insulin concentrations μU/ml					
	Serum I	II	III	IV	V	VI
Undiluted	350	285	224	152	196	415
1:2(0.05ml)	345	290	223	150	190	418
1:4(0.025")	335	280	220	158	197	410
1:10(0.01ml)	348	287	225	154	201	425

TABLE 3. Serial concentration of standard ox insulin and mouse serum

ml.	Co/ci value	Mouse serum insulin μUnits/ml						Mean and S.E.M.
	Insulin stds.	Serum I	II	III	IV	V	VI	
0.10	2.25	33	25	42	27	45	38	35 ±3.2
0.20	3.75	70	52	89	50	90	75	70.2±6.7
0.30	4.55	100	75	126	80	138	115	105.6±10.2
0.40		134	98	170	110	175	150	139.5±12.7

Standard used- 100 μUnits insulin/ml.

TABLE 4. Reproducibility of the insulin determinations (μU/ml) and the stability of insulin in serum

A. repeated determinations in the same assay

B. determination during different separate assays

C. after prolonged freezing - 6 months, 12 months, 18 months.

	Day 1	Day 2	Day 3	Day 4	6 mths.	12 mths	18 mths
1	35	36	35	40	35	33	36
2	33	35	35	38	34	37	35
3	34	36	34	33	37	35	34
4	35	36	33	30	35	35	35
5	35	34	36	35	36	35	35
6	34	33	35	37	35	36	38
Mean	34.353	35.00	34.666	35.50	35.333	35.1666	35.5
SEM	0.330	0.5162	0.4215	1.4775	0.4215	0.5424	0.5626

Packed cell volume 41.92 ± 0.69 (n = 12)

Total serum protein 7.35 ± 0.18 mg/ml. (n = 10)

Insulin content of insulin-free serum 6.0 ± 0.33 μU/ml (n = 3)

TABLE 5. RECOVERY OF ADDED INSULIN

Serum insulin alone (μU/ml) (A)	Added insulin (μU/ml) (B)	Serum + added insulin (μU/ml) (C)	Percentage total recovery $\frac{C}{(A)+(B)} \times 100\%$
21	20	50	125
18	20	40	105
32	20	50	96
45	20	60	92
63	50	110	97
25	50	80	106
37	50	85	98
47	50	100	103
46	100	145	100
31	100	130	99
28	100	130	101
23	100	115	94

Mean 101.33%

S.E.M.± 2.468

CHAPTER IV

THE DEVELOPMENT OF THE OBESE HYPERGLYCEMIC SYNDROME

Introduction

The obese hyperglycemic mice and their non-American strains (they all originated from the same colony at Jackson Laboratories) are probably the most extensively studied variety of animals with respect to this syndrome. As may be observed from the foregoing review a great deal of information on various aspects of metabolism is available but there is not sufficient knowledge about the actual mechanism responsible. At the time this work was begun it was felt that a proper understanding of the metabolic abnormalities of these animals could be furthered by establishing whether obesity or the impaired carbohydrate metabolism or a defective mechanism of the islets of Langerhans was the primary aberration and in what order these occurred. This involved an analysis of the sequential development of the syndrome and the relationship of obesity and diabetes.

It was felt that the best approach would be preliminary studies of body weights, blood glucose and serum insulin levels followed by glucose tolerance and insulin tolerance tests. The work of Gershoff et al. (1966) suggested that an investigation into the forms of circulating insulin would be worthwhile. These parameters were chosen because they were the simplest to study *in vivo* over the period of the life time of the animals and their determination would be the least traumatic. What could be more suitable? Blood glucose concentrations have been known

for decades to be subject to any alteration of carbohydrate metabolism. Insulin secretion is apparently regulated fundamentally by blood glucose concentrations. Increase in blood glucose results in increased pancreatic release of insulin and therefore an increased quantity of circulating insulin. A drop in blood glucose brings about the reverse of the above sequence and presumably a decreased release of insulin into the blood stream. Any number of a great variety of factors can influence blood glucose concentrations which in turn affects insulin secretion. However up to the present there is no evidence to suggest that blood glucose is not the only important physiological mechanism to influence insulin secretion directly. Thus the above feedback mechanism is of fundamental importance. Insulin in its turn has overall effects on nearly all aspects of metabolism and is essential to life.

At the time that these investigations were carried out the effects of various drugs and hormones on the blood glucose concentration had been studied but changes in blood glucose concentrations subject to the animal's own physiological make up had not been looked at; indeed it has never been observed in any other species. It has however apparently become the current practice today to observe blood glucose concentrations with regard to the development of the syndrome studied.

Thus at first an initial study was carried out with regard to body weight, blood sugar and insulin levels in animals of both sexes between the pre-obesity stage of three weeks up to sixty-four weeks. An investigation was carried out to see if

it was possible to detect the obese hyperglycemic homozygote early and reliably in the pre-obesity and pre-hyperglycemic stage before it showed the characteristics of the syndrome. The glucose tolerance test was used for this purpose in these young animals, glucosuria being the parameter that was measured. Care was taken to ensure that the animals used were not glucosuric before the experiment. The blood glucose concentrations were estimated using the glucose oxidase method.

RESULTS

Body weights

Obese homozygote

Figure 11 shows that there are 4 or 5 phases in the growth and weight pattern of the obese mouse. The female obese animals are consistently heavier than the males from the beginning (3 weeks to death) (Figure 19).

Phase 1

From birth to the fourth week of life when the obese animals are indistinguishable from their normal littermates.

Phase 2 - The Active Phase

This period is characterized by a very rapid increase in weight between weeks 4 and 24 when weight gain is from 15 ± 7·8g. to 93 ± 8·3g. The largest weight increase of 15g. occurred between weeks 4 and 5 followed by smaller weight increases of about 6g./week until about week 9 and then an average increase of about 3g./week until the maximal average weight is reached at week 24 when the weights of the animals range between 83g. and 103g.

Phase 3 (between weeks 24 and 31)

Here there is a gradual small decrease in weight between 93 ± 8·3g. and 77 ± 15·8g. the biggest decrease being between weeks 24 and 25.

Phase 4 (weeks 31 to 50)

This is characterized by a fairly static phase when the weights are generally stable.

Phase 5 (weeks 50 to 64)

This is characterized by a general weight decrease. The weight rise at week 59 is explained by the demise of two animals who had become extremely underweight even in comparison to their lean littermates just prior to death. One animal weighed only 20g.

Some animals not used in these investigations reached 140g. in weight.

Heterozygote Lean-Littermates (ob/+)

As seen in Figure 15 the weights of these animals increased fairly rapidly until week 12 when they weighed 275g. This was succeeded by a more gradual weight increase until week 39 (40g.) and a very slight insignificant decrease until week 60 (38g.). Figure 20 shows that the female heterozygote animals are heavier between weeks 3 and 12, but between weeks 12 and 30 they are lighter than the males. By week 40 both males and females are of the same weight but by week 50 the females are once more heavier than the males.

TO white mice (figure 17)

In this strain of mice there is an increase in weight until week 21, the most rapid phase being until week 13. As there was no reason to expect marked weight fluctuations

the experiments were discontinued. The male animals tend to be about 5g. heavier and the growth until the 13th week is much faster compared to the females after which growth in both sexes level off (Figure 21). Both the males and females weigh about 11g. at week 5 but by week 8 the females are only 24g. in comparison to the males which are 30g. In the female the growth rate is more gradual between weeks 8 and 21 when the maximum weight of 32·5g. is reached.

Blood glucose concentrations

Figure 12 shows that in the obese animals blood glucose levels are extremely variable. Their erratic behaviour is emphasized even more after an analysis of the percentage change in blood sugar concentration from week to week (Figure 14).

However, in spite of this great variation, the whole picture may be said to fall into a patternof four phases. The first three phases between weeks 3 and 24, 24 and 39, 39 and 50 are fairly similar in that they show a rise in blood sugar to a peak succeeded by a gradual fall, i.e. they are characterized by peaks and troughs which bear resemblance to a sine wave or a sinal oscillatory curve. Phase 4, from week 50 to week 64, represents a decrease in the glucose concentration to hypoglycemic values of 53mg%. Further analysis using the "moving average" principle whereby the great percentage changes between the weeks are compensated, confirmed this picture (Figure 13).

The lean heterozygote animals generally exhibited a very stable picture of blood glucose concentrations, variations being between 100mg.% and 140mg.%. A few animals did show blood glucose levels of about 160mg.% on occasion (Figure 16).

The albino TO mice had a steady picture variations being between 85mg.% and 140mg.%. (Figure 18).

Serum insulin concentrations

Figure 22 shows that at 4 weeks the obese animals had insulin levels of 90± 13 μUnits/ml. i.e. almost double that of the lean TO albino mice of the same age (50 ± 10·5 μU/ml.). There is a rapid steep increase in the serum insulin concentrations of the obese animals until 20 weeks of age (741 ± 196·83 μU/ml.) There is a rapid drop by week 24 by nearly 50% (461 μUnits/ml.) followed by a gradual and steadier decrease until week 60 (67μU/ml.).

The serum insulin in the normal mice fluctuated between 40 μU/ml. and 68 μU/ml. throughout, i.e. between weeks 4 and 52.

The blood glucose concentrations of these mice (Figure 23) are a good reflection of their serum insulin.

Glucose Tolerance Tests

Table 6 shows that the obese young give a 100% positive result with the clinistix test at 23 days and a third of the animals are glucosuric at 20 days. However, the heterozygote lean littermates give a total positive result of about 42% over the whole experimental period and 33 1/3% at 20 days. The glucosuric lean animals were mated with known heterozygotes later on. Seven of them produced obese animals in the F1 generation and one of them in the F_2 generation. Thus these

animals were proven to be heterozygotes.

In the sixteen week old animals glucose tolerance is definitely impaired as may be observed from the high fasting blood sugar and the decreased rate of glucose disappearance (Figure 24).

In the fourteen month old obese animals a picture of a similar but very much greater impairment of glucose tolerance is seen. (Figure 24). The normal control animals do not show any significant impairment at the fasting, one and two hours values.

The percentage change in the glucose concentrations with respect to basal or fasting levels in the obese mouse is surprisingly and coincidentally the same in the 4 month old and 14 month old animals, i.e. 200% at one hour and 184% at two hours. However, the basal levels of the older animals was 166% of the younger obese group. In the lean 4 month old animals it was 120% and 80% at one and two hours and the corresponding figures in the 14 month old normal mice were 130% and 96%. The basal levels of the mature controls was 105% of the younger ones. However regrettably these observations were carried out on different groups of animals, i.e. the 4 month and 14 month old sets of mice are not the same.

<u>Serum insulin levels after glucose tolerance under fed and fasted conditions</u>: (Figures 25A and 25B)

The serum insulin response at one hour and its disappearance

rate after two hours in the normal animal is very much higher than in the obese animal.

This difference in response and characteristic pattern is confirmed by the graph of insulin response in terms of percentage increase over fasting levels. (Figure 25B). The high fasting or initial glucose and insulin levels and the decreasing rate of disappearance, as observed by the two hour figure, appear to be characteristic of both the parameters measured.

Under conditions of starvation the insulin response of the lean animals was 540% of its fasting value at one hour and 275% at two hours while the obese animal had a response of 350% at one hour and 300% at two hours. Under fed conditions when the animals were allowed to feed ad. lib, the basal insulin levels were raised by 200% in the normal animal and its insulin response in relation to the new basal levels was 280% at one hour and 140% at two hours. In the obese animal the basal insulin levels were raised by 150% and the insulin response in relation to this basal level was 185% at one hour and 162% at two hours.

DISCUSSION

Insulin and Glucagon

The phases observed for body weight, blood glucose and serum insulin concentrations approximately coincide with each other particularly the first two which were observed in the same animals.

When this pattern, which resembles a sine wave, is considered against the background of the general instability of blood glucose values in the obese animal, it may be purely incidental and meaningless. However it may reflect the activities of glucagon and insulin and indirectly perhaps that of growth hormone. A similar oscillatory pattern of blood sugar may be observed in the results of Westman (1968). It is known that in man the rise in plasma glucose concentration after the oral administration of glucose is accompanied by a two to three fold rise in plasma glucagon and a four to ten fold rise in plasma insulin (Samols et al. 1965), and a fall in plasma growth hormone (Glick et al. 1965). Thus the secretion of large amounts of glucagon would elevate blood sugar and in turn insulin secretion. We have here two opposing effects - those of insulin and glucagon. The pattern of oscillation in blood sugar perhaps may be a reflection of which of these hormones is being more effective at any one time. Thus the alpha cells of the islets may be stimulated to hyperactivity which is followed by a phase of at least partial exhaustion, indicated by the falling blood glucose concentrations. During this period the cells are

regenerated at least partially and stimulated to hyperactivity once more as shown by the increasing concentrations of blood sugar. At the same time the beta cells show a similar cycle out of phase with the alpha cells, insulin being high when glucagon is low and vice versa. The three phases of the blood glucose oscillatory curve last 21 weeks, 15 weeks and 10 weeks respectively. In the first two phases, the recovery periods, if such they may be called, take 7 and 8 weeks respectively. The periods of hyperactivity take 14 weeks (probably longer since glucose measurements were made initially at 3 weeks) 7 and 9 weeks respectively in the three phases. The third cycle apparently has no recovery period as it is just a downward trend (Phase 4, weeks 46 to 64). Thus patterns of hyperactivity and hypoactivity alternate until finally the islets succumb and secrete decreasing quantities of glucagon. Blood glucose concentration too falls and insulin secretion falls, partially in response and partially from beta cell exhaustion. (Christophe et al. 1959). Insulin is lipogenic and is also antilipolytic in action. Thus when reduced quantities of insulin are secreted the balance of lipid metabolism is reversed, i.e. lipogenesis is decreased and lipolysis is increased. This is manifested by the loss in weight observed in these animals. The difference in time between the phases is probably indicative of the losing battle of the cells to secrete the hormones. This hypothesis is supported by the studies of serum insulin in these animals which have dropped below the levels that were measured in the young four week old animals and are within the upper

limits of the normal mice. At twenty weeks the insulin concentration in the obese was more than ten times that of the non-obese. Some obese individuals at this stage had serum insulin values at least twenty times that of their lean controls. This hypothesis also receives further support in that, Gepts et al. (1960) found that obese animals had a greater proportion of beta cells to alpha cells - they had 8% of alpha cells compared to the 17% in the lean animals. In addition, the alpha cells also showed signs of hypertrophy and degranulation. (Mayer et al. 1951.b.). Hellman (1965) found that a short fast of a few hours was sufficient to lower blood glucose and to bring about the regranulation of the beta cells.

The insulin resistance of the obese animals was noted by Mayer et al. (1953b) and was attributed to the excessive adipose tissue by Batt and Miahle (1966). Silides and Mayer (1956) demonstrated that the administration of insulin to obese and lean animals increased the incorporation of acetate into fatty acids and also abolished the differences in lipogenesis between the two groups of animals. Bates et al. (1955 d) found that although the rate of lipogenesis in both the obese and the lean animals was reduced after a fast of 24 hours, the obese animal still converted significantly more radioactive label into lipids than did the non-obese animals. This is probably the reflection of the very high concentrations of circulating insulin in the obese hyperglycemic animal. Hyperinsulinism has been associated with obesity by several investigators, but it has never been clear whether abnormality in insulin

secretion was the result of obesity or whether it preceded it. However, from the present investigation the indication is that hyperinsulinism probably precedes obesity and in fact it may actually be the cause. Hyperinsulinism and obesity are both present in four week old mice. However the circulating serum insulin is already 150% of the normal animals and onset of obesity is only just recognisable, although there is no appreciable difference in weight between the obese and non-obese. Hyperglycemia is sometimes but not commonly detected in the young obese animals at 4 weeks of age. By 5 weeks the serum insulin and weights of the obese animals are double those of the lean controls. Stauffacher et al. (1967) too found the presence of both hyperglycemia and hyperinsulinemia in young obese animals but these authors and Malaisse et al. (1968) found normal or reduced concentrations of pancreatic insulin in 5 week old mice. Stauffacher et al. (1967), Genuth (1969) and Wrenshall et al. (1955) found pancreatic insulin was high in mature obese animals. Westman (1968 a.) too found that circulating insulin was decreased in old animals. Westman (1968 b.) demonstrated the insulin degrading activity of the obese. Adipose tissue homogenates were eight times that of the controls. Thus hyperinsulinemia in these animals co-exists with the increased breakdown of insulin. The question arises what is the cause of the hyperinsulinism in these animals? The extreme resistance (up to 20 units of insulin had no effect) was demonstrated by Mayer et al.(1951 b.) and has been attributed

to muscle (Stauffacher et al. 1967) and adipose tissue (Leboeuf et al. 1961). However, it appears that insulin resistance may be prevented by food restriction right from the time of weaning and under these conditions the response of the obese animals to insulin is similar to that of the normal animals. (Batt and Miahle 1966). The presence of high or normal blood sugar together with hyperinsulinemia as observed in this investigation further illustrates the presence of some form of resistance in these animals. However the nature of this resistance has still to be characterized.
Goldstein (1961) described a factor which is produced by muscle in exercise which has insulin-like properties as seen by its ability to lower blood sugar. Exercise has been known to lower blood glucose and to cause hypoglycemia in insulin - dependent diabetic patients. However the nature of the factor is not known but its insulin-like property is demonstrated when the blood of exercising dogs was transfused into eviscerated nephrectomized resting dogs.

Stauffacher et al. (1967) also elucidated the problem of "bound" and "free" insulin. Their work suggests that "bound" insulin plays no significant role in this syndrome, and that the serum from these animals does not have any circulating antagonist or inhibitor of insulin activity.

There is evidence for the direct effect of glucagon on insulin release (Vecchio et al. 1966, Samols et al. 1966). Glucagon itself activates liver phosphorylase which results in the breakdown of glycogen to glucose, though this

effect has not been observed in muscle (Sutherland and Butcher 1965). Thus a functioning liver is necessary to maintain hyperglycemia. Shull and Mayer (1956b)did find evidence of a higher liver phosphorylase activity in the obese mouse. Williamson (1966) and Rizack (1964) demonstrated the gluconeogenic and lipolytic activities of glucagon, which are believed to be via cyclic AMP. Thus glucagon is able to elevate blood glucose which is useful during fasting. Like growth hormone it has diabetogenic properties.

Although insulin secretion is primarily regulated by glucose, it is also subject to other factors, e.g. growth hormone and adrenal glucocorticoids. Thus insulin hypersecretion may not necessarily be the result of a malfunctioning pancreas. It has often been observed in this laboratory that the adrenals of the obese animal are at least twice the normal size and this was also noted by Carstensen et al. (1961).

Growth Hormone

Defective growth hormone secretion has been suggested by several observations in these animals. Obese animals had less body protein and more adipose tissue even under conditions of food restriction (Alonso and Maren 1955). Growth hormone is an important lipid mobilizer under conditions of stress, e.g. food restriction and cold. In comparison to normal animals the obese are extremely sensitive to cold. If the laboratory temperature fell slightly, they appeared "shivery" and unhappy. In extreme cold (3°C) their body

temperature fell rapidly and they died within two hours. (Mayer and Barnett 1953). Even under conditions of starvation or food restriction, lipogenesis was significantly greater in the obese (Bates et al. 1955d). Under normal conditions the obese are resistant to ketosis. Clarke et al. (1956) found that the obese animals, already known for their hypersensitivity to growth hormone reacted with a further rise in blood glucose levels, glucagon and reduced pancreatic insulin within 24 hours of growth hormone therapy. Shull and Mayer (1956) found that fasting completely abolished growth hormone response and carbohydrate-free regimens significantly lowered it. The diabetogenic action of this hormone was measured in terms of blood sugar changes. Blood glucose plays an important regulatory role in growth hormone secretion. The lowering of blood sugar or hypoglycemia brought about by e.g. tolbutamide therapy (Hunter and Greenwood 1964) or insulin (Roth et al. 1963 b), or ordinary physiological conditions, e.g. exercise, fasting, exposure to cold, increases the circulating growth hormone concentrations. Thus the obese animal, e.g. under fasting conditions is probably protected against ketoacidosis by its relative lack of growth hormone response.

Growth hormone is also known to increase the breakdown of neutral fat to fatty acids and glycerol in the adipose tissue (Raben and Hollenberg 1963). These fatty acids are further oxidised by the liver to ketone bodies, these in turn cause insulin antagonism. (Bennet et al. 1948) (Randle et al. 1965)

by decreasing muscle uptake of glucose and enhancing the dependence of the muscle on the metabolism of fatty acids.

The effects of growth hormone on insulin synthesis or release are still obscure. Wagle (1966) demonstrated that hypophysectomy reduced the incorporation of amino acids into crude-insulin in vitro. The ability of growth hormone to raise blood glucose in the obese mouse (Clarke et al. 1956) man (Kipnis and Stein 1964) dog (Campbell and Rastogi 1966) cats and rats (Randle and Young 1956) might be the result of its action directly on the beta cell or just a secondary response to blood glucose changes. Whatever its action may be, the effects take sometime to develop. The sensitivity of some species or strains to growth hormone is probably the result of the ketosis caused by fat mobilization similar to that seen in diabetes. The hyperglycemia observed after growth hormone injection may be the consequence of the inability of the islets to continue secreting sufficient insulin.

Obesity and starvation may be said to be the opposite sides of the same coin in eliciting response from insulin, glucagon and growth hormone for metabolic regulation. Roth et al. (1963 a) and Beck et al. (1964) found that growth hormone response after glucose ingestion was reduced or non existent in obesity. This diminished response was also observed after exercise, after a period of prolonged fasting and in insulin-induced hypoglycemia in the obese. Decreased growth hormone secretion is conducive to obesity in that the lipolysis of adipose tissue particularly during fasting

is not elevated, thus plasma free fatty acids and ketones are not raised and the animal in turn is resistant to ketosis. In normal adults HGH secretion is stimulated by hypoglycemia induced by insulin (Roth et al. 1963). However, deficiency of growth hormone secretion is probably not a permanent feature of obesity; this was demonstrated by Lessof et al. (1966) in that growth hormone response to insulin induced hypoglycemia was normalized by weight reduction. Thus reduced growth hormone secretion appears to be a metabolic adaptation which could enhance the resilience of the animal under adverse conditions of food deprivation. The growth hormone, glucagon and insulin effects have had only preliminary attention in animals which are both obese and diabetic. The histological work done on these animals by Bleish et al. (1952) proved negative. However, it is not certain whether hyper or hypo-secretion, particularly in the early stages, is necessarily reflected or recognised histologically. This, however, helps to explain the lethal effects of exogenous growth hormone. It would be interesting to study the relationship between the onset of the syndrome and growth hormone concentrations. Growth hormone has also been attributed hypoglycemic or insulin-like properties (Ottoway 1953).

Development of the syndrome and selection of animals

It also apprears that the onset of the syndrome coincides with the weaning of the animals. When they were allowed a free choice of food they showed a preference for a high fat,

low carbohydrate diet which is strikingly characteristic of the composition of milk.

Once growth has ceased there is a diminishing of hyperinsulinism, weight stablizes and decreases accompanied by a decrease of hyperglycemia. It would be tempting to suggest the amelioration of the syndrome; however, when one considers merely the appearance of the animals just prior to death, one is inclined to attribute the cause of death to ketosis. The animals become exceedingly lethargic, the pelage had the appearance of dullness and dampness and the eyes took on a dim somewhat opaque appearance. Thus it seemed that the animals finally succumbed to diabetes per se. This corresponds very well with the observations of Christophe et al. (1959) and is confirmed by the increased impairment in glucose tolerance in the older animals. The evidence seen here lends further support to the concept that the diabetes which occurs in these animals is secondary to hyperinsulinism if not the result of it. Thus one wonders if this apparent amelioration of the syndrome during the static phase signifies a successful but temporary adaptation of the animal to the increased peripheral demand for insulin. This ability to meet this demand results in stabilization at hyperglycemic levels and the downward trend of blood glucose, insulin and weights suggests spontaneous remission of the syndrome but in fact is really representative of the losing battle. The primary cause or one of the major causes contributing to this picture is, as pointed out earlier, the insulin resistance of both adipose tissue and muscle of

these animals (Leboeuf et al. 1961; Stauffacher et al. 1967). Thus the peripheral demand finally results in the exhaustion of the pancreas.

The weights recorded for this strain of obese mice are higher than any recorded by any other laboratory but this is not surprising considering their genetic history. Diet has been shown to be an essential factor in the development and life span (Lane and Dickie 1958). The decrease in weight and hyperglycemia in the aged animals has not been reported before. In fact, Christophe et al. (1959) on the other hand reported pronounced hyperglycemia in the mature animals. Glycosuria even in traces was not a very frequent finding in this colony even at the climax of the syndrome (16 to 20 weeks). This could be suggestive of an increased renal threshold for glucose. Thus screening our colony for the early identification of these animals using this criterion was not infallible. The glucose tolerance tests were successful between the 23rd and 27th day. Danielsson et al. (1968) reported similar findings. However, positive results were obtained from a number of animals which later turned out to be heterzygotes instead of obese. This stresses that age is not the only criterion for the selection of experimental animals, and that the type of animal used as a control is also important. At this age it is not possible as yet to distinguish reliably between the lean homozygote (+/+) and heterozygote (ob/+). Thus it is obvious that this recessive obese gene shows either variable penetrance or is affected by other factors. However,

it is more probable that the heterozygotes are partially defective in some way, while the obese animals are totally defective. The above possibilities are further confirmed by the onset of hyperglycemia at variable times or the lack of onset of hyperglycemia observed in a few cases. Thus it is most important to select the homozygote (+/+) as the control animal to be absolutely safe. However, time did not allow for the selection of the lean homozygote, as this may be done only by breeding experiments, and commercially obtained albino mice (TO) were, therefore, used as controls for all the other experiments in this chapter and Chapters V and VI. Assurance was obtained that this strain was free of inherent obesity and diabetes. However, a slight degree of obesity was observed in a few old animals (not used experimentally) but this was probably due to the effect of aging.

Glucose tolerance and circulating insulin

The 16 week old obese mice showed moderate impairment of glucose tolerance and the 14 month old animals demonstrated a much more severe impairment. The normal animals however did not show any significant change. In this particular experiment the deterioration of carbohydrate metabolism in the old obese animals was observed in the diminishing control of basal blood glucose, Although the magnitude of the response at one hour and two hours is apparently the same. However, experiments to correlate the endogenous insulin response to the changes in blood sugar concentrations should be performed to predict with accuracy the actual progress of the impairment of glucose uptake in vivo,

i.e. there is a need to characterize this state of hyperinsulinism even further. This state is obvious in the young weanling animals of 4 weeks, whose insulin values are already nearly 200% of the lean controls. It is also equally obvious that endogenous glycemia is not necessarily a reflection of the degree of insulinaemia. This is observed particularly in the young mice where hyperinsulinism co-exists with normal blood sugar. However, the reverse may be observed in Figures 22 and 23.

The mean fasting serum insulin in the obese was ten times that in the non-obese. Although the absolute insulin response of the obese animals exceeds that of the non-obese during an oral glucose tolerance test, the relative response is diminished in the obese mouse. This is true also of a glucose tolerance test carried out after the animals were allowed to feed ad. libitum.

This places emphasis on the excessive circulating endogenous insulin in the obese animals in the presence of lower blood sugar concentrations and, therefore, its inability or lack of need to respond to increased exogenous glucose and the apparently reduced rate of insulin disappearance in comparison to the lean animals. It also illustrates an obvious point, i.e. the higher the starting level, the higher will be the absolute response, although the relative response is diminished.

We confirmed that the insulin response in terms of percentage increase over fasting levels (0 value) was greatly reduced in the obese animal. This also confirms that a deteriorating glucose tolerance is associated with a diminishing insulin response. The insulin response is similar both under fed and fasted conditions. Hyperinsulinemia is prevalent in both fasting and fed animals.

Conclusion

Unfortunately, we do not have obese non-diabetic animals and lean diabetic animals as part of the same strain as our studies could not dissociate the separate effects of obesity and diabetes.

Our results clearly indicate that hyperinsulinism definitely precedes hyperglycemia and probably obesity. The onset of the obese hyperglycemic syndrome is rapid. Glucose tolerance impairment is detectable in some animals including the heterozygotes as early as 20 days. This denotes tissue resistance to insulin. Hyperinsulinism and sometimes hyperglycemia are present in 4 week old obese animals, and increased weight gain is just observable. However, the young heterozygote animals which displayed impaired glucose tolerance were of normal weight and were no heavier than the lean normal littermates (+/+) up to the age of 12 weeks. Heterozygotes have so far been indistinguishable from the normal homozygote except by breeding experiments. Increased circulating insulin in the presence of normal blood glucose is already suggestive of the onset of relative tissue resistance. The hypersecretion of insulin is essential for increased body weight, deposition of triglycerides in adipose tissue and the prevention of the onset of ketosis for as long as possible. The cause of this hyperinsulinemia apparently lies with tissue resistance to this hormone and, therefore, the excessive peripheral demand. However the nature and cause of this resistance still needs to be defined and characterized. Further investigations at the

pre-obesity stage (i.e. between weeks 2 and 5) at the climax of the syndrome, (weeks 16 to 20), during the transistion period, (weeks 20 to 24) and at the terminal stage of the syndrome (approximately a year and later) might prove fruitful in enhancing our understanding of this syndrome.

Thus on the basis of these investigations it appears that the fundamental abnormality of insulin resistance is probably a phenomenon of obese tissue. It may indeed be the cause of obesity. Stauffacher et al. (1967) agree with this hypothesis while Chlouverakis and White (1969). Chlouverakis et al. (1970) on the other hand attribute insulin resistance to be secondary to obesity in this syndrome. Their conclusion is based on evidence suggesting increased fat deposition in 21 day old mice in the absence of increased immunoreactive insulin and blood sugar concentrations.

The symptomsof diabetes mellitus are probably the natural consequence of obesity resulting from the excessive and persistent imposition on the beta cell. This is clearly illustrated by a common observation that not all obese individuals are diabetic while insulin resistance is a feature of obesity. It has also often been observed that carbohydrate tolerance may be improved by weight loss or mere restriction in carbohydrate intake. Serum insulin response to carbohydrate challenge also improved in obese, non-ketotic diabetics on a controlled diet. Thus the pancreatic function is improved with regard to insulin secretion. The tissue resistance to insulin, however, is not absolute.

It is reversible in obese animals on a restricted diet and maintained at normal body weight. Batt and Miahle (1966) Chlouverakis and White (1969). Thus tissue resistance is apparently an adaptive feature with regard to excessive weight increase. It must obviously be related to some metabolic change or changes occurring during weight gain but which is apparently absent during weight loss. However, the impaired glucose tolerance in the young heterozygotes further suggests that insulin resistance in this strain of animals is a question of degree rather than whether the abnormality is existent or not. Obesity itself may, therefore, be merely a symptomatic manifestation of the factors involved.

From these observations it would appear that diabetes is the function of obesity. However, one may regard the whole problem in another light, in that the mature overweight diabetic individual is not so much diabetic because he is obese, rather he is obese because he is of a particular diabetic genotype.

FIGS.11 and 12. The weekly body weight and blood sugar of obese animals during their life time.

FIGS.13 and 14 The analysis of the above blood sugar estimations using the "Moving average" principle and the percentage change of blood sugar against the mean of weeks.

FIGS.15 and 16 The blood sugar and body weights of the heterozygote lean littermates

FIGS.17 and 18 The blood sugar and body weights of TO albino mice.

FIGS.19 - 21 The comparison of body weight of the two sexes of the obese homozygote, lean heterozygote and TO mice.

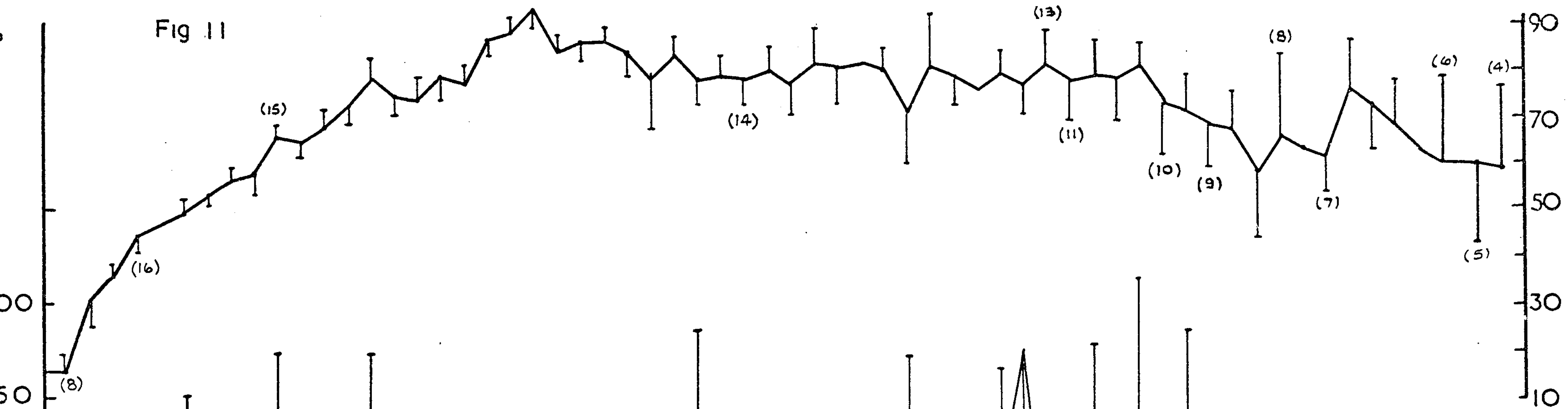


Fig. 11 & 12. Obese homozygote — blood sugars and weights with time

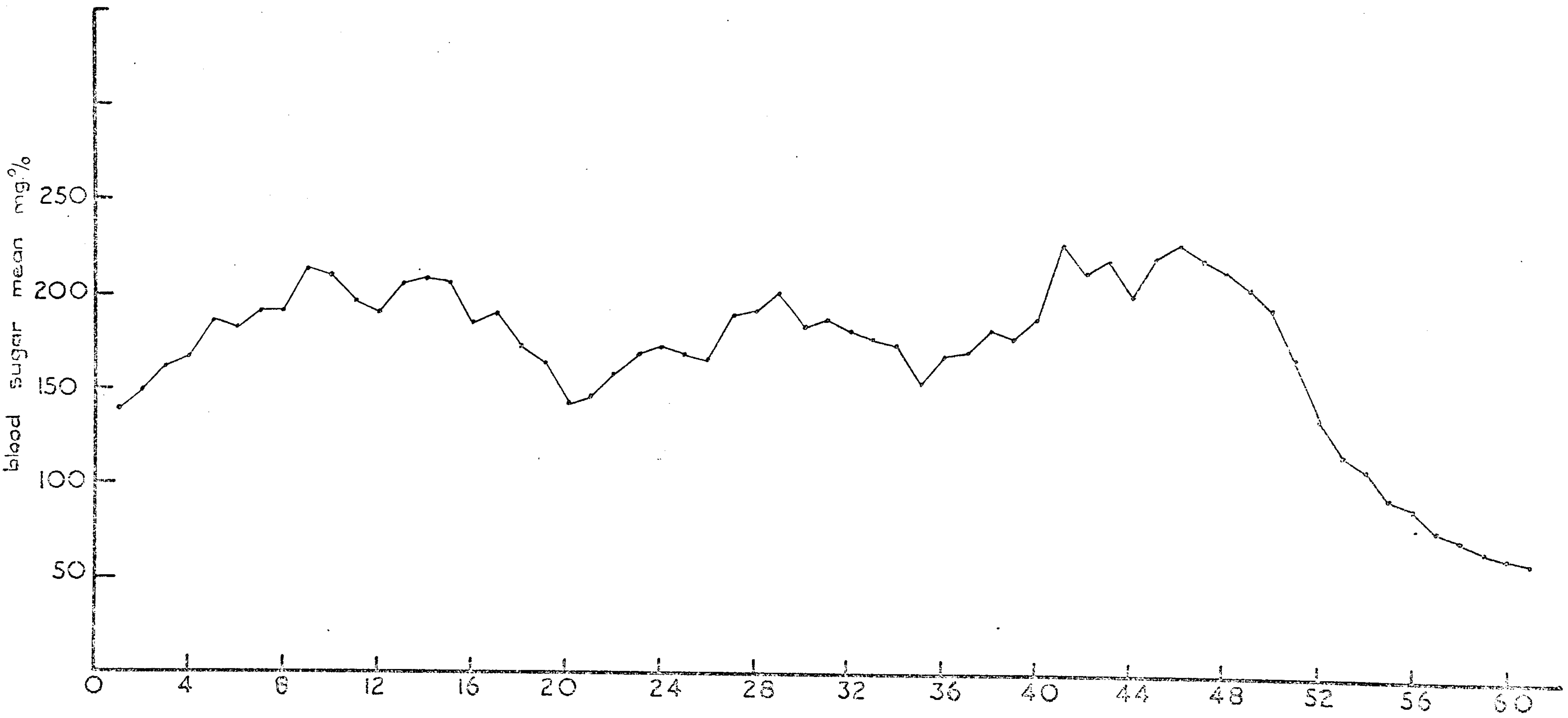


Fig 13 Obese mouse blood sugdr analysis - 'moving average' principle

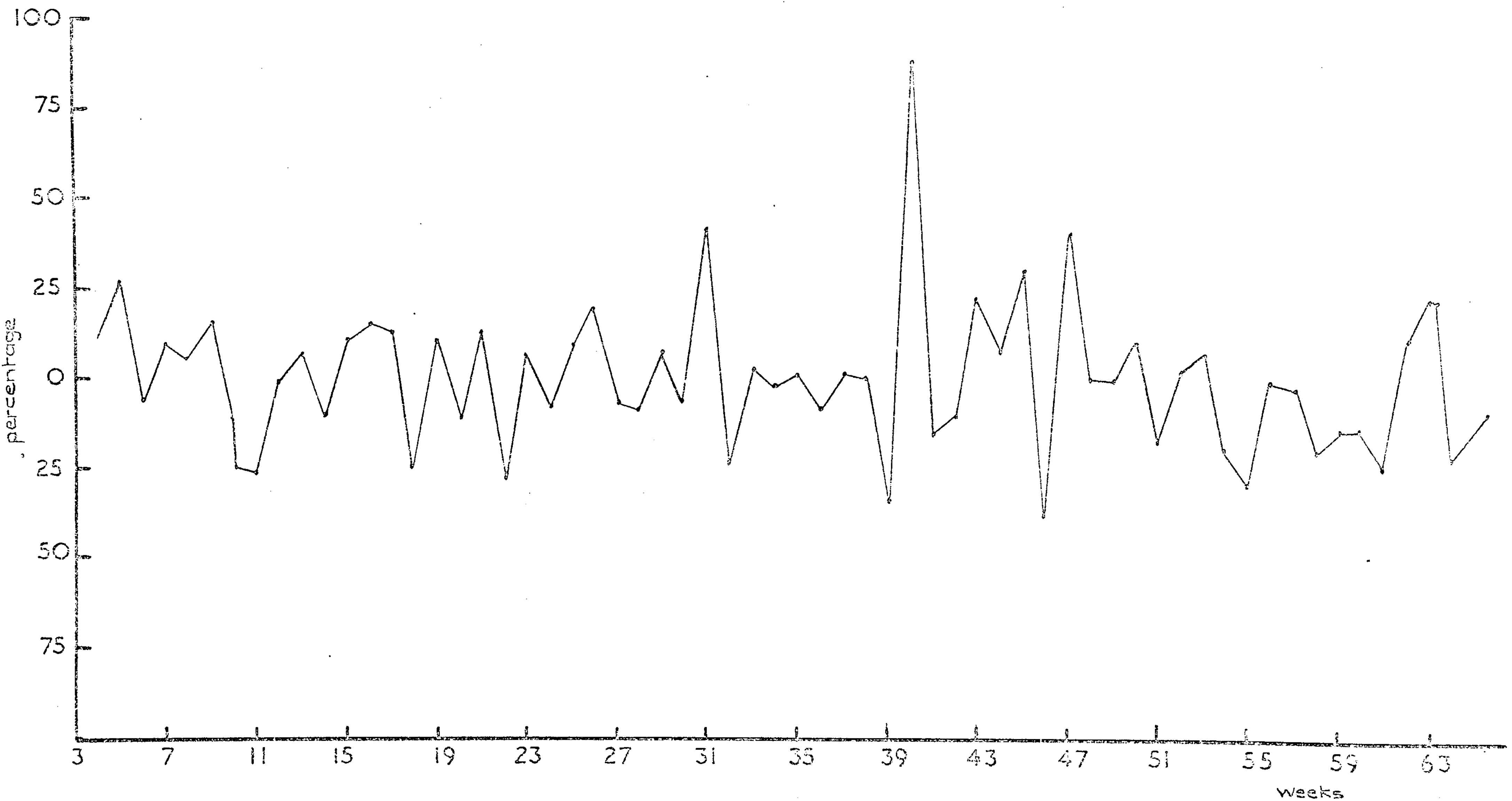


Fig 14 Percentage change of the mean blood sugar against weeks

Fig 15

Fig 16. Heterozygotes :— blood sugars and weights with time

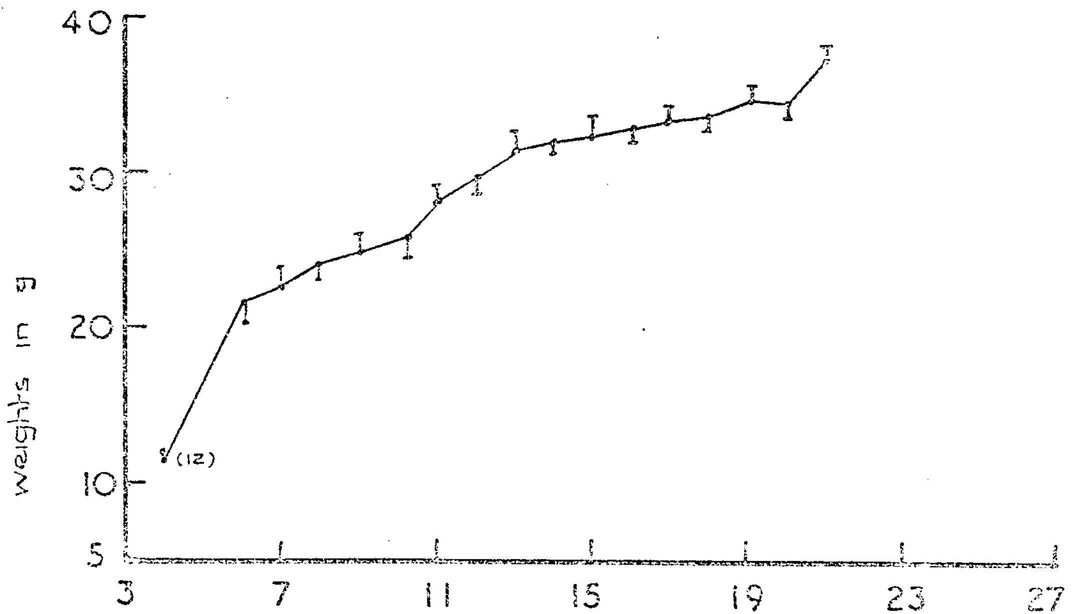


Fig 17.

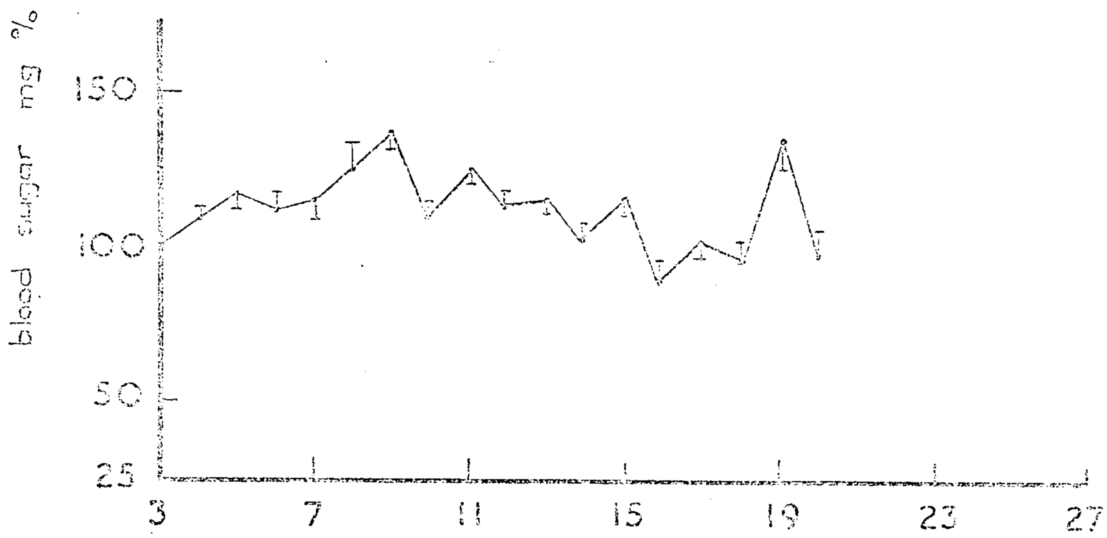


Fig 18 TO mice blood sugars and weights with time

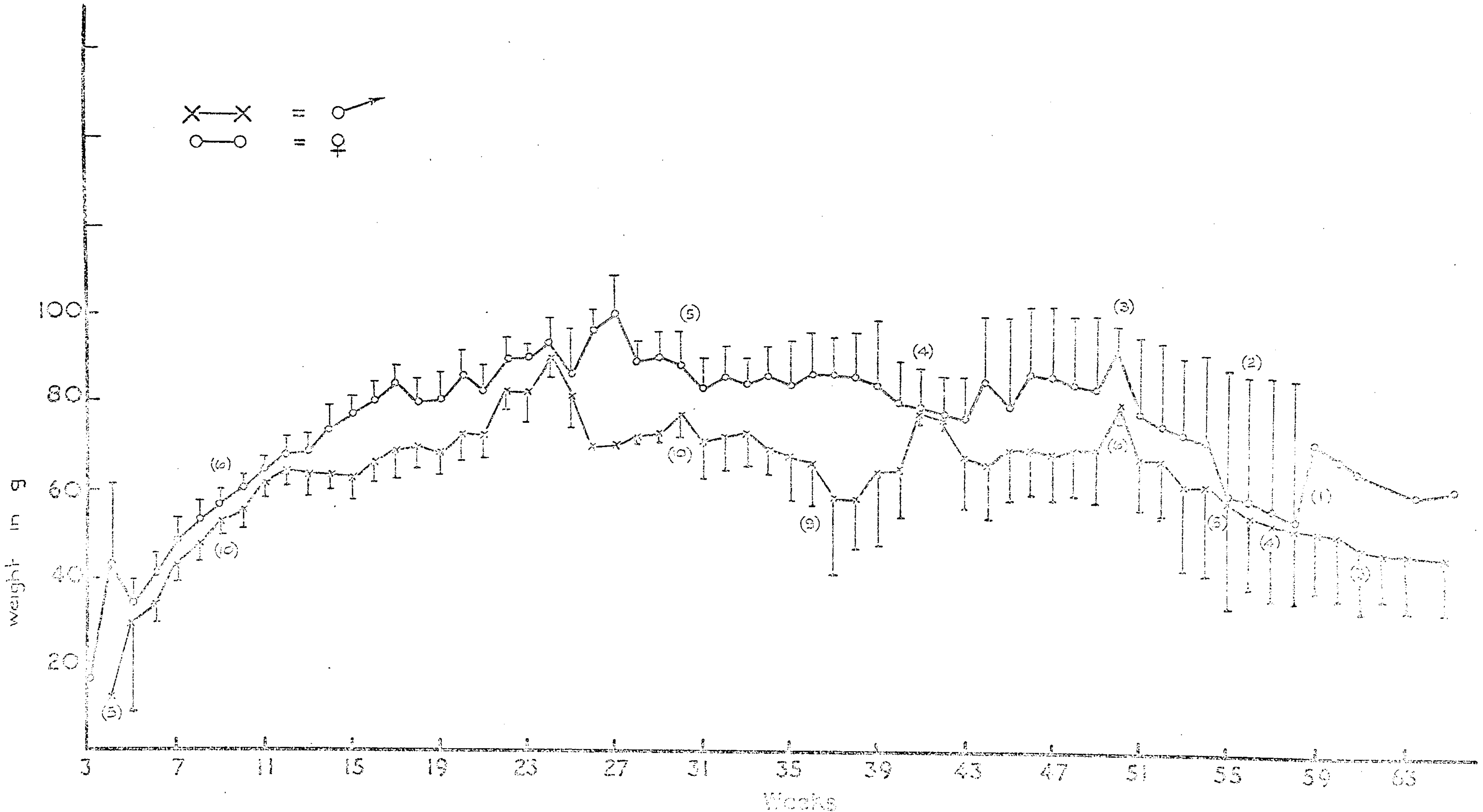


Fig 19 Obese mice – comparison of weights in the two sexes

Weights in g
40
30
20
10
3 7 11 15 19 23 27 31 35 39 43 47 51
Weeks
(9)
(1)
(4)
(5)
(3)
(5)
(2)
(4)
Heterozygote mice

Fig 20

x——x = ♂
o——o = ♀

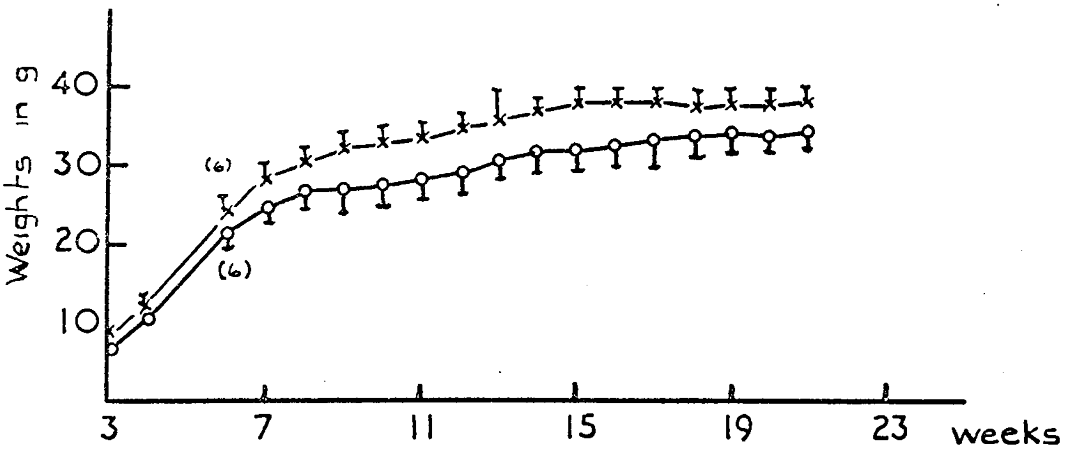


Fig 21 Comparison of weights in both sexes

FIGS. 22 and 23 The serum insulin and blood glucose estimations in the obese and TO albino mice between 4 weeks and 60 weeks.

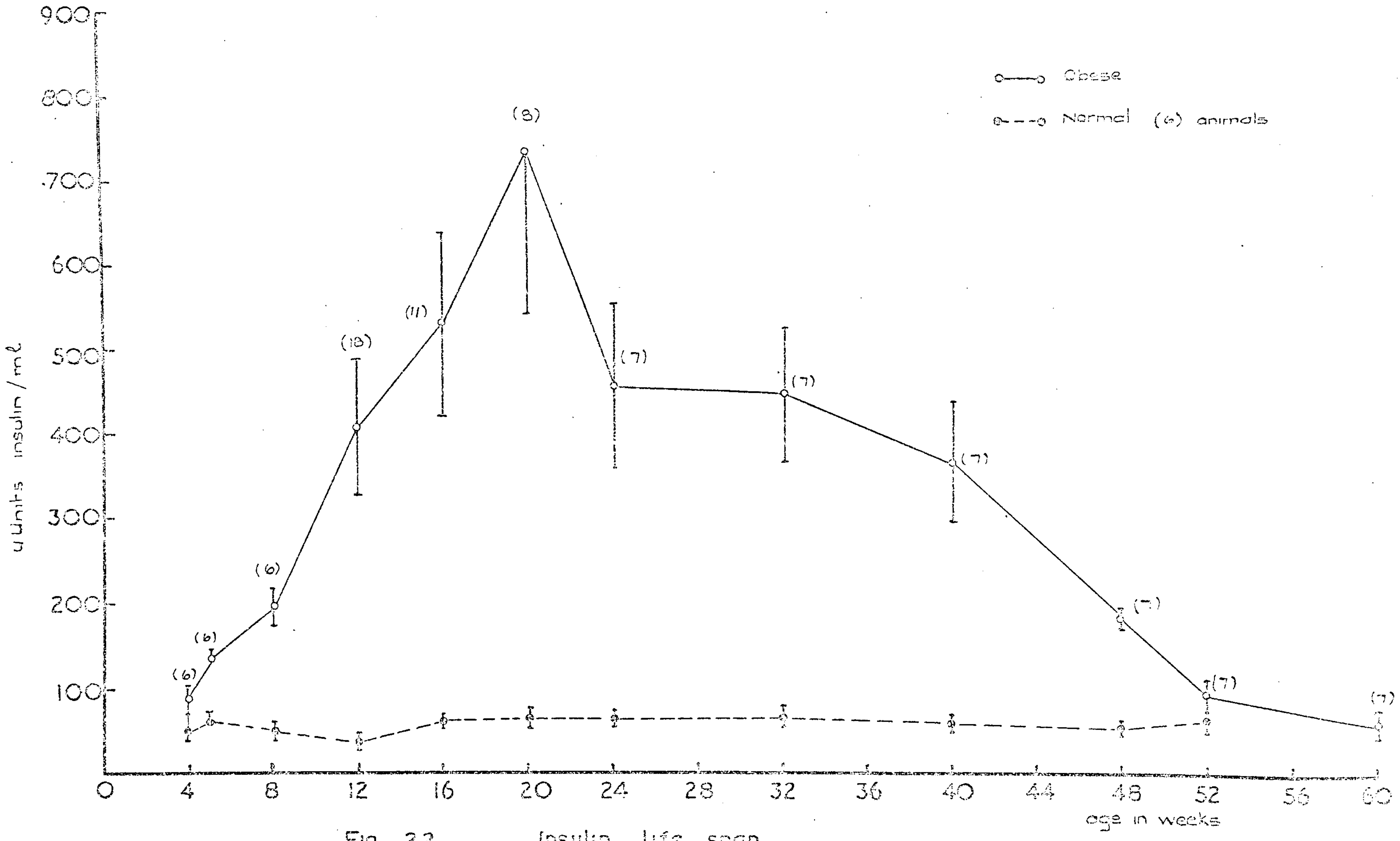


Fig. 22 Insulin life span

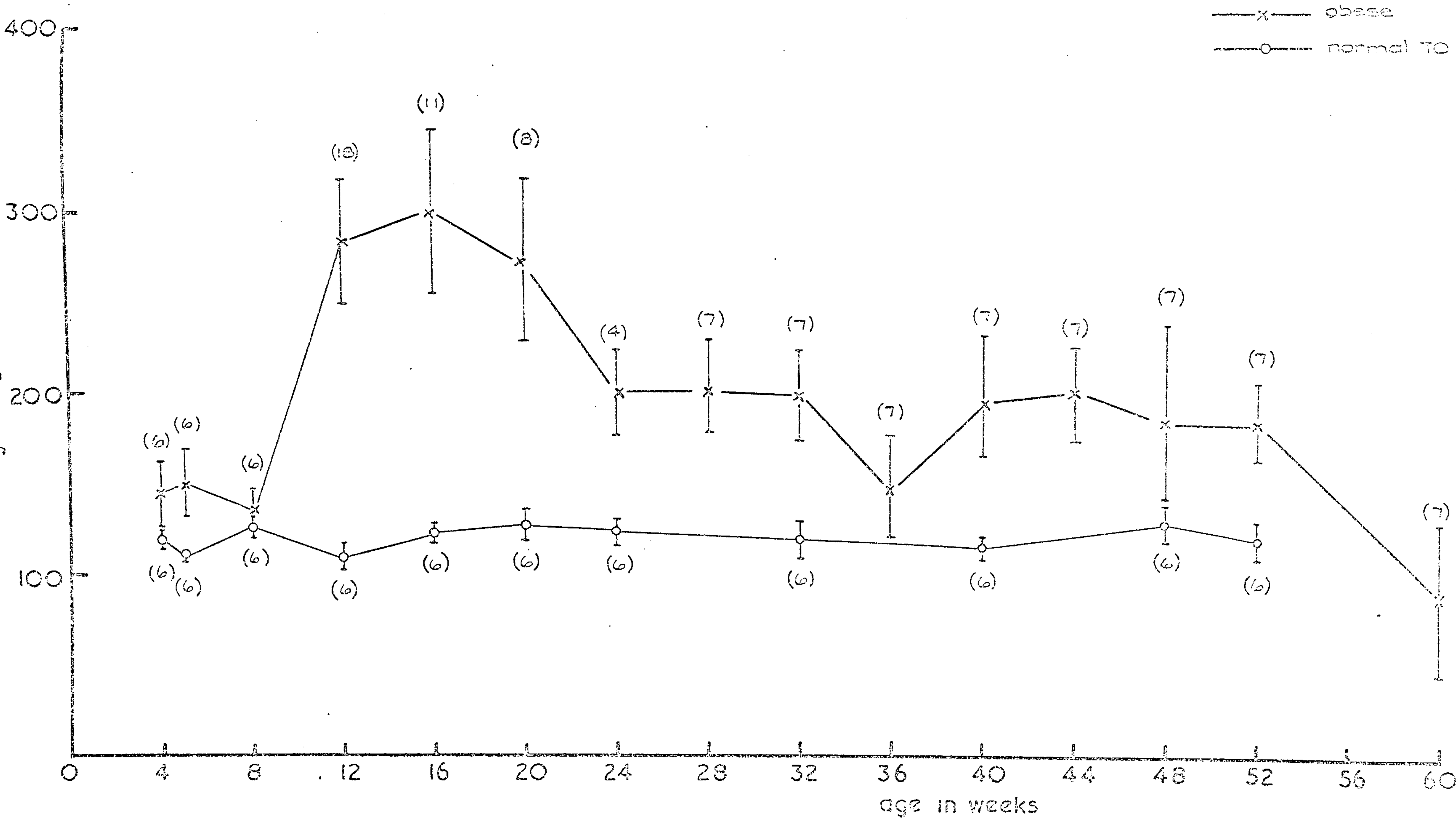


Fig. 23 Blood glucose concentration of mice used for insulin assay

FIG. 24. Glucose tolerance in 4 month and 14 month old animals.

FIGS.25 and 25A. Serum insulin measured after a conventional glucose tolerance test and one performed after *ad lib.* feeding. Insulin response measured as percentage increase over fasting values (0 value).

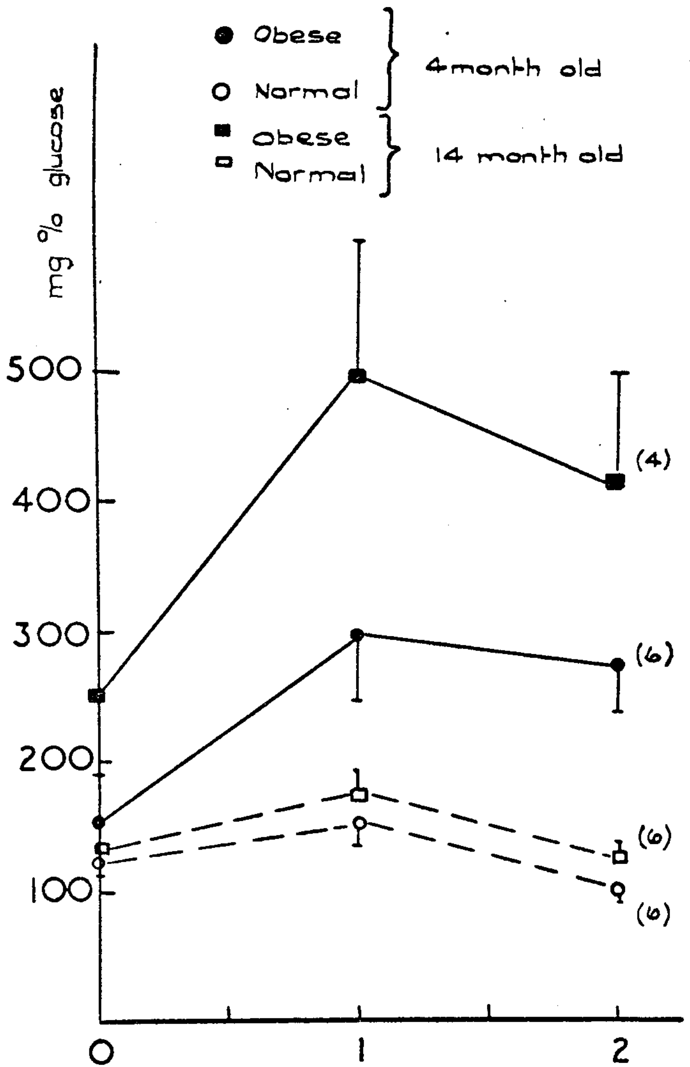


Fig 24
Glucose tolerance in 4 month old & 14 month old animals

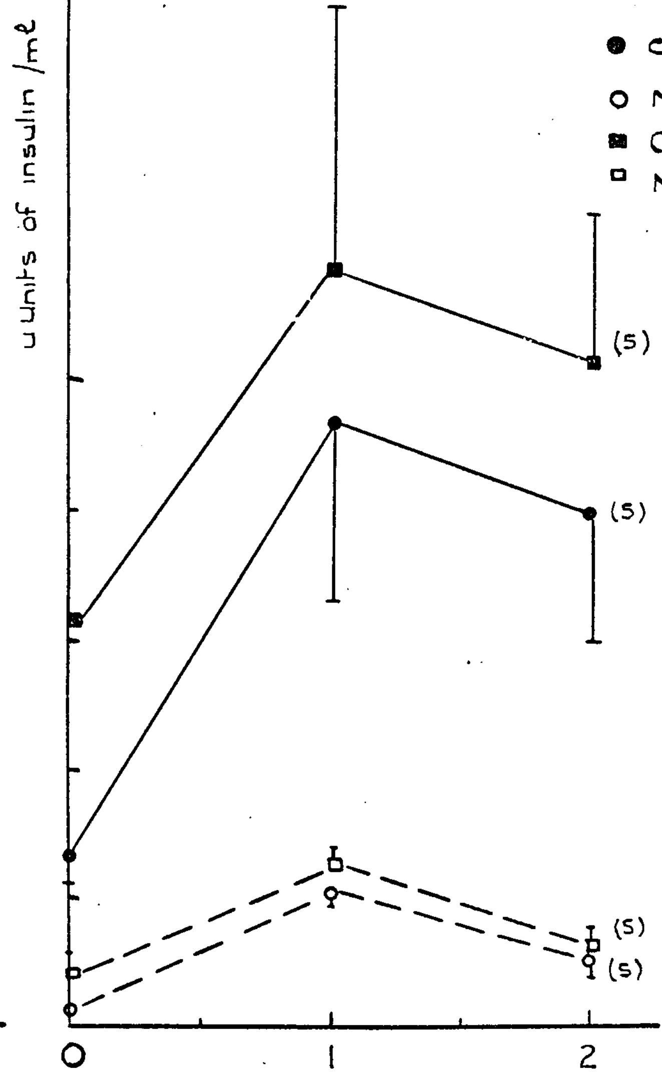


Fig 25
Serum insulin levels after a conventional G.T.T. & a G.T.T. performed after ad lib feeding.

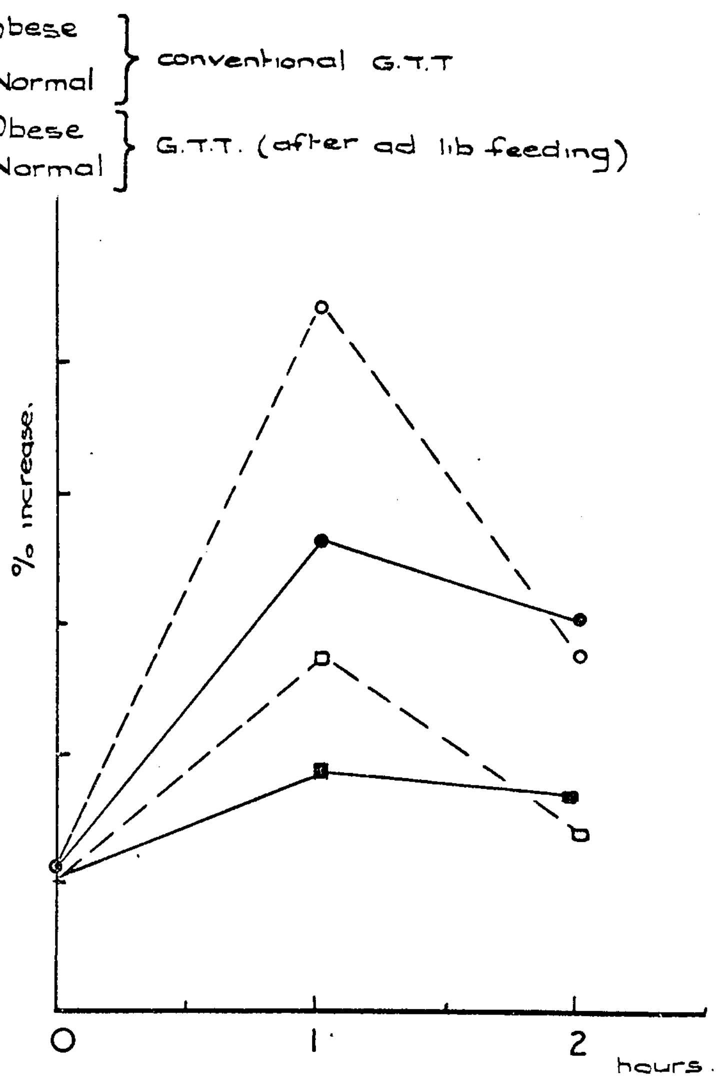


Fig 25 a.
Percentage insulin responce % increase over fasting levles (O value).

TABLE 6. Glucose Tolerance in the Young Obese Animals

Obese

Age in Days	Clinistix				Weight of Animals	Numbers of Animals
	+++	++	+	0		
20	0	0	2	0	11.0±0.8	6
23	3	2	1	0	11.5±0.7	6
25	5	1	0	0	11.8±0.9	6
27	6	0	0	0	12.0±1.3	6

Lean

Age in Days	Clinistix				Weight of Animals	Numbers of Animals
	+++	++	+	0		
20	0	0	2	4	10.6±0.78	6
23	0	0	2	4	11.4±1.2	6
25	1	1	1	3	11.8±0.9	6
27	1	0	2	3	11.5±0.8	6

CHAPTER V

ELECTROLYTE STUDIES IN ASSOCIATION WITH CARBOHYDRATE METABOLISM

Introduction

Sodium is the chief cation of extracellular fluid and potassium is the chief cation of intracellular fluid. Chloride is the principal anion of both the extracellular and intracellular compartments. The osmotic uniformity of the cells and body fluids is, therefore, dependent upon the movement of these electrolytes between the extracellular and intracellular compartments of the body as well as the balance between the intake and excretion chiefly of sodium and potassium. The movement of potassium into the extracellular compartments, including plasma, and back again into the cell compartment in exchange for sodium is a normal occurrence. However, under abnormal conditions, e.g. diabetic acidosis, abnormal renal loss, diarrhoea and dehydration, there is a loss of intracellular fluid, involving the mobilization of intracellular potassium to the extracellular compartments and then it is promptly excreted by the kidneys. This deficit is partially compensated for by the migration of sodium into the cells. However, any appreciable increase or decrease of potassium in the cells makes itself evident by the production of serious and even fatal disturbances in the body, e.g. myocardial function, muscle irritability and respiration. Potassium is irreplaceable as far as the functional activity of the cell is concerned, and it is certainly no exaggeration to state that the very basis of life itself is dependent on the maintenance of the balances between sodium and potassium in the cells and tissues of the body.

Electrolytes and the Pancreas

The role of ions including calcium in the secretion of a variety of proteins, both exocrine and endocrine has been known of for some time (e.g. Vogt 1952; Woodin and Wienke 1964) but Grodsky and Bennet (1966) were the first to draw attention to its direct action at the pancreatic level.

Until recently it has been generally accepted that glucose is both the normal and direct stimulus for insulin secretion, for which it was in fact believed to be indispensable. The hormone release is determined by the amount of glucose perfusing the islets of Langerhans (Filed 1964). The amount of insulin secretion is a function of glucose concentration, the minimal requirement being 50mg.% of glucose and the maximal requirement being between 170mg.% and 500mg.% glucose. The β cell degranulation produced has also been observed to be maximal in this range. (Grodsky et al. 1963).

Grodsky and Bennet (1966) demonstrated that the effects of potassium on insulin secretion were independent of glucose. Milner and Hales (1969) also showed that glucose was not a requirement for the action of various stimuli on insulin secretion. In recent years a number of other workers have demonstrated that insulin secretion is stimulated by fatty acids (Lefebore 1966,) amino acids (Sokal et al. 1964), keto acids (Sokal 1966) sulfonylureas (Levine and Mahler 1964) and in this last case tolbutamide is probably the best known (Coore and Randle 1964) glucagon (Turner and McIntyre 1966) and a variety of other agents.

In 1966 Grodsky and Bennet studied insulin secretion using the perfused rat pancreas and a synthetic perfusate of albumin buffer which enabled the controlled addition and omission of cations. They observed that no insulin release occurred when the potassium concentration in the perfusate was normal i.e. 4mEqK/L. However, when the concentration was raised up to 8mEq/L there was an immediate and sustained release of 8·5% of the pancreatic content of insulin. This was approximately 66% of the release obtained with 200 mg% glucose. Their results also established that calcium was essential to insulin release whereas magnesium was not. They found the calcium requirement for partial insulin release to be only 0·2mEqCa/L. Their results concerning potassium are at slight variance with those of Howell and Taylor (1968) who found that alteration of the potassium concentration of the medium between 3 and 8mEq/L had no effect on the rate of insulin release, but if the potassium concentration of the medium was decreased to 1mM/L inhibition of secretion in response to both tolbutamide and glucose was observed. However, in the presence of 10mM potassium the rate of insulin secretion was significantly increased. Howell and Taylor (1968) also demonstrated that when the glucose concentration of the medium was increased from 0·50mg/ml to 2·50mg/ml there was an increased uptake of K^{42} by the isolated islets of Langerhans (obtained by collagenase digestion). However, this was not the case with the tolbutamide - induced insulin secretion. Potassium uptake by the islets was inhibited by ouabain. Thus while Grodsky and Bennet (1966) feel that both

calcium and potassium act at the membrane Howell and Taylor (1968) are not entirely in agreement. They feel that there is the absence of conclusive evidence demonstrating the importance of electrical activity iniating insulin release and that the potassium concentrations required are unphysiological.

Milner and Hales (1967a) using incubated rabbit pancreas worked on the hypothesis that if insulin stimulation was due to the inhibition of the sodium pump then the absence of potassium from the medium should effectively inhibit the sodium pump and thereby stimulate insulin secretion. They found that the entry of sodium into the β cell seemed fundamental to the stimulation of insulin release because stimulation by L-leucine glucagon, tolbutamide, ouabain and glucose are all dependent on the presence of sodium. This restimulation by ouabain by reconstitution of the medium and stimulation of insulin release in a potassium-free medium has been interpreted as only secondary to a rise in the intracellular sodium concentration. Milner and Hales (1967 b) (in a brief communication) confirmed the importance of sodium in insulin secretion. They reported that potassium stimulation which was maximal at 55mEqK/L was reversible. When all the extracellular sodium was replaced by potassium there was an initial stimulation followed by an inhibition. On the basis of these observations the authors postulated that the β cell membrane is polarized and a rise of extracellular potassium depolarizes the cell membrane resulting in an influx of sodium into the cells and thus

stimulating insulin secretion.

Milner and Hales (1967 c) confirmed the work of Grodsky and Bennet (1966) about the absolute requirement of calcium and the non-requirement of magnesium for insulin secretion. They found the optimal requirement of calcium to be 2·64mM and also discovered that 10mM Magnesium actually inhibited insulin secretion.

In 1968 Milner and Hales also found that barium stimulated insulin release to be sodium dependent and that ouabain and potassium to be dependent on extracellular calcium for insulin secretion. Hales and Milner (1968 b) found that insulin secretion by barium was inhibited by 5·1mEq/L calcium. Barium acts as calcium analogue entering the cell in the same way and leaving the cytoplasm more slowly or by displacing membrane - bound calcium with the resulting increase of membrane permeability to calcium or barium. On this basis the authors felt that both sodium and calcium have an interrelated role since so far one or the other has been an absolute requirement for secretion. The authors suggested that they both act on the cell membrane or must enter the cell before insulin is able to leave the cell in response to stimuli. However, this cannot apply to the granules containing insulin. This has been demonstrated by Milnes and Hales (1967 c) who carried out their investigations on the foetal rabbit pancreas which contains insulin but not in granules and in this case too insulin secretion is dependent on the presence of calcium.

Bennet et al. (1969) studied calcium-magnesium antagonism in insulin secretion and found that magnesium is inhibitory or completely abolishes secretory response to calcium. This inhibitory effect can be counteracted by increasing the calcium concentration. Inhibition can be brought again by increasing the magnesium concentration. This behaviour supports the view that both ions must compete for the same active site on the granule membrane or cell membrane and when these sites are bound by magnesium the stimulatory or facilitating effects by calcium or insulin release are not permitted.

Curry et al. (1968) demonstrated that although calcium by itself did not stimulate insulin secretion it was indispensable for secretion in response to glucose and tolbutamide and that glucose induced secretion was dependent on calcium concentrations up to 4mEq/L. At levels greater than this insulin secretion seemed to become independent of calcium concentration and levelled off.

Milner and Hales (1969) in their investigations into the interactions of various inhibitors and stimuli of insulin release to further the understanding of their mode of action found adrenaline to be the most potent inhibitor. Unfortunately they did not study the stimulating effect of calcium in relation to various stimulatory and inhibitory agents used. From the preceding work it appears that calcium

is vital to insulin release. The effects of sodium have not been studied either although its importance has been demonstrated in the past. However their studies indicate that insulin release is probably not mediated by the nerves as demonstrated by the failure of tetrodotoxin to inhibit the various stimuli studied.

Malaisse-Lagae et al. (1969) in a short communication presented evidence that adrenaline inhibited calcium^{45} uptake by the isolated islets incubated in the presence of glucose and the authors feel that this finding supports their hypothesis that this inhibitory effect on insulin secretion could be due to a change in the metabolism of calcium in the β cell.

Thus it seems that many different things can stimulate or inhibit insulin release and the importance of electrolytes in insulin release has been established. The requirements of these various stimulants and inhibitors are not all the same but so far it appears that the presence of calcium may be essential to all. The role of calcium is unclear, since the lowest plasma concentration compatible with life may be much higher than that required to maintain the secretory system - hence the difficulty of experimental work <u>in vivo</u>. Sodium is important but it has not been shown to be essential and its relation with calcium is obscure. The whole subject is still not fully clear in

spite of a great deal of work in vitro. There is a need to look at the effects in vivo in the intact animal.

Glucose utilization

It is known that the stimulation of glucose utilization is accompanied by the shift of potassium from the extracellular fluid into the intracellular fluid. This is effectively demonstrated when insulin and glucose are administered to subjects with ketosis when hypokalemia can result in the early stages of treatment or after a period of carbohydrate restriction. The fall in potassium is also accompanied by the fall in inorganic phosphate in blood. The closely parallel changes of blood glucose, potassium and phosphate that follow the administration of insulin are dependent upon common cellular metabolic mechanisms involving phosphorylation processes and the formation of hexose phosphates during the course of glucose utilization and metabolism. (Briggs and Koechig. 1923. Harrop and Benedict 1924).

It is also known that when glycogen is deposited in the liver it is accompanied by potassium, water and acid soluble phosphate (Fenn 1939). The intimacy of the relationship between potassium and carbohydrate metabolism has also been demonstrated by Gardner et al. (1950). Potassium shifts into the cells also take place during protein anabolism and potassium moves into the extracellular fluid during protein catabolism. This situation also becomes

evident, e.g. in uncontrolled diabetes mellitus, when glycogen breakdown is increased due to the lack of insulin or lack of effective insulin action, and potassium is transferred from the intracellular to the extracellular compartments. This is characteristic in ketoacidosis-resistant maturity onset diabetes where peripheral utilization of glucose is impaired. The body attempts to increase glucose utilization by increasing blood glucose through hepatic overproduction of glucose or glycogen breakdown. Thus this hepatic gluconeogenesis and glycolysis and impaired peripheral uptake result in a rising blood glucose level. Then at least a relatively minor degree of glucose utilization is achieved (Butterfield et al. 1958). Thus changes in body water and electrolyte metabolism can be attributed to carbohydrate protein and fat metabolism with resultant hyperglycemia and accumulation of ketoacids and ketones. Thus on the basis of this picture it will not be unreasonable to expect potassium depletion in diabetics to be higher than normal.

Apart from the metabolic activities of the cells and tissues the electrolyte homeostasis of the blood is determined primarily not by what is ingested but by what the kidney will retain.

Renal regulation of the ions

Body sodium, potassium and chloride are derived solely

from the diet and are practically completely absorbed from the gastro intestinal tract, less than 2% sodium and 10% potassium being eliminated with the faeces. In subjects with diarrhoea large amounts are lost with the faecal matter, owing largely to the failure of reabsorption of the constituents of the digestive fluids. Sodium and chlorides are eliminated chiefly in the urine and to a lesser extent in the perspiration. Potassium is normally eliminated almost quantitatively in the urine. The quantity ingested is generally in excess of the normal body requirements and the constancy of the plasma levels of these electrolytes is primarily dependent on the capacity of the kidney to vary the excretion of these ions. These ions are removed from the circulating plasma by glomerular filtration.

Under normal physiological circumstances 99% of the sodium is reabsorbed into the blood stream by the tubular epithelium. The reabsorption is thought to take place in two stages.

1. A proximal process involving a reabsorption of 80-85% of sodium.

2. the distal tubule reabsorbs the rest and thus exhibits a limited maximal capacity of sodium reabsorption.

The proximal reabsorption of sodium is believed to be an active process and that of chloride a passive process. (Pitts. 1960-1961; Giebisch and Windhager 1964). In common

with the rest of the body cells, the cells of the proximal tubules have a low sodium content and a high potassium content. Thus any sodium which diffuses into the cell must be pumped out to keep the internal sodium concentration low. Simultaneously the potassium which diffuses out must be pumped back into the cells again, to maintain the high internal potassium concentration. A potential difference exists across the tubular wall, the luminal wall being relatively negative to the outside wall. The chloride migrates passively along this electrical gradient from negative to positive.

However, when the solute load and therefore osmotic diuresis increases the usual rapid passive reabsorption of water is inhibited and thus sodium reabsorption is impeded due to dilution. Thus the distal tubule is faced with excessive sodium. (Smith 1951). The adrenocortical hormones, particularly aldosterone increases the reabsorption of both sodium and chloride and the excretion of potassium. In the distal tubule 1-2% of the sodium reabsorption occurs in exchange for the hydrogen ion or potassium ion. This is believed to be a competitive process. Thus the amount of potassium excreted is dependent upon the requirement for urine acidification, e.g. potassium excretion is increased during alkalosis (extracellular) when the requirement for urine acidification is low and decreased potassium excretion occurs e.g. during acidosis when the

requirement for hydrion excretion becomes urgent and the requirement for urine acidification increases.

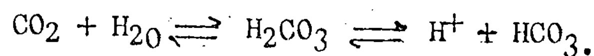
During a period of sodium restriction the urinary sodium excretion falls to minimal levels. It has also been demonstrated that high plasma sodium results in decreased tubular reabsorption of sodium. (Mudge 1967). Recently it has been demonstrated by Rector et al. (1966) that increased sodium reabsorption is brought about by an increase in the diameter of the proximal tubule whereas a decrease in diameter results in a diminished reabsorption of sodium. (Brunner et al. 1966). Thurau and Schneenan (1965) demonstrated the glomerulo-tubular relationship when a microinjection of hypertonic saline in the distal tubule led to the diminished filtration of sodium in the corresponding glomerulus, indicated by collapse of the proximal tuble.

However, in contrast to the effective renal conservation of sodium, potassium conservation is relatively poor. Appreciable amounts of potassium are still excreted during the dietary restriction of potassium and this increases body potassium deficits. Potassium is completely filtered at the glomerulus, virtually reabsorbed during the proximal process and actively secreted into the distal tubule, unlike sodium. (Berliner 1950 Giebisch and Windhager 1964). Some potassium reabsorption is also believed to take place along the collecting duct. (Hierholzer 1961). Urinary potassium is ascribed an intracellular rather than an extracellular source. Black and Emery (1950) give

evidence that urinary potassium concentration resembles the potassium present in the renal cells. In potassium depletion the renal cells show changes in potassium concentration similar to that of other tissue, so that a regulation of potassium excretion based on renal-cell potassium would not be far removed from the general state of potassium balance.

Hydrogen ion

In considering the electrolyte balance of the body, the hydrogen ion homeostasis cannot be ignored. The oral intake of hydrogen ion is negligible and it is generated during the oxidation of tissue and food. Unlike the above mentioned elements it is constantly emerging or disappearing during the multitude of complex biochemical reactions in the body. Carbon dioxide is produced by the combustion of proteins, carbohydrates and fats. This carbon dioxide is generally got rid of by the lungs but, if too much carbon dioxide is produced or if respiration is impaired it can be retained. It dissolves in plasma to form a weak acid, carbonic acid which on dissociation forms hydrogen and bicarbonate ions-



$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3.$$

However, the incomplete oxidation of fats and carbohydrates gives rise to organic acids - aceto acetic acid, β-hydroxybutyric acid, pyruvic and lactic acids.

An influx of these acids into the blood is at first neutralized by the blood buffer (bicarbonate principally) so that the blood pH remains reasonably constant. An excess of acid will ultimately exhaust the buffering capacity and at this point blood hydrogen ion concentration will increase and the pH will fall. The responsibility of removing hydrion associated with the metabolic production of these weak acids and others, e.g. sulphuric acid, from degradation of cysteine falls upon the kidney.

Thus these anions are normally present in the body as salts chiefly of sodium and potassium in the body fluid and are therefore balanced by an equivalent concentration of the cations. These anions do not combine significantly with hydrion while the bicarbonate and proteinate anions do within the normal physiological range of pH (pH7.3 - pH.7·52) (Shock and Hasting 1934).
However carbonic acid, protein (albumin, oxyhaemoglobin, plasma proteins) etc. which act as buffers in the body easily dissociate and reform within the normal physiological range of plasma pH. Consequently, the concentration of these anions change when the plasma pH is altered and hence the concentration of the cations they balance also changes. Therefore, the anions are present in the body fluids as both undissociated acids and salts. The bicarbonate anion (partial carbon dioxide gas pressure of about 40 mm. Hg and pH.7.4) acids and salts. The bicarbonate anions

represent the difference between the cations and the other anions e.g. protein chloride etc. Thus a constant bicarbonate concentration and therefore a constant H^+ concentration is dependent on the regulation of these electrolytes, chiefly sodium and chloride.

Metabolic acidosis caused by the accelerated breakdown of fatty acids can occur in starvation and uncontrolled diabetes. Hepatic production of these ketoacids exceeds urinary excretion which is only $^1/7$ of the total production. Utilization of acetoacetic and beta-hydroxybutyric acid by the tissue, accounts for the difference. Acetoacetic acid also spontaneously decarboxylates to form acetone to a limited extent. The diabetic animal can also suffer a moderate impairment in the oxidation of these acids.

Thus if the organic acid cannot be oxidised the disposal of hydrion as explained previously rests with the kidney while the organic anion is excreted along with an equivalent quantity of sodium or potassium. Thus in the normal animal the hydrogen ion is not retained in the body to any appreciable extent and if it is, it is removed either as carbon dioxide or in preferential exchange for potassium in the distal tubule.

The renal excretion and retention of hydrion by the normal kidney is associated with the selective excretion of sodium, chloride and bicarbonate ions and is directed

towards the maintenance of the normal ratio of sodium to chloride or, in other words, the maintenance of constant HCO_3^- and H+ concentrations is dependent on the regulation of the sodium and chloride concentrations. As already stated before the source of H^+ and HCO_3^- may be of metabolic origin and their quantity is subject to changes. The H^+ reacts with the urinary HCO_3^- to form carbonic acid, and this gives carbon dioxide which diffuses out of the tubular fluid and into the peritubularblood. This is one form of urinary acidification: (Pitts and Alexander 1945; Brodsky and Carrasquer 1962; Pitts 1961). Hydrion secretion also involves an exchange for sodium ions which are reabsorbed in the proximal tubule. The reabsorption of filtered bicarbonate ions in the proximal and distal tubules too depends on this exchange of hydrion for luminal sodium. (Rector and Carter 1963, Rector et al. 1965, Gottschalk 1963, Clap et al. 1963).

The ability to secrete hydrion also depends on the renal capacity to form ammonia, primarily from glutamine. Ammonia is also formed in muscle and gut and is converted to neutral urea, mainly by the liver and excreted. Ammonia accumulates in the blood in the event of liver failure. The kidney forms ammonia in response to acidosis. The ammonia combines with the hydrion and is excreted as NH_4^+. This forms a major vehicle for H^+ excretion.

The capacity of H^+ excretion is dependent on ammonia formation and on the amount of buffer excreted, principally phosphate. Thus the phosphate and ammonium buffer system as the resultant of the formation of conjugate pairs effectively bring any hydrion excretion and pH remains stable provided the hydrion supply or depletion is not excessive.

Maxwell and Kleeman (1962) assumed that approximately half of the infused ion is buffered intracellularly and the rest is buffered extracellularly. (Tobin 1956 and 1958, Winters et al. 1958, Forbes et al. 1959). Nichols and Nichols (1956) found that skeletal muscle and bones were the main tissues concerned with pH stability. During the infusion of acid there was a fall of plasma pH and bicarbonate ion and a rise in Na^+ and K^+ resulting probably from the movement of the hydrion intracellularly. When acid loading is prolonged the bone releases chiefly Ca^{++} (Leman et al. 1965 and 1966).

Thus Hydrion excess contributes to the clinical picture of acidosis by the loss of intracellular electrolytes i.e. potassium, magnesium, phosphate and calcium and sodium and extracellular sodium in the urine. The cell is surrounded by an environment of increased hydrion concentration and as the result of faulty ion transfer mechanisms, it has difficulty maintaining the extracellular-intracellular gradients.

The lowering of intracellular pH releases bound potassium and magnesium and hydrolyses certain organic phosphates.

Raison d'être

Although the relationship between carbohydrates and potassium has been under investigation for about half a century, there is still some misunderstanding and uncertainty as to the result of the existing controversial reports on the subject. In addition, comparatively very little work has been done in this connection in terms of obesity and diabetes, and there is no available data on electrolytes in mice.

In order to study the relationship of the potassium ion and carbohydrate metabolism in vivo one must consider the effects of the sodium ion since the behaviour of these two bulk ions in the body are very closely linked and dependent on each other.

As a result of the lack of information the present work began very basically by studying several parameters:-

1. the daily blood glucose acetoacetate and weight response of animals on varying diets
2. glucose tolerance
3. serum insulin, sodium and potassium
4. the response of serum insulin to exogenous potassium chloride over a time period of 30 minutes, one week and two weeks.
5. The disappearance of serum potassium resulting from the adminstration of insulin

6. the response of blood glucose to exogenous potassium chloride

7. the sodium, potassium, glycogen and water content of liver, muscle, pancreas and kidney

Materials and Methods

16 week old obese hyperglycemic animals and lean TO animals were maintained on a normal diet (41B), or low potassium and sodium diets, high potassium and sodium diets. The low potassium diet caused more gastrointestinal disturbances, so that the animals suffered malnutrition, even though the diet was nutritionally adequate. Low potassium intake was, therefore, simulated by feeding a normal diet (41B) with 2% sodium chloride. In this way it was hoped to achieve depletion of body potassium without the gastrointestinal disturbances and to separate the effects of potassium and of malnutrition. By the use of these diets primarily three situations have been created - normal, high potassium and low potassium. The forty-eight hour fast was initiated to facilitate depletion. The details of the diets are given in Chapter II (pages 56-58).

The urine was collected using the metabolic cages described in Chapter II (page 46). Glycogen was hydrolysed to glucose and measured by the fericyanide method. Blood glucose and blood acetoacetate were simultaneously determined chemically on the autoanalyser (Chapter II page 60). Urine blood and tissue electrolytes were determined on the flame photometer (Chapter II (page 64).

Qualitative and semi-quantitative urine analysis for blood, ketones, glucose, protein and pH changes were carried out with Labstix strips.

Results

Daily Blood glucose, acetoacetate levels and body weight (Fig.26 - 29).

41B Diet

The experiment was carried out for eleven days. The animals, after the normal forty-eight fast carried out on all the treatment diets were refed the 41B control diet. As seen from Fig.26, during the forty-eight hour fast, the blood glucose concentrations and body weights dropped and acetoacetate went up as expected and on refeeding blood sugars and body weight went up to their original levels and acetoacetate dropped again as expected in the normal group of animals. In the obese animal the same effects are seen except that the aceto-acetate levels dropped during the fasting period.

The blood glucose range in the normal animal is very narrow in comparison to the obese animals inclusive of the fasting sugars. The range in body weight of the normal animal is half as much as the obese animal. The body weights of the obese animals of 16 weeks of age range between 55g and 75 g. There is not much difference in the acetoacetate levels between the two groups, although the concentration in the obese animal in the fed state on the second day is higher.

Sodium Deficient Diet (Fig.27)

As seen in Fig.27 the blood glucose and acetoacetate do not show very much change except at fasting when blood glucose falls and acetoacetate goes up at forty-eight hours, for the

normals particularly. They both show an initial fall at twenty-four hours. Body weights too remain fairly constant, falling during the fasting period. In fact body weights have almost gone back to what they were before treatment and the blood glucose of the obese animals has not returned to its high pre-treatment values. The blood glucose in the normal animals shows a slight increase.

<u>High Sodium diet</u> (Fig.28.)

As seen in Fig.28 the blood glucose values of the non-obese group drop with fasting. However, the acetoacetate concentration of the normal animals dropped during the first twenty-four hours and then rose. The acetoacetate levels of the obese animals rose with fasting at twenty-four hours and the acetoacetate of both groups dropped when they were refed. However they tend to fluctuate greatly from day 7 to day 16 when the experiment was terminated. The body weights fell as expected with fasting and weight gain thereafter was constant and slow but the pre-treatment levels were never reached.

<u>Low Potassium diet</u> (Fig.29.)

As may be seen in Fig.29 the blood glucose levels of the obese animals rose after the first twenty-four hours of fasting and then fell again and continued to the end of the experimental period without too much fluctuation. The acetoacetate rose during fasting and then fell when they were refed but showed a gradual rise again from day 6 to day 16 when the experiment was terminated. Body weights fell during

the fasting period and continued to fall steadily but almost unnoticeably till the end of the experiment, 30% in the obese and 20% in the non-obese. At the end of the experiment the obese animals were about 75% of their original weight and the normal animals were 80% of their pre-treatment weights. The normal animals exhibit a tendency towards hyperglycemia and an increase in acetoacetate concentrations. As for the obese group, the blood glucose has not reattained pre-treatment values. The increase in the blood aceto-acetate is about 70% in comparison to the normal animals.

Glucose Tolerance Tests (Figs.30A & B - 34 A & B)

In this series of experiments the animals have been used as their own controls. The post-treatment (experimental test diet) graphs are compared with the pre-treatment ones (41B control diet) as well as, for example, fasting values with the one and two hour values of the same graph.

The control or handling experiments, Fig.30 C & D - Fig.34 C & D have been carried out similarly except that the animals have been administered water instead of glucose.

41B Diet

The normal lean TO mice showed the normal response (Fig.30B). The two hour value is 8% lower than the zero or fasting value. The peak reached at 60 minutes is 160 mg% which in terms of humans is approaching the margin between diabetics and non-diabetics, the range of sugars in the animals being between 140 mg% and 200 mg%. The acetoacetates however do not show any particular pattern during the glucose tolerance tests. The glucose tolerance curve is characteristic for each animal at least between 16 weeks and 18

weeks of age. The weights have remained constant. The urine pH is normal ranging between pH 5.5 and pH 6.5. Two of the animals had transient glucosuria at the 60 minutes period, otherwise blood, ketones and glucose were not present in the urine. Proteins are present in trace quantities.

In the handling experiment or the control experiment (Fig. 30 C & D) the glucose and acetoacetate curves show great stability. The acetoacetate also exhibits a gradual increase from 0.19 mM (fasting values) to 0.22 mM at two hours.

The obese animals (Fig.30A) exhibit the pathologically diminished glucose tolerance curves of the diabetic. The fasting values were about 160 mg% with the individual values ranging between 104 mg% and 220 mg%. The peak values are reached at 60 min. at about 360 mg%, the values ranging between 200 mg% and 560 mg% and the two hour value is 280 mg% which is 75% higher than the fasting values. The individual animals here too tend to exhibit a characteristic glucose curve, each peculiar to itself. The acetoacetates range between 0.19 mM and 0.21 mM but the individual values of each animal during the test tend to fluctuate a great deal. Weights have remained constant during the two-week period. Urine pH is betwenn 5.5 and 6.5. Slight proteinuria is seen in all animals. Haemoturia was present in one animal only but it had the lowest glucose tolerance curve. Urine ketones were absent in all. Glucosuria was present to a varying degree in a few of the animals at zero hours but at one hour was present in all the animals.

Sodium deficient diet (Fig.31)

The glucose tolerance of the obese animals (Fig.31A) showed a marked improvement after being put on a low sodium diet - Fasting ($p<0.05$),8%; one hour ($p<0.2$), 3%; two hours ($p<0.001$), 29% in comparison to the pre-treatment values. The post treatment fasting value however represents a deterioration.

The glucose tolerance of the non-obese, normal controls (Fig.31B) showed a similar if not a greater improvement - Fasting ($p<0.3$), 7%; one hour ($p<0.001$), 25%; two hours ($p<0.001$), 33%.

The post treatment acetoacetate in the obese group (Fig. 31A) decreased slightly and in the normal animals by almost 50% at one and two hours and by about 36% at the fasting or zero value in comparison to the control pre-treatment curve.

In the obese animals the pre-treatment urine pH 6.0 rose to pH 6.4 post-treatment and in the case of the normal animals, pre-treatment urine pH 6.2 rose to post-treatment values of pH 6.6. The weights of both normal and obese animals were constant. Glucosuria was present in three of the obese animals on the pre-treatment 41B (normal) diet, and glucosuria was absent in the other three animals. Of these one animal obviously had a high renal threshold. After being maintained on the experimental diet two of the animals showed a marked rise, between 25% and 50%, in the fasting blood sugar. One of these animals showed a deterioration of glucose tolerance throughout the test, i.e. at one hour and two hours as well while the other animal

showed an improvement at two hours. All the other four obese animals improved to a varying degree on the experimental diet. Proteinuria showed a slight improvement with treatment and ketonuria was absent in all the individuals including the normal animals. Glucosuria was present in two of the normal animals on the 41B diet one hour after glucose administration and these animals also suffered an impaired glucose tolerance. The glucose tolerance however improved in the animals maintained on the low sodium test diet and one of the mice was no longer glucosuric.

In the handling experiments (Fig.31 C and D) where water was administered instead of glucose there was a small and insignificant improvement in the fasting blood glucose of both the obese and lean TO animals. Acetoacetate in the obese decreases while it increases in the normal animals.

Three of these control obese animals had glucosuria. In the obese animals the pre-treatment urine pH was 6.1 and after maintenance on the test diet it was pH 6.3. In the normal animals it was pH 5.8 and pH 6.0 in exactly the same order. The weights of both groups of animals have been constant. Proteinuria was more pronounced in the obese animals and did not appear to change with treatment.

High Sodium diet (Fig.32)

The glucose tolerance of the obese animals (Fig.32A) showed further impairment on the experimental diet particularly two hours after the administration of glucose ($p<0.001$). Most of the animals suffered glucosuria even before glucose was injected intraperitoneally. One of them gave evidence of a reduced renal threshold for glucose in that glucosuria was existent together with a fasting blood sugar of 130 mg%. Proteinuria increased after treatment with the high sodium diet and urine pH decreased from pH 6.5 to pH 5.5. Blood aceto-acetate increased by about 113% at fasting, by almost 143% at one hour and by approximately 50% at two hours. However, ketonuria was absent except for the suggestion of its development in two animals. Body weight remained constant.

The glucose tolerance of normal animals (Fig.32B) showed marked deterioration after treatment with the test diet - Fasting ($p<0.001$), one hour ($p<0.001$), two hours ($p<0.02$). This was observed particularly at one hour where the blood sugar was approximately 70% higher post-treatment. One animal exhibited moderate glucosuria on the pre-experimental diet (41B) at one hour where its blood glucose reached 200 mg% and its post-treatment blood sugar concentration rose even further by more than 150% at one hour. All the animals with the exception of one showed marked glucosuria after treatment. Urine pH fell from pH 6.5 to pH 5.5. Fasting blood acetoacetate showed an increase of 38% after treatment and a decrease of approximately 25% at one hour and 15% at two hours. On the whole there is

no consistent relevant pattern in the acetoacetate response with the exception of the post-treatment increase in fasting concentrations. Ketonuria was absent but a slight increase of proteinuria was evident. Weights remained constant.

In the control experiments of both the obese and lean animals (Fig.32 C & D) the post-treatment blood sugars are slightly higher than those estimated on the 41B diet. The pH value of urine was reduced from pH 6.7 to pH 6.5 only. Glucosuria and ketonuria were not observed. Proteinuria was evidently increased in one animal only. Weights were still constant. The pre-test diet acetoacetate remained fairly steady in the obese but increased markedly by about 42% in the normal animals. The post-experimental values too were fairly steady in the obese animals but in the non-obese underwent a reduction of approximately 20% at one hour and 8% at two hours.

Low potassium diet (Fig.33)

As may be observed from Fig.33A the obese showed further significant impairment, post-test diet, of the already impaired glucose tolerance - Fasting ($p<0.02$), 25%; one hour ($p<0.001$), 33%, two hours ($p<0.02$), 18%. The acetoacetate concentrations underwent a slight reduction.

The normal mice (Fig.33B) too suffered impaired glucose tolerance after being put on a low potassium diet - Fasting ($p<0.01$), 9%, one hour ($p<0.001$), 47%, two hours ($p<0.001$), 27%. The aceto-acetate concentrations too exhibited a small rise post-treatment, but as in the case of the above obese group they remained fairly stable both pre and post-treatment.

The normal animals all enjoyed normal glucose tolerance before treatment and suffered neither glucosuria or ketonuria. They all had traces of proteinuria and the urine pH was 6.7. After having been maintained on the test diet for 10 days, the glucose tolerance became markedly impaired as pointed out above. Two of the six animals developed glucosuria and in one it was very marked. The other three animals appeared to have a high renal threshold because they all had blood sugar concentrations between 190 mg% and 200 mg% but had no glucosuria. Ketonuria was not detected. Urine acidification was observed (pH 5.1). The lean animals also suffered a weight loss of about 30%.

In the case of the obese group, all of them had glucosuria before treatment and this was further accentuated post-treatment. Proteinuria was worsened in two animals while the other four mice did not suffer further deterioration. An average weight loss of almost 50% was estimated. The urine pH fell from pH 7.0 (41B diet) to pH 6.4.

Ketonuria was absent in all the animals on the 41B diet but traces were observed post-treatment.

In the handling experiments (Fig.33 C & D) where the animals have water administered instead of glucose the obese showed an increase in their post-test diet fasting sugars of 9%, and 10% and 13% at one and two hours. The post-experimental diet aceto-acetate showed an increase in fasting concentrations and then a decrease at one and two hours. However, all the values are still higher than the pre-treatment ones. Urine pH fell from 7.1 to pH 6.3. Glucosuria was absent but suggestive traces of ketonuria were observed.

The normal animals (Fig.33D) too showed a very slight increase in their fasting sugars and no significant change at one and two hours. Urine underwent acidification from pH 6.8 to pH 6.2. Weight loss of about 28% was noted. Post-treatment proteinuria was increased in three of the five animals. Ketonuria and glucosuria were both absent in all the animals.

High potassium diet (Fig.34)

The obese animals (Fig.34A) showed a pronounced improvement in their glucose tolerance after having been put on a potassium supplemented diet, - Fasting ($p<0.1$) by 6%, one hour ($p<0.001$), 25%, two hours ($p<0.001$), 10%. The acetoacetate levels did not show much difference before and after treatment.

The normal animals (Fig.34B) too showed improvement in their glucose tolerance particularly at the one-hour stage ($p<0.001$) i.e. by 25%. At the fasting level and 2-hour levels there was a small but insignificant improvement ($p<0.5$ and $p<0.3$) respectively. The acetoacetate had shown a general decrease in levels of about 50%.

The weights of both obese and normal animals were constant. Urine acidification increased with treatment in the case of both obese and normal animals: obese animals pre-treatment urine pH 6.4, post-treatment pH 5.6; normal animals pre-treatment pH 6.0, post treatment pH 5.5. Ketonuria was absent in both groups. Proteinuria as usual was more pronounced in the obese animals. Glucosuria was present in all the obese animals.

In the handling experiments (Fig.34 C & D) the blood glucose values were steady as they should be. The sugar curves suggested greater tolerance after treatment. The acetoacetate values decreased with treatment. Weights remained constant. Urine acidification increased with treatment in both sets of animals, pre-treatment pH 6.0 in both cases to pH 5.4 in the obese group and pH 5.5 in the normal group post-treatment. Three of the obese animals out of five had glucosuria. One of

them improved with treatment. Obese animals as usual are more prone to ketonuria. Haemaeturia and ketonuria were absent again in both groups of animals.

Serum insulin, sodium and potassium (Figs.35 and 36).

41B diet

In the obese animal the insulin values range between 170 μUnits/ml and 1090 μUnits/ml. The sodium concentration ranges between 130 mEq/L to 190 mEq/L and the potassium levels occur between 4.9 mEq to 7.5 mEq.

In the normal animals the serum insulin ranges between 36 μUnits/ml and 84 μUnits/ml., the sodium concentration is between 144 mEq/l and 170 mEq/l and potassium values are between 4.5 and 6.5 mEq/L.

Sodium Deficient Diet

In the obese animals the serum insulin has been reduced by 39%, the sodium by about 10% and the potassium by about 13%. The insulin values range from 100 μUnits /ml to 750 μUnits/ml., the sodium between 94 mEq/L and 150 mEq/l and the potassium between 4.5 mEq and 6.5 mEq/L.

The normal animals however showed an increase in serum insulin of about 8%, sodium is reduced by 7% and potassium by about 2%. On this treatment diet however insulin varies between 32 μUnits/ml and 8 μUnits/ml., sodium between 130 mEq/L and 160 mEq/L and potassium between 4.4 and 7.8 mEq/L.

High Sodium Diet

The serum insulin concentrations of the obese animals showed a drop of 37% from 600 μUnits/ml to 438 μUnits /ml and the insulin levels of the normal animals showed a decrease of

10% from 70 μUnits/ml to 63 μUnits/ml.

The obese animals showed a reduction of 7% of serum sodium and the normal animals a reduction of 3%. The serum potassium was reduced by 3% in the obese animals and increased by 11% in the normal animals.

Low Potassium diet

The insulin concentrations in the obese animals had fallen by 71% and in the normal animals it had fallen by about 40%. The serum sodium values of both the obese and normal animals have remained virtually unchanged. The serum potassium of the obese animals fell by 17% and that of the normal non-obese creatures fell by 8%.

High Potassium diet

The serum insulin shows an increased of 4% in the obese group and 17% in the normal animal. The serum sodium shows a decrease of 7% in the obese group but shows little change in the normal group. Likewise the serum potassium is reduced by 13% in the obese and is unchanged in the normal animal.

Serum insulin after the oral administration of 1% Potassium chloride.

There was a 208% increase in the serum insulin of normal mice that had been administered with one ml. of 1% KCl. The control animals which had been loaded with water had serum values of 60 μUnits/ml. of insulin but those that had been loaded with potassium chloride gave a value of 200 μUnits/ml.

These animals were killed 30 minutes after the administration of water or potassium chloride. The obese showed an increase of 35%.

Animals which had been allowed to drink 1% w/v KCl solution for 7 days showed a 92% increase, i.e. animals fed ordinary tap water had a serum insulin value of 60 μUnits/ml and those fed KCl solution had 115 μUnits/ml. The obese animals showed an increase of 9%.

Disappearance of serum potassium after the administration of insulin (normal animals 0.5 U and obese animals 2.5 U).Fig.38.

Potassium depletion induced by saline

The reduced uptake or peripheral resistance in the obese animals on a 41B diet is made obvious by the slower rate of disappearance of potassium after insulin administration and in the still reduced rate after the animals had been depleted of potassium. The same was observed with blood glucose levels.

Potassium supplemented diet

The serum potassium levels flatten out almost parallel to the levels estimated on a 41B diet. The diminished rate of disappearance is obvious in the obese animal.

The disappearance of Blood glucose after KCl administration in Animals on 41B diet followed by a low potassium diet. (Fig. 39).

In the obese the disappearance of blood glucose is obviously slower and the rate of disappearance has not changed. The zero level has deteriorated by about 10%. In the normal

animal however potassium supplementation on a low potassium diet has increased the rate of disappearance significantly. The fasting level has however deteriorated by about 20%.

Urine analysis (Figs.40 - 43)

The sodium, chloride and potassium curves run fairly parallel to each other in both types of animals. The absolute values particularly of potassium are higher in the obese animal. However on examination, the Na/K ratios of both the obese and normal animals (Fig.42) are not very different. The variability in the obese animals is greater.

Tissue analysis.

The tissue content of sodium and potassium and Na/K ratios are summarised in tables 7 - 10 while the glycogen and water content are given in tables 11 - 14.

Signs and Symptoms

Sodium deficient diet

Blood appeared to increase in viscosity and the animal became increasingly difficult to bleed in the second week. Faecal matter was black.

Low potassium diet

Extreme diarrhoea, abdominal distension, anorexia were manifested. The fur took on an extremely shaggy appearance and the animals themselves became extremely listless.

Discussion

In analysing the results one must consider the large standard error of the mean that the data obtained particularly from the obese animal is often subject to. Data from haematuric animals were not included in the experiments.

Blood sugar, acetoacetate and weight changes.

Blood glucose, acetoacetate and weight changes were studied over a short period of time (two weeks). On the 41B diet, fluctuation of daily blood glucose, particularly in the obese, is seen and a fluctuation of acetoacetate in both groups until the third post-fasting day. The fasting acetoacetate in the normal animals is elevated and that of the obese animal is depressed. This indicates that breakdown of fatty tissues increases during periods of food deprivation in the normal animal, but apparently does not in the obese animal, in the initial stages at least, (Hollifield et al. 1962; Bates et al. 1955 b). Lipogenesis from glucose is decreased under fasting conditions by 90% in the lean animals and only 70% in the obese (Jansen et al. 1967). There is a reduction in blood glucose during the fasting period. The obese animals show a slight insignificant increase on the second day.

On the sodium deficient diet weights in both groups of animals are stable, the blood glucose of the obese and aceto-acetate levels of both groups fluctuate more. The character-istic elevation of acetoacetate in the normal animals and fall in the obese animals may be generally observed on all the diets.

The slight drop in weight in the animals on the high saline diet may be the result of a slight reduction in food intake.

The increase in acetoacetate in both groups on the low potassium diet and the obese animals in particular, the hyperglycemic trend of the normal controls and the weight reduction is probably a function of the high fat and starch diet. Lipolysis is increased significantly as may be observed from the acetoacetate levels. There is no simple direct relationship between blood glucose levels and ketonemia. In the situation where there is a lack of insulin, glucose penetration into the cell through the cell membrane is reduced. As a result there arises a situation characterised by a lack of glucose-6-phosphate and glycogen and therefore pyruvic acid. Pyruvic acid is metabolised to form acetyl CoA and oxaloacetate. When insufficiency of acetyl CoA results then fat is catabolised to acetyl CoA. However, since oxaloacetate can only be provided from pyruvic acid, acetyl CoA cannot be metabolised to completion via the Krebs cycle. Thus two molecules of acetyl CoA combine together to form acetoacetyl CoA which in turn is degraded to form acetoacetic acid. Acetoacetic acid in turn forms acetone and beta hydroxybutyric acid (under conditions when liver glycogen is high) which can be resynthesised to form long chain fatty acids. Acetone is formed under starvation conditions. However if both acetone and acetoacetic acid are formed faster than they can be metabolised or excreted in the urine, their concentration in the blood increases and that of bicarbonate is reduced. The carbonic acid formed decomposes to carbon dioxide

and water. Respiratory compensation (or hyperventilation) ensues to effect the removal of excess carbon dioxide and the blood pH drops.

However, a tendency for higher acetoacetate levels in the presence of low blood glucose levels may be discerned though it is not statistically significant. However, the obese hyperglycemic animals are resistant to ketosis although extreme hyperglycemia may be present (100 mg%). This is almost certainly explained by the increased rate of lipogenesis even under conditions of ordinary food restriction (Bates et al. 1955a). However, on a rich diet such as the low potassium diet the animals are unable to adapt and increasing hyperketonemia ensues. For converse reasons low or normal blood glucose levels may coexist with severe hyperketonemia.

Potassium and carbohydrate metabolism

The relationship of potassium and carbohydrate metabolism has been confirmed in both groups of animals, in that potassium depletion induced by the low potassium diet and further induced by the diarrhoea resulted in glucose intolerance to almost the same extent in both types. Impaired glucose tolerance also exists in animals fed the high sodium diet. In the obese animal it is particularly evident at two hours when the blood sugar is still high. The impairment is exceedingly accentuated throughout in the normal TO animals. The fasting concentration is increased by about 150%, that at one hour by 228% and at two hours by 320%. The animals were on the high sodium test diet for a fortnight and were in good condition. The animals

on the low potassium diet were ill although they were on it for only ten days. Previous experience indicated that survival after the twelfth day was a struggle. Glucosuria was a frequent accompaniment of the glucose intolerance in the normal animals. Proteinuria increased in the obese animals. Urine acidification on the low potassium diet occurred in both groups although urine pH remained within the normal physiological range. Urine pH of animals on the high sodium diet showed too a trend towards acidity but still remained within normal physiological limits. The acetoacetate during the glucose tolerance test showed no recognisable trend.

Animals maintained on a potassium-supplemented diet showed a definite improvement in their glucose tolerance throughout, and particularly at one hour in the obese animals (25%) and in the normal animals (22%).

Animals maintained on the low sodium diet showed an improvement significantly at two-hours (28.5% for the obese and 50% in the normal) and 25% at one hour in the normal animals.

The normal animals are apparently subject to the biggest changes as far as glucose tolerance is concerned. Their sensitivity or the relative lack of sensitivity in the obese animal could be the result of the inability of the obese animal to respond. The sixteen week old obese animals are at, or almost at, the climax of the dynamic phase of development characterised by weight increase and excessive insulin secretion. At this time peripheral tissue resistance and the ability of the islets to synthesize and store insulin may also be at its

peak (Chap.IV). Therefore any possible improvement in the response of the animal must also be minimal and any further deterioration too must be minimal in this respect as exacerbation of this syndrome is at its very height. In the normal animal the ability to respond in either direction must be considerable. These animals have been stressed only during the experimental period. The glucose tolerance test is a measure of the ability of the animal to return its blood sugar to normal after challenge with a glucose load. The response of the animal is at least in part a reflection of both the ability of the beta cells to secrete insulin and the sensitivity of the target tissues to that insulin. This test therefore represents a form of stress.

As explained before the relationship between blood glucose and blood acetoacetate is not simple or direct. Acetoacetate is also obviously very much more variable. The G.T.T. response is also apparently characteristic within limits for the animal provided it is repeated within a reasonable time.

The slower rate of glucose disappearance after potassium depletion could be the result of diminished insulin secretion and storage, a reduction in the rate of peripheral cellular uptake of glucose or from increased hepatic glycolysis or in part due to all three. It is well known that the administration of food or glucose inhibits hepatic glucose output and increases hepatic gluconeogenesis, but it is uncertain during the state of negative potassium balance whether one, or the other or even both these effects are diminished or abolished. Insulin promotes the entry of glucose through the

cell membrane and glucose entry is accompanied by potassium and phosphate; therefore the rate of glucose disappearance is partly a reflection of tissue sensitivity to insulin and partly a result of insulin release. Presumably these effects are reversed if potassium is high. This situation was investigated by the measurement of four parameters (Nos.3,4,5,6, as detailed previously, p.139).

(a) Serum insulin, sodium and potassium

The highest elevation of serum insulin in both groups is seen in the animals on a high potassium diet, (obese 4%, normal, 17%). The greatest decrease is observed in animals maintained on a low potassium diet (obese 70%, normal, 40%). The normal animals apparently had an increase of 8% on the low sodium diet and a decrease of 10% on the high sodium diet. The obese animals on the other hand exhibited a decrease in serum insulin levels of 37% on the high sodium diet and 39% on the low sodium diet.

The serum sodium appears to be reduced by varying degrees on all diets and the serum potassium too seems to follow the same pattern except for the normal animals on a high sodium diet which show a small but insignificant elevation.

(b) The response of serum insulin to exogenous potassium chloride.

The highest elevation of serum insulin in animals on a high potassium diet was obtained 30 minutes after a potassium chloride (1%) load,(obese animals, 35%, normal animals 208%). Animals which had been maintained for one week had an elevation

9% (obese) and 92% (normal). Those that were maintained during a two-week period showed an elevation of 4% (obese) and 17% (normal). Thus it is obvious that the longer the animal is maintained on a potassium supplemented diet the greater the reduction in the elevation of the serum insulin levels in both groups of animals. However the insulin levels at the two-week period are still elevated in comparison to the animals on a normal 41B diet.

(c) Response of serum potassium to insulin

The disappearance of serum potassium after insulin administration was evident in both groups of animals which had been maintained on a potassium depleted diet for ten days. However, the recovery of serum potassium levels to the pre-insulin levels seems to be greater in animals on the test diet. The magnitude of this fall in serum potassium and its subsequent recovery in the obese animals is half that of the normal animals on the test diet and one-third that of the lean animals on the normal 41B diet. This is in spite of being administered five times the insulin that was injected into the normal animals.

(d) Response of blood glucose to exogenous potassium chloride

Potassium chloride loading provoked a fall in blood glucose in both groups of animals both on a 41B diet and on a low potassium test diet. This fall in blood glucose is more accentuated in the normal group maintained on the test diet. The low potassium diet has not influenced the magnitude of the fall in the obese animal significantly and its level at zero time

on a potassium deficient diet is higher.

Thus from the above experiments there is no doubt about the role of potassium in carbohydrate metabolism. It provokes a rise in serum insulin which in turn may be considered as an index to the pancreatic release of insulin. The increased storage and secretion of insulin in turn must be secondary to increased synthesis. The decline in the elevation of serum insulin with time in animals on a potassium supplemented diet could mean that insulin secretion although increased is adapting itself to the high potassium environment and the needs of the animal in both groups. In the obese group the changes observed are generally relatively smaller simply because, as pointed out earlier, insulin synthesis and secretion are maximal and further stimulation is only minimally possible. Besides, this small percentage increase or improvement as in the glucose tolerance which is observed may probably represent a decline in insulin secretion. This possibility has been confirmed in the above experiment.

The coexistence of effective hypoinsulinism with absolute hyperinsulinism, i.e. the relative insensitivity of obese tissue to insulin is clearly demonstrated in the magnitude of the fall of serum potassium and its restoration following insulin administration and is further confirmed by the disappearance of blood sugar after potassium chloride administration. The serum potassium disappearance in animals on a high potassium

intake is not remarkable in comparison to the same animals on a normal 41B diet. In fact the serum potassium levels in the obese animals on this test diet are lower than when they were on the normal laboratory diet.

The utilisation or assimilation of glucose takes place in three successive stages:

1. Transport to the cells
2. The passage through the cell membranes
3. Intracellular metabolism.

It is not possible to comment on the first point on the basis of these results simply because the rate of glucose disappearance from the blood needs to be known and this is most rapid within the first few minutes of administration. However, these experiments confirm that this disappearance is definitely linked with that of the potassium ion. The passage of glucose through the cell membrane is insulin dependent, the hormone increasing the permeability of membrane (e.g. muscle cells) to glucose. This was confirmed by the work of Levine (1966) who demonstrated that the volume of galactose distribution is approximately equivalent to that of extracellular fluid in the absence of insulin, but in the presence of this hormone its volume of distribution was further increased. This illustrates that this sugar was enabled to cross much faster a barrier, i.e. the cell membrane which it normally crosses very slowly.

Also the work of Crofford and Renold (1965) demonstrated that intracellular glucose content at physiological glucose concen-

trations is low and metabolism is regulated by transport and not by phosphorylation. Insulin levels are reduced with potassium depletion and increased with potassium supplementation. As the consequence of potassium depletion fasting blood glucose was higher the rate of disappearance of glucose was slower. This was demonstrated by the elevated levels at one and two hours after glucose administration which is characteristic of diminished insulin responsiveness. The improvement observed in animals on a high potassium diet suggests that the rate limiting factor for glucose uptake is its transport through the cell membrane. The present experiments have also further established that this rate limiting factor is even more rate limiting in the obese hyperglycemic animal. Although the work done on these animals confirms the peripheral tissue resistance to insulin in the obese animal and establishes the importance of potassium in insulin secretion, it obviously does not under physiological conditions cause any deterioration or problems, although it is responsible for the production of decreased peripheral uptake when the animal is probably in considerable negative balance.

The reduced rate of glucose disappearance following potassium deprivation might conceivably result from increased hepatic glycogenolysis. This process is also apparently stimulated by glucagon and results in hyperglycemia. How it would alter the response of the animal (if it does) to potassium depletion or supplementation is not certain, or in other words, the relationship of the potassium ion and

glucagon is not known, neither is its relationship with growth hormone. Potassium and phosphate retention associated with growth hormone is probably the result of increased cell mass but its role in sodium retention in the presence of increased glomerular filtration rate is not certain. However the role of growth hormone in various species and in this syndrome has still to be elucidated.

Thus the present investigation further supports the work (Bennet , 1966, Milner and Hales, 1967 etc.) on the perfused rat pancreas and isolated rabbit pancreas. This work established that insulin secretion increased when the extracellular potassium increased. Milner and Hales (1967) also pointed out that this effect is sodium dependent. These observations suggest that an increase in intracellular sodium may also be involved in the secretion of insulin. Milner and Hales also demonstrated the dependence of the effects of glucose, L-leucine, glucagon and tolbutamide on sodium. Another method of increasing the intracellular sodium was by inhibiting the sodium pump. The role of sodium in the secretion of insulin is however difficult to study in vivo. The sodium requirement is probably far smaller than the minimum sodium levels that the animal will be able to tolerate. The differentiation of the separate effects of sodium and potassium in vivo is even more difficult because the effects of both these physiological antagonists are so very interdependent on each other. This very relationship is itself sufficient to make sodium indispensable to life let alone merely essential to any one function of

the organism. This balance is so very important that the body is geared to adjust this balance under ordinary physiological conditions.

The findings of Milner and Hales (1967) suggest the following possible sequence by which increases in extracellular glucose could lead to an increase in insulin secretion:

1. Increased intracellular sodium and calcium
2. Increased secretion of insulin.

Glucose transport by intestinal epithelium is coupled with increased sodium transport (Csaky and Thale, 1960) but how glucose uptake produces an increase in intracellular sodium is uncertain.

Perhaps at this point it would be beneficial to consider the theoretical relationship of these two bulk ions under the conditions of the experiment.

Relationship of Sodium and Potassium under experimental conditions.

When maintained on a low sodium diet the body attempts to conserve sodium and in so doing there is an increased excretion of water and potassium and efficient sodium retention. This is the extracellular situation. However, intracellular sodium may move extracellularly in exchange for the movement of extracellular potassium intracellularly. Thus the intracellular situation in the tissue is that of high potassium and low sodium content. In the case of severe depletion the blood potassium would increase in spite of concurrent sodium depletion.

This could result from increased breakdown of tissue and more potassium being set free into the extracellular fluid. Thus there is a reduction of extracellular fluid and possibly an increase of intracellular fluid. Urine acidification and chloride depletion would also occur.

On a high sodium diet, water retention takes place in an attempt to dilute out the sodium. Intracellular potassium is depleted in exchange for extracellular sodium. Sodium excretion by the kidneys is increased. Plasma potassium may remain normal or low. Thus this leads to a situation of low potassium content in the tissue. Extracellular acidosis results as the bicarbonate ion (HCO_3^-) is increased and this ionises to produce hydrogen ion resulting in acidemia. Oedema may be observed in severe cases.

On a low potassium diet only partial potassium retention takes place as the kidney is not very efficient in conserving potassium. Sodium and water depletion takes place resulting in dehydration of extracellular fluid. Intracellular potassium is depleted and replaced by both extracellular sodium and hydrogen ion resulting in intracellular acidosis. The accompanying extracellular alkalosis is characterised by a high plasma bicarbonate and low plasma chloride. Intracellular acidosis stimulates the hydrogen ion secretion in the proximal tubule and the increased absorption of bicarbonate and decreased reabsorption of chloride. Thus aciduria may be observed and serum potassium may be high while the tissues are depleted of potassium but high in sodium content.

In the converse situation of a high potassium diet, on a similar basis intracellular potassium is increased and the sodium content is decreased. Thus there is a tendency to extracellular acidosis accompanied by a low plasma bicarbonate and increased plasma chloride. Potassium excretion is increased and sodium retention is effected.

However, in a situation involving hyperglycemia as in the case of the obese animals one cannot ignore the effects of glucose. In the obese animal, as a result of tissue resistance to the action of insulin, glucose must increase the effective osmotic pressure of the extracellular fluid, primarily blood. The increased concentration of glucose and therefore hypertonic extracellular fluid causes a transfer of water from the intracellular to the extracellular compartment. Thus prolonged hyperglycemia as in the case of these animals may represent a dilution effect and result in lowered serum sodium content. Polyuria which is associated with glucosuria results in the loss of water and sodium. However, if this hyperglycemia is corrected, for example by insulin administration, then the extracellular fluid becomes hypotonic, This causes water to move intracellularly and with this reduction of extracellular fluid volume hypernatremia results. If and how insulin itself directly affects excretion of the ions is uncertain. It has been confirmed by this investigation that serum potassium decreases after insulin administration and blood glucose levels decrease with potassium administration in animals which have been maintained on a potassium deficient diet,

although due to the existing peripheral tissue resistance,in the obese animals it is less rapid. It is also known that glycogen deposition is accompanied by potassium and water deposition. Liver and muscle glycogen were found to be higher in the obese animals in comparison to their lean litter mates (Shull and Mayer, 1956). Toreson (1951) observed glycogen infiltration in the pancreas of both humans and experimental animals. Carpenter and Lazarow (1967) observed that immediate glycogen infiltration of the beta cell resulted from merely transitory production of hyperglycemia in normal and sub-diabetic rats. Lowering of the blood glucose levels conversely removed the glycogen from the beta cell. Thus in the presence of degranulation of the beta cells (Gepts et al. 1960) and in the presence of persistent hyperglycemia it will not be unreasonable to expect to find increased levels of glycogen content in the beta cell. However one must remember that this increased glycogen content is brought about by the hyperglycemic state in the animal. This hyperglycemia is caused at least partially by extra-pancreatic factors. Thus the changes in the beta cell must be secondary. However, one cannot be certain that changes in the other islet cells and pancreatic cells are not contributing to that of the beta cell on to the whole body *in vivo*. Thus glycogen, water, sodium and potassium ions were measured in pieces of whole pancreas and also in muscle, liver and kidney. An evaluation of urinary electrolytes, sodium, potassium and chloride was carried out for other reasons mentioned earlier.

The estimation of sodium and potassium levels in serum cannot be relied on to give an accurate picture of the situation in the body. Blood which is the main extracellular fluid of the body forms the link, so to speak, between the tissue (intracellular compartment) and the kidneys which are mainly responsible ultimately for the maintenance of the balance in the body. Thus the serum potassium and sodium, because of the existing compensatory mechanisms in the body, are subject most to transitory or temporary changes. Sodium loss is accompanied by dehydration. This is partially compensated by an increase of water excretion. Thus this sodium loss or decrease may not be evident and sodium levels may even be elevated as the result of the concentrating effect of the body unless this loss is severe. This depletion however, will be evident when rehydration occurs. However, in the presence of hyperglycemia (as pointed out) under ordinary conditions, hyponatremia may result. This could result in potassium depletion of the tissues and increased excretion of potassium.

The sodium and chloride levels follow each other fairly closely in both groups while the potassium is more elevated particularly in the obese animal. The increased excretion of potassium in the obese animal is confirmed by the increase in the sodium:potassium ratio in comparison to its lean control animals. However, the total volume of urine excreted during the experimental period of 17 days by the normal animals (2.7 ml) is greater than that of the obese animals (2.4 ml.) The general variability in the excretion

of the ions and water seems to follow a random up and down pattern.

Electrolytes and glycogen under experimental conditions in tissue.

Liver

As may be observed although the absolute levels of sodium and potassium are subject to a great deal of variation the effect is a little less dramatic when the sodium:potassium ratios are considered instead. The ratio, as well as the absolute level is virtually identical in both groups of animals on a 41B diet. The increase of the ratio in liver seen on the low sodium diet is the result of the absolute elevated levels of potassium while sodium shows little change. This applies to the obese animal in particular. This effect is also seen in both groups on the high potassium diet. However there is a reduction in the absolute values of sodium in both groups and potassium in the normal animals and the greatest increment of potassium in both groups is manifested in animals on the low sodium diet. The ratio of glycogen to water is 1:10 in the obese and 1:20 in the normal and the glycogen content of the obese is twice that of the normal. On the low sodium and high potassium diets a general decrease of both glycogen and water content occurs in the obese. The normal animals however manifest an increase in the glycogen content and a decrease in the water content. The decreased glycogen content of the obese liver may be the result of increased glycogenolysis in response to increased insulin

secretion.

The Na:K ratio is reduced in the two strains of animals on the saline diet and the low potassium diet, particularly in the obese mice. The glycogen levels and water content are reduced in the obese animals on both the above diets. However the normal animals show an increase of glycogen levels on both diets and water on the low potassium diet.

At this point one is made to remember that hepatic glycogen is subject to various other factors like glycolysis, gluconeogenesis, glycogen breakdown and synthesis which will help to explain the all-round increase in glycogen levels in the normal animals and conversely the all-round decrease in the obese animals. The livers of the obese animals too are bigger than the normal animals although this factor has been accounted for in the calculations.

One must also consider the results in the light of the methods used for estimating these parameters. One is measuring not only the cellular sodium and potassium but to some extent extracellular sodium and potassium. This method gives an excellent indication of intracellular potassium which is high compared to the extracellular content. However, the reverse applies to sodium which makes the sodium estimations far more inaccurate. The water content measured too is a combination of intra-cellular and extracellular water. The glycogen that is estimated is first hydrolysed to glucose and blood in particular is a rich source of glucose especially in the obese hyperglycemic animals. During the handling procedure of

the tissue all precautions were taken to standardise and eliminate surface extracellular fluid and blood, to eliminate the above errors as far as possible. However, these results may only be treated as a reflection of the changes taking place in the organism.

Muscle

In the case of muscle the Na:K ratio in the obese is lower than in the normal animal and this is due to the increased sodium levels in the obese animal. The muscle glycogen in the normal animal is almost twice that of the obese animal. This may be considered as another indirect indication of the decreased peripheral uptake of glucose or impairment of glycogen storage by obese animals. These results are also in direct contradiction to those of Shull and Mayer (1956b) who found significantly higher levels of glycogen in the muscles of obese animals.

On a low sodium intake the obese group showed an increase in the Na:K ratio by more than 200%. This was caused by a 200% increase of potassium and 22.5% rise in sodium. The normal animals showed a decrease in the levels of both ions but managed to maintain a similar Na:K ratio to that of the control value. Both groups show a significant increase in glycogen.

On a high potassium intake the increase in the Na:K ratios in both groups is almost exactly of the same magnitude.

This is caused by a fall in both the ions in the obese group. In the normal group this is manifested as an increase in the potassium and a decrease in the sodium. An increase in the glycogen and water is seen in both groups but a slight drop in water is seen in the normal group.

In the case of animals on a high sodium diet, the Na:K ratios have decreased in both groups. There is a slight decrease in muscle glycogen in both groups. Water content is slightly reduced in the obese group but the same in the normal group.

On the low potassium diet a reduction in the Na:K ratio in both groups is seen once more. This seems to be the function of the increased sodium levels. The glycogen levels are reduced in both groups of animals. Water content remained the same in the obese but was elevated in the control animals.

Liver and muscle

Although the obese hyperglycemic syndrome is characterised by hyperinsulinemia which appears to be at its very climax in these animals at this stage (16 - 20 week old animals - Chapter IV), the utilisation of glucose by the peripheral tissue is low due to its resistance to insulin. Thus the tissue may be said to be in a "state of starvation" and "effective hypoinsulinism" may be said to coexist with hyperinsulinism. Thus in effect, as far as the peripheral tissue is concerned the situation is very much like that of

diabetes per se which is characterised by the relative lack of insulin.

From the above work we are enabled to infer, on some aspects of metabolism of the obese hyperglycemic mouse in comparison to the normal control animal, on the changes resulting from primarily two situations, the low potassium surroundings (low potassium and high sodium diet) and the high potassium environment (low sodium and high potassium diet) and secondarily the low and high sodium content of the animals of both groups. Finally we have been able to observe the effects of a highly nutritious diet on these animals.

In the liver the concentration of glycogen is twice as great in the obese animal. The water content of the liver is greater than the normal animals by 5%. This again is contrary to the findings of Hellman et al. (1962). These authors had attributed the low water content of the liver to fatty infiltration. This symptom has been clearly observable in some of the obese animals but not in the animals used in this experiment. The liver unlike muscle and adipose tissue is freely permeable to glucose. Insulin apparently does not affect entry of glucose into the liver cells although it has the usual significant effects on the metabolism of glucose within the cell. The reduction of glycogen levels in the obese animal on high and low sodium diets and the high and low potassium diet and the increase of the glycogen content in normal animals on all these experimental diets perhaps reflects a change in permeability of liver cells to glucose. It also

reflects the efficiency of the liver in conserving potassium. In fact the absolute levels of potassium in the liver of animals on the experimental diets were often elevated particularly the normal animals. The obese animals appear to have a relatively reduced efficiency. As the potassium and water content of the liver are partially associated with the glycogen content, the reduction of all three is possibly a reflection of impairment of glycogen storage. The levels of hepatic glycogen are generally dependent on the state of nutrition of the animal. Why exactly the livers in the obese group differ so greatly from the normal control group is not clear at the moment. Burton et al. (1967) in their work on rats demonstrated that 8% of the liver potassium was dependent on insulin for its intracellular retention and recovery. 70 - 90% was associated with the protein content and the rest with glycogen. Thus, further investigation is obviously necessary to clarify and verify this situation in the obese animals.

The muscle content of glycogen in both groups reflects the trend in insulin levels in that increased incorporation of glycogen is observed in the high potassium and reduced glycogen content in the low potassium environment. The water content is variable but water content is also very much influenced by the salt content of tissue.

Pancreas

In the pancreas most of the glycogen content may be attributed to islet tissue, i.e. it is about 7.2-fold greater

and the water is 75% (Matchinsky and Ellerman, 1968). Our estimations were carried out on whole pancreas. The glycogen content of the obese animal was higher than the control animal by more than 200% and this also probably reflects its higher metabolic activity. The general pattern of elevation and reduction of glycogen levels is seen in both groups except on the low and high sodium diets. The generally increased glycogen content of the pancreas therefore must represent the adaptation of the islets or beta cells to the hyperglycemia of these animals. The work of Hellman and Idahl (1969) further suggests that insulin secretion may well be sensitive to the islet glycogen levels under certain circumstances. The obese animals on a high sodium diet also appear to have a decreased Na:K ratio. This appears to be a function of elevated potassium and sodium. It is not certain to what extent the glycogen content of islets themselves is influenced by the insulin content of the islet cells.

Kidneys

The kidneys, like the liver, are not subject to insulin where glucose transport is concerned, and, unlike the liver, muscle and pancreas, they have probably very little if anything to do directly with intermediary metabolism. Their primary importance is in the regulation of metabolism by maintenance of osmotic equilibrium in the extracellular and intracellular fluids and the excretion of unwanted waste products. The extracellular compartment of this tissue is

very much greater. In the kidney the Na:K ratios are generally low. This is however not unexpected. The absolute values of sodium are higher than in the other tissues. The glycogen values do not differ greatly in either groups of animal in any of the experimental diets.

General Comments.

Thus, with the exception of liver, the response of both groups of animals on the various experimental diets is generally similar. The main difference is in the absolute levels and the magnitude of the response. However, the insulin resistance of muscle is again made obvious. The sensitivity of adipose tissue in the obese animal is well illustrated by the increased lipogenesis and the decreased lipolytic activity of this tissue. The epididymal adipose tissue too has a degrading activity of insulin which is eight times higher in the obese animal (Westman, 1967). Unfortunately apart from uncovering still further indirect evidence illustrating this peripheral resistance to insulin one is still almost as far away from the answer as to its cause. Several factors all of which are still obscure and not properly understood are suggested, for example, the mysterious muscle factor which increases glucose uptake after exercise. This was postulated partly to explain the reduced insulin requirement of diabetic patients during exercise. In the obese animal it has been noted that decreased activity precedes obesity. Another theory that has been postulated is the existance of a

"bound" or "non-suppressible" insulin. This issue has been in the past decade and still is the subject of controversy. Antibodies to exogenous insulin are probable but when no exogenous insulin has been introduced into the body one is left at a loss to explain the existence or source of antibody. Whether this resistance is a function of the increased adipose tissue only is not certain, but from our studies (Chapter IV) onset of obesity and hyperinsulinism seem to take place almost simultaneously. This requires further confirmation. Insulin resistance however probably has an earlier onset. Hyperinsulinism may be the response to hyperglycemia and effective hypoinsulinism and to the increased degrading activity of obese adipose tissue. The exhaustion of the beta cells is clearly the result of this unknown factor.

Most of the investigations in this particular field have been carried out *in vitro* and the various contradictory results and unphysiological conditions make their relevance to the living animal questionable, e.g. the insulin degradation activity must be verified *in vivo*.

The low potassium diet is a powdered diet whose rich nutritional content caused gastrointestinal disturbances resulting probably from the inability to absorb these nutrients. This in turn resulted in a greater malnutrition. Besides the animals on this diet ate less than they did on their previous laboratory diet or the others on any of the experimental diets. The reduction of plasma insulin, and an increase in plasma glucose and hepatic glycogen accompanied by a reduced muscle

glycogen in animals on a high fat diet was observed by Blázquez and Quijada (1968). This was observed in our animals too besides elevation in acetoacetate levels, particularly in the obese, indicating increased lipolytic activity. However decreased liver glycogen levels were observed in the obese animals. The high fat diet used by Blázquez and Quijada (1968) has a different composition from the rich but low potassium diet. That the above effects seen in our animals were partially due to the richness of the diet was verified by inducing potassium depletion in animals by replacing the drinking water with saline.

FIGS. 26 - 29 Daily blood glucose, acetoacetate and body weight in animals maintained on a 41B control diet, and experimental diets of low and high sodium, low and high potassium.

↑ indicates when the animals were put on a 48-hr. fast after caudal blood collection for the above biochemical estimations

↓ indicates when the animals were refed the experimental or control diet after the 48-hr. fast which was terminated after blood collection.

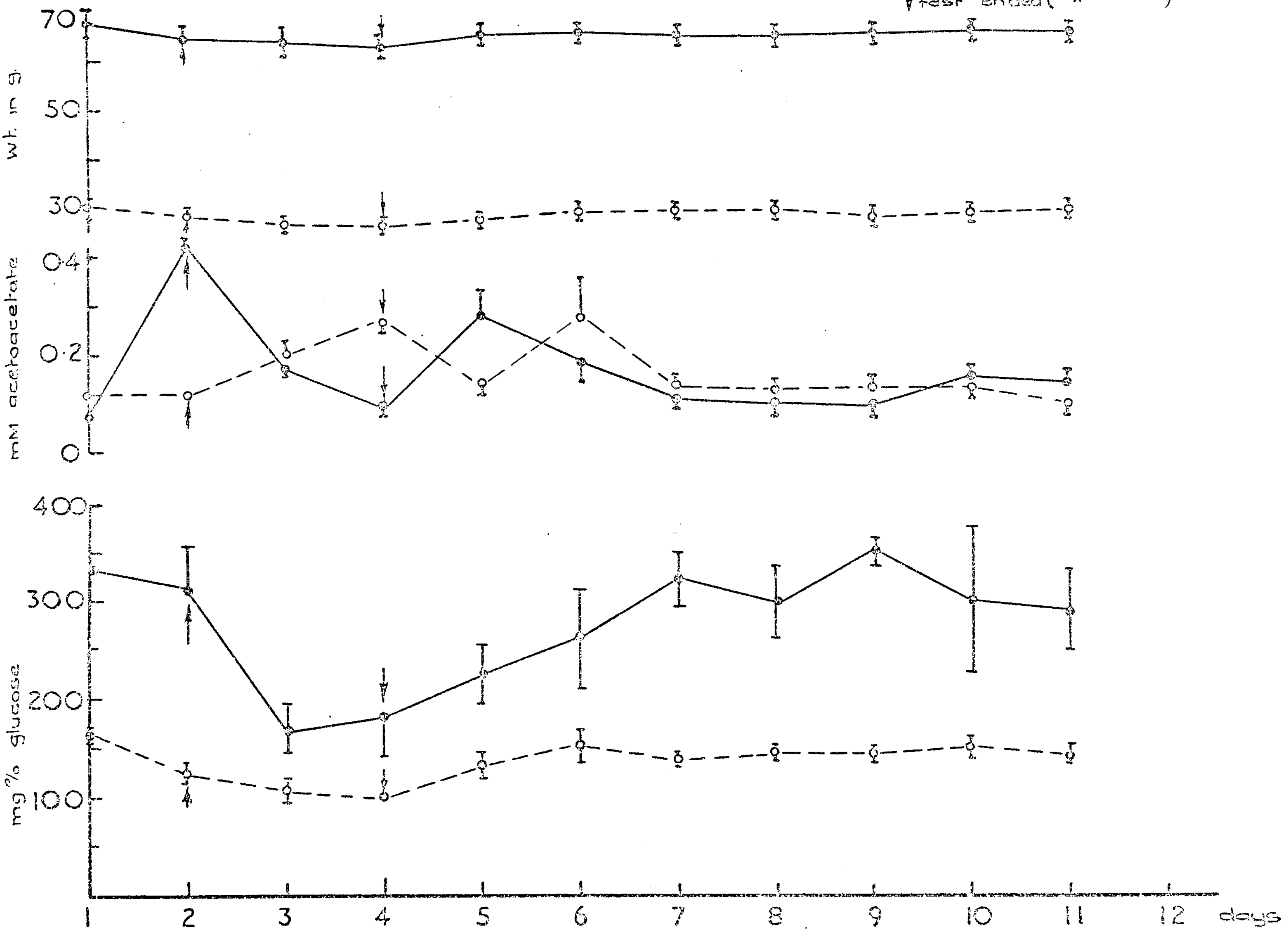


Fig 26 Control 41 B diet

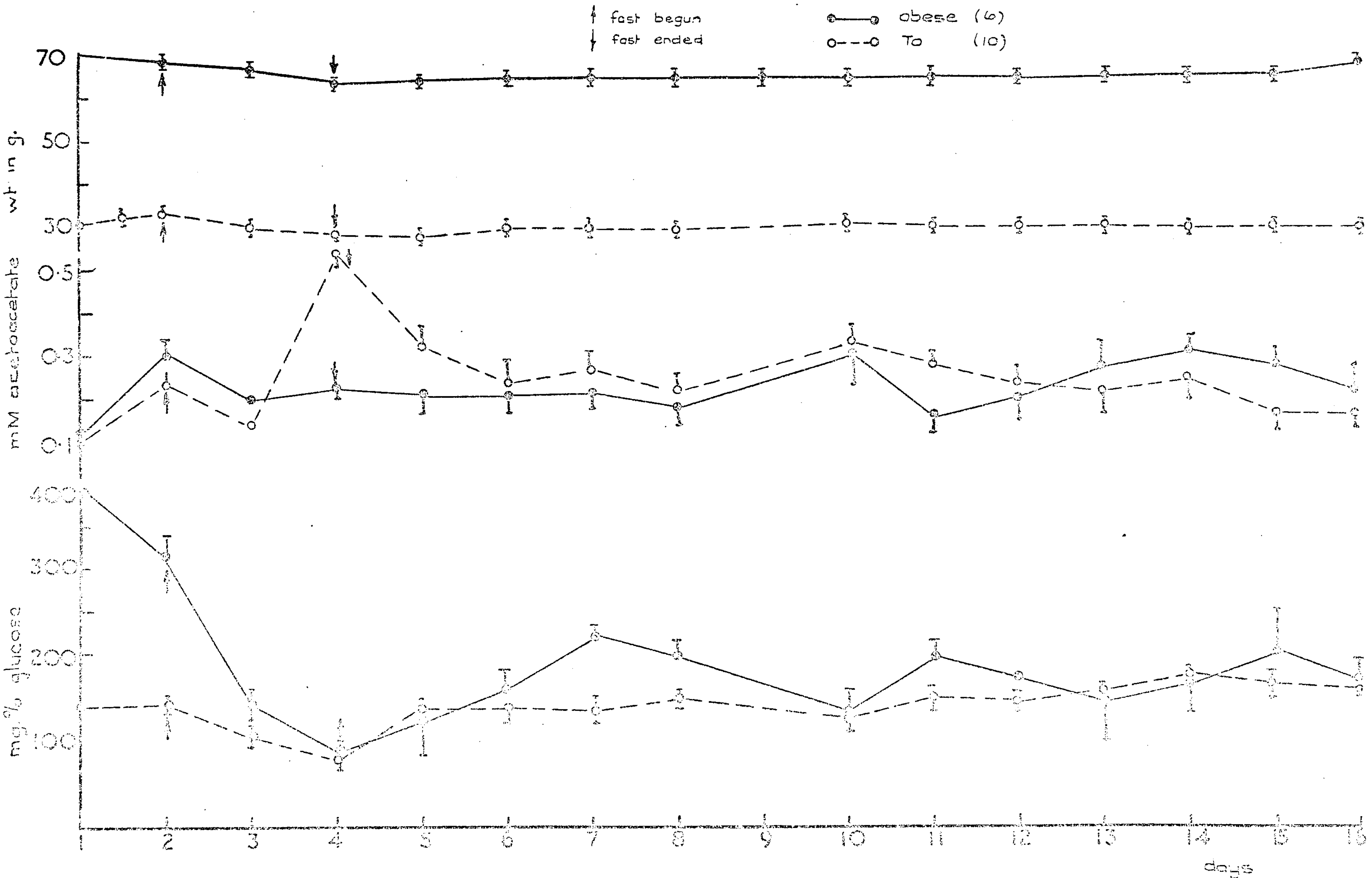


Fig. 27 Sodium deficient diet

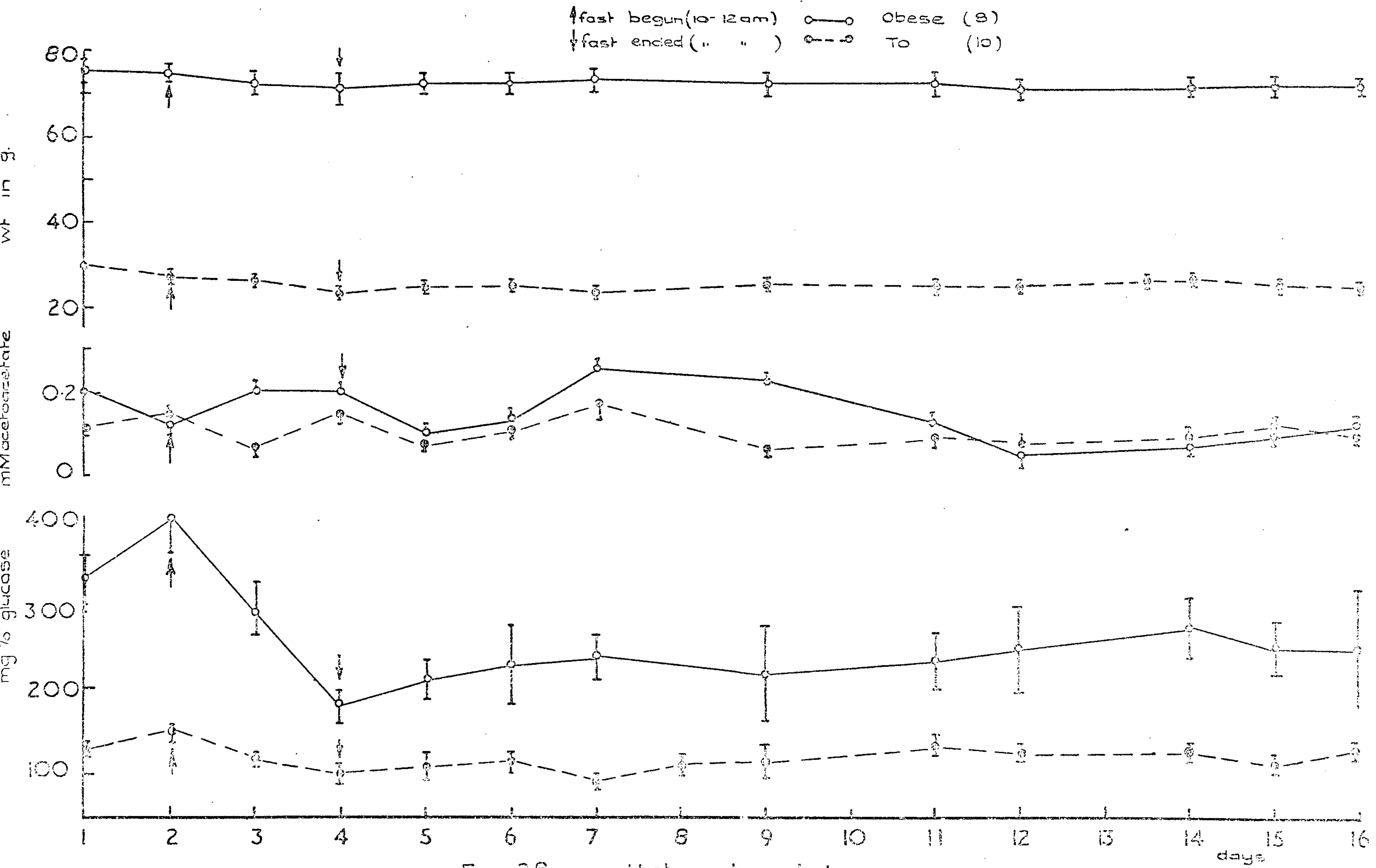


Fig 26 High sodium diet

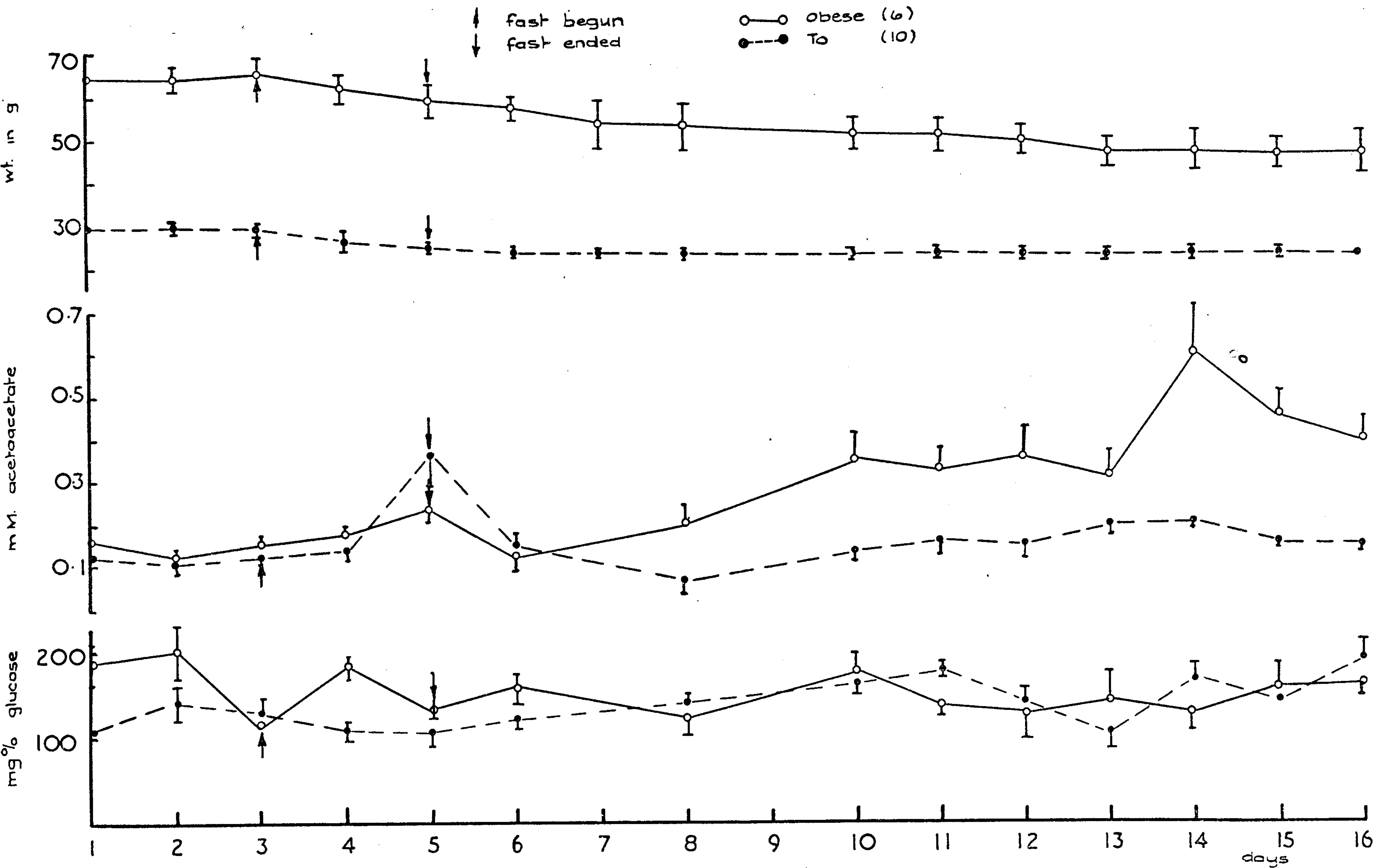


Fig. 29 Low potassium diet

FIGS. 30 - 34 (A,B,C,D)

The glucose tolerance tests were performed twice on each animal, once on the control 41B diet and the second time on the experimental diet. (FIGS. A and B).

Figs. C and D are the control experiments where distilled water instead of glucose was administered.

Control expt.
Obese.

Control expt
TO.

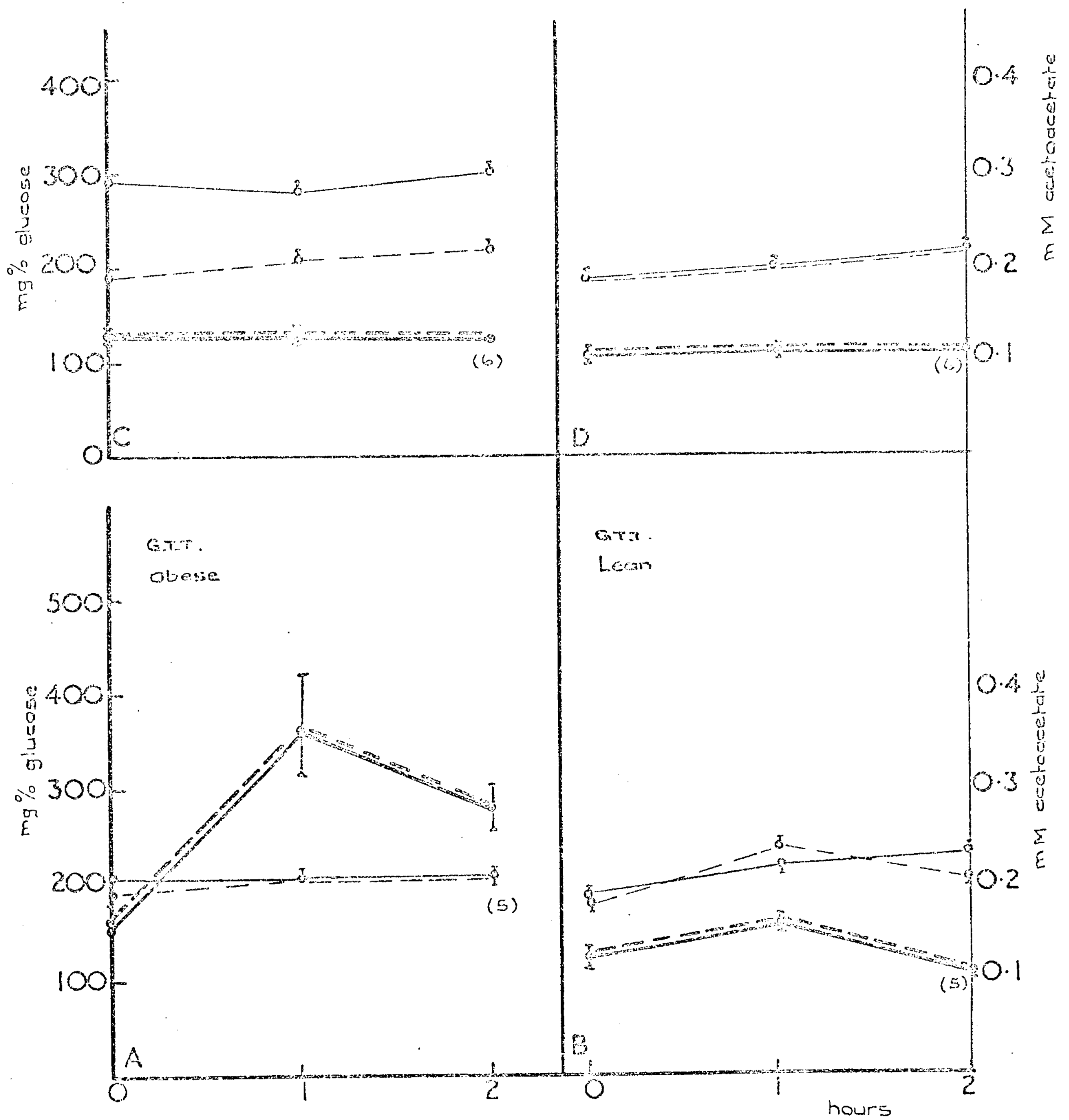


Fig 30 41 B Diet

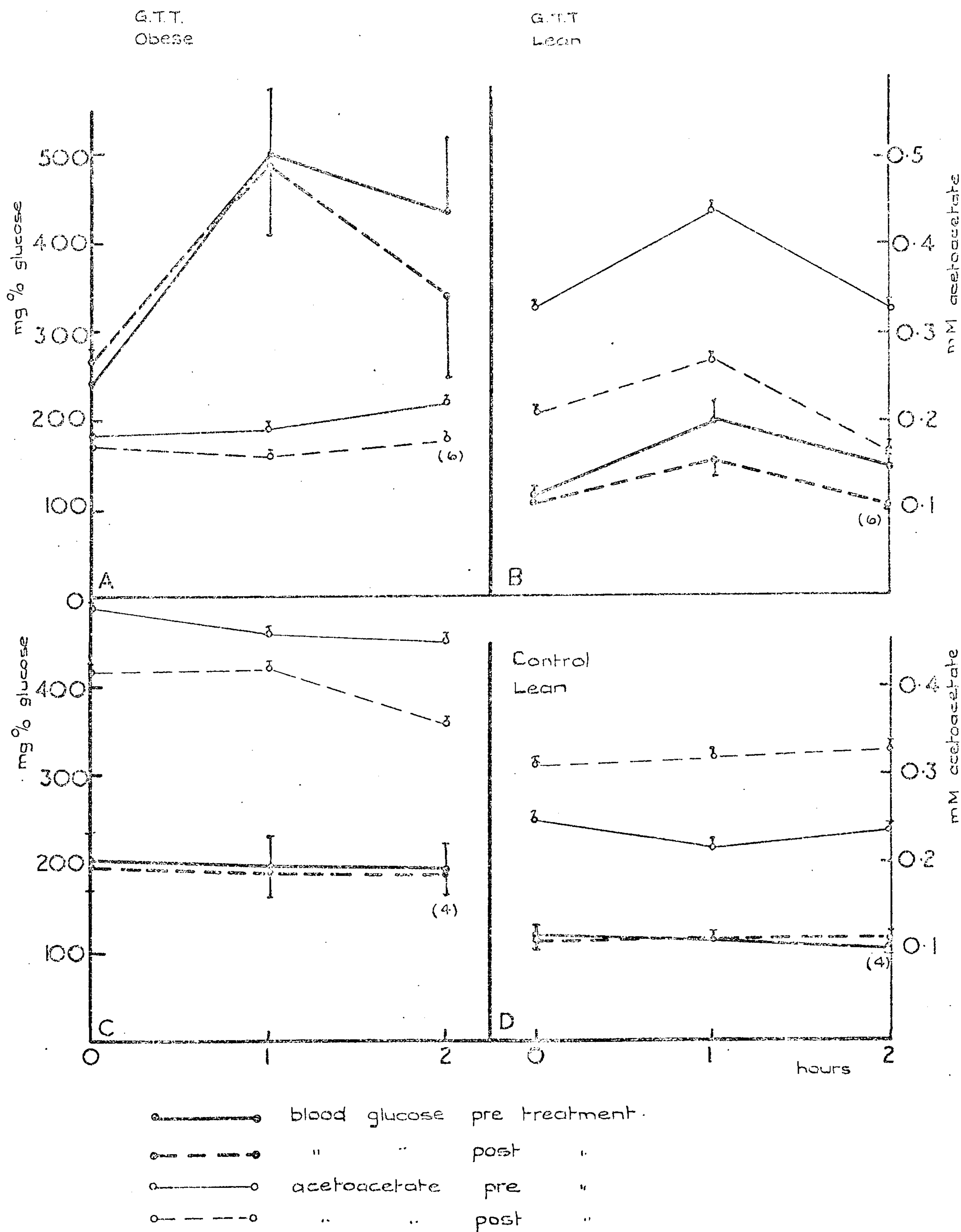


Fig 31 Sodium deficient diet

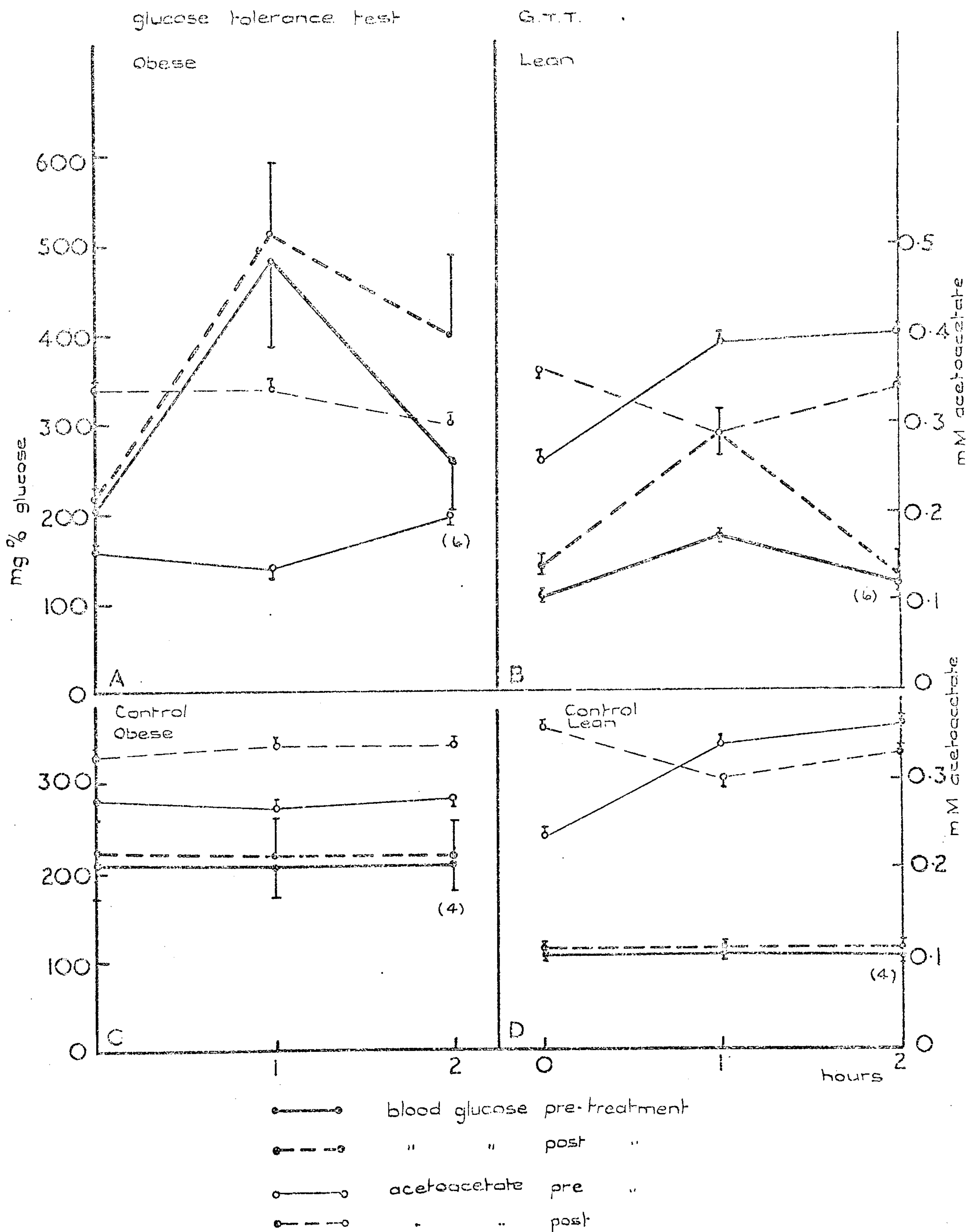


Fig 32 High sodium diet

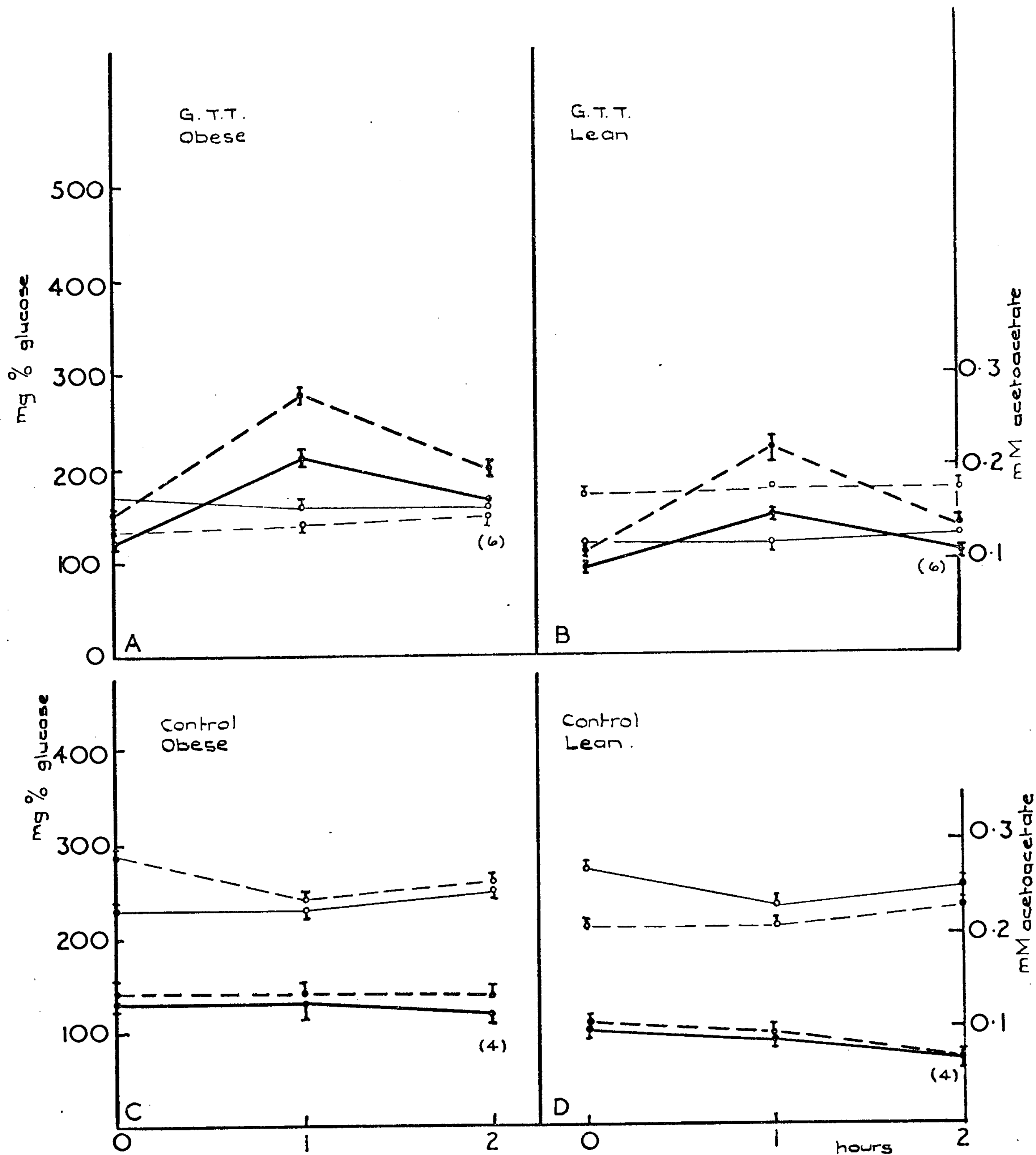


Fig. 33 Potassium deficient diet

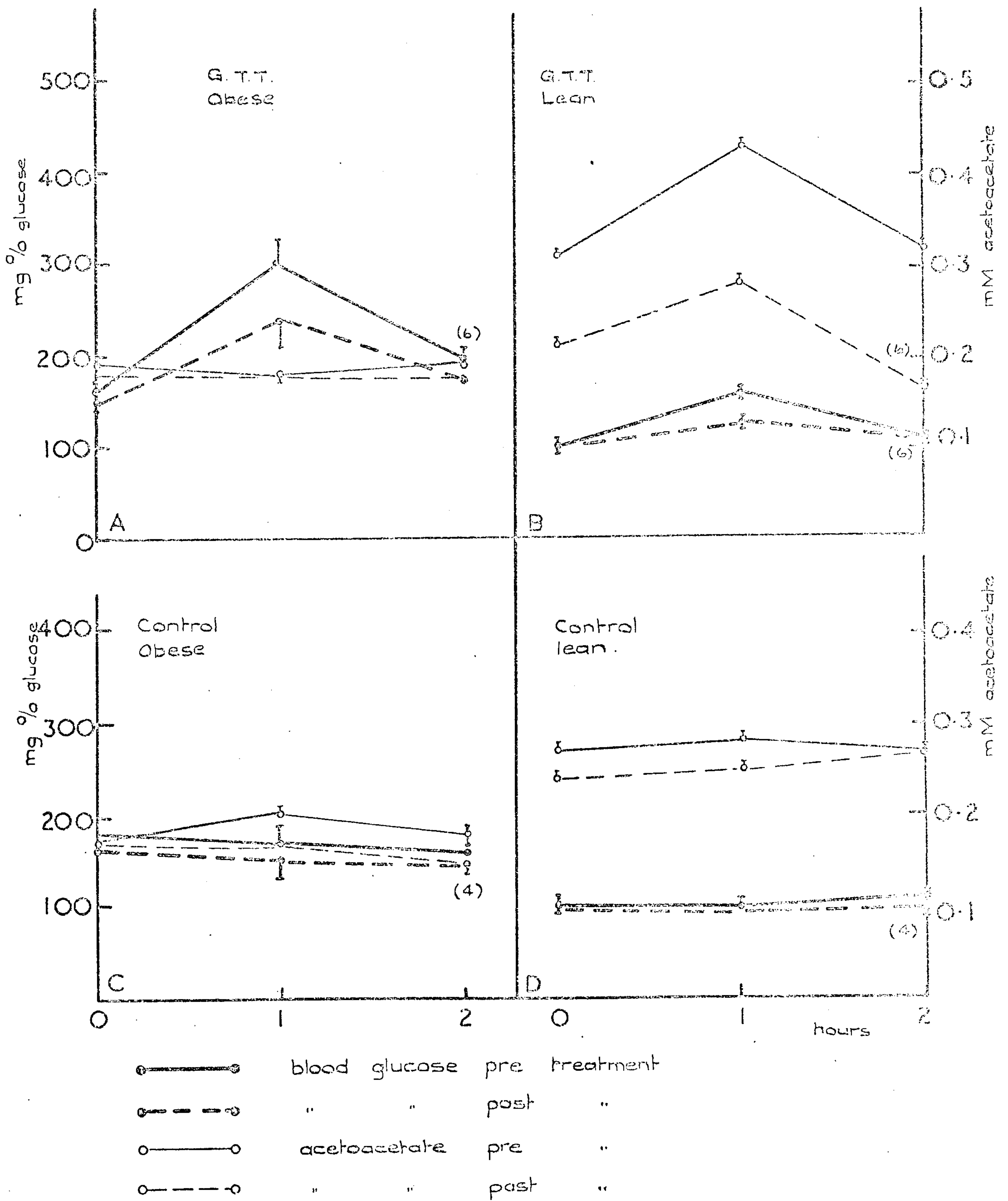


Fig. 34 High potassium diet

FIGS. 35 and 36. The serum insulin and the serum electrolytes of the animals used for the biochemical estimations. (Fig.26 - 29).

FIG. 37. Serum insulin of animals administered potassium chloride (30 min.) or maintained on potassium chloride for 1 week and two weeks.

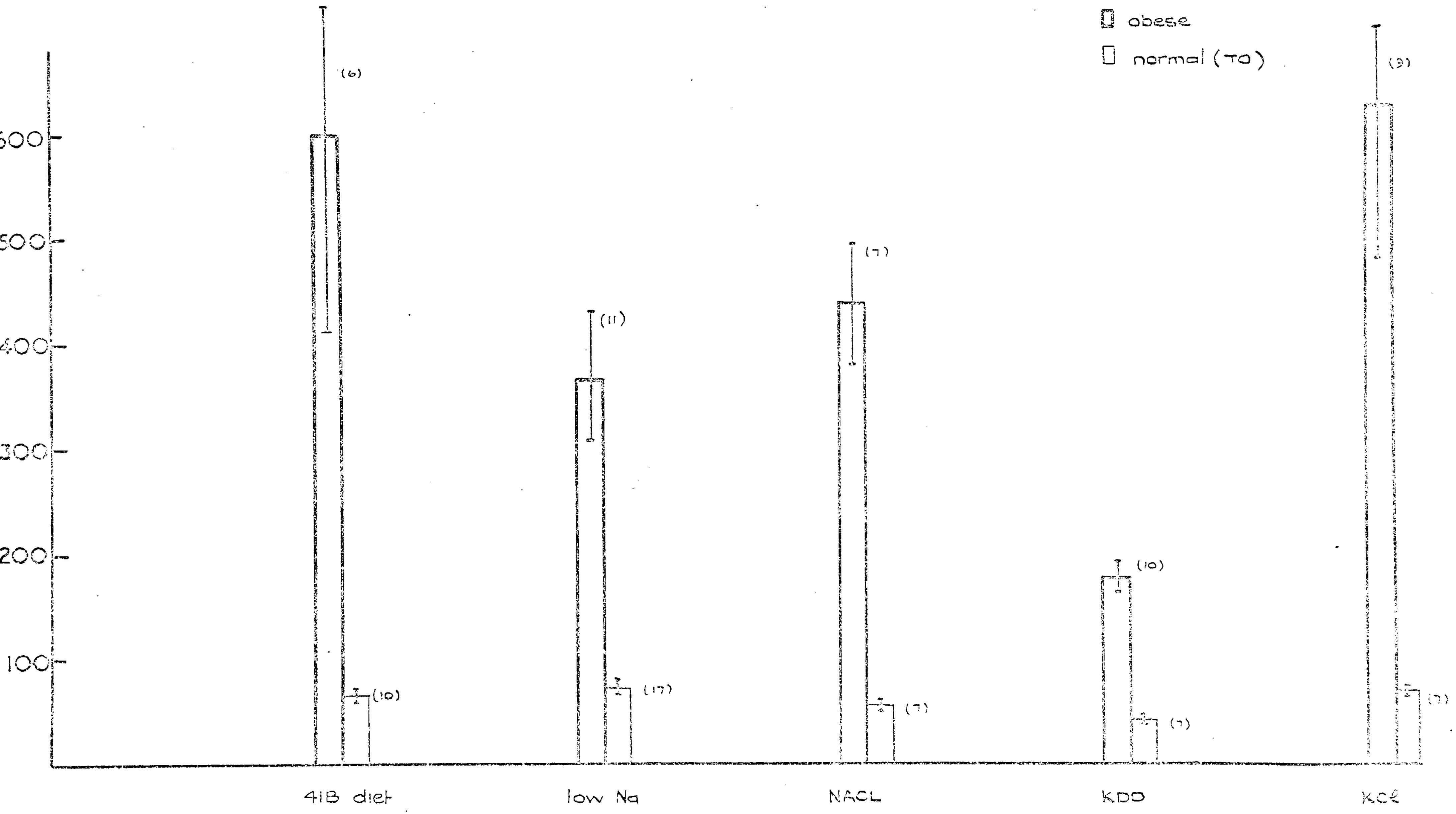


Fig. 35 Insulin - treatments - diets

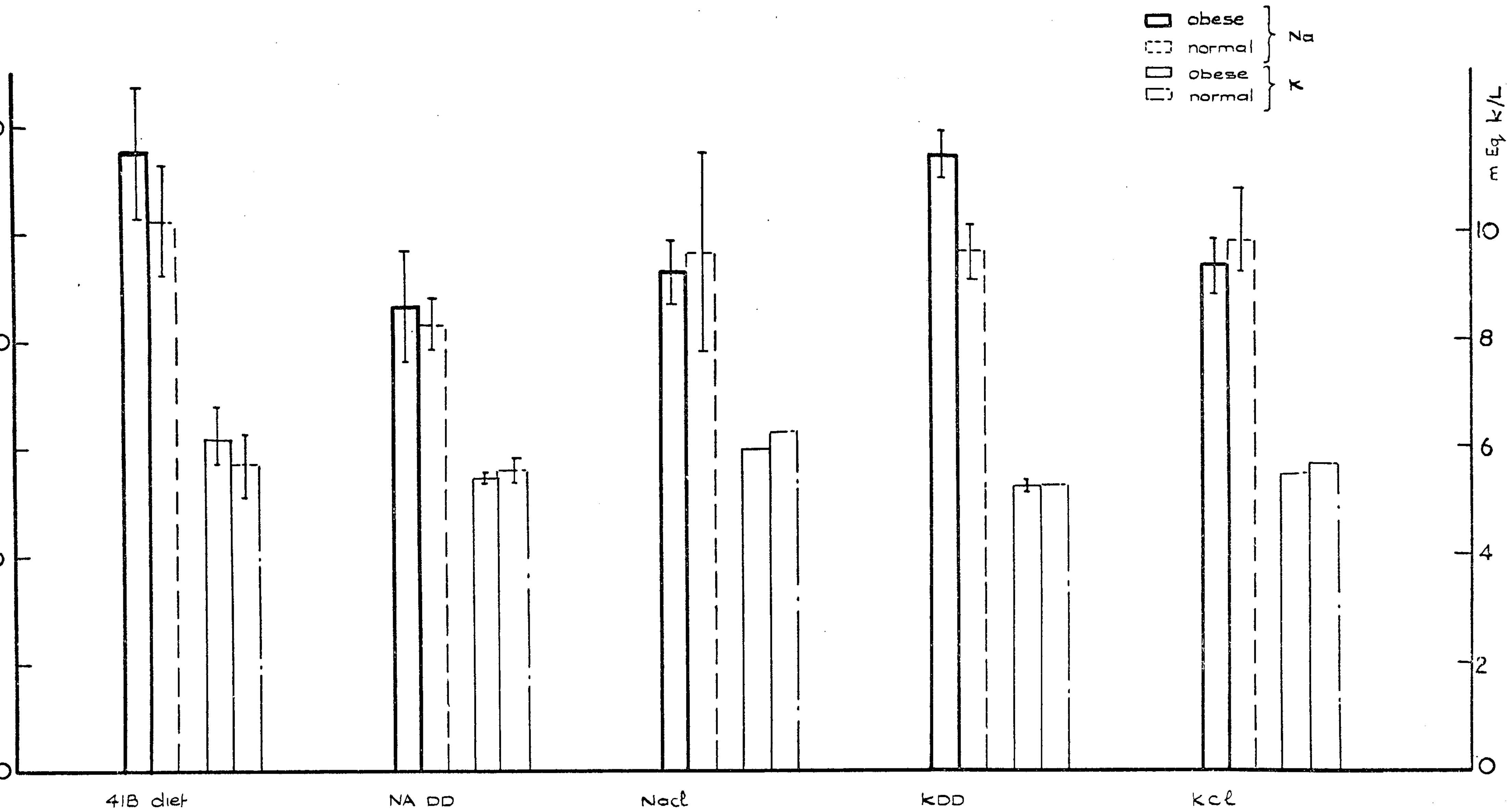


Fig 36 Serum electrolytes

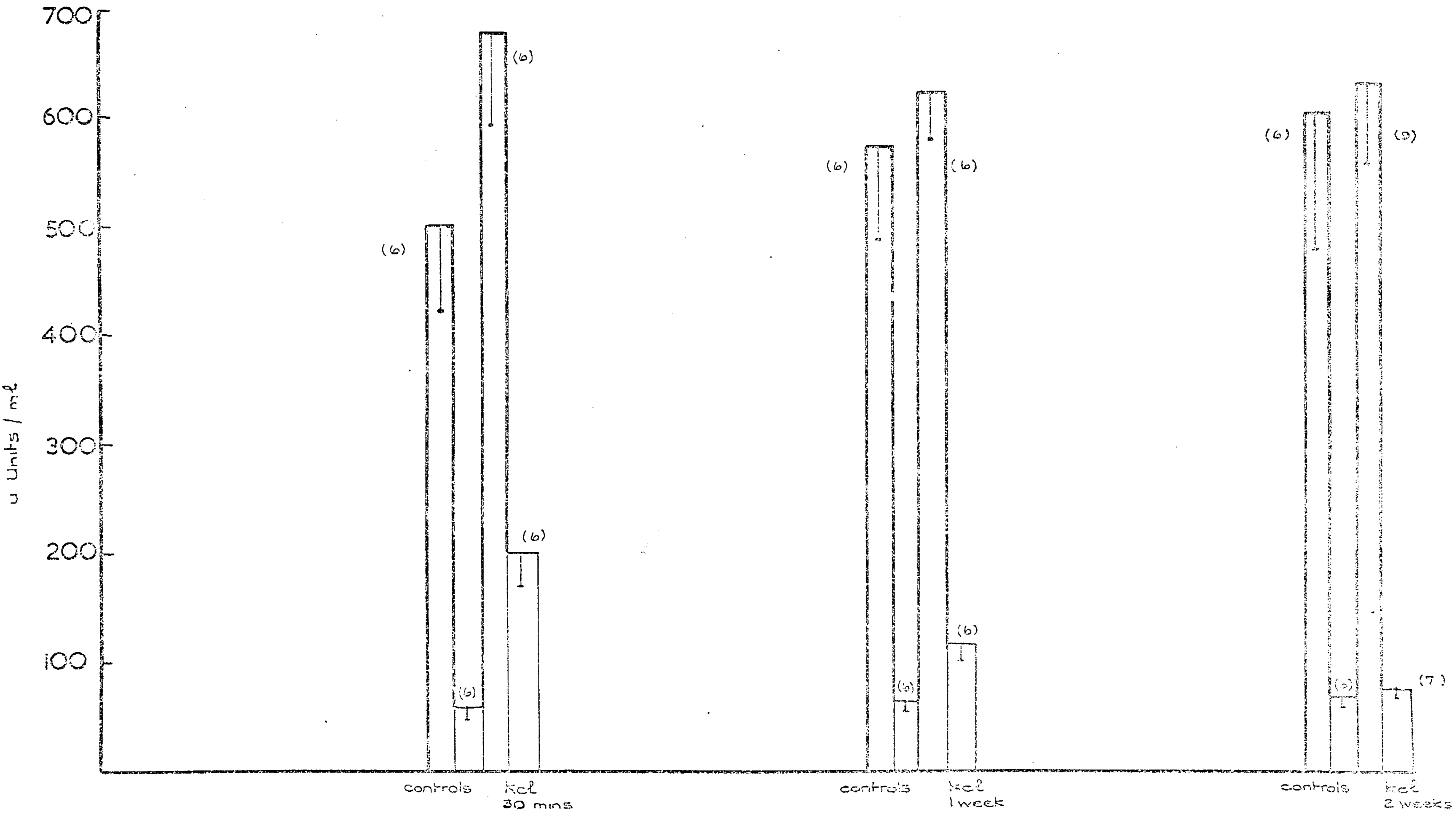


Fig. 37 Serum insulin after the oral administration of 1 % Kcl with time

FIG. 38. The serum potassium concentration after the administration of exogenous insulin. This was performed twice on each animal, once on the 41B control diet and the second time after the animals had been maintained on the high potassium diet or the low potassium diet (potassium depletion was induced with saline, i.e. high sodium diet).

FIG. 39. The blood glucose concentration was measured after the oral administration of potassium chloride. This experiment was again performed twice on each animal, once on a 41B control diet and for the second time after the animals had been maintained on low potassium (i.e. the saline diet).

Normal

high potassium diet

Obese

7

6

5

(6)

(6)

mEq K/L

low potassium diet

6

5

4

(6)

(6)

Fig 38 Disappearance of serum potassium after insulin administration.

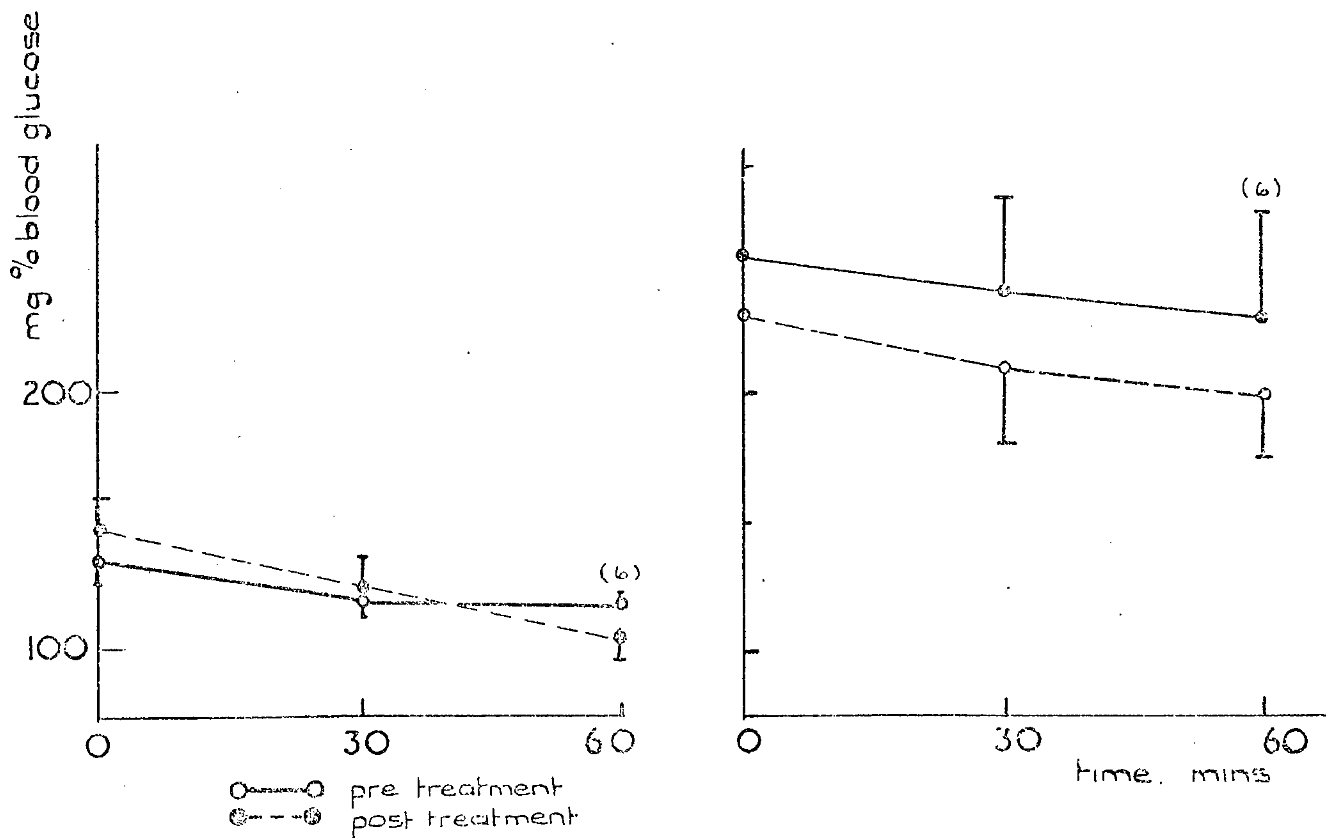


Fig 39 Disappearance of blood glucose after potassium chloride administration in animals on 41B diet followed by a low potassium diet.

FIGS. 40 - 43. Daily electrolyte excretion of the obese and TO animals.

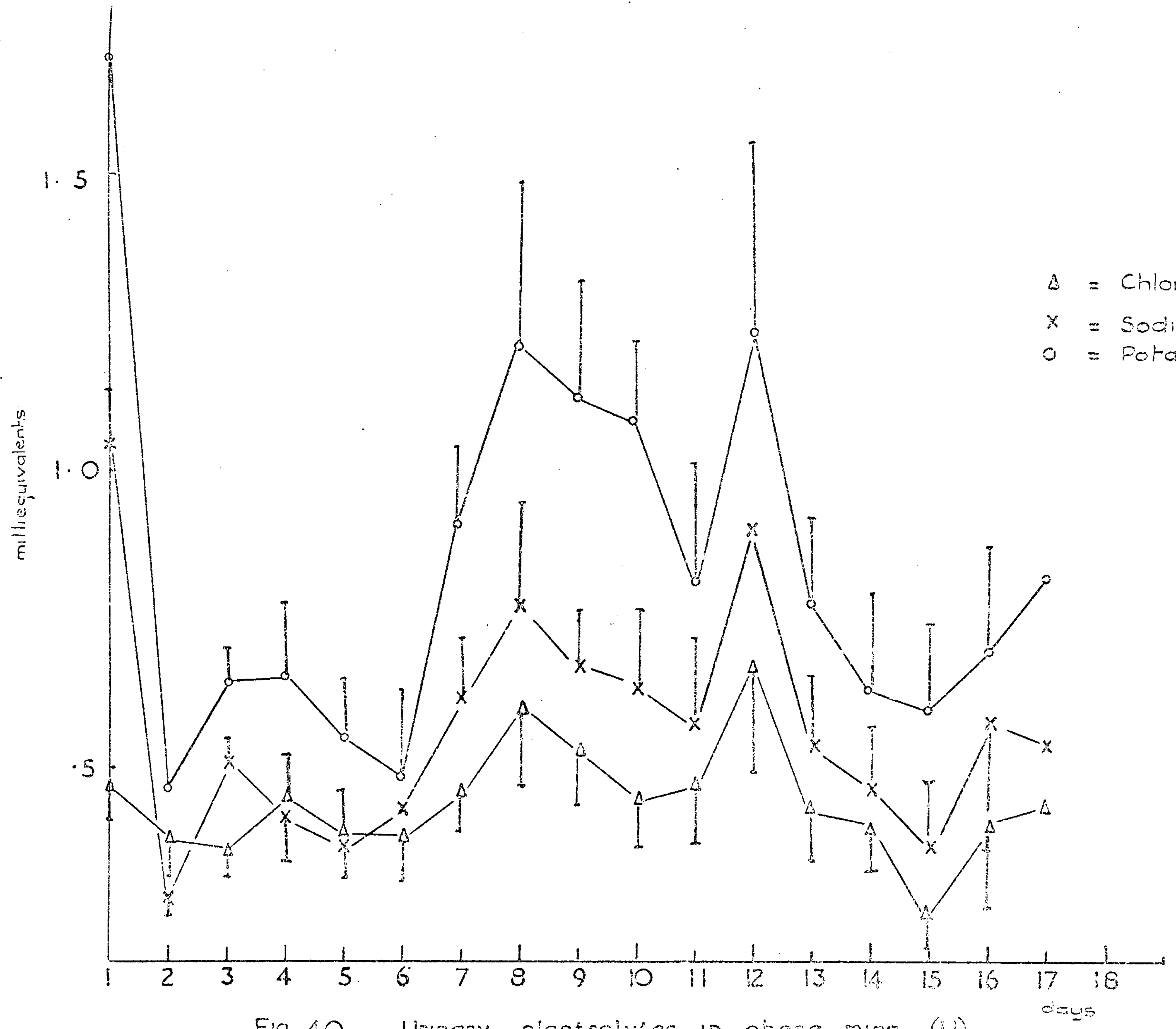


Fig. 40 Urinary electrolytes in obese mice (II)

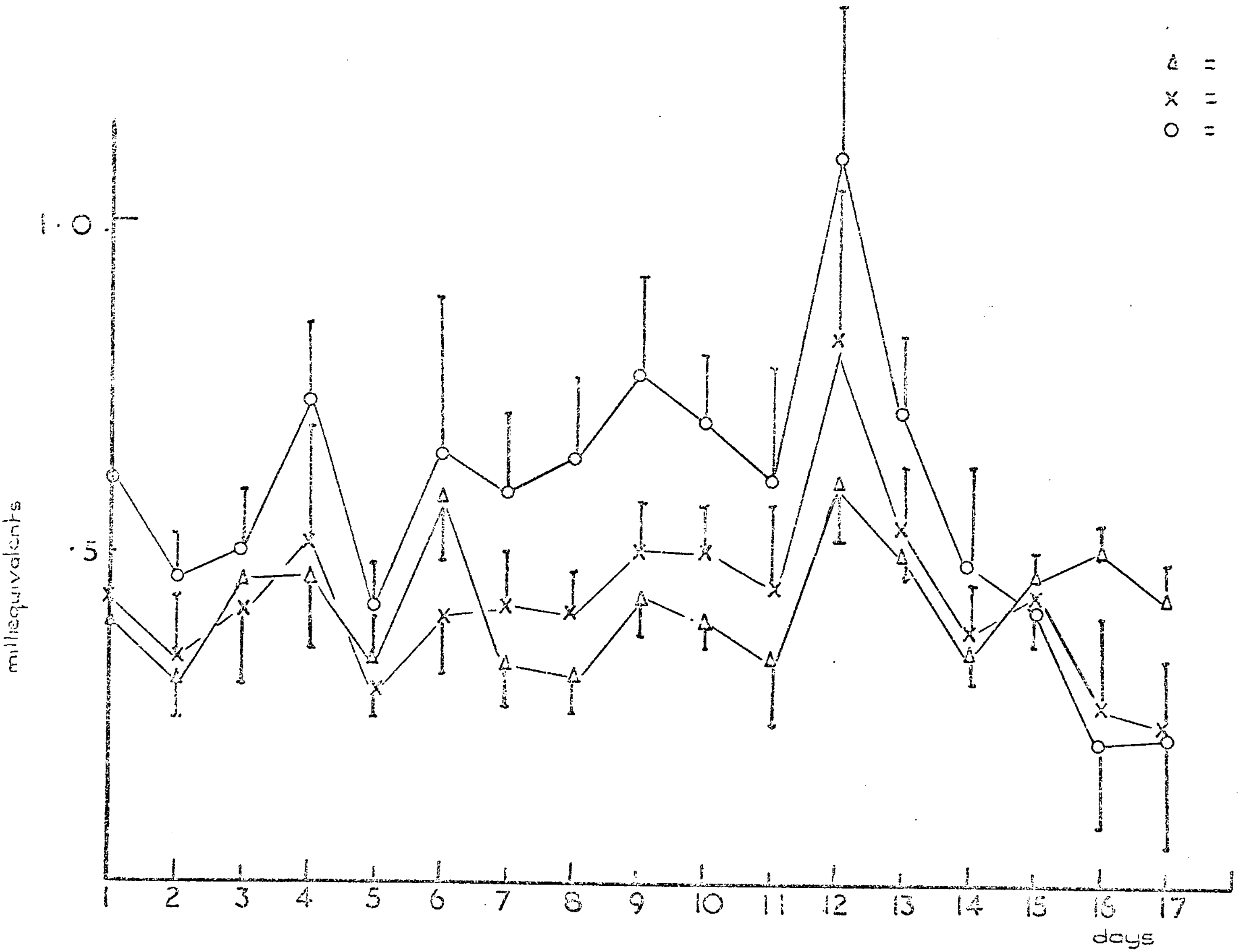


Fig. 41 Urinary electrolytes in normal To mice (10)

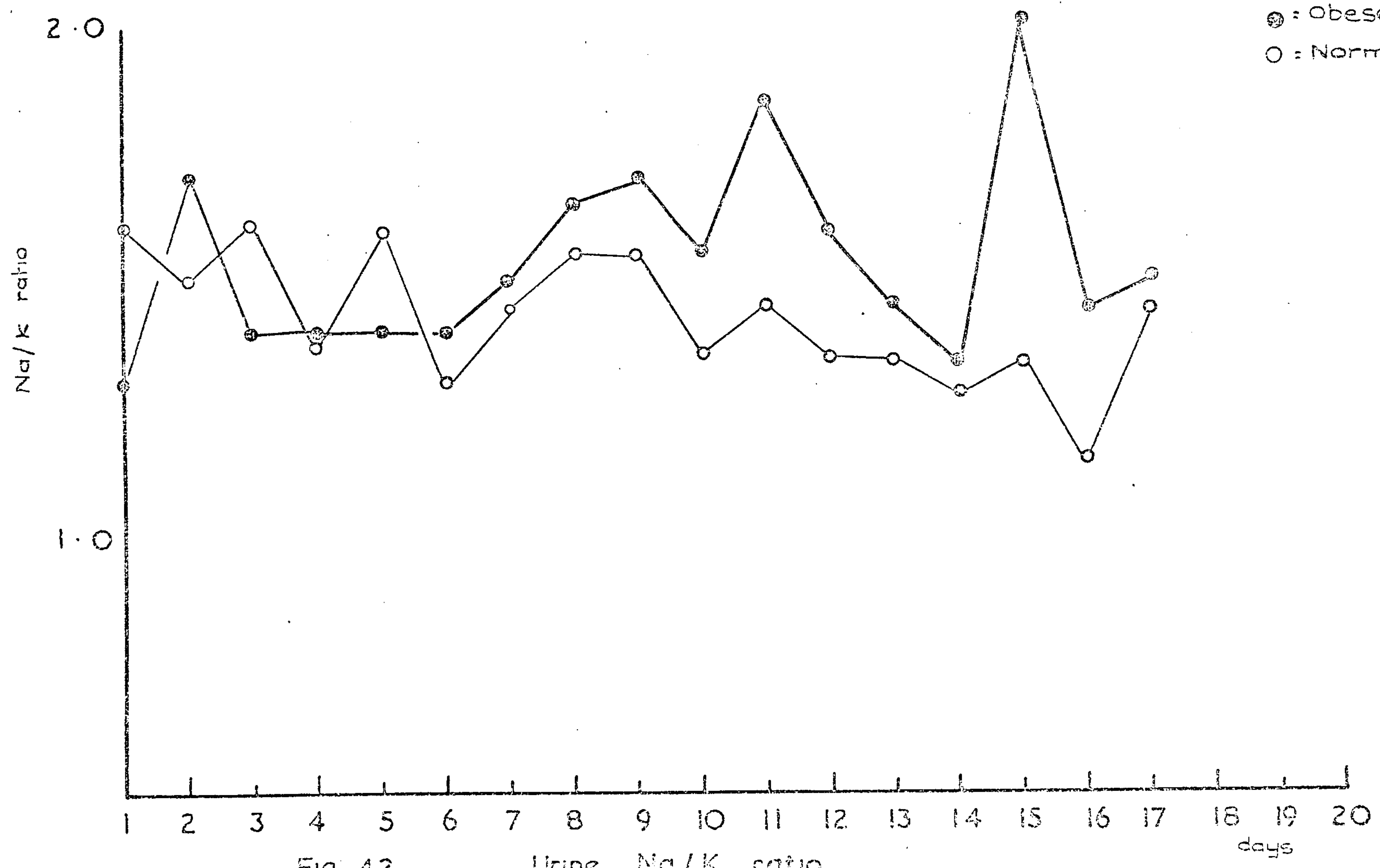


Fig 42 Urine Na/K ratio

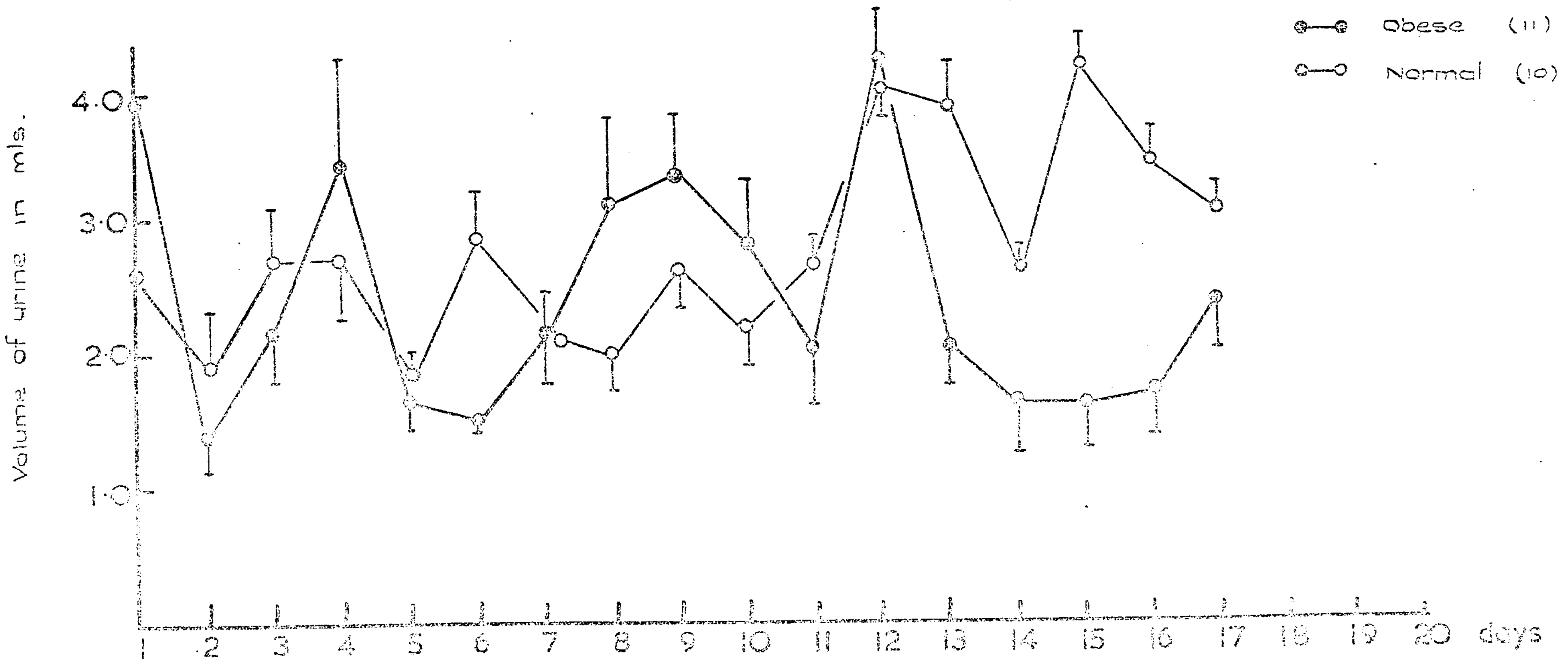


Fig 43 Twenty four hour urine volume

TABLES 7 - 10. Tissue sodium and potassium and the Na:K ratio in animals fed the control diet (41B) and experimental diets (low and high sodium, low and high potassium).

TABLES 11 - 14. The glycogen and water content of the above tissue.

ELECTROLYTES IN TISSUE

TABLE 7. Liver

	OBESE					NORMAL				
	Na in m.Eq/ Kg.dry fat free tissue	P value <	K in mEq/ Kg.dry fat free tissue	P value <	Na:K	Na in m.Eq/ Kg.dry fat free tissue	P value <	K in mEq/ Kg.dry fat free tissue	P value <	Na:K
41B diet (control) (n = 6)	100 ± 6.3	0.6*	397.5 ±7.1	0.6*	1:3.97	95.8 ±7.1		390.8± 7.7		1:4.08
Low sodium diet (n = 6)	102 ± 5.83	0.9	782 ±23.3	0.001	1:7.67	88 ± 4.7	0.5	590 ± 95.4	0.05	1:6.71
High sodium diet (n = 6)	128 ± 8.6	0.05	289 ± 5.1	0.001	1:2.26	140 ± 9.45	0.01	496 ±127.5	0.4	1:3.54
Low potassium diet (n = 6)	150.8±6.5	0.001	400 ± 8.4	0.9	1:2.65	146.8 ±6.34	0.001	450 ± 31.3	0.1	1:3.07
High potassium diet (n = 6)	90 ± 3.1	0.3	528 ±10.1	0.001	1:5.87	72.6 ±11.9	0.01	370 ±107.8	0.9	1:5.10

* Obese and normal are compared

ELECTROLYTES IN TISSUE

TABLE 8. Muscle

	OBESE					NORMAL				
	Na in m.Eq/ Kg.dry fat free tissue	P value <	K in mEq/ Kg.dry fat free tissue	P value <	Na:K	Na in m.Eq/ Kg.dry fat free tissue	P value <	K in mEq/ Kg.dry fat free tissue	P value <	Na:K
41B diet (control)	100.6 ± 9.2	0.3*	400 ± 13.1	0.8*	1:3.77	85.1 ± 2.9	-	408.3 ±16.3	-	1:4.80
Low sodium diet	122.5 ±12.2	0.3	990 ±161.6	0.01	1:8.08	73 ± 3.4	0.1	346.2 ±57.9	0.3	1:4.74
High sodium diet	159.7 ±25.6	0.1	384.2±45.4	0.8	1:2.41	139.7±12.2	0.001	400 ± 19.9	0.8	1:2.87
Low potassium diet	238 ± 51.22	0.02	374 ± 10.3	0.2	1:1.57	236 ± 17.1	0.001	458 ± 19.1	0.1	1:1.94
High potassium diet	91.6 ± 5.1	0.6	369 ± 28.4	0.4	1:4.03	85.3 ± 6.1	> 0.9	433 ± 12.7	0.2	1:5.08

* Obese and normal are compared

ELECTROLYTES IN TISSUE

TABLE 9. Pancreas

	OBESE					NORMAL				
	Na in m.Eq/ Kg.dry fat free tissue	P value <	K in mEq/ Kg.dry fat free tissue	P value <	Na:K	Na in m.Eq/ Kg.dry fat free tissue	P value <	K in mEq/ Kg.dry fat free tissue	P value <	Na:K
41B diet (control)	98 ± 22.9	0.7*	323.3±23.5	0.1*	1:3.30	92.8 ±4.9	-	411.6±34.5	-	1:4.44
Low sodium diet	91.3 ± 7.3	0.7	293.5±47.6	0.6	1:3.22	85.8 ±11.3	0.7	429.3±67.2	0.9	1:5.00
High sodium diet	158.8 ± 8.5	0.001	514.6±232.5	0.4	1:3.24	201.5±5.5	0.001	351 ±39.97	0.3	1:1.74
Low potassium diet	185.7 ±26.9	0.01	377.8±16.2	0.1	1:2.03	125 ± 15.3	0.05	487.61±97.2	0.5	1:3.90
High potassium diet	82.2 ±4.1	0.3	404.7±44.7	0.2	1:4.94	85.6±5.2	0.7	475±14.9	0.2	1:5.49

* Obese and normal are compared

ELECTROLYTES IN TISSUE

TABLE 10. Kidneys

	OBESE					NORMAL				
	Na in m.Eq/ Kg.dry fat free tissue	P value <	K in mEq/ Kg.dry fat free tissue	P value <	Na:K	Na in m.Eq/ Kg.dry fat free tissue	P value <	K in mEq/ Kg.dry fat free tissue	P value <	Na:K
41B diet (control)	271.6 ± 16.7	0.2*	417.5±10.0	0.4*	1:1.54	226.6±23.4		436 ±16.9		1:1.93
Low sodium diet	202 ± 10.2	0.01	334±20.9	0.01	1:1.65	172.7±15.2	0.1	326.4±2.1	0.001	1:1.89
High sodium diet	270 ± 23.6	>0.9	340 ±17.0	0.01	1:1.26	360±114.4	0.001	332±12.4	0.001	1:1.08
Low potassium diet	280 ± 34.5	0.8	411 ±9.5	0.7	1:4.7	280±77.8	0.5	378 ±14.4	0.05	1:1.35
High potassium diet	168.8±11.7	0.001	378 ±19.2	0.1	1:2.24	166±18.0	0.1	356 ±22.8	0.02	1:2.23

* Obese and normal are compared

GLYCOGEN AND WATER CONTENT IN TISSUE

TABLE 11. Liver.

	OBESE				NORMAL			
	Glycogen/ glucose g. equivalent/ Kg. wet tissue	P value	Water content g/Kg wet tissue	P value	Glycogen/ glucose g. equivalent/ Kg. wet tissue.	P value	Water content g/Kg wet tissue	P value
41B diet (control) (n = 6)	79.44±13.3	<0.2*	797.5±21.4	<0.3 *	39.88±3.59		760.9±21.7	
Low sodium diet (n = 6)	48.31±14.31	<.2	603.2±33.1	<0.001	52.23±10.61	<0.3	677.2 ±5.4	<0.01
High sodium diet (n = 6)	66.9 ±10.71	<.5	683 ± 5.1	<0.001	60.61±21.21	<0.1	746.9±54.9	<0.9
Low potassium diet (n = 6)	49.98±9.83	<0.2	778.58 ±7.03	<0.5	43.65±11.8	<0.8	802.1 ± 16.5	<0.2
High potassium diet (n = 6)	76.35±16.51	>.9	695.7 ±2.5	<0.01	51.59± 9.24	<0.3	713.4±48.9	<0.4

*obese and normal are compared

GLYCOGEN AND WATER CONTENT IN TISSUE

TABLE 12. Muscle

	OBESE				NORMAL			
	Glycogen/ glucose g. equivalent/ Kg. wet tissue	P value <	Water content g/Kg wet tissue	P value	Glycogen/ glucose g. equivalent/ Kg. wet tissue.	P value <	Water content g/Kg wet tissue	P value
41B diet (control)	4.17±0.60	0.02 *	628.5±26.1	<0.2 *	7.5 ± 0.97		696.2 ± 36.9	
Low sodium diet	8.58 ±1.62	0.05	552±65.6	<0.3	13.35 ± 7.07	0.1	697.1± 4.9	>0.9
High sodium diet	3.87 ± 0.53	0.8	551.1±56.3	<0.3	6.87 ± 0.88	0.7	663.4± 20.3	<0.5
Low potassium diet	1.74 ±0.31	0.01	626.2±15.2	>.9	6.18 ± 1.21	0.5	794.3 ± 1.9	<0.05
High potassium diet	7.01 ±0.98	0.05	662.3±47.1	<0.6	13.35 ± 3.23	0.1	679.3 ±55.9	<0.05

* Obese and normal values are, compared

GLYCOGEN AND WATER CONTENT IN TISSUE

TABLE 13. Pancreas

	OBESE				NORMAL			
	Glycogen/ glucose g. equivalent/ Kg. wet tissue	P value <	Water content g/Kg wet tissue	P value <	Glycogen/ glucose g. equivalent/ Kg. wet tissue.	P value <	Water content g/Kg wet tissue	P value <
41B diet (control)	4.61 ± 1.81	0.1*	595.6 ± 39.8	0.9*	1.91 ± 0.20		607.3± 33.2	
Low sodium diet	2.33 ± 0.41	0.2	529.2 ± 52.5	0.4	1.39 ± 0.22	0.2	628.6 ± 37.8	.7
High sodium diet	4.54 ± 1.02	>0.9	637.2 ± 46.3	0.6	2.42 ± 0.42	0.3	694.1 ± 88.7	.4
Low potassium diet	2.75 ± 0.65	0.3	555.7 ± 55.9	0.6	1.89 ± 0.10	>0.9	692.1 ± 73.9	0.3
High potassium diet	3.58 ± 0.68	0.6	627.1 ± 12.2	0.6	1.87 ± 0.22	0.9	661.3 ± 21.9	.3

* Obese and normal are compared

GLYCOGEN AND WATER CONTENT IN TISSUE

TABLE 14. Kidneys

	OBESE				NORMAL			
	Glycogen/ glucose g. equivalent/ Kg. wet tissue	P value <	Water content g/Kg wet tissue	P value <	Glycogen/ glucose g. equivalent/ Kg. wet tissue.	P value <	Water content g/Kg wet tissue	P value <
41B diet (control)	1.8658±0.415	0.2*	838.8±35.9	0.2 *	1.35±0.00		724.6±61.9	
Low sodium diet	1.96 ± 0.30	>0.9	712.6±16.8	0.02	1.42±0.22	0.8	671.6±17.1	0.5
High sodium diet	2.49±0.20	0.4	653.0±36.6	0.01	1.62±0.22	0.3	698.4±7.6	0.8
Low potassium diet	2.44±0.44	0.3	799.6±4.8	0.04	1.45±0.00	0.5	797.8±3.9	0.3
High potassium diet	1.93±0.42	0.9	672.8±35.7	0.01	1.35±0.10	0.0	661.0±35.1	0.5

* Obese and normal are compared

CHAPTER VI

ALLOXAN AND CAFFEINE IN THE OBESE HYPERGLYCEMIC MOUSE

INTRODUCTION AND LITERATURE REVIEW

Alloxan

Alloxan was first made by Brugnateli (1818) by the oxidation of uric acid. Structurally it is a 6-membered organic compound with 4 keto groups and 2 imino groups. Lang (1866) reported the presence of alloxan in the urine of a patient with cardiac failure. Archibald (1945) suggested its presence in blood. Jacobs (1938) first described its hypoglycemic effects and Dunn et al. (1943) were the first to discover its specific effects on the islets of Langerhans in the pancreas of rabbit, thereby introducing the first chemical tool by which diabetes could be selectively produced in experimental animals. Hughes et al. (1944) discovered that the effects of alloxan were virtually confined to the β cells of the islets thus making the compound both a favourite and valuable instrument in the field of experimental diabetes. However, it is now known that alloxan causes damage to the liver, kidneys and adrenals as well (Lukens, 1948).

Ruben and Yardumian (1945) observed that alloxan produced diabetes in the rat even when orally administered. Jones (1947) observed that severely diabetic rats (blood sugar 900 mg%) remained diabetic but rats with initial and mild diabetes (blood glucose 250 mg%) had a tendency to improve when alloxan was administered. Sub-diabetogenic doses of alloxan produced only transitory diabetes and minimal lesions of the larger islets in an alloxan resistant variety of the hooded rat. The animals proved resistant to any continued administration

of alloxan (Duff, 1945). The guinea pig is also believed to be resistant to alloxan (West and Highet, 1948). Wraisben (1948) first used it on mice. Solomon and Mayer (1962) found that alloxan administration produced β cell regranulation and normalized blood glucose. Almost every species of laboratory animal and man has been subjected to this compound.

Mechanism of diabetogenic action

Reaction with sulfhydryl groups

There is overwhelming evidence to demonstrate the importance of glutathione, a naturally occurring sulfhydryl tripeptide in the maintenance and protection of the sulfhydryl group of some enzymes in the cells of the body including the pancreas from the diabetogenic action of alloxan (Hopkins et al. 1938; Pur, A. 1935), e.g. succinic dehydrogenase, glucose-6-phosphate dehydrogenase, glucose-6-phosphatase. It can protect provided it is administered shortly before alloxan. If the enzymes are already inactivated by alloxan, reactivation is possible only within the first three minutes. This hypothesis that alloxan may act by the inactivation of SH-groups was first advocated by Lazarow (1946; 1947) and Lazarow and Patterson (1948). These enzymes which constitute free SH-groups reduce alloxan to dialuric acid, a non-diabetogenic compound, provided it is not reoxidised to alloxan. Besides alloxan may react even further with glutathione to form an "addition compound" with an absorption spectra maxima at 305 nm. This substance may be involved in diabetogenic mechanisms (Patterson et al., 1949).

This is confirmed by the drop in glutathione content of various tissues of the body after alloxan administration (de Carro and Rovida, 1937). Leech and Bailey (1945) found that blood glutathione reached almost zero within five minutes of alloxan treatment and did not return to normal even after five hours. This is hardly surprising because when alloxan is administered it first comes in contact with blood and is reduced by the glutathione present to dialuric acid and only secondarily comes into contact with other tissues which consequently will show a smaller fall in glutathione content. This is obviously dependent on the dosage of alloxan. Labbe et al (1936) found a decrease in blood glutathione in diabetics. Conn et al. (1948) demonstrated the relationship between diabetes and blood glutathione when they found a temporary reversal of hyperglycemia and glycosuria in response to the intravenous administration of glutathione in patients receiving ACTH. Lazarow (1949) gives a comprehensive review on the subject. Treatment of serum with reduced glutathione or cysteine has been reported to destroy the ILA (Saman et al. 1963).

Hammarström and Ullberg (1966) found that alloxan accumulated in the islets to a far greater extent than any other tissue with the exception of an initial renal concentration. In the β cell, the SH-groups necessary for insulin synthesis are provided by cysteine, glutathione and enzymes containing free SH-groups. Therefore glutathione concentrations in the beta cells may be lower than in the other tissue and thus these cells are more susceptible to alloxan. Hammarström et al.(1966)

found that alloxan concentrated in the beta cells of adult mice but not in young alloxan-resistant mice.

The GSH values of tissue in inherent obesity and diabetes are not yet available. In this situation presented by the obese animals, hyperinsulinism is a characteristic feature and one might expect the GSH concentrations of the pancreas to be at a critical level in which case these animals will be even more susceptible to insulin.

Griffiths (1948) and Mukerjee et al. (1955) also demonstrated that the experimental lowering of GSH content of the blood increased the susceptibility tc various forms of diabetes. This stresses the importance of measuring GSH values in blood. It is doubtful if measuring GSH concentrations in the islets will reveal much unless it is possible somehow to selectively destroy or completely inhibit the acinar tissue and the alpha cells of the islet which are believed to be impervious or unharmed by alloxan. Another alternative may be to compare the GSH values of whole intact healthy β cells and of necrotic beta cells after alloxan treatment and thus by so doing, to infer on the GSH variations.

Alloxan and glucose

It is also interesting to note the antagonistic effects of alloxan and glucose. Bhattacharya (1952) found that mannose and fructose protected the rat against the diabetogenic effects of 40 mg/kg alloxan while galactose seemed quite ineffective. Ordinary mammalian hexokinase is a non-specific enzyme for

glucose and fructose. It is also a sulfhydryl enzyme and is inactivated by alloxan and reactivated by glutathione. It also exists in the beta cells. Bhattacharya (1954) found that the protective effectiveness of glucose, mannose and fructose was 100%, 50% and 15% respectively. However the presence of glucose did not protect the succinic oxidase from alloxan. His results further demonstrated that the protection against a given dose of alloxan by a certain dosage of glucose may be counteracted by increasing the dose of alloxan and similarly the diabetogenic effects of alloxan may be suppressed by increasing the dose of glucose administered. Bhattacharya therefore postulated the interference or inactivation of hexokinase by alloxan.

Watkins et al. (1964a and b) and Cooperstein and Lazarow (1964) in their studies on toadfish islets demonstrated that alloxan was confined to the extracellular space and proposed that the primary site of action was the beta cell membrane. They also showed that alloxan damaged the islet cell membranes and that pre-treatment of tissue slices with GSH or cysteine before incubation with alloxan protected them from the action of alloxan but did not affect the permeability to Mannitol-C^{14}. Cooperstein and Lazarow (1969) also suggested that alloxan may act by binding to the cell membrane at the site of glucose transport and it therefore follows that the protective action of glucose is dependent on its greater affinity for this receptor. These authors also proposed the existence of a

stereospecific D-glucose carrier. Alloxan may well use this same carrier. This receives support from the observation that fasting increased the toxicity of alloxan (Mayer, 1966).

Histological studies after alloxan administration

Hughes (1946, 1947) demonstrated that the large islets of the pancreas contain small beta cells and the small islets contain large beta cells. By the use of graded doses of alloxan he produced a differential necrosis of the small beta cells in the large islets. He suggested that these small diameter beta cells were senescent and were replaced in time by the transformation of acinar cells into large diameter beta cells. This was thought to be the situation in 10% of the normal islets but to be much more common after the administration of alloxan. It was also believed to be indicative of the occurrence of a cycle of growth followed by death or decay of the beta cells and their replacement by the exocrine acinar tissue. This transformation of acinar tissue was thought to be stimulated by the necrosis of the β cell and that the acinar cells were already in a resting state, pre-destined for transformation into the islet beta cells. This hypothesis was based on the fact that ligation of the pancreatic duct can stimulate this acino-insular transformation because the exocrine functions of the cells are inhibited. Often acinar cells appear to be stimulated by the breakdown or atrophy of the mature islet and the need for β cell replacement results. When the supply of

acinar cells becomes exhausted the animals will begin to demonstrate the signs of insulin deficiency and this requires a period of 6 - 12 months in man and dog.

Hughes also suggested that the regenerated cells secreted some insulin which in turn was partially responsible for the lowering of blood sugar. This however resulted in the exhaustion of the beta cells because they were still in insufficient numbers to cope and hyperglycemia resulted once more. This process was repeated several times until the normal situation was reached. It was further suggested that proliferation and transformation took about eight weeks before normal beta cell granulation was established. This is due to the fact that initially at least, the rate of insulin secretion is at least as fast as the rate of insulin synthesis and accumulation is therefore impossible.

House (1958) reported similar findings in the alloxan diabetic hamster. He suggested that intermittent and permanent diabetes improve as the result of the proliferation, transformation and incorporation of acinar cells into beta cells in the islet tissue. Transient diabetes is therefore the consequence of β cell recovery, beta cell regeneration and replacment taking place sometime after beta cell destruction.

More recently Patent and Alfert (1967) studied histology and cytology of the mouse pancreatic islets, ducts and acinar tissue after the administration of alloxan (0.5 mg/10 g body weight in 2% physiological saline into the left external jugular vein). They found the sequence of degenerative changes which occurred in the islets following a single injection of alloxan

comparable to those described by other workers (Bencosome, 1955; Lazarus, 1962; Lazarus and Volk, 1962). Williamson and Lacy (1959) found evidence which seemed to show that alloxan was initially involved in granule formation in that they found an initial increase in β cell granules before their loss.

Patent and Alfert (1967) observed that by 24 hours the centre of the islet was filled with debris of fragmented, pycnotic nuclei and necrotic cells which gradually disappeared within 48 hours. This left the islet populated principally by the alpha cells and the islet was now ready for the regeneration of beta cells from the acinar tissue. This was heralded two or three days after alloxan administration by the loss of chromidial substance or basophilic endoplasmic reticulum and zymogen granules which characterise the acinar cells and the acquisition of the beta cell granules and the marked acidophilia of the acinar tissue. The fully transformed acinar cells resemble an over-large beta cell.

Patent and Alfert (1967) further suggested that ductal proliferation, for example by the budding of ductal epithelium, could also be the source of islet cells. Volk and Lazarus (1960a and b; 1963) demonstrated in rabbits treated with glucagon and cortisone or cortisone alone that β cell neo-genesis arose from hyperplastic ductules and also the presence of intracellular ducts in these animals.

Patent and Alfert (1967) did not find islets containing the normal α:β cell ratio. Théret and Tamboise (1963) from their investigations on the guinea pig and Bjorkman and Hellman (1964)

from their work on duck felt that the alpha cells too are derived from the acinar tissue. If alpha cells do proliferate, their source is unclear, or mutually agreed upon by other workers. Patent and Alfert (1967) too are unclear about whether the alpha cells actually proliferate or simply become prominent as the consequence of β cell disintegration and disappearance and they therefore appear prolific.

Solomon and Mayer (1962) found the islets of alloxan-treated obese mice to be strikingly more granulated than the control mice. The β cells were healthy and the granules varied from coarse to fine and were evenly dispersed.

Thus, despite the countless and intensive investigations made utilising alloxan in this field, its actual mode of action still remains obscure and the discovery of its unusual and paradoxical effects on the obese animal did not help to elucidate it.

Caffeine

This is the best known and popularly considered the most potent component of coffee. It constitutes at least 1% of this beverage (Report of the Chemical Laboratory of the American Medical Association, 1928). It is also present in other popular drinks, e.g. coco-cola and cola flavoured drinks. It is generally considered to be a mild cerebral stimulant with mild diuretic properties. It is also believed to increase the basal metabolic rate. Coffee is commonly believed to be harmful, nevertheless it is a beverage consumed in vast

quantities by a very large proportion of the world populace including the diabetics and the obese; however its physiological role has as yet received very little attention. Caffeine is completely metabolised by man (Cornish and Christenson, 1957) and the ill effects observed after the ingestion of about one gram are the result of the stimulatory effects on the central nervous system. Roth et al. (1944) felt that caffeine may be contributory to peptic ulcers.

Kuftinec and Mayer (1964) demonstrated that caffeine administration increased β cell degranulation and further aggravated and prolonged the hyperglycemic state in the obese animals while it left normal animals unaffected. Coffee has recently become associated with cardiovascular diseases (Little et al. 1966) but he felt that the relationship between coffee intake and serum lipid concentrations was significant only in patients suffering from heart diseases. Brown (1962) and Little (1965) did not feel that coffee ingestion necessarily led to the development of coronary thrombosis. Bellet et al. (1965, 1966) reported the rise of serum-free fatty acids in normal men, diabetic and artherosclerotic patients after the administration of caffeine sodium benzoate.

Lambert et al. (1967) demonstrated in vitro that caffeine (10 mM) stimulated insulin release, even in the absence of glucose by about 15%. Caffeine and glucagon together brought about a stimulation of 55% while caffeine and tolbutamide resulted in a 205% increase.

Jankelson et al. (1967) in their studies on maturity-onset diabetic patients found that blood glucose was significantly

raised following two cups of coffee. The serum insulin was variable and showed no significant change. Cheraskin et al. (1967) demonstrated a hyperglycemic effect following the administration of pure caffeine in normal healthy males.

Naismith et al. (1970) in their studies using coffee and decaffeinated coffee on normal people concluded that the habitual use of caffeine had no influence on fasting blood sugar and that the small but significant reduction of blood glucose and the failure of cholesterol to return to pre-experimental values was believed to be due to some component of coffee solids other than caffeine. This is in contradiction to Bellet et al. (1965) who concluded that the physiological effects of coffee were due entirely to its caffeine content.

The interest in caffeine and diabetes arises from the work of Kuftinec and Mayer (1964) and Mayer (1966). Mayer's interest in the subject was aroued by the structural similarity of caffeine to alloxan, although there are differences in structure, notably the second pentagonal ring in caffeine which is not present in alloxan. Histological studies in the treated obese animals did not throw any light on the hyperglycemic effect of caffeine except that the islets were more degranulated in the treated animals.

Alloxan and Caffeine in the obese hyperglycemic mouse

Contrary to the numerous previous investigations on normal animals Solomon and Mayer (1962) observed that alloxan produced an immediate and long lasting decrease in blood sugar accompanied by a massive increase in beta cell granulation in the obese mouse. This corpulent animal also exhibited a very great

sensitivity to caffeine (Kuftinec and Mayer, 1964). The simultaneous administration of caffeine and alloxan either preserved or increased the state of degranulation, or decreased the extent of degranulation, i.e. the regranulation which took place, depending on the proportion of caffeine to alloxan that was administered in both the normal and obese animals. Thus it appears that alloxan actually protected the beta cells from the effects of caffeine in the obese animal while in the normal animal the beta cells are protected by caffeine from the diabetogenic action of alloxan. Alloxan and caffeine are therefore direct antagonists and Mayer has explained this antagonism by the close relationship of their chemical structure and that they probably act on the same cellular structure. Fasting seemed to increase the toxic effects of caffeine. After a twenty-four hour fast there was a 72% survival rate which was only 10% after a 48-hour fast. This is in contrast to alloxan where tolerance is apparently increased with fasting.

This is also the first time that alloxan has been studied in terms of inherent diabetes and obesity and Mayer has based his few conclusions on the cursory studies of the response of blood sugar, glucose and insulin tolerance tests and some histology.

Thus there are several ifs and buts about this subject. Are the observations made by Mayer and co-workers on the alloxan-treated obese animal the result of beta cell regeneration or are the obese genuinely more resistant to alloxan because of the high concentrations of circulating blood glucose which are sufficiently high to protect the beta cells from the diabetogenic

action of alloxan? (Bhattacharya, 1954; Carter and Ezzat, 1962). The islets of the obese are already hyperplastic and necrosis is not uncommon and therefore a small dose of caffeine (2 mg) may be the final straw. That extra stimulation or stress on the islets may have actually brought upon premature necrosis, degranulation and therefore secretion. Perhaps also the beta cell and islet turnover may be more rapid in the obese animal or acinar cell transformation into the beta cell is inhibited and thus the source of the beta cells is limited. Which becomes evident first - physiological or morphological abnormality?

Gepts et al. (1960) also had evidence which they interpreted as acinar cells differentiating into islet tissue. They also thought that another source of islet tissue might be the end of the secretory ducts. Hellman et al. (1960) and Gepts et al. (1960) observed a diminished proportion of alpha cells in the obese. Hellman and Petterson (1966) obtained significantly higher values for nuclear and nucleolar size in the β cells of obese mouse islets in comparison to the lean littermates. According to Casperson (1950) nucleolar size depends on protein synthesis and according to Kracht (1958) and Sandritter et al. (1951) is relevant to insulin synthesis. Thus this reflects on the increased metabolic activity of the obese islets in response to hyperglycemia which is characteristic of this syndrome.

Reliable GSH values in inherent obesity and diabetes are not yet available. In this situation where hyperinsulinism is a characteristic feature, one might expect the GSH concentrations to be low in these animals in which case they would be even more

susceptible to alloxan. However the results of Solomon and Mayer (1962) and Mayer (1966) have demonstrated these animals to be more resistant. GSH is essentially intracellular and leaves the cells with great difficulty.

Regranulation too appears extensively (Solomon and Mayer, 1962) and from the morphological studies available one presumes that acinar cells are being rapidly transformed into beta cells. The question now arises that if this is the case, how has alloxan triggered off this transformation and what influence has it got on this rate of transformation? Is it merely a response to β cell requirement? What is unique about the pancreas or islets of these obese animals? Morphologically the islets of the obese mice consist of larger beta cells and the ratio of beta cells to alpha cells is greater and these animals have six times as much islet tissue as the non-obese (Gepts et al. 1960). The apparent primary cause of the syndrome appears to lie with an impaired peripheral uptake of glucose (Chapters IV and V) but the morphological evidence suggests that the obese mouse pancreas is not normal. However, it is uncertain whether this state of the pancreas is merely the result of an increased demand on the islets or whether it is part of the syndrome.

Alloxan inhibition of adenyl cyclase was reversible. Caffeine inhibited phosphodiesterase but alloxan had no effect. Thus, although both caffeine and alloxan act as antagonists to cyclic AMP they do so at different points in the system and are independent of each other (Cohen and Bitensky, 1969). Therefore this does not explain the results of Mayer's group of

investigations (1962, 1964, 1966). Thus it appears probable that another mechanism involving alloxan and caffeine as direct chemical antagonists must be responsible. The effect of caffeine on GSH is not known. Perhaps caffeine inhibits the oxidation of GSH to GSSG or stimulates the reduction of GSSG to GSH or as Mayer (1966) explained they simply neutralized each other's effects. Likewise the oxidation of cysteine to cystine may be stimulated. Cysteine too protects the cells from alloxan.

The relationship between alloxan and caffeine and that of alloxan and glucose has been established but nothing has been reported involving alloxan, caffeine and glucose. It appears possible that alloxan and glucose compete at the same site of glucose transport or metabolism. Alloxan and caffeine probably act at a common site or merely with each other but their relationship has not been characterised as yet. Is it possible that all three substances act at the same site?

Thus in the light of all the uncertainties and obscurities of previous work utilizing alloxan and caffeine and also since the investigation involving inherent obesity and diabetes is so very limited it was decided to start at the beginning i.e. by repeating Mayer's work.

<u>Methods and Materials</u>

The daily blood glucose response, aceto-acetate and weights of the alloxan treated and control animals were studied. Alloxan in citrate buffer pH 3.2 and pH 4.0 was used (Chapter II. p.53). The control animals were administered with buffer alone. Treatment was carried out after a forty-eight hour fast. The animals

were killed 6 days after treatment and their serum insulin was measured using the radioimmunoassay method (Chapter III). Blood glucose and acetoacetate were measured simultaneously on the auto-analyser (Chapter II, p.60).

The same procedure was followed using caffeine (Chapter II, p.54). A glucose tolerance test was performed on the animals both before treatment and after treatment. As usual acetoacetate was measured and the urine analysed with the aid of Labstix. 16 week old animals were used for all the experiments.

RESULTS

The response of the animals during the post-treatment period (the interval between alloxan administration on the day that the 48-hour fast was terminated and the end of the experiment, i.e. 6 days after alloxan administration) is compared with the response produced during the pre-treatment period before the fast was commenced. Thus the animals are used as their own controls.

Alloxan pH 3.2 (Figs. 44 and 45)

The blood sugars of the alloxan-treated obese animals reached only 63% of the pre-treatment value on Day 6 after alloxan treatment, i.e. day 15 of the experiment (mean value 400 mg% pre-treatment - 252 mg%) while the sugars of the control obese animals were 120% of the pre-fasting values on Day 6 (mean value 338 mg% pretreatment - 405 mg%). In the experimental group the maximum blood glucose concentration was observed on Day 13, i.e. 4 days post-alloxan treatment (275 mg%).

Pre-test blood sugar values were generally seen to show less fluctuation than post-treatment values. In the case of the control group the post treatment sugars showed a gradual increase from the fasting sugar values during the first three days and then increased rapidly by more than 50% on the fourth day followed by a further 20% on Day 11 to 120% of the pre-fasting values.

The normal mice injected with alloxan showed an increase in blood glucose values of 81% of the pre-experimental values (mean value 145 mg%, pre-treatment - 263 mg%) by Day 15. The maximum increase of 155% (370 mg%) was seen on Day 2 post treatment. The maximum fluctuation in the pre-test values was 40 mg%

(i.e. Day 3 - 4). In the non-obese control animals injected with 0.2 ml. of buffer alone the fluctuation in blood glucose did not show any significant difference. The control acetoacetate concentrations rose with fasting as expected and then appeared generally to be steady. After treatment with alloxan the acetoacetate in the obese mouse fell on the first day and then in both the obese and the normal animals it rose to a mean peak of 0.35 mM on day 2 post alloxan treatment.

Half of the obese animals had died by day 6 after the administration of alloxan and in six of them there was marked ketosis of up to 0.66 mM acetoacetate. The blood of these animals also showed visible signs of haemolysis on the day prior to death as was confirmed by the packed cell volume measurements which were 25% to 50% of the pre-treatment figures (mean pre-treatment values - 40% p.c.v).

The extent of haemolysis was further increased simply by pipetting with a blood pipette, emphasising the increased fragility of the erythrocytes. The animals themselves displayed visible signs of ill health, e.g. extreme lethargy, a cold clamminess of the whole body and dimness of the eye. Exactly the same symptoms were observed in the non-obese mice prior to death. Blood collection too proved exceedingly difficult as compared to their healthier mates. These symptoms became obvious only in the final twenty-four hours of life. Hyperglycemia was highest on the day before death in every animal.

Alloxan pH 4.0 (Fig.46 and 47)

Alloxan treatment of obese animals caused a depression in blood sugar in two days (i.e. on day 10) followed by a rise. On Day 12, i.e. 4 days after alloxan administration the maximum blood sugar level of 234 mg% was reached. This value was 92% of the pre-treatment levels. The maximum blood sugar fluctuation was 50 mg% (i.e. days 9 - 10 and 11 - 12). Alloxan in buffer pH 4.0 too is able to suppress the slow, steady rise of blood sugar to its pre-fasting values. This effect may also be observed with alloxan pH 3.2 although it is slightly less effective.

In the normal alloxan treated non-obese groups the maximum post-treatment level was reached on Day 3 after the administration of alloxan, i.e. 446 mg%. This was approximately 200% of the pre-treatment levels (Mean value 153 mg% pre-treatment - 446 mg%). The maximum pre-treatment fluctuation was 40 mg% (i.e. day 1 - 2).

In the control experiments the blood glucose values remained fairly constant except for a sharp rise in the obese group on day 10. Some of the individual non-obese animals achieved blood glucose values of up to 850 mg%. Acetoacetate rose a little during the fasting period. In this experiment the only fatalities were one of the eight obese animals and two of the eight non-obese animals. The normal mice had acetoacetate levels of 0.5 mM and 0.69 mM on the day prior to death but the previously mentioned haemolytic condition of the blood was not observed. The packed cell volumes averaged 40%. As usual all the non-obese animals developed various degrees of glucosuria and two of the individuals developed mild signs of ketonuria. Evidence of glucosuria and ketonuria was not observed in the control group.

Histological and histochemical investigations by another worker on specimens of liver, pancreas, heart, aorta, lung and kidneys obtained from both sets of investigations involving the buffer of different pH did not reveal anything of significance (Dr. D. Urbanova, personal communication).

Caffeine (Figs.48 and 49)

The results of the blood glucose concentrations in the obese animals show that caffeine produces a rise of the blood sugar to its pre-fasting values by day 11. After the administration of water to the control animals it was 100%, whereas in the caffeine treated animals it was only 74% of the mean fasting value (326 mg% -> 240 mg%). Thus the blood sugar of the control group of animals which received merely 0.2 ml. of water instead of caffeine reattained pre-treatment values while caffeine appears to be suppressing blood glucose. These results obtained for the obese mice are completely at variance when compared to Mayer's results.

Glucose Tolerance test (Fig.50)

The control glucose tolerance test was performed on mice maintained on a 41B diet. The animals were injected with caffeine immediately afterwards and the test repeated 6 days later.

The non-obese (Fig.50B) show no change in the glucose tolerance on day 6 but in the obese (Fig.50A) a slight deterioration of about 10% was observed. The acetoacetate decreased during the test in the obese both pre- and post-caffeine but followed no pattern in the non-obese. Weights were constant.

There was neither change in urine pH nor in the degree of proteinuria. Glucosuria was present in all the obese animals and absent in all the lean mice. Haematuria and ketonuria were not detected in any of the animals used in this experiment.

In the control experiments (Fig.50 C & D) where 0.2 mls. of distilled water alone had been administered, the blood glucose was very stable. The acetoacetates rose or fell or remained level as they have already been observed to do previously (Chapter V).

Serum insulin concentrations (Fig.51)

The serum insulin of the obese animals treated with caffeine is virtually unchanged compared with the normal mice where there was a decrease of about 15%.

Among the alloxan treated animals of both strains the greatest decrease in serum insulin was brought about in the mice treated with alloxan in buffer pH 3.2 (54% in the obese and 67% in the non-obese). On the other hand in those treated with alloxan in buffer pH 4.0 the non-obese show a reduction of serum insulin by 64% and the obese show a decrease of 30%.

In the control mice administered buffer alone, the greatest elevation may be seen in those treated with citrate buffer pH 3.2 (19% in the obese) while the non-obese seemed to suffer a decrease of about 31%. With buffer pH 4.0 the serum insulin concentration increased by 11% in the obese and decreased by 17% in the normal.

DISCUSSION

From the results it is obvious that the diabetogenic effects of alloxan are more potent in the citrate buffer at pH 3.2 than in the same buffer at pH 4.0. This is made evident especially by the increased number of fatalities and the increased incidence of haemolysis in animals treated with alloxan in buffer pH 3.2 in comparison to that of animals treated with alloxan at pH 4.0. Serum insulins in normals are unaffected by buffer alone but are elevated in the obese mice. It is not certain whether this represents an increased secretion of insulin in response to an increased need for insulin particularly in animals treated with buffer pH 3.2.

In this particular experiment the post-treatment blood glucose levels in the obese animal seem to be steadily rising as might be expected. The acetoacetate levels are steady.

From one point of view these results also contradict those of Solomon and Mayer (1962)and Mayer (1966). Their investigations imply an actual improvement in the diabetic condition of the obese mice in that hyperglycemia is lowered over a long period of time. In the present investigations half of the animals died and in 37.5% of the animals acetoacetate was elevated and unmistakable signs of ketosis were observed. Whether this difference may be attributed to the difference in solvents used for alloxan is not certain. Mayer (1966) injected alloxan intraperitoneally in saline. Apparently rapid inactivation of alloxan occurs in solutions of pH 7.0 or above whereas ice-cold citrate buffer pH 4.0 is able to preserve its potency (Falkmer, 1961).

Our experiments definitely suggest that alloxan is more potent at pH 3.2. Nevertheless this may not entirely explain the discrepancy between these results and those of Mayer (1966).

Acetoacetate levels too have risen by day 2 post-treatment in both types of animals and fallen later. However from merely the hyperglycemic point of view these results appear to be in agreement with those of Mayer (1966) and Solomon and Mayer (1962). The blood glucose of alloxan treated animals is held steadily at a lower concentration in comparison to its pre-treatment levels particularly in the animals treated with alloxan in citrate buffer pH 4.0.

With regard to the reduced insulin levels in the obese animals treated with alloxan the greatest reduction is seen in animals treated with alloxan at pH 3.2 (55%) compared to a 30% reduction in those given alloxan at pH 4.0. This reduction could partly be due to increased degranulation of the beta cells as well as the reduced blood sugar levels. Unfortunately histological and histochemical investigations proved inconclusive.

The obese animals did not seem particularly sensitive to caffeine. This is at variance with Kuftinec and Mayer (1964) and Mayer (1966). The drug had no significant effect on serum insulin in either the obese or normal, non-obese mice. There is a deterioration in glucose tolerance of the obese animals by about 12.5% at zero time, 13% at one hour and 6% at two hours. Thus it appears that the rate of glucose disappearance is slightly slower after treatment with caffeine.

Thus the results of our investigations on this particular

aspect in the obese animals appear to be generally at variance with the quoted works above. The obese mice appear to be less resistant to alloxan and not so sensitive to caffeine. The exact cause for this difference is not clear though the modified genetic traits in our stock could possibly be the cause. It is possible that alloxan administered in citrate buffer pH 3.2 is very much more stable. It is stable at least till the moment of administration. Then it may be circulated fast enough for it to exert more of its diabetogenic action. Mayer et al. administered alloxan in saline which is approximately at pH 7.0. Alloxan is highly unstable and would have lost more of its potency in comparison to that in buffer even before it was injected into the animal. In fact if the serum insulin values of alloxan treated obese animals are compared with their controls treated with the respective buffer the greatest decrease is observed in the obese animals, although one must remember the large standard error of the mean which generally characterises the results obtained from obese animals. Acetoacetates present a similar picture to those already discussed in Chapter V.

In fact only the physiological relationship between alloxan and caffeine has been established (Mayer, 1966) and as yet there is little indication as to why or how this is so. It might also prove fruitful to study alloxan and caffeine in relation to glutathione. The relationship if any between caffeine and glucose and between caffeine and glutathione is not known. Therefore further preliminary work to elucidate the above is necessary.

FIGS.44 and 45. Daily blood glucose, acetoacetate and body weight in animals maintained on a 41B diet and administered either alloxan in citrate buffer pH 3.2 or citrate buffer pH 3.2 alone. At the end of the 48-hr. fast terminated after the biochemical estimations (↑)

↓ indicates when the 48-hr. fast.was commenced again after blood collection.

FIGS.46 and 47. Similar experiment to the above except that alloxan was administered in citrate buffer, pH 4.0.

FIGS.48 and 49. The same experiment was repeated with caffeine in distilled water.

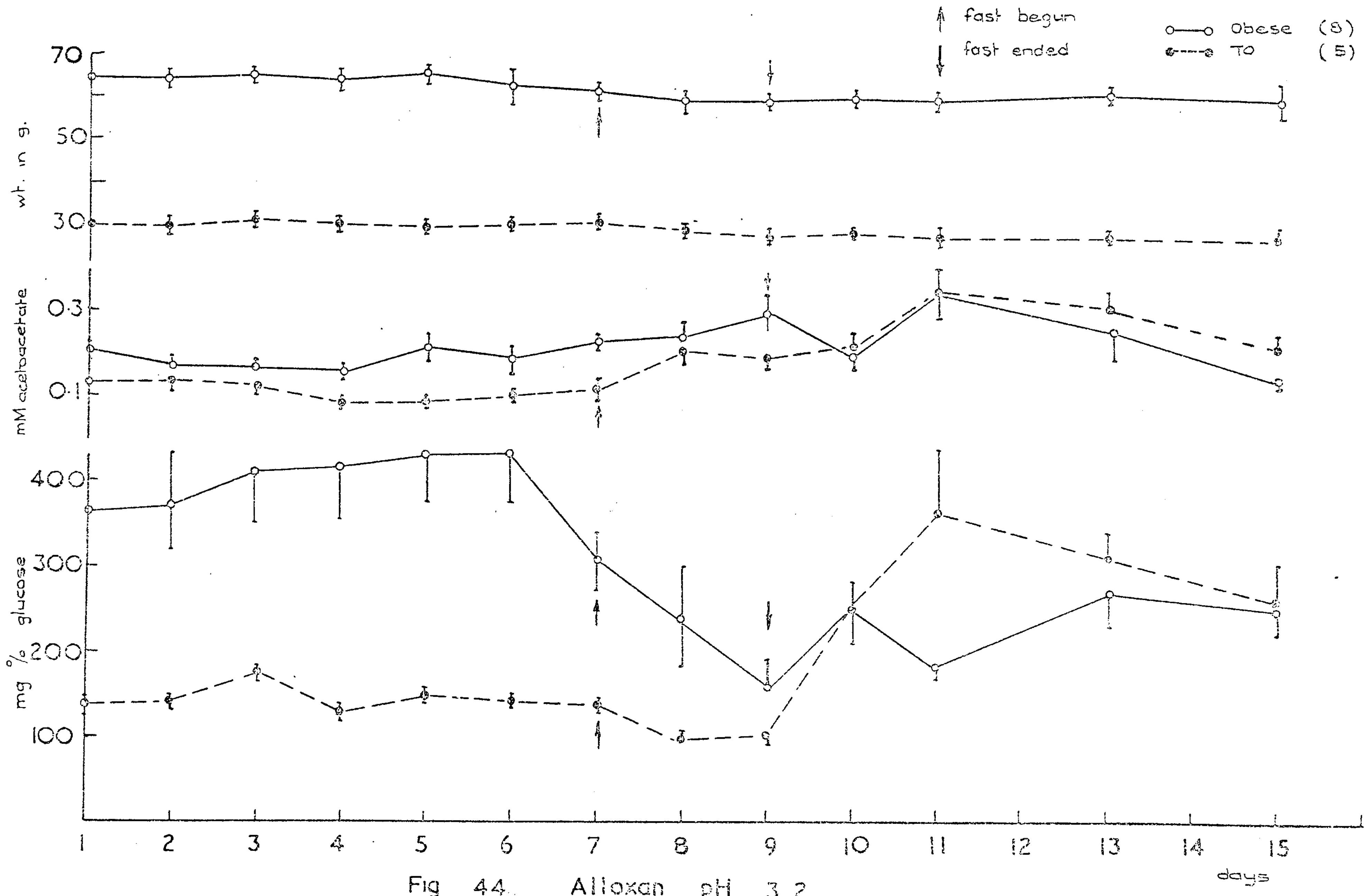


Fig 44. Alloxan pH 3.2

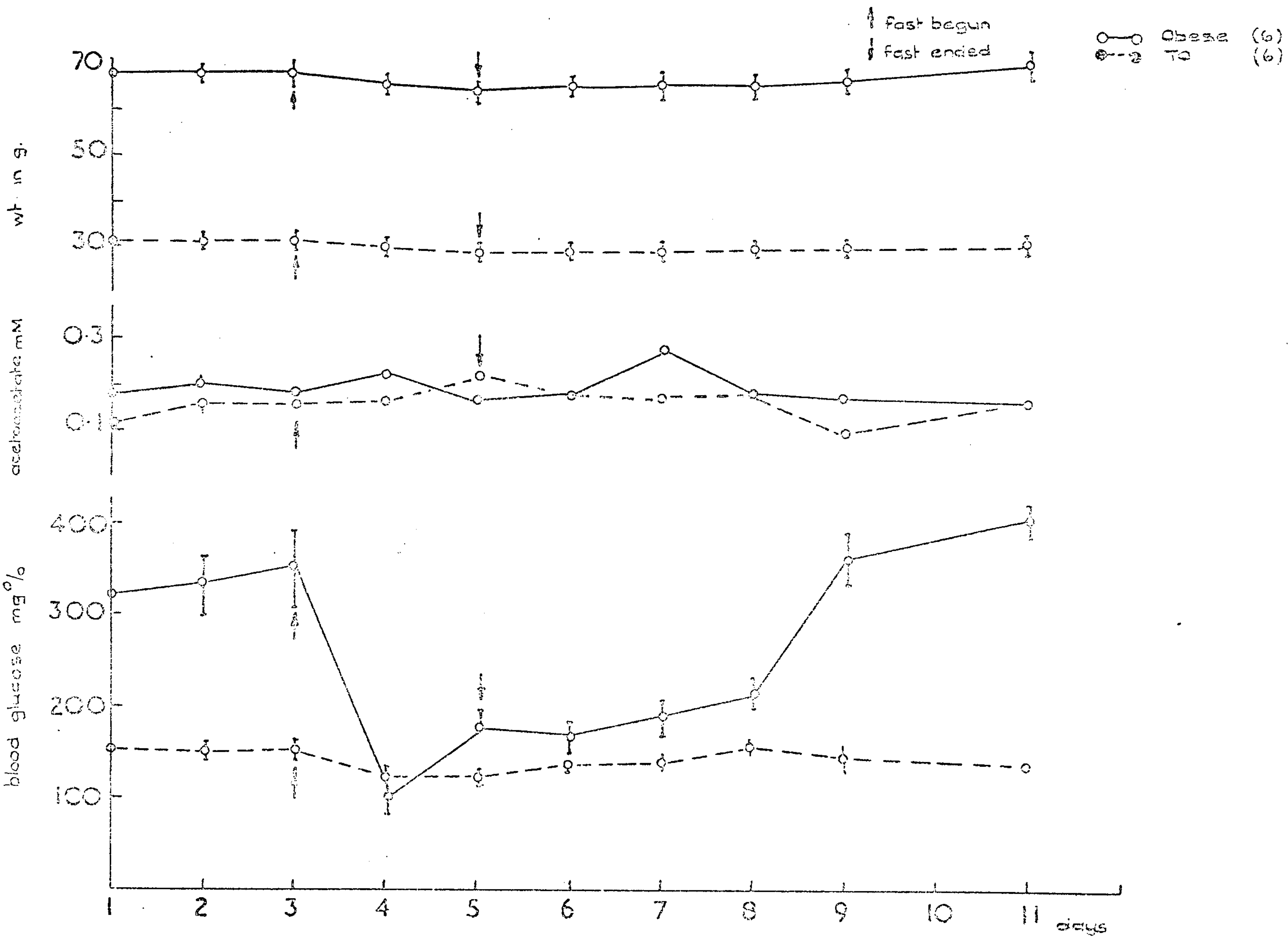


Fig 45 . Citric acid buffer pH 3.2 controls

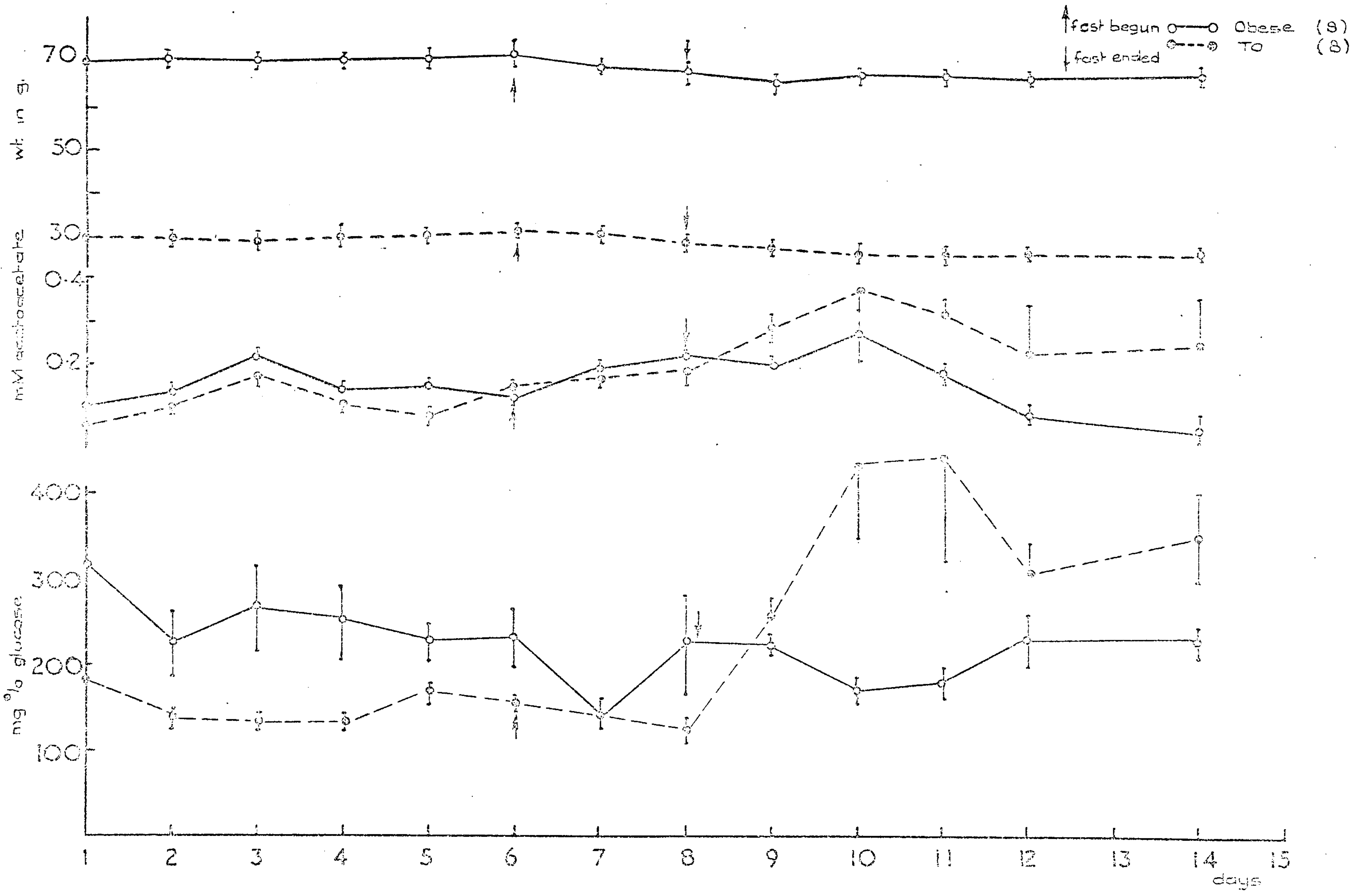


Fig 46 Alloxan citric acid buffer pH 4.0

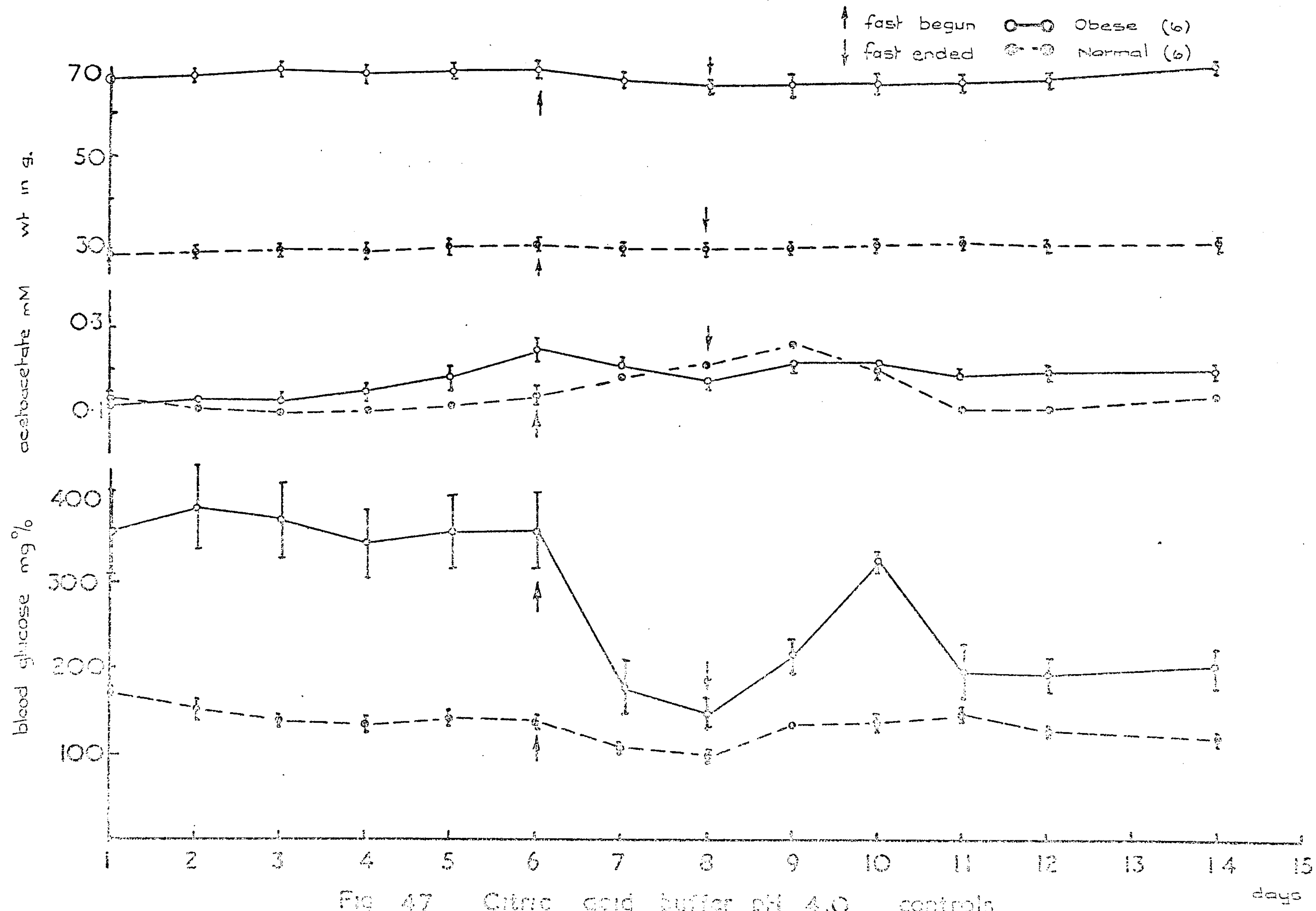


Fig 47 Citric acid buffer pH 4.0 controls

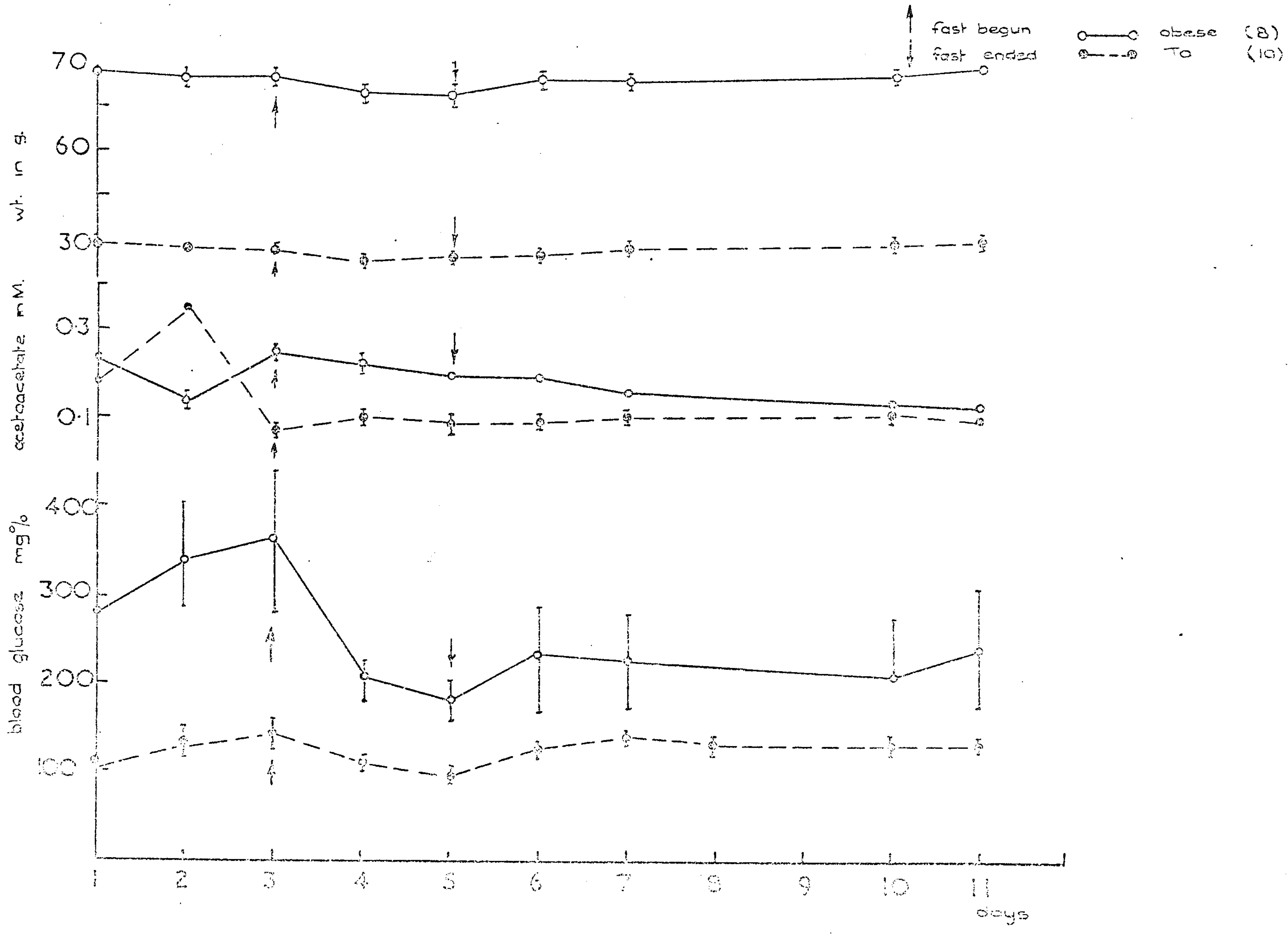


Fig. 43, Caffeine

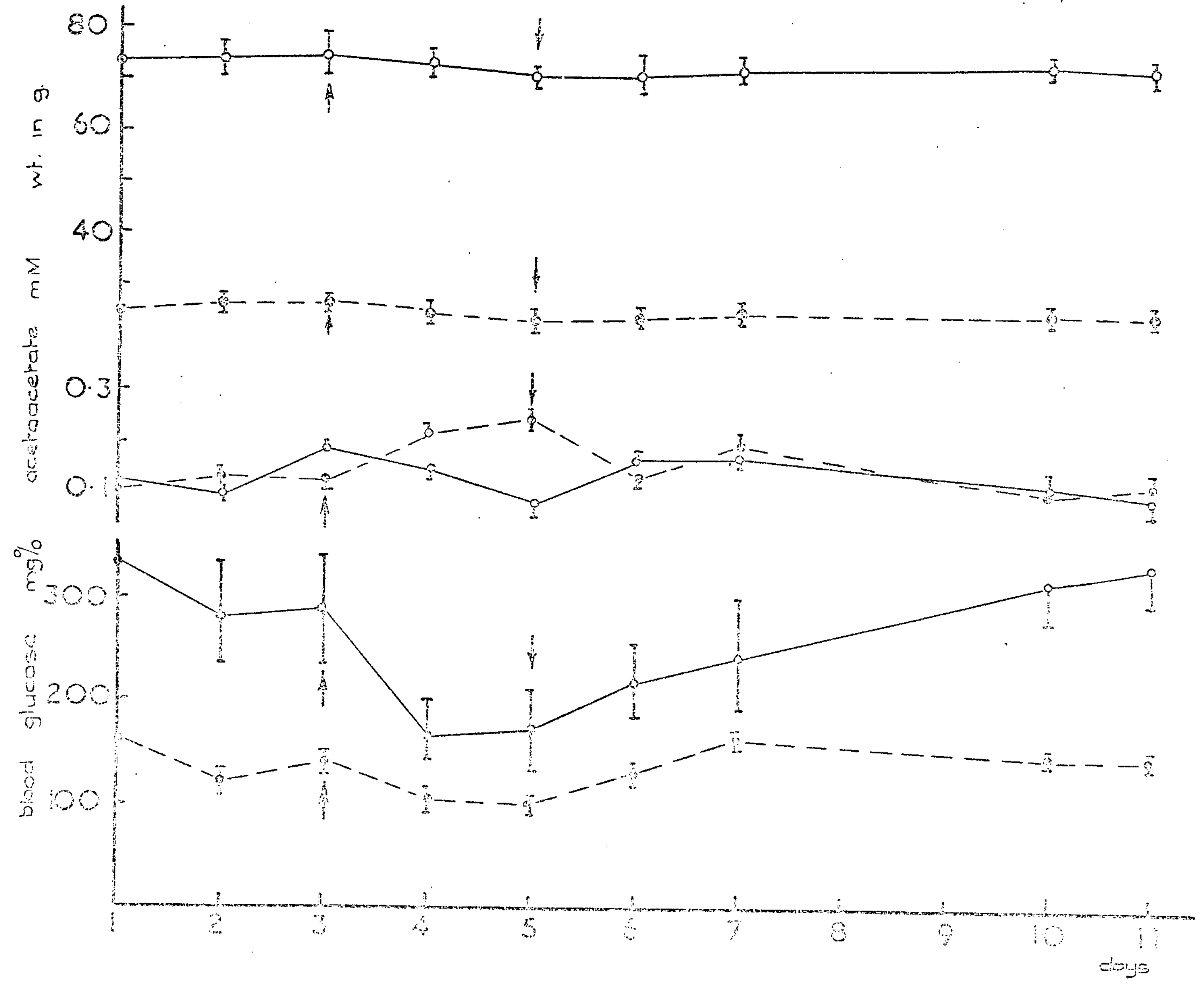


Fig 49 Caffeine - controls

FIG.50. The glucose tolerance test (Fig.A and B) was performed on caffeine treated animals first before the administration and next after the injection of caffeine.

Fig.C & D are the control experiments where distilled water was used instead of glucose.

FIG.51. The serum insulin of animals used for the alloxan and caffeine experiments and depicted in Figs. 44 - 49.

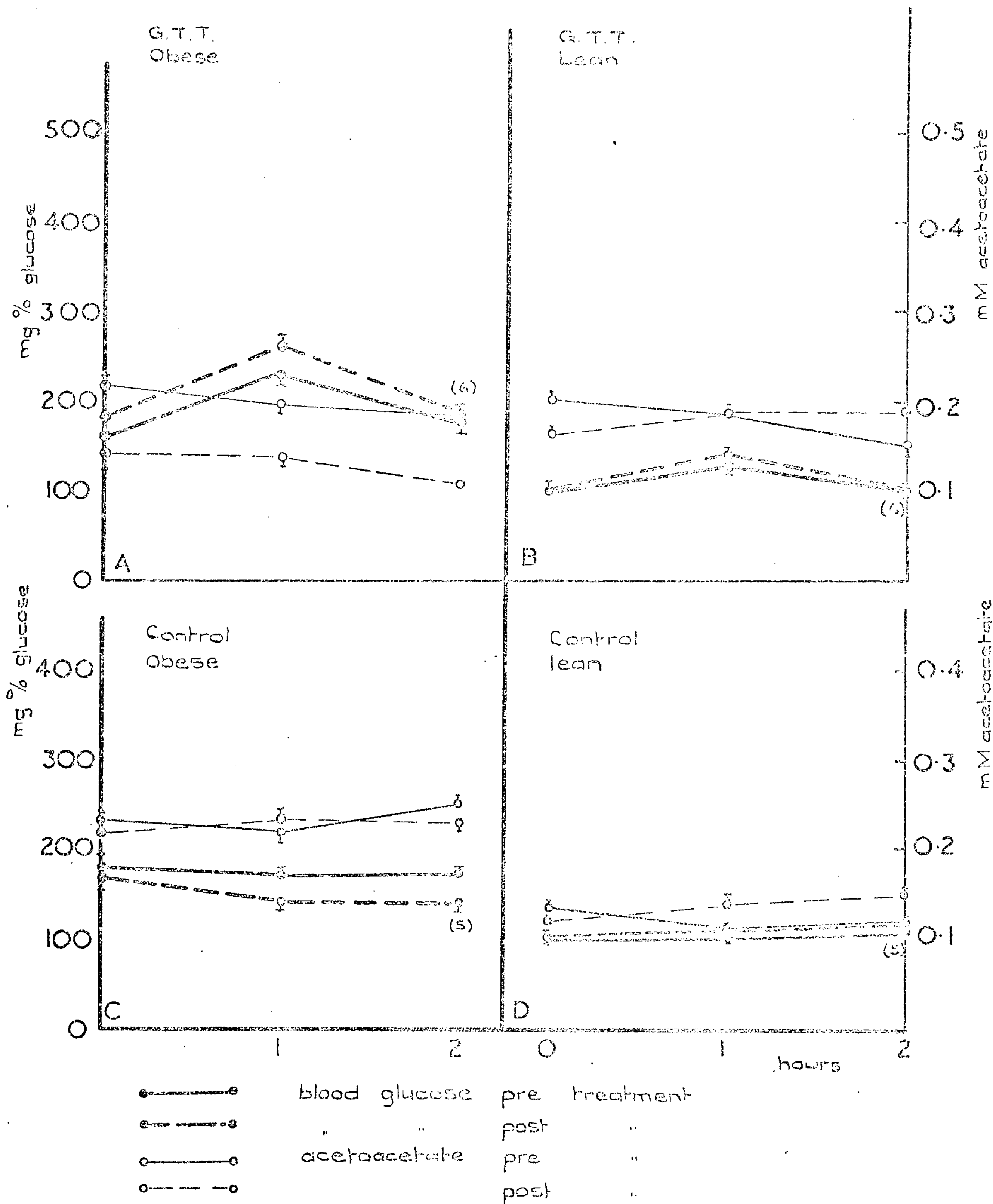


Fig 50 Caffeine

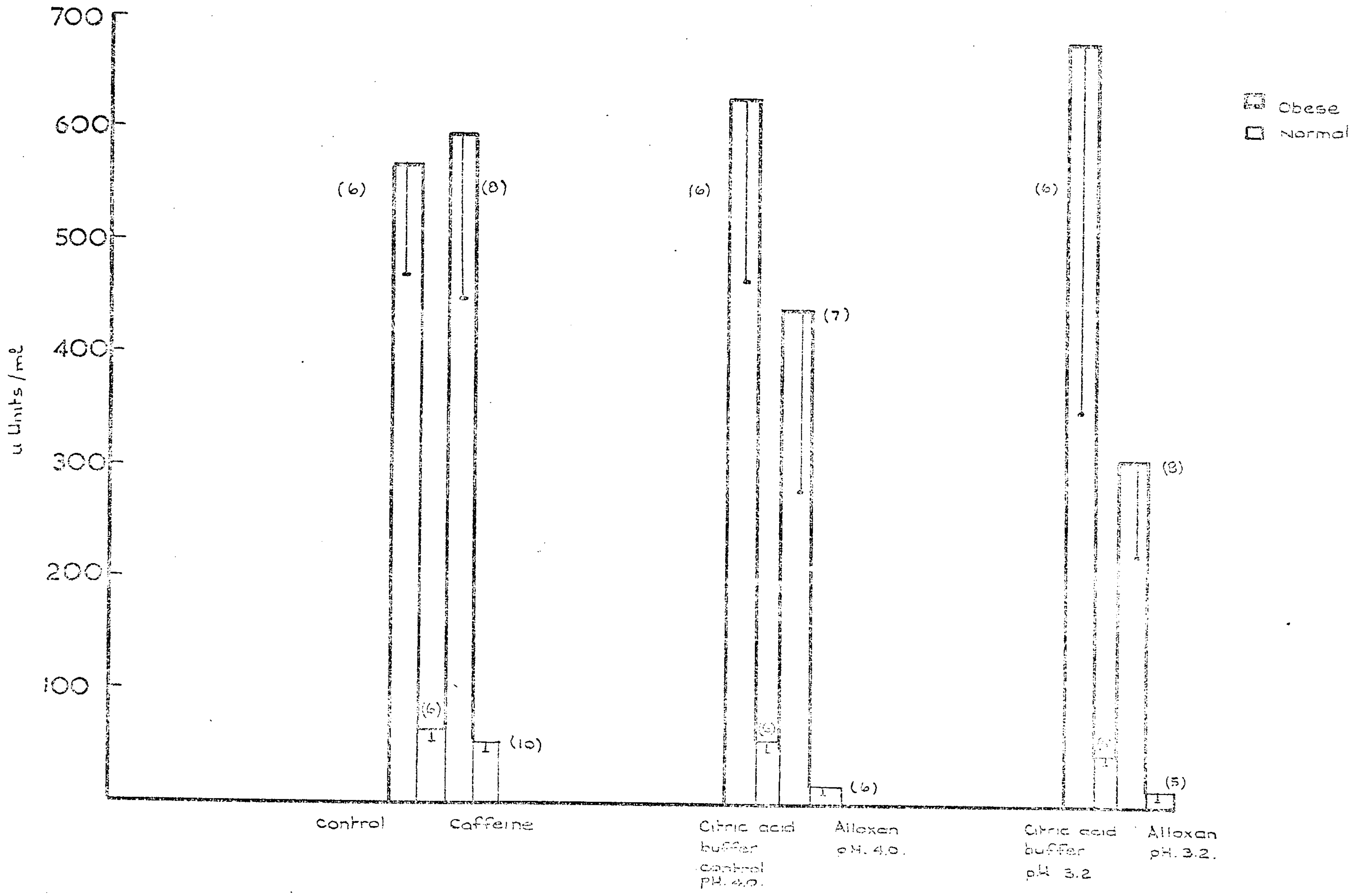


Fig 51 Serum insulin - Alloxan and caffeine

CONCLUDING REMARKS

This work was originally stimulated by the awareness that although certain changes occurred in the obese diabetic mouse the sequence of these changes had never been investigated. At the time this investigation was commenced there was a great deal of vagueness about the relationship between obesity and hyperglycemia. The major question in the understanding of obesity was whether or not the obese individual was characterized by some basic abnormality or whether in accordance with the classical attitude he was merely an under-exercised compulsive glutton.

The comparative deficit of information on the electrolytes in obesity and diabetes and particularly in mice was most surprising since mice are one of the major experimental types of animal that are used throughout the world. The importance of potassium in carbohydrate metabolism was well known and thus maintenance of the balance of sodium and potassium in the body must be essential. Thus this lack of information necessitated a great deal of preliminary work in the obese hyperglycemic mouse and its comparison with normal mice. The unexplained paradoxical behaviour of both alloxan and caffeine was intriguing.

The obese hyperglycemic colony was set up, established and investigated. The radioimmunoassay of insulin was explored and modified to suit the special requirements of these studies.

The development of the obese syndrome in the obese animal went through a number of phases, a phase in the young animals where they were indistinguishable from their lean littermates, followed by a dynamic phase characterized by a very rapid

increase in weight, a fairly static phase where the weights were stable or increase was minimal and finally a stage characterized by a loss in weight. These phases may also be observed in the blood glucose and insulin relationships, the periods corresponding to each other approximately. Hyperinsulinism and onset of obesity are already evident in the 4 week old animals. Onset of hyperglycemia may be observed in a few mice of the same age. Peripheral tissue resistance to insulin has been detected in 3 week old animals including the lean littermates. The age of onset and degree of hyperglycemia are subject to a great deal of variation. This may be due to the influence of other genes or the obese recessive gene itself.

Every biological system is subject to a certain amount of inherent variability. As Tepperman (1958) stated "In the light of what we know about the complexity of the nature of the intermediary metabolic processes and about the obvious variability of individuals with respect to many visually apparent traits we know to be genetically linked, it would be nothing less than astonishing if every person in the world derived precisely the same amount of utilizable energy from a molecule of glucose." In the same way, obesity and lean ess are the genetic expressions of the two extremes of a very wide range of somatotypes.

From the present investigations it appears that the basic abnormality lies with the insulin resistance of peripheral tissues. At first it appeared that obesity or rather the excessive increase of adipose tissue was the result of increased insulin secretion.

However the evidence obtained here conclusively indicates that hyperinsulinemia is probably a resultant rather than the cause of the corpulence in this animal. Thus obesity may be said to be a secondary metabolic consequence of tissue resistance to insulin. Superimposed on this picture is the terminal loss of pancreatic reserve of insulin resulting probably from excessive demand and giving rise to deficiency in insulin production. However this does not yet absolve the islets of Langerhans, an aberration of insulin metabolism, or the pituitary, from the genesis of obesity. Further investigations involving G.H. and glucagon could prove most interesting.

Although the potassium ion is related to carbohydrate metabolism, little is known about its uptake and hormonal regulation. Although we have established that reduced potassium uptake after insulin adminstration occurs in the obese hypergylcemic mouse, we are still ignorant of what causes this diminished uptake. Is it, like glucose, subject to this mysterious muscle factor, or is it dependent on something else as well? Potassium uptake is also dependent on sodium and calcium uptake. If this is essential in vivo does it represent a separate effect of insulin or does any other hormone influence its activity in this respect? Another approach may be to study the relationship between blood acetoacetate and disappearance or uptake of potassium. Potassium uptake is so closely associated with glucose uptake that one may presume in a sense that if glucose uptake was minimal, e.g. as in starvation, potassium uptake too would be minimal. However, these circumstances

favour lipolysis which gives rise to increased levels of acetoacetic acid and acetone in the blood. Thus an inverse relationship may possibly be said to exist between potassium and acetoacetate. Under these circumstances the levels of ATP or energy are reduced due to the impairment of metabolism. One cannot forget that potassium movement intracellularly or extracellularly is accompanied by the phosphate anion. Insulin not only stimulates lipogenesis but also inhibits lipolysis, and lack of it, either absolutely or effectively in peripheral tissue has the reverse effects so that the system overflows with free fatty acids from adipose tissue breakdown. The impaired glycolytic mechanism also limits the supply of alpha - glycerophosphate which is required for the esterification of the fatty acids into triglycerides. The above reactions exist to a lesser degree in the normal animal and are generally able to compensate each other. However, in a diabetic or undernourished animal the culmination of the above derangement if left unchecked may be observed as the manifestation of diabetic acidosis. Potassium, being the principal intracellular cation, is obviously essential for intracellular metabolism. Perhaps it is also essential for optimal lipolysis. If so, potassium deficiency may represent another autoregulatory mechanism for fat mobilization in the normal animal. The protocol of these experiments was designed to achieve depletion of the potassium stores in the body. We have succeeded in stressing the importance of this ion but we have still far to go before we are able to verify its regulation and its regulatory role.

As for the effects of alloxan and caffeine in the obese animals, unfortunately very little can be concluded from our few basic preliminary investigations except that the obese animals are not as insusceptible to alloxan or as sensitive to caffeine as suggested by the work of Mayer and associates (1962, 1964, 1966). This aspect of the subject needs to be investigated further before anything conclusive may be said.

Thus it would appear from these investigations that the real problem is probably not merely a question of the presence or absence of an "abnormality" but the degree to which it is present. Up to the present, most of the changes including obesity itself appear to be the secondary consequence of this syndrome. The genetically transmitted defect has not yet been identified with any certainty.

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