The Development of Certain Enzymes and Isoenzymes During Normal Pregnancy

Denzil Dilwyn Jones, M.Sc.

A thesis submitted to the University of Aston, Birmingham in fulfilment of the requirements for the degree of

Doctor of Philosophy

1973.

CERTIFICATE

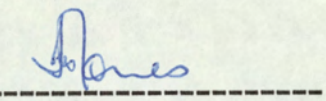
I hereby certify that the work embodied in this thesis has not already been submitted in substance for any degree, and is not being concurrently submitted in candidature for any degree.

Signed---------- Flores

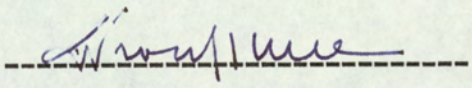
Date ---------- 20/9/73

DECLARATION

I hereby declare that the whole of the work now submitted in this thesis is the result of my own investigations except where reference is made to published literature and where assistance is acknowledged.



Candidate



Director of Studies

ACKNOWLEDGEMENTS

I am indebted to Dr. B. Prochazka for valuable supervision and constructive criticism throughout the course of the work described in this thesis. I would also like to express my gratitude to Mr. E. Wintle for assistance with photography and to Miss E. Phillips for typing the manuscript.

I wish to thank Mr. G. F. Williams, Consultant Obstetrician and Gynaecologist without whose helpful discussion and advice on all aspects of the work, would have made this investigation impossible.

In addition I would like to thank Dr. W. H. Beasley, Consultant Pathologist for his encouragement and for providing facilities for this research project.

Finally I wish to place on record my gratitude to the Welsh Regional Hospital Board for a grant in support of this work.

SUMMARY

During normal human pregnancy the maternal serum activity of carboxypeptidase, leucine aminopeptidase, alanine aminopeptidase, cystine aminopeptidase, γ-glutamyl transpeptidase, alkaline phosphatase, glucose-6-phosphatase, 5′-nucleotidase, n-acetyl-β-glucosaminidase, n-acetyl-β-galactosaminidase and β-glucuronidase, have been found to progressively increase. Dipeptidase and tripeptidase were not found to increase during pregnancy.

A wide scatter of results was found during each week of pregnancy which was due in part to poor reproducability of the techniques. The enzyme assays were automated which improved the reproducability and also afforded a means of quickly screening maternal serum enzymes in all pregnant women attending the ante-natal clinic. With the exception of γ-glutamyl transpeptidase which was found to originate from liver the source of the increase in maternal serum enzymes is the placenta.

Placental specific isoenzymes of leucine aminopeptidase, alanine aminopeptidase, cystine aminopeptidase were characterized by electrophoresis and by the use of activators and inhibitors. The isoenzymes of liver also found present in pregnancy serum were found to increase in maternal serum.

L-methionine was found to selectively activate the placental isoenzyme and to inhibit isoenzymes of non-placental origin.

The apparent increase in glucose-6-phosphatase and 5′-nucleotidase was found to be due to the non-specific action of alkaline phosphatase and results are presented which suggest that both enzymes are not detectable during normal pregnancy.

The increase in n-acetyl-β-glucosaminidase and β-glucuronidase was found immunologically to be of placental origin. Specific anti-enzyme sera were produced in rabbits which showed that the placental isoenzymes were immunologically distinct from that found in foetal and maternal tissues.

In the light of the results obtained the estimation of certain

maternal serum enzymes or isoenzymes may be of clinical value as a test of foeto-placental function.

CONTENTS

I. THE FOETUS, PLACENTA AND MEMBRANES

I. THE FOETUS, PLACENTA AND MEMBRANES

An investigation into the enzymology of the foeto-placental unit requires a basic understanding of the physiology anatomy and development of the unit. The descriptions that follow have been taken from the published work of Boyd and Hamilton (1970), Thomsen and Hiersche (1969) and from Batstone et al. (1971).

I. 1. The foetal-placental unit.

Implantation is a term used for the period during which the mammalian blastocyst becomes attached to the endometrium. A blastocyst estimated to be 108 hours was found by Boyd and Hamilton (1970) to consist of 107 cells which included eight formative cells and the rest were trophoblastic cells. A part of the zona pellucida was also present. The establishment of a receptive endometrium and the invasive properties of the trophoblast results in the implantation, which is under the fundamental control of the hormone progesterone. Between the seventh and twelfth days after ovulation the blastocyst becomes completely embedded in the stratum compactum of the uterine endometrium. During the next few days the mural cells of the blastocyst become the primitive cytotrophoblast and from then on all later varieties of trophoblastic tissue arise. Initially the trophoblastic cells are uninucleated but gradually become multinucleated and are termed syncytial masses. Once the syncytium becomes established the uninucleated cells and their derivatives are called cytotrophoblasts.

During the period between thirteen and twenty-one days both the syncytium and cytotrophoblast increase considerably in amount and at the same time become reorientated in relationship to each other so as to constitute the earliest villi which are outgrowths from the chorion. With continuing development and expansion of the implantation site the primary villi increase in length. Coincidentally with the extension of the cytotrophoblast the primary villi gradually develop mesenchymal cores converting them into secondary villi. Fig. 1 illustrates the development of the secondary villi and the cytotrophoblastic shell.

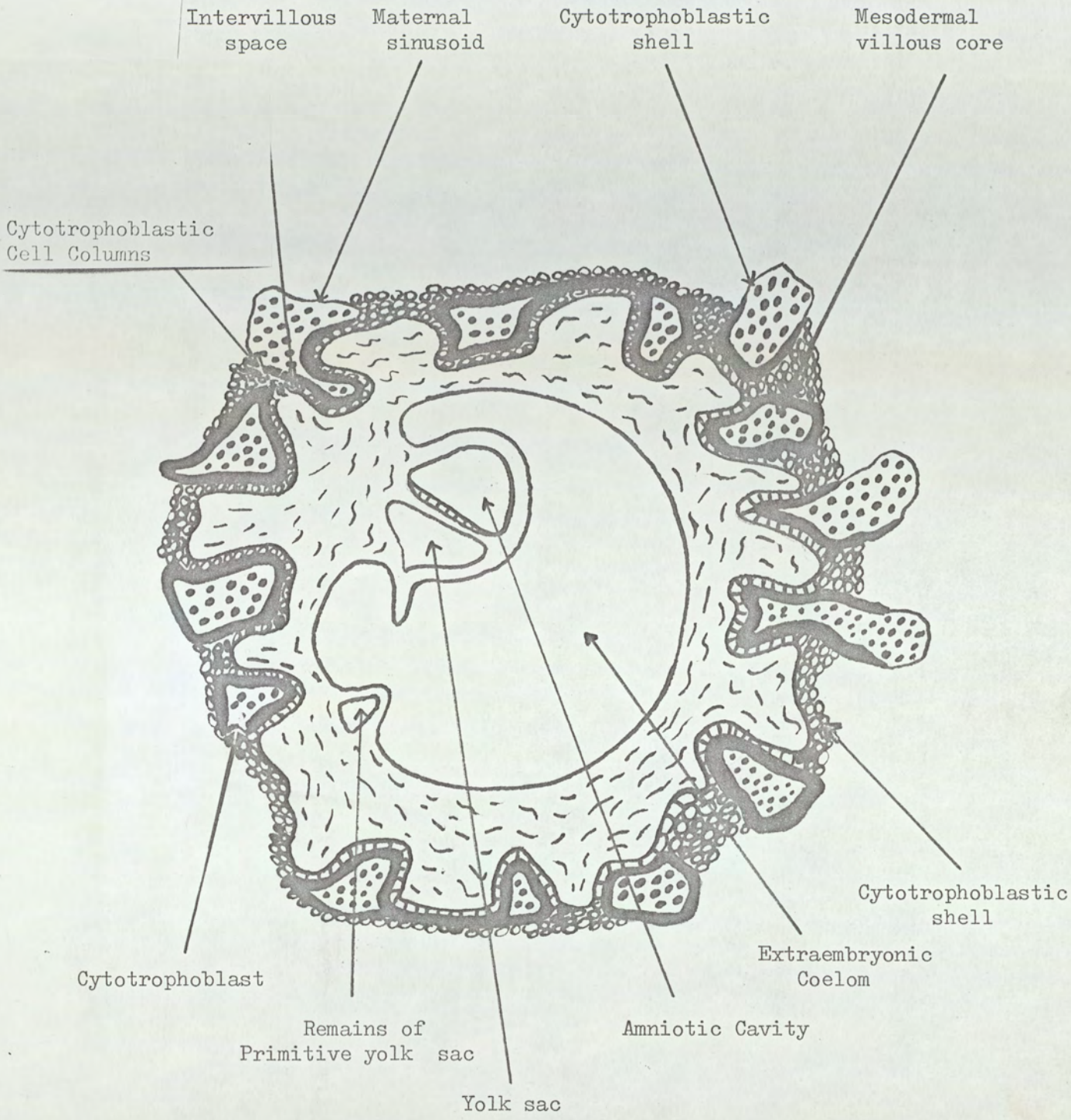


Fig. I The development of secondary villi and the cytotrophoblastic shell.

The appearance of mesenchymal cores of embryonic blood vessels and the secondary villi gradually transform into tertiary villi. Because of the depth of implantation in human development a complete decidua capsularis is formed surrounding the whole of the conceptus. The term chorion frondosum is used to describe the membrane enclosing the conceptus.

The mesenchymal cores and associated blood vessels do not extend to the tips of the tertiary villi and hence the extremities of these villi remain trophoblastic with a thin covering of syncytium and a central core of cytotrophoblasts which constitute the cytotrophoblastic cell columns. Gradually the cytotrophoblastic columns break through the syncytial layer and expand laterally to enclose the whole conceptus. This cytotrophoblastic layer is known as the cytotrophoblastic shell. The cytotrophoblastic shell splits the primitive syncytium in an irregular fashion, into an inner layer that constitutes the lining of the basal aspect of the intervillous space and an incomplete layer separating the shell from the decidua. The outer layer ultimately becomes intermingled with the decidua rendering it difficult to define a foeto-maternal junction.

The syncytial trophoblast at this time exhibits alkaline phosphatase activity and has been suggested by McKay et al. (1958) that the enzyme plays a role in placental transfer. There was no indication of the presence of acid phosphatase in the trophoblast but surrounding the implantation site there was an intense positive reaction. The presence of the enzyme adenosine - 5́ - phosphatase in the invading syncytium by Hertig et al. (1958) may involve this enzyme in digestion of decidual elements to enable the conceptus to obtain access to the maternal blood.

During the fourth week of development the spiral arteries of the decidua parietalis pass radially from the myometrium through the stratum spongiosum to the compactum. The chorionic plate consists of two layers of trophoblast, - a continuous Langhans layer and a thinned syncytium. Both layers are illustrated in Fig. 2. The cytotrophoblastic shell is intimately related to the decidua compacta and it is difficult to establish the precise line of junction between foetal and maternal tissue.

The size of the embryo during the second lunar month varies between

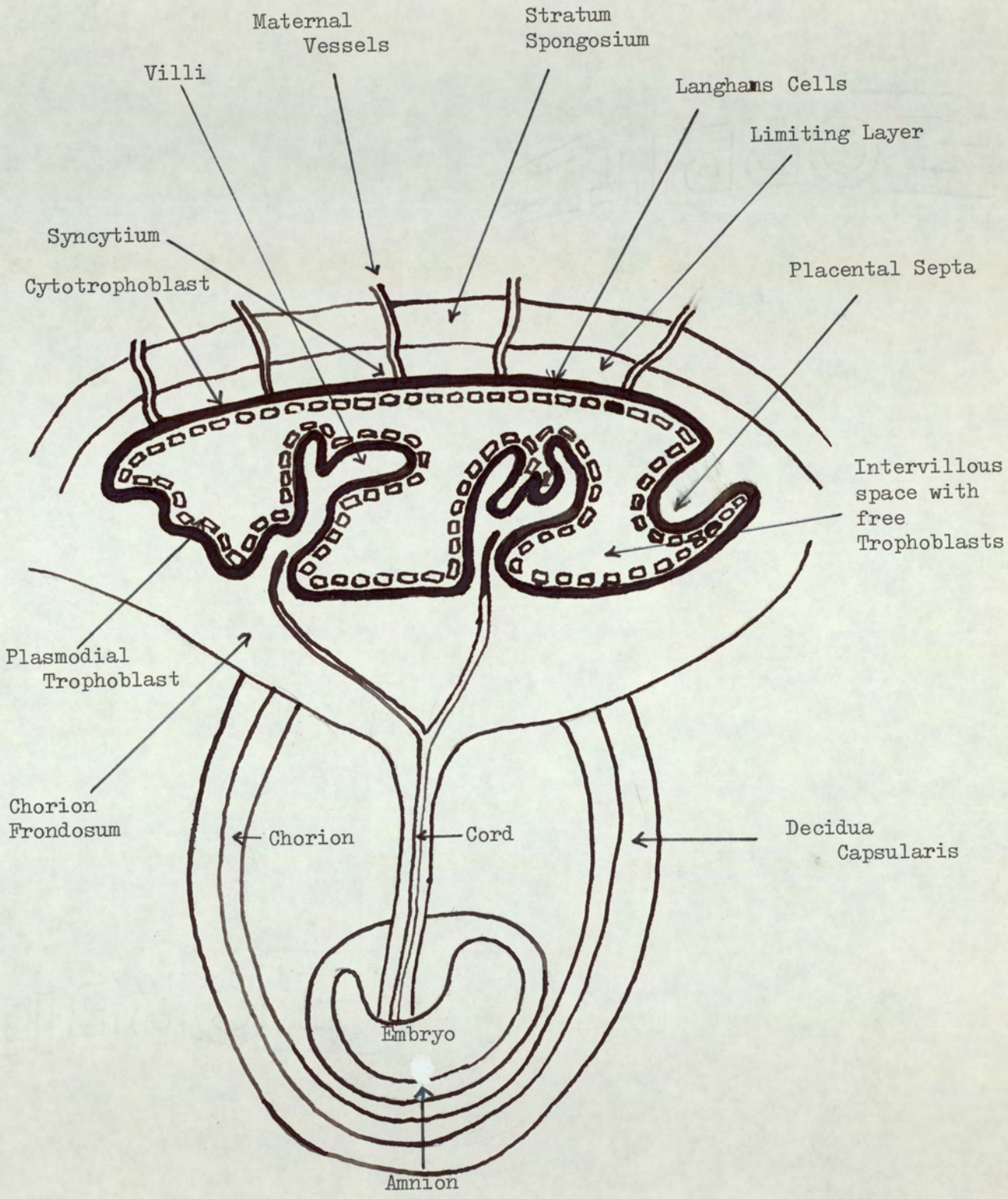


Fig. 2. The foeto-placental unit.

5 mm. and 30 mm. and the embryonic weights ranged from 0.5 g. to 2.9 g. The corresponding placental weights were between 5 g. and 27 g. During this time the decidua basalis is reduced in thickness and the decidua parietalis is thicker. The villi are distributed over the entire surface of the chorionic sac and a definitive placenta can be identified.

Fig. 3 illustrates the foeto-placental unit in relation to the uterus. During the 12th week of pregnancy, the chorionic sac progressively becomes larger but has not fused with the decidua parietalis. The villi related to the decidua capsularis show signs of retrogression and as a result a chorion laeve is gradually formed. The cytrotrophoblastic shell is attenuated and may be reduced to no more than one single layer of cells.

The crown-rump lengths of foetuses between the 85th day to the 112th day ranged between 61 mm. and 100 mm. and the foetal weights ranged from 11 g. to 135 g. Placental weights varied between 28 g. and 134 g. The villi show increased branching and are separated by the inter-communications of the intervillous space. This space begins to show some indications of the subdivision that will result in the formation of the lobes and lobules termed cotyledons. The decidua capsularis not yet fused with the relatively thick decidua parietalis appears as a layer of retrogressing cells covering the chorion.

By the end of the fourth lunar month of gestation the decidua capsularis and the attenuated chorion laeve have become intimately fused. The foetus measures at this time between 90 mm. and 100 mm. in length and weighs approximately 100 g. The placenta is of approximately the same weight and has a diameter between 80 mm. and 85 mm.

In association with the increase in the size of the placenta during the 5th month there is greater connective tissue differentiation in the villi. There is also a gradual increase in the number of the small villous branches. It is through the coverings of the villi that all exchanges between maternal and foetal blood takes place. The layers separating the foetal blood from the maternal blood are often called the placental barrier. The thickness of this membrane is about 0.025 mm., in the early stages of pregnancy,

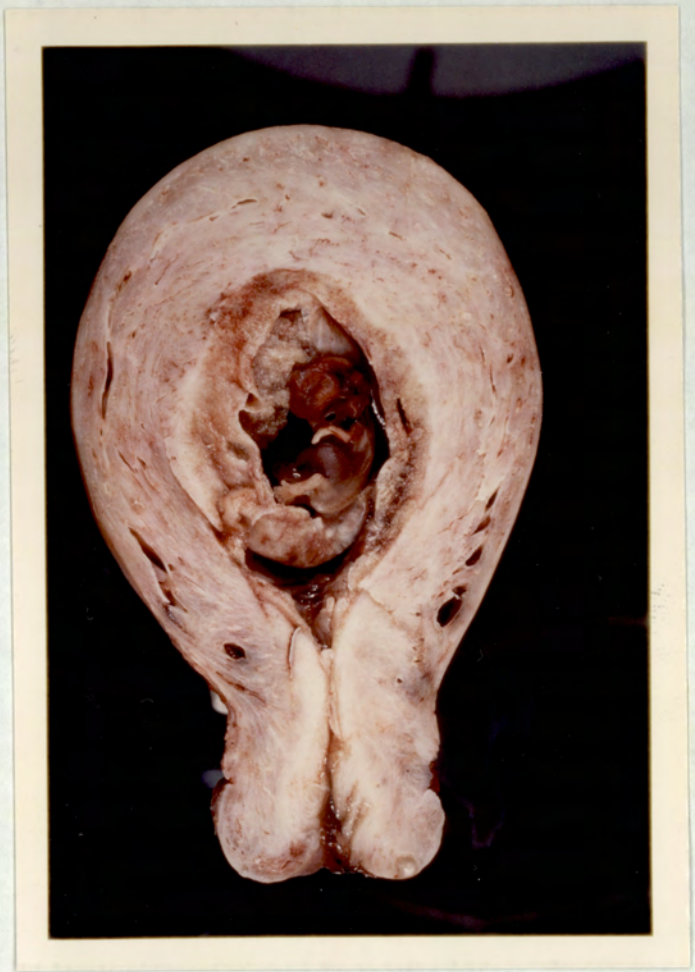


Fig. 3. An in situ foetus at 12 weeks of pregnancy.

and not more than 0.002 mm. in the later months. During this period the so-called 'subchorial lake' is established. When fully developed the subchorial lake may occupy from one sixth to one third of the thickness of the placenta. The chorion laeve has become more estensive and is no longer in contact with the decidua parietalis. The trophoblasts of the chorion laeve consist of several layers of cytotrophoblastic cells. The amnion is also increasingly brought into contact with the interior of the chorion. The chorio-amniotic type of placentation would allow electrolytes from the maternal vessels of the parietal decidua pass into the amniotic fluid. Plentl (1958), Sugawa et al. (1963) and Braune et al. (1968) have suggested that some dissolved substances could be exchanged through this area.

Foetuses during the sixth lunar month weigh between 259 g. and 807 g. and the crown-rump length between 151 mm. and 200 mm. An increase of 1 mm. in crown-rump length corresponds to an increase of 0.025 g. in placental weight. The placental septa becomes well established and divides the placenta into larger lobes and smaller lobules.

During the last four lunar months of gestation the structure of the mature placenta has been fully established as illustrated in Fig. 2. The intervillous space decreases in volume relative to the space occupied by the villi. The average weight of 1,000 placentae delivered at full term is 508 g. whilst the average diameter is 185 mm. The maternal surface of the placenta at term is a dull red colour and often a greyish layer of decidua basalis may be seen covering the lobes. Although the placenta has grown considerably since the twelfth week it has not changed its basic structure.

The decidua basalis and the blood in the intervillous space are of maternal origin whereas most of the placenta is of foetal origin. The foetal vessels (normally two umbilical arteries and one vein) beneath the amnion, pierce the chorion and finally break up into small branches which enter the stems of the chorionic villi. The intervillous space is in continuity with the maternal blood vessels and uterine arteries.

Degenerative changes are found in the placenta at term, one of the

most prominent of these is the presence of fibrin or fibrinoid. The syncytium covering the villi and extending into the decidua undergoes degeneration into fibrinoid, of which large masses may be produced. Such masses are frequently called white infarcts.

The blood in the intervillous space is kept from clotting by some inherent power of the trophoblast and towards term clots may occur which are described as red infarcts. The villi depend on maternal blood for their nutrition rather than on the foetal blood within their own vessels hence it is possible for chorionic villi to survive after the embryo has been destroyed.

I. 2. Yolk sac and allantois.

The yolk sac develops from the endodermal layer of the inner cell mass, and after the appearance of the extra-embryonic coelom it is covered by a layer of extra-embryonic mesoderm. From the yolk sac, the allantois grows into the mesoderm of the connecting stalk. Part of the yolk sac is enclosed within the embryo to form the gut which is in communication with the yolk sac through the vitelline duct.

I. 3. The Amnion.

Between the ectodermal cells of the embryonic disc and cells derived from the trophoblast the amniotic cavity develops. The edges of the ectoderm of the embryo are continuous with the cells forming the amnion. Soon after the formation of the amniotic cavity amniotic fluid appears in it so that the ectodermal surface of the embryo is bathed in this fluid. When the amnion first appears the amnion is attached by mesoderm to the inner aspect of the chorion. When the extra-embryonic coelom develops the amnion is progressively separated from the chorion except at its posterior part where the mesoderm persists as the connecting stalk. Fig. 4 illustrates the development of the amnion and the formation of the connecting stalk.

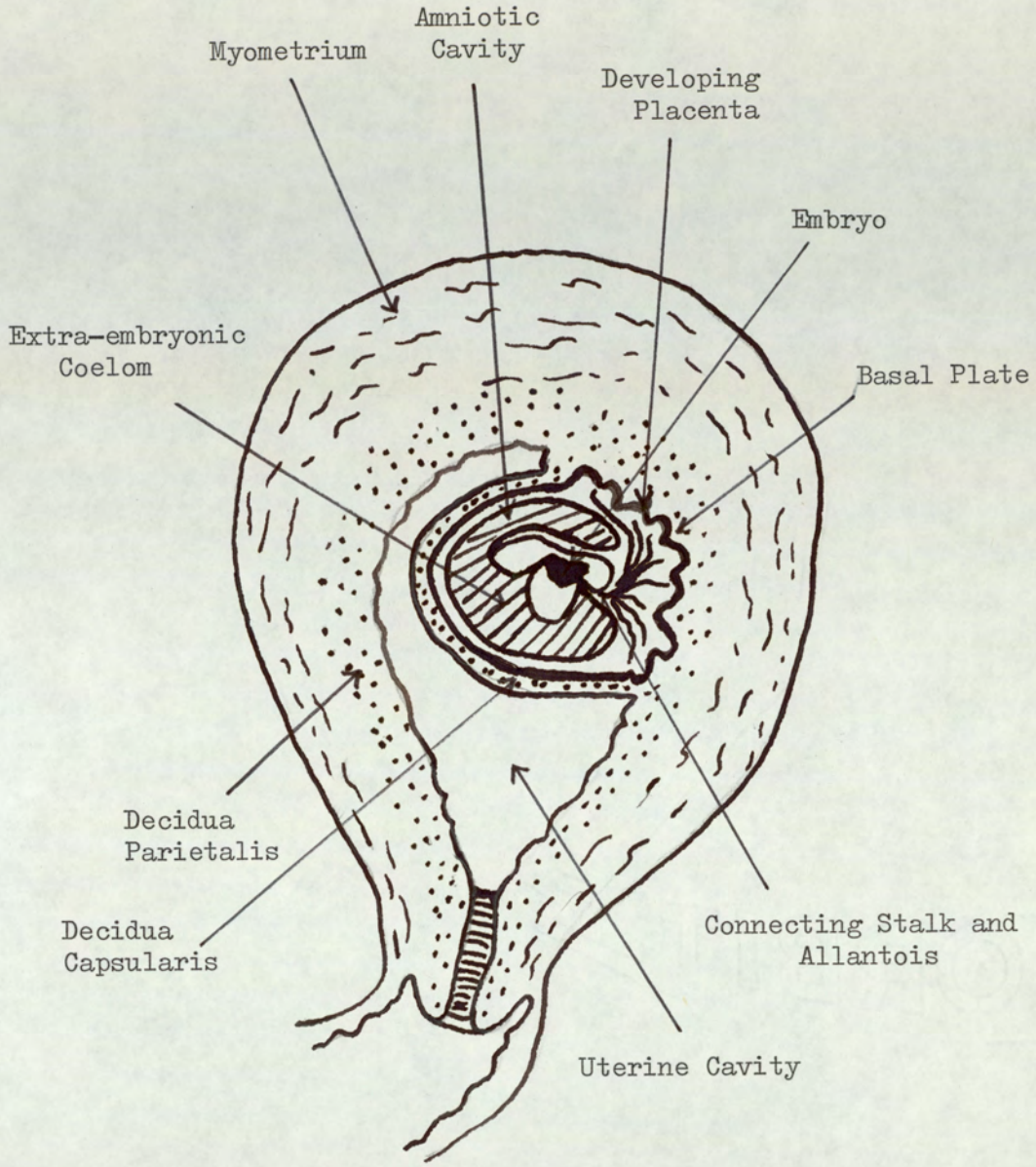


Fig. 4 The development of the early placenta.

I. 4. The umbilical cord.

The connecting stalk, allantois and vitelline duct become ensheathed by a covering of amnion to form the umbilical cord. The surrounding mesoderm becomes Wharton's jelly which surrounds the cord. The umbilical chord at term averages 50 cm. in length. It is commonly flattened and twisted in a spiral manner. The umbilical arteries are branches of the iliac arteries of the foetus and carry deoxygenated blood from the foetus to the placenta. The umbilical vein carrie oxygenated blood from the placenta to the foetus.

I. 5. The amniotic fluid.

The liquor amnii is slightly turbid with a specific gravity of about 1010 and a volume of about 1,500 ml. at term. It contains meconium, inorganic salts, albumin and urea. The amniotic fluid is first produced by the amniotic membrane by filtration or secretion. When the foetal kidneys function, foetal urine is added to the fluid. Some of the fluid is swallowed by the foetus which is absorbed in the foetal intestine and is ultimately excreted by the placenta. The liquor acts as a protective buffer for the foetus. It also guards the foetus from injurious effects of jars or shocks.

2. INTRODUCTION

2. INTRODUCTION

'A notable change in obstetric thinking in recent years, has been a greater concern for the welfare of the foetus in utero' (B.M.J., 1972), and in consequence, a variety of biophysical and biochemical techniques for measuring foeto-placental function have been developed.

The methods most commonly used are hormone assays of maternal blood and urine, ultrasonic cephalometry, uptake tests, aminoscopy foetal scalp blood analysis, amniotic fluid estimations, cardiotocography and measurements of maternal weight and girth. The object is to try and forecast those pregnancies which are likely to produce conditions leading to difficulty for the growth of the foetus and possible intrauterine death or will lead to foetal distress during labour.

The urinary excretion of oestrogens have been studied by many workers during the last three decades. Coyle and Brown (1966) Green and Touchstone (1963) and Klopper and Billewicz (1963) have estimated oestriol. Other workers Bradshaw and Jessop (1953), Brown (1956) and Hopkirk et al. (1960) have studied the three classical oestrogens oestriol, oestradiol and oestrone. The urinary excretion of oestriol increases a thousand fold and almost one-hundred-fold in the case of oestrone. At term approximately 1.5 mg. of oestrone, 0.5 mg. of oestradiol and 25 mg. of oestriol are present in urine. A number of investigators (Frandsen and Stakemann, 1963; Green and Touchstone, 1963; Wray and Russell, 1964) have found that serial estimations of urinary oestrogens are of value in patients where intra uterine death is imminent. Curzen and Varma (1971) considered oestrogen assays unreliable in predicting foetal distress and a low Apgar score at birth although it may be of some use in predicting dysmaturity. Oestrogen metabolism in pregnancy is a function of both the foetus and the placenta and may be abnormally low in anencaphaly, placental sulphatase deficiency, congenital adrenal hyperplasia and patients on corticosteroids, ampicillin and methenamine mandelate.

The large amounts of progesterone produced by the placenta are excreted in the urine as pregnenediol which has not provided a good assessment of foetal welfare. This has been in the main due to the great variation in excretion patterns (Batstone et al., 1971a). These workers also point out that the foetus also contributes to the production of pregnanediol and contributes up to 20% of the steroid in maternal urine. The technique used for the estimation of pregnanediol are cumbersome and the inherent error in collecting twenty four hour samples of urine have ruled out its use for large scale screening of patients. More recently human chorionic somatomammotropin (placental) lactogen has been used as an index of foeto-placental function. Letchworth et al. (1971) used a radioimmunoassay method to estimate the hormone. A number of workers have described the clinical value of placental lactogen estimations in foetal distress and neo-natal asphyxia (Letchworth and Chord, 1972) in placental function (Genazzani et al., 1971) and in the management of high risk pregnancies (Spellacy et al., 1971). Individual reports of the value of placental lactogen have been conflicting and as recent studies indicate (Teoh et al., 1971; Genzzani et al., 1971) its value in predicting impending foetal death has not yet been established. Although it does appear to be of prognostic value in threatened abortion and in pregnancies complicated by diabetes, toxaemia or postmaturity where a low value would be indicative of subsequent inevitable abortion or of serious degrees of foetal jeopardy later. Low values are also found in dysmaturity.

Ultrasonic cephalometry is a useful index of dysmaturity but it requires expensive equipment and a high degree of expertise in its use. Kukard and Freeman (1973) found ultrasound scanning to give dangerous results in multiple pregnancy. Ultrasound has been shown to produce chromosome breakages in cell nuclei during experimental preparations (Batstone et al., 1971a).

Van der Crabben, (1970) has described a load test by administering dehydroepiandrosterone sulphate, an oestriol precursor, and estimating the oestrogen excretion during the next eight hours. In severely affected placental function the maximum excretion occurred after 6 hours.

Liggins (1972) used a single dose of 100 mg. of dehydroepiandrosterone sulphate (DHAS) administered intravenously and measures 24 hr. urine oestrone and oestriol estimations. Foetal survival for a further two weeks has invariably followed DHAS loading in which the increase in excretion of oestrone and oestriol totalled more than 1 mg. per 24 hrs. regardless of how low the basal oestriol excretion may be at the time of the test. Another uptake method has been described by Garrow and Douglas (1968) where the quantity of amino acid transferred to the foetus was measured. An intravenous dose of ^{75}Se-selenomethionine is given and an uptake ratio calculated from the count rate over the sternum to that of the uterus. During late pregnancy, in at risk patients aminoscopy is useful to look for meconium staining. During labour direct methods by scalp blood analysis give good indications of foetal well being. Foetal electrocardiography has also been found to be valuable to the clinician in assessment of the foetus during labour.

Despite the pre-occupation with complex tests of placental function it has been recognized that simple observations of maternal weight and girth changes are of considerable value in assessing foetal wellbeing. Sustained failure to gain weight or sustained loss of weight after 34 weeks of pregnancy was found to be significant (Batstone et al., 1971). The results of all these attempts to measure foeto-placental function have been on the whole disappointing. This fact has served to stimulate the present work in an attempt to assess estimations of various enzymes in maternal blood as an indication of foeto-placental function. A further objective was to evolve automated methods so that large numbers of specimens could be estimated,

leading to these tests being used as a routine on all ante-natal patients.

An increase in enzyme activities above normal are found in diseases where sufficient number of cells are damaged and which in turn lose their constituent enzymes to the serum. No enzymes are released when slightly damaged cells are capable of regeneration, and furthermore the amount of increase depends primarily on the extent and the severity of cell damage. A number of enzymes estimated in clinical laboratories fall into this group and include serum lactic dehydrogenase (EC 1.1.1.27) which is increased after myocardial infarction and alkaline phosphatase (EC 3.1.3.1) found increased in liver diseases.

The diagnostic specificity of these enzymes have been improved by examination of their isoenzymes which are found to be tissue specific. In liner disease the isoenzyme LDH_1 is increased and in myocardial infarction the fast moving LDH_5 is found to increase. Similarily alkaline phosphatase isoenzymes are fractionated into bone and liver fractions.

It was felt that a similar situation could exist during pregnancy where maternal serum may reflect an increased enzyme activity derived from the foetus or placenta. Furthermore a tissue specific isoenzyme could be fractionated, the estimation of which could be of clinical value in assessing foeto-placental function.

Before applying these ideas to the foeto-placental unit one must consider aspects of placental function which are not found in other tissues. The placenta simultaneously performs a number of diverse tasks such as respiration, transport of nutrition to and excretion from the foetus, and endocrine exchange. Other characteristics include its dual origin from foetal and maternal tissue and the intimate contact between the surfaces of the foetal chorionic villi and the maternal blood which effects exchange between substances in the maternal blood and foetal blood vessels.

Page (1957) classified substances crossing the placental barrier to the foetus into four groups, on the basis of the physiological significance of the material to be transferred. The first group includes substances essential to life such as water and oxygen which are transferred by simple

diffusion. The second group consists of substances important in foetal nutrition, glucose, amino acids, lipids and vitamins, the transport of which may include active processes. The third group was composed of substances regulating metabolic activity including hormones and are believed to diffuse slowly across the barrier. The fourth group includes macromolecules of immunological importance.

Since Page's presentation there has been some alteration in the emphasis laid on the manner in which materials are transported across the placenta. Some of these changes are due to increased knowledge provided by electron microscopy of the structures traversed during placental passage. It has been suggested that many substances are present in higher concentration in the foetal blood than in that of the mother e.g. sodium, Miller (1941), inorganic phosphate, Fuchs (1957), calcium, D'Agostino Barbaro (1952) and iron, Widmer (1948), and amino acids, Clemetson and Churchman (1954).

Schreiner (1965) has pointed out that the higher concentration of these substances in foetal circulation must be of advantage to the foetus. These substances would appear to be transported against a concentration gradient which would involve active trans-placental transport and hence require the expenditure of metabolic energy.

In addition there has been electron microscopic evidence of transfer by pinocytosis, a 'drinking in' of whole droplets by the microvilli of the syncytiotrophoblast which constitutes the primary layer of the placental membrane (Page, 1969). Pinocytiosis may be of importance to maternal-foetal immunologic relationships but does not appear to be of significance for foetal nutrition. Page has also described three groups of compounds which would not cross the placental barrier. The first group would contain molecules too large for diffusion, the second compounds which would be firmly bound to a larger molecular complex and thirdly compounds which are altered by the placental membrane e.g. the deamination of amines by placental monoamine oxidase (EC 1.4.3.4). In addition to its duties as a transport organ the placenta has a second function with major metabolic responsibilities. Hagerman (1964) has suggested that the placenta serves early in gestation as a metabolic organ in lipid

and protein metabolism for the developing foetus and later in pregnancy when the foetal liver becomes more competent, the metabolic functions gradually cease. In laboratory animals it has been shown that for some metabolic reactions the liver does not acquire the necessary enzymes until the time of parturition Nemeth (1954) and Nemeth (1959).

Hagerman (1964) suggested that the placenta should contain three kinds of enzymes: those enzymes that maintain the cells of the placenta in the living state: enzymes that primarily catalyze reactions necessary for the transport of materials and would include enzymes involved in active transport and in furnishing energy for cellular processes: enzymes with specific metabolic functions of intermediary metabolism with specialized activities in protein and steroid hormone synthesis.

The histochemistry of the placenta can give an approximate account of the distribution of enzyme activity in the placenta and hence a guide to which enzymes are likely to be organ specific. Page and Glendenning (1955) tabulated forty five known enzymes of the placenta. A similar tabulation made in 1963 by Hagermann contained sixty three enzymes. Klopper and Diczfalusy (1968) found a total of 85 enzymes which did not include those whose existence had been inferred from histochemical or metabolic studies. Approximately 1,000 enzymes from all sources have been listed by Dixon and Webb (1964).

Wachstein et al. (1963) examined fourteen different enzymes by histochemical techniques in the term human placenta. These workers found that while general patterns of staining could be established, a certain variability was noted. Alkaline phosphatase (EC 3.1.3.1) was found to be non-homogenous by enzymatic staining. In general they found that alkaline phosphatase was most distinct on the outer surfaces and less intense at the inner margins of the syncytiotrophoblastic cells. Alkaline phosphatase was also found in the cytoplasm of these cells and very marked in the cellular membranes.

The functional significance of an increase in alkaline phosphatase during late pregnancy has not been definitely established. Curzen (1967) suggested that the increase was due to increased calcium metabolism necessitated by foetal osteogenesis. Increased calcification

of the placenta has been shown to occur in late pregnancy by Fox(1964).

Acid phosphatase (EC 3.1.3.2) first identified in both syncytial nuclei and syncytioplasm by Wislocki and Dempsey (1948). The enzyme was found to have the greatest activity during the third month of pregnancy and tended to decrease with advancing placental age. Thomsen (1955a) (1955b) suggested that alkaline phosphatase and acid phosphatase have different functional significance because their cellular location was different.

Wachstein et al. (1963) suggested that the enzyme reaction observed at an acid pH was not caused by acid phosphatase, but rather by non-specific alkaline phosphatase activity. These workers also found complete abolishment of granular and diffuse staining in histiocytes, fibroblasts and the endothelial cells by sodium fluoride, a specific inhibitor of acid phosphatase. The reaction found in trophoblastic cells was only slightly depressed by sodium fluoride indicating that the enzyme or enzymes responsible were not in the nature of non-specific acid phosphatase.

A number of different dehydrogenases have been detected in placenta, Verlado and Rosa (1963) and Curzen, (1964). Only traces of succinic dehydrogenase (EC 1.3.99.1) were found to be present in the syncytium and cytotrophoblast.

Curzen (1967) examined the distribution of other oxidative enzymes including lactic dehydrogenase (EC 1.1.1.27) malic dehydrogenase (EC 1.1.1.37), and glycerophosphate dehydrogenase (EC 1.1.95.5) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) all of which were found in the trophoblast. These results confirmed the work of Lobel et al. (1962) and Wachstein et al. (1963). Furthermore Curzen found no variation in the amount of the oxidative enzymes with ageing or with impairment of placental function.

Cerletti and Zichella (1960) using ultra centrifugation studies found that the hydrolysis of adenosine triphosphate and of adenosine monophosphate by placental tissue was not caused by a non-specific alkaline phosphatase, but rather by substrate specific enzymes. Similar results were found by Ahmed and King (1959). The reaction in the muscularis of large arteries was noted to be exclusively due to

adenosine triphosphate as substrate.

The histochemical distribution of placental 5́-nucleotidase (EC 3.1.3.5) has been investigated by a number of workers, Thomsen and Netz (1955) and Wielenga and Willighagen (1962). Thomsen and Netz (1955) found strong nuclear reaction in many cells with the calcium phosphate method but this has not been demonstrated using the lead phosphate method used by Wachstein et al. (1963).

Villee (1953) concluded that the placenta in the early phases of gestation, but not at term, contained glucose-6-phosphatase (EC 3.1.3.9). The activity of glucose-6-phosphate dehydrogenase was (EC 1.1.1.49) found by Lobel et al. (1962) to be stronger in the trophoblastic epithelium than in other components.

Leucine aminopeptidase (EC 3.4.1.1) was located by Curzen (1967) in the connective tissue stroma of the chorionic villi and in the walls of the larger blood vessels. The function of this enzyme was uncertain but it could be responsible for the breakdown of oxytocin. Page (1946) found that oxytocin was a polypeptide containing at least five amino acids. It was shown to be destroyed by dipeptidases and aminopeptidases.

Klopper and Diczfalusy (1969) have demonstrated the enzymes of the energy producing systems of glycolysis, the tricarboxylic acid cycle and the triglyceride oxidizing complex. Furthermore they point out if the foetal enzyme capability is lacking then the placental enzymes would provide the capability. Villee (1953) found that prior to the fourteenth week of gestation the human placenta could secrete glucose while the foetal liver could not; thereafter the placenta was found to lose this ability while the foetal liver acquired it.

Boyd and Hamilton (1970) point out that the results of histochemistry require careful assessment before final acceptance. Difficulties can arise from uncertainties in the chemical methods when the results are to be estimated microscopically. Furthermore they point out that the changing nature of the placenta and regional differences in the organ pose problems of interpretation.

In the 'Critique on the histochemistry of the female reproductive tract' Verlado and Rosa (1963) ask the pertinent question 'what does this information

tell us or where can it lead us?' They point out that the question is easily answered on purely histochemical grounds. This gives the biochemist an approximate account of the distribution of enzymic activity, but in terms of the physiological state the answer is difficult in an organ like the placenta where the architecture is rapidly changing from day to day. The writers state that histochemical and cytochemical analysis gives an incomplete answer to the whole problem of placental enzymes and they wonder whether optimum conditions of pH, substrate, co-enzyme, salt concentration which are used for enzymes in vitro, really represent the actual conditions under which the particular enzyme operates.

During gestation maternal serum is known to contain enzymes that progressively increase during the last two trimesters. Serum β-glucuronidase (EC 3.2.1.31), hyaluronidase (EC 3.2.1.35) and β-acetylglucosaminidase (EC 3.2.1.30) have been described as increasing during pregnancy (Platt and Platt 1968). Plasma renin (EC 3.4.4.15) was found to increase during the third trimester of pregnancy by Geelhoed and Vander (1968).

Three enzymes have been used to assist the management of pregnancy, diamine oxidase (EC 1.4.3.6), cystine aminopeptidase and the heat stable isoenzyme of alkaline phosphatase all of which increase during pregnancy. Using putrescine labelled with isotope as substrate Southren et al. (1966a), Southren et al. (1966b) and Southren et al. (1968) have studied the serum enzyme levels of diamine oxidase. Serial changes in the serum enzyme were found to be related to threatened abortion, history of habitual abortion or missed abortion. A falling or a low level of diamine oxidase was associated with an increase in foetal wastage. In pregnancies complicated by hydatiform mole serial enzyme estimations remained within the normal range during the first trimester but fell to zero during the second trimester Torok et al. (1970).

Numerous substrates have been employed to assay the enzymes aminopeptidase and include L-leucyl-β-naphthylamide (Kleiner, 1969; Kleiner et al. 1969), leucine-p-nitroanilide (Rinbach and Sehreiner, 1967)

or L-cystine-di-β-naphthylamide (Babuna and Yenen 1966a and b; Klimek, 1969; Poch et al., 1970; Hensleigh and Krantz, 1970). The serum levels of aminopeptidase increase during normal pregnancy and have been shown to have a wide scatter. Lambrinopoulos (1964) found significantly higher levels in patients who were seven to nineteen days past term than in controls at term. High levels have also been found in twin pregnancies (Ichaliotis and Lambrinopoulos, 1965; Babuna and Yenen, 1966b). Serum 'oxytocinase' levels have also been shown to correlate with placental weight (Klimek, 1969; Poch et al., 1970), foetal weight (Poch et al., 1970), and foetal surface area (Klimek, 1969). Cystine aminopeptidase was estimated in maternal serum by Hensleigh and Krantz (1970) on 186 pregnant women with a variety of clinical abnormalities. Twelve patients were found to have abnormal enzyme values and who showed inadequate placental function. In 174 patients the values were normal and seven of these showed placental disfunction.

Beckman et al. (1966) have studied leucine aminopeptidase variations in different human tissues by starch gel electrophoresis. Extracts of placentae showed four different components, two of which reacted differently when treated with neuraminidase. It has been suggested that the different forms were under genetic control (Beckman, 1966). An extra zone of leucine aminopeptidase activity has been found in sera of pregnant women by Wintersberger and Tuppy (1964). The source of this zone was suggested by Page et al (1961) to be the placenta. Beckman et al. (1966b) found a further isoenzyme in sera from pregnant women which had the ability to hydrolyse γ-L-glutamyl-β-napthylamide.

The placental content of serum alkaline phosphatase has been shown to increase during pregnancy (Curzen and Morris 1965) and to be relatively higher in pregnancies complicated by hypertensive disorders than in normal pregnancies (Jeacock et al., 1963; Curzen, 1964; Curzen and Morris, 1966). The serum placental isoenzyme was found by Neale et al. (1964) to be heat stable. McMaster et al. (1964) demonstrated that the incubation of maternal serum at

56°C for 30 mins. destroys the alkaline phosphatase from other sources. Hunter (1969) showed that heating serum at 65°C for 30 mins. instead of 56°C for 30 mins. inactivated all non-placental isoenzymes of alkaline phosphatase. Using this technique he showed that serial heat stable alkaline phosphatase estimations underwent an abnormal rise in patients with pre-eclampsia.

Fishman and Ghosh (1967), Fishman et al. (1968a and 1968b) and Ghosh and Fishman (1967) have investigated the isoenzymes of alkaline phosphatase and found that the optimum heating temperature was 65°C for 5 mins. Furthermore they found that the placental fraction had an optimum pH and substrate concentration higher than that found for other alkaline phosphatase isoenzymes.

The serum of fifty pregnant women thought to be at risk from placental dysfunction were assayed for heat stable alkaline phosphatase by Curzen (1970) using the method of Ghosh and Fishman (1967). Oakey et al. (1967) also estimated the urinary oestrogen output on the same patients. From these results they found that urinary oestrogens correctly predicted foetal dysmaturity in 64.7% of cases and foetal distress in 64.3% of cases. In contrast serum heat stable alkaline phosphatase were of little value. The correlation between the serum enzyme and urinary oestrogen was poor ($r = 0.13$) and the correlation between the enzyme and birth weight or placental weight was even worse.

The maternal serum enzymes so far investigated have wide scattergraphs. Watson et al. (1973) found that term values for cystine aminopeptidase ranged between 60 and 240 I.U. per litre. The wide range of values found both for the placental alkaline phosphatase isoenzyme and for cystine aminopeptidase in normal term pregnancy made their discriminative use difficult in abnormal pregnancy. They further concluded that both enzymes had no prognostic value in cases of mild placental inadequacy.

The wide scatter of results during normal pregnancy could in part be caused by poor methodology or due to the presence of other isoenzymes from other tissues contributing to the total enzyme activity. To examine these possibilities both automated equipment and enzyme

fractionations by electrophoresis would be ideal tools for such an investigation.

The increase in maternal serum enzymes over the normal value has been found to occur between 16 and 20 weeks of pregnancy which could be attributed to cellular changes in the placenta and or foetus. Villee (1953) found that the placenta plays the role of a liver for the developing embryo until about the 10th to 12th week of gestation. Although little is known it would not be surprising if later in pregnancy as the foetal liver becomes more competent the placenta may relinquish some of its metabolic functions.

Before a full clinical evaluation can be made a detailed study would be required of the maternal serum enzymes, their isoenzymes and their source of origin. To complete the investigation the tissue of origin together with cellular and subcellular localization of the enzymes would contribute to the understanding of the pregnancy enzymes and their functions.

The placenta is a convenient and readily available source of human enzymes and is conveniently available in large quantities in the fresh state for experimental investigation. It was also felt that a great deal of information of interest to comparative enzymology would be obtained by a study of placental enzymes compared with maternal serum enzymes and to enzymes from other tissues.

The development of a specific foeto-placental test must be capable of detecting abnormal conditions early in pregnancy because by the time signs or symptoms appear, intrauterine death may be imminent. Such data could help to reduce perinatal mortality and morbidity, and contribute to the solution of problems of mental retardation, cerebral palsy and congenital malformations.

3. MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Sera.

Bloods were taken without anticoagulant between the twelfth week of pregnancy and term. All patients attending the ante-natal clinic were bled. Each patient attended the clinic at least twice during the pregnancy. Approximately 10 ml of blood was taken by venepuncture and allowed to clot. The blood was centrifuged at 3,000 rpm for 10 min. and stored at -20°C. For the estimation of n-acetyl-β-glucosaminidase and β-glucuronidase all sera were prepared by 'fast' centrifuging (30 min. at 2,000 g) as described by Woollen and Walker (1965). The 'fast' centrifuging was required to remove platelets.

3.2 Tissue extracts.

Fresh placentae obtained from the patient within 1 hr. of delivery were washed with pre-cooled 0.9% saline (4°C) and about 0.5 g of tissue were taken for extraction. The tissue was homogenized with 10 ml of saline in an all glass hand homogenizer.

Liver, intestine, kidney, pancreas and bone were obtained from fresh post mortem material and about 0.5 g of tissue was homogenized as described for placenta . The bone extract was obtained by crushing 0.5 g of sternum in a pestle and mortar containing 10 ml of saline.

The liver from foetuses obtained during hysterotomy was removed washed in 0.9% saline and 0.5 g taken for extraction as described above.

3.3 Standardization of enzyme assays.

Each batch of enzyme assays was controlled by calibration with a known set of standards and by using a freeze dried serum with a known enzyme value. Control serum with known enzyme activities for alkaline phosphatase, γ-glutamyl transpeptidase and leucine aminopeptidase was purchased from Hughes and Hughes Ltd., Elms Industrial Estate, Harold Wood, Romford, Essex. All other enzyme estimations were controlled

with two freeze dried sera obtained by pooling pregnancy serum between the 16th and 26th week of pregnancy and between the 26th week and term. Aliquots of 1 ml of each serum pool were pipetted into 5 ml glass ampoules and freeze dried. Both pools used as controls checked the lower and higher enzyme activities found during pregnancy.

The three aminopeptidases leucine, alanine and cystine were estimated using the corresponding salts of either α or β naphthylamine which are carcinogenic and may cause tumours of the urinary tract. The recommendations of the 'code of practice for laboratory workers who handle carcinogenic amines' (obtainable from the Chester Beatty Research Institute) was adhered to throughout this work. To decrease the frequency of weighing and the use of glassware the day to day standardization of all methods where salts of β-naphthylamide were used, were made using the two 'pregnancy' control sera. Initial calibration of the enzyme activity in the control serum was made using β-naphthylamine as standard for the calibration curve. The mean and standard deviation were calculated for each 'pregnancy' control serum.

3.4 Methods of enzyme assays.

As far as possible fluorimetric methods of enzymes assay were chosen which were capable of very high sensitivities. The fluorescent compound in each fluorimetric assay was either β-naphthylamine or methylumbelliferone. The latter compound has the further advantage in that its derivatives 1-naphthal, 2-naphthol and naphthol - AS-BI are commonly used as histochemical substrates which offers the possibility of correlating fluorimetric enzyme estimations with the results of enzyme localization techniques in histochemistry or with iso-enzyme location procedures after electrophoresis.

The reproductability of each enzyme assay was investigated by twenty duplicate estimations on a serum pool containing ten sera taken between the 28th week and the 34th week of pregnancy. Table 2 lists the mean, standard deviation and variance of each enzyme. The results of each enzyme estimation have been listed in Appendix A.

All colorimetric assays were read on an 'Eskalab' spectrophotometer

Table 2. The means, variances and standard deviations of each enzyme investigated calculated from twenty enzyme assays. The actual enzyme values are listed in Appendix A.

	Mean ($\bar{x}$)	Variance (S^2)	Standard Deviation (S)
Cystine aminopeptidase	8.6	0.4	0.64
Carboxypeptidase	610	3518	59.1
Dipeptidase	3.7	0.04	0.2
Tripeptidase	34.5	3.43	1.8
γ-Glutamyl transpeptidase	20.2	2.03	1.4
Alanine aminopeptidase	157	7.6	2.8
Leucine aminopeptidase	64.8	1.9	1.4
Glucose-6-phosphatase	0.7	0.04	0.2
5´-Nucleotidase	3.3	0.6	0.4
Alkaline phosphatase	13.4	0.54	0.3
n-Acetyl-β-glucosaminidase	12.8	0.64	0.4
n-Acetyl-β-galactosaminidase	4.7	0.53	0.3
β-Glucuronidase	1.7	0.1	0.1

Alpha and fluorimetric assays on a Locarte Mk 4 fluorimeter.

3.4. 1 Cystine aminopeptidase. The micro-fluorimetric method of Wintersberger et al. (1966) was used in which the substrate L-cystine di-B-naphthylamide is hydrolyzed, liberating B-naphthylamine. In alkaline solution the β-naphthylamine may be estimated fluorimetrically using an activating wavelength of 335 nm and a secondary wavelength of 410 nm.

Slight modification of the method was made when turbidity was found to occur in the serum blank tubes. The substrate was seen to precipitate from the addition of alkali but this problem was overcome by the addition of alkali to the serum dilution prior to the addition of substrate.

Results were expressed in I.U. (μM β-naphthylamine liberated per min per litre).

3.4. 2 Carboxypeptidase. The technique of Erdos et al. (1965) was employed, in which the splitting of lysine from the substrate hippuryl-L-lysine was followed in an ultraviolet spectrophotometer at 254 nm. Since the enzyme has been found to be different from pancreatic carboxypeptidase A and B it has been provisionally named carboxypeptidase N by Erdos and Sloane (1962).

Results were expressed in I.U. as μM lysine liberated per min per litre and not as described by Erdos et al. where μM of substrate hydrolysed per min per ml was used.

3.4. 3 Dipeptidase. The term dipeptidase was given by Smith (1948) to enzymes capable of hydrolysing sarcosylglycine, as well as glycylglycine, but not N-dimethyl-glycylglycine, diglycylglycine, N-acyl substituted glycylglycine, glycylglycinamide or β-alanyl dipeptide. The enzyme that hydrolyses glycylglycine will be described here as dipeptidase. Cobalt has a pronounced activating effect on the enzyme. The method used for assay was that of Fleisher et al. (1964) where the increase in amino groups after hydrolysis of glycylglycine is estimated

by the ninhydrin method. Results are expressed in I.U. as μM of glycine liberated per min per litre.

3.4. 4 Tripeptidase. The colorimetric method of Placer and Horky (1962) was employed in which a copper suspension both deproteinates and gives a soluble blue copper complex. The original substrate has a violet biuret colour and the hydrolysate a blue amino complex.

Since there are two possible products of the reaction glycine and glycylglycine it was impossible to express the results in I.U. Therefore the units of Placer and Horky (1962) have been used and the results were expressed as the percent hydrolysis of the substrate under conditions of the test.

3.4. 5 γ-Glutamyl transpeptidase. The assay involved the hydrolysis of γ-glutamyl-β-naphthylamide which was simultaneously accompanied by the transfer of the glutamic acid released to the acceptor glycyl-glycine forming a new peptide. The technique of Swinnen (1967) was used without modification. Results were expressed as μM β-naphthyl-amine liberated per min per litre (I.U.).

3.4. 6 Alanine aminopeptidase. The β-naphthylamine liberated from DL-alanine-β-naphthylamide was estimated fluorimetrically. Conditions for the test were as described by Farr et al. (1968). Enzyme activity was recorded as μM product per min per litre.

3.4. 7 Leucine aminopeptidase. The ultramicro fluorimetric procedure of Rockerbie and Rasmussen (1967) was employed. The natural fluores-cence of β-naphthylamine in an alkaline medium was measured. Re-sults were expressed as I.U. per litre at 37°C.

3.4. 8 Glucose-6-phosphatase. The inorganic phosphorus liberated from glucose-6-phosphate was estimated by the method of Fiske and Subbarow (1925). The technique of enzyme assay was that of Koide and Oda (1959). Results were expressed as μg inorganic phosphorus liberated during 1 hr.

at 37°C.

3.4. 9 5́-Nucleotidase. The method involved the measurement of the phosphorus liberated by hydrolysis of 5́-adenosine monophosphate at 37°C as described by Belfield and Goldberg (1969). The enzyme activity was expressed in terms of phosphorus liberated in mμM per min per ml (m I.U./ml).

3.4. 10 Alkaline phosphatase. The technique used was that of Kind and King (1954) where the phenol liberated from disodium phenol phosphate was estimated colorimetrically. Results were expressed as King Armstrong units (mg of phenol liberated per 100 ml per 15 mins at 37°C.

3.4. 11 N-acetyl-B-glucosaminidase. The enzyme activity was estimated fluorimetrically using methylumbelliferyl n-acetyl-β-glucosaminide as substrate. The method was that described by Woollen and Walker (1965). A unit of enzyme activity was expressed as I.U. (μM per min per litre).

3.4. 12 N-acetyl-β-galactosaminidase. The method was basically similar to that used for n-acetyl-β-glucosaminidase using methylumbelliferyl n-acetyl-β-galactosaminide as substrate, but due to the low solubility of the substrate a saturated solution was employed.

3.4. 13 β-Glucuronidase. The substrate methylumbelliferyl β-glucuronide was employed and the methylumbelliferone liberated by enzyme hydrolysis was estimated fluorimetrically. The technique described by Woollen and Walker (1965) was employed.

3.5 Automated methods of enzyme assay.

During the course of this work it was decided that some of the enzymes should be automated with the object of improving precision and to increase the number of sera assayed during the working day. The enzymes

automated were cystine and leucine aminopeptidase, n-acetyl-β-glucosaminidase, n-acetyl-β-galactosaminidase and β-gluc ronidase.

The continuous flow of instrumentation used to automate the enzyme assays consisted of a sampler, pump and visual colorimeter obtainable from Technicon Instruments Ltd., Hamilton Close, Haundmills, Basingstoke, Hants. In addition a model 2700 recorder from Bryans Ltd., Mitcham, Surrey and a Grants variable temperature water bath from Grants Instruments Ltd., Barrington, Cambridge were used.

The glass incubating coil was 50' long with a diameter of 1.6 mm which was completely immersed in a water bath maintained at 37°C. The optimum dilution of serum in substrate for both cystine and leucine aminopeptidase was similar and a similar situation was found to exist with the three enzymes n-acetyl-β-glucosaminidase, n-acetyl-β-galactosaminidase and β-glucuronidase. Therefore, the automation of the two groups of enzymes are described separately.

3.5 1 Automated method for cystine and leucine aminopeptidase. The manual techniques for both enzymes required the use of salts of naphthylamine and it was considered unwise to use such a carcinogen in an automated system. The kinetic determination of serum cystine aminopeptidase has been described by Oudheusden(1971) and for leucine aminopeptidase by Jösch and Duboch (1967). Both techniques used the salts of nitroaniline.

The nitroaniline liberated from leucine-p-nitroanilide or from L-cystine-bis-p-nitroanilide was estimated colorimetrically at 405 nm.

Reagents. Phosphate buffer 0.2 M, pH 7.5.

Substrate (leucine aminopeptidase), 32 mg leucine-p-nitroaniline per 100 ml of distilled water.

Substrate (leucine aminopeptidase), 48 mg L-cystine-bis-p-nitroanilide in 15 ml of 2-methoxyethanol with the addition of 100 ml of distilled water.

Method. The automated manifold is shown diagrammatically in Fig. 5. The baseline was set by aspirating buffer and substrate and water through

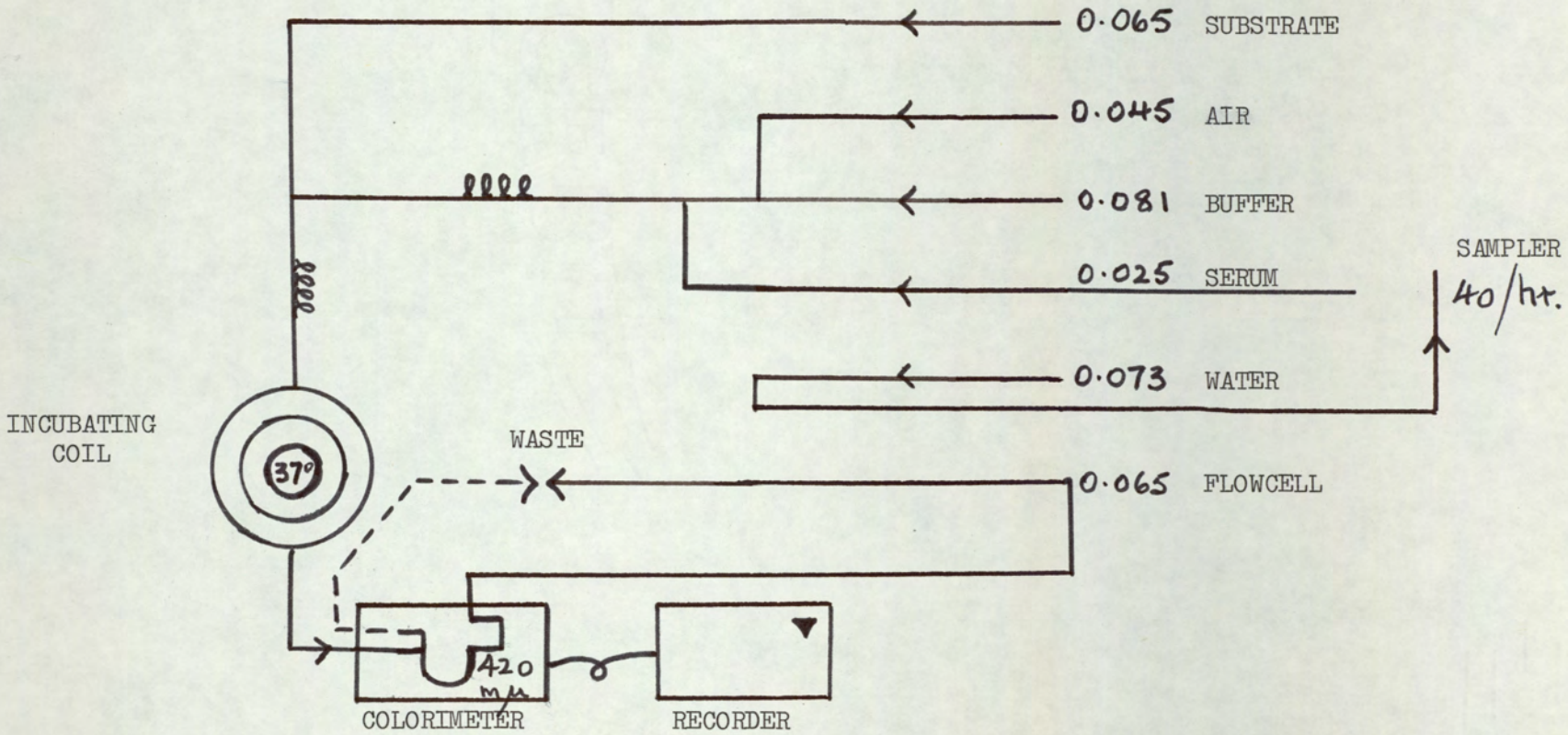


Fig. 5. Flowdiagram for the automated assay of leucine or cystine aminopeptidase.

the sample line. A series of nitroaniline standards ranging from 0.5 to 3.0 mM per litre were then aspirated. With each batch of estimations a serum of known enzyme activity was also assayed.

Calculation. A graph was constructed of the nitroaniline standards and the nitroaniline equivalent of the test peaks obtained. The time of incubation of serum and substrate was estimated. Using a 40 foot glass coil the incubation time was 6 min 55 sec. No serum blank values were obtained.

Enzyme activity was calculated from the formula

$$\text{I.U.} = \frac{\text{Test}}{\text{Time}} \times 1000$$

where Test = μM nitroaniline in unknown serum

Time = incubation time at 37°C.

3.5. 2 Automated method for n-acetyl-β-glucosaminidase, β-glucoronidase and n-acetyl-β-galactosaminidase. The manual techniques described by Woollen and Walker (1965a) for n-acetyl-β-glucosaminidase and Woollen and Walker (1965b) for β-glucuronidase were automated. The methylumbelliferone liberated on hydrolysis of n-acetyl-β-glucosaminide, methylumbelliferyl β-glucuronide and n-acetyl-β-galactosaminide was measured fluorimetrically. The primary wavelength was 375 nm and secondary 455 nm.

Reagents for n-acetyl-β-glucosaminidase and galactosaminidase. 0. M citrate buffer, pH 4.75. Substrate (1) 4-methylumbelliferyl-2-acetamido -2-deoxy-β-D-glucopyranoside 0.026 mM (0.1 mg per 1.0 ml).
(2) 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-galactopyranoside.
Glycine buffer, - 0.4 M glycine containing 0.4 M NaCl added to 0.4 M NaOH to a pH of 10.3.
Stock standard, - 100 mg/100 ml 4-methylumbelliferone. Dilute standards are equivalent to 3.0, 6.1, 9.2, 12.3 and 15.4 I.U. of enzyme activity.

Reagents for β-glucuronidase.
Acetate buffer, 0.2 M pH 3.6.

Substrate, - 4-methylumbelliferyl-β-D-glucuronide 0.057 mM Glycine buffer, - as described above.

Stock standard solution - 100 mg/100 ml. 4-methylumbelliferone.

Dilute standards - 1, 2, 3 and 4 mg per litre and are equivalent to 0.5, 1.0, 1.5 and 2.0 I.U. of enzyme activity.

Method. The automated manifold is shown diagramatically in Fig. 6. The baseline was set by aspirating buffer and substrate. The sensitivity was set by aspirating the highest standard for the enzyme being run and setting the instrument to give approximately 90% deflection. A control serum of known enzyme activity was assayed with each batch of tests.

Calculation. A graph was constructed of the methylumbelliferone standards and the equivalent concentrations obtained for the test peaks. The time of incubation for the serum substrate mixture was estimated and using the 40 ft. coil the approximate time was 6·4 mins. No serum blank values were obtained.

Enzyme activity was calculated from the formulae

$$\text{I.U.} = \frac{\text{Test (mg/100 ml methylumbelliferone)}}{\text{Time of incubation}} \times \frac{10}{1000}$$

$$\text{I.U.} = \frac{\text{Test}}{\text{Time}} \times \frac{1}{100}$$

3.6 Iso-enzyme fractionations.

Three supporting media for enzyme electrophoresis were employed, 'Cellogel' membrane, disc polyacrylamide gel and 'Gradipore' gradient polyacrylamide gel.

3.6. 1 'Cellogel' is the registered name of a gel form of cellulose acetate which contains between 65 and 70% of chemically bound water. 'Cellogel' has advantages over cellulose acetate membranes which have no chemically bound water. The pore size of 'cellogel' ranges between 0.1 and 0.5 nm. and cellulose acetate pores vary between 1.0 and 5.0 μm. The gel was purchased from Reeve Angel Scientific

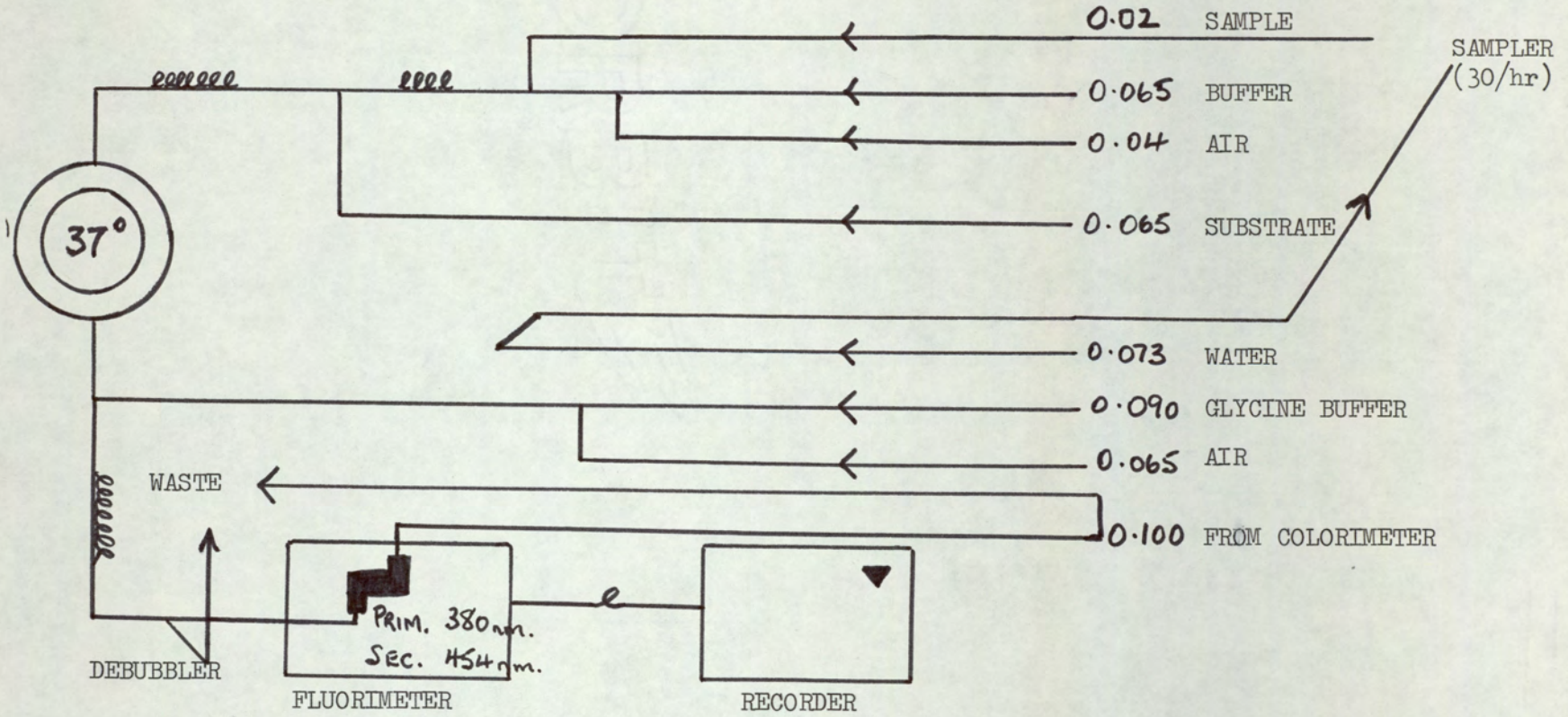


Fig. 6. Flow diagram for automated assay of n-acetyl-β - glucosaminidase, n-acetyl-β -galactosaminidase and β -glucuronidase.

Ltd., 14 New Bridge St., London EC4.

'Cellogel' rather than cellulose acetate was chosen as supporting medium because the bands were more discreet with sharper separations and less diffusion of the enzyme fractions occurred. Alkaline phosphatase isoenzymes were investigated using 'cellogel' as supporting medium.

A tris-borate buffer was used (Tris 22.75g dissolved in 900 ml. of water, titrated to a pH of 9.5 with a saturated solution of boric acid and diluted to 1 litre).

A Shandon horizontal electrophoresis tank was employed throughout. 'Cellogel' RS strips measuring 5 x 23 cm. stored in 30% methanol were submerged before use in the electrophoresis buffer for 2 hrs. The strips were lightly blotted before placing in the electrophoresis tank. Samples were applied with a Phoroslide applicator obtained from Millipore (UK) Ltd., Heron House, 109 Wembley Hill Rd., Wembley. Four samples were applied to each strip one of which was a serum containing bromophenol blue so that the migration of the albumin could readily be detected. Two applications of sample were made when applying serum obtained during the first two trimesters of pregnancy. The total alkaline phosphatase activity of tissue extracts was estimated prior to electrophoresis and extracts were diluted to approximately 20 K.A. units/100 ml.

Electrophoresis was carried out at 75 V per strip for 90 mins or until the albumin had migrated a distance of 8 cm.

3.6. 2 Disc polyacrylamide electrophoresis. The analytical polyacrylamide electrophoresis apparatus supplied by Quickfit Co. Ltd., Stane, Staffs was employed. This technique was used because better resolution of isoenzyme bands was obtained. A Tris (hydroxymethyl methylamine buffer was prepared by dissolving 45.5 g of Tris in 900 ml of water and 1.2 ml of N,N'-tetramethylethylenediamine (TEMED) added. The solution was then titrated to pH 9.7 with boric acid and the whole diluted to 1 litre. This buffer was used to prepare the gels. The buffer used in both electrode vessels was prepared as above

but omitting the TEMED. For use this buffer was diluted 1 in 4.

The gel monomer contained 19 g. acrylamide and 1 g. of N, N^1- methylenebisacrylamide per 100 ml. of distilled water. To 15 ml. of Tris-TEMED buffer was added 15 ml. of gel monomer and 10 ml. of 0.4% persulphate. The mixture was immediately added to the gel tubes and allowed to stand for 1 hr. for polymerisation. Gel tube size was 6 mm. x 65 mm. More consistent results were obtained when the gels were used after standing for 3 hr. or overnight at 4°C. After polymerisation the gels were covered with 40 µl of water.

Before use the water layer over each gel tube was discarded and 5 µl. of tissue extracts or serum applied to a filter paper disc, the diameter of which was slightly less than the diameter of the gel tube. A solution containing 2.5% polyacrylamide was used to fill each tube prior to electrophoresis. Current was adjusted to 5 mA and 30 V per tube. Gels were run at 4° and the run was terminated when the albumin of a control serum containing bromophenol blue had reached the bottom of the gel tube and required approximately 45-60 min. to complete. The bromophenol blue stained serum sample was subsequently used to stain the protein fractions with ponceau S.

3.6. 3 'Gradipore' polyacrylamide electrophoresis. During the course of this work Universal Scientific Ltd., 231 Plashet Rd., London E13 marketed an electrophoresis system suitable both for analytical and preparative use. This system was found to give better fractionation and resolution without diffusion of the bands than any other technique examined. The system is based on the use of continuous gradient gels as support medium. The concave gradient gels contain approximately 4 to 26% acrylamide. Sodium azide used as preservative was removed from the gels by electrophoresis for 1 hr. and the buffer discarded.

10 µl of tissue extracts or sera were introduced to the gel

through an acrylic comb by the gel slot technique. Each gel slab accomodated during preparative electrophoresis 2 ml. of tissue extracts which were applied to the gel slabs without use of the acrylic comb. Three gel slabs approximately 70 x 70 x 3 mm were electrophoresed at 80 mA for seven hours. With each batch, a serum stained with bromo phenol blue was incorporated enabling the albumin fraction to be visually located during the run. During the electrophoresis the buffer was cooled to 5°C by circulation through a refrigerated cooling bath.

3.7 Visualization and localization of iso-enzyme fractions.

The techniques used in the visualization of the iso-enzyme fractions differed with each electrophoresis supporting medium employed, and the visualization methods have therefore been grouped under their respective supporting media.

3.7. 1 'Cellogel' electrophoresis. Alkaline phosphatase was the only enzyme fractionated into its isoenzymes using 'cellogel' as supporting medium.

Location buffer, - boric acid, 3.74g, magnesium chloride, 2.04g dissolved in 900 ml. of water and the pH adjusted to 9.7 with a potassium hydroxide solution and diluted to one litre.

Substrate, - β-naphthyl phosphate, 2 mg/ml dissolved in location buffer.

Location reagent, - diazonium salt, fast blue RR, 30 mg per 100 ml. of location buffer.

Both substrate and location reagent were freshly made before use.

Immediately at the end of the electrophoresis run the cellogel strips were removed and placed horizontally in a moist chamber. Strips of Whatman No. 1. filter paper freshly submerged in substrate solution with the excess substrate drained were layered onto the cellogel strip taking care to avoid bubble formation between the two layers. The strips were then incubated in the moist chamber for 1 hr. at 37°C. At the end of the incubation period the 'cellogel' strips were removed

and immersed in the location reagent for 15 mins. The strips were then washed in distilled water for 1 hr. prior to scanning. A Chromoscan obtained from Joyce Loebl and Co. Ltd., Princesway Team Valley, Gateshead was used in the reflectance mode (at 490 nm) for quantifying the isoenzyme fractions. The migration of each batch of isoenzymes was referred to a serum protein fractionation stained with ponceau S.

3.7. 2 Disc polyacrylamide electrophoresis. Two methods were used for polyacrylamide gel rods. When fractionating the isoenzymes of cystine, alanine and leucine aminopeptidases the gel rods were directly scanned in the Chromoscan. To increase sensitivity in the detection of γ-glutamyl transpeptidase fractions the gels were cut into slices and the enzyme content of each slice estimated colorimetrically.
Substrate for leucine aminopeptidase, - 0.68 mM L-leucyl-β-naphthylamide hydrochloride in 0.1M phosphate buffer pH 7.0.
Substrate for alanine aminopeptidase, - 125 mg of DL-alanine-β-naphthylamide in 100 ml of 0.1 M phosphate buffer, pH 7.0.

Cystine aminopeptidase substrate, - 30 mg. of L-cystinyl-di-β-naphthylamide dissolved in 10 ml. of dimethylformamide and 90 ml. of Tris-maleate-NaOH buffer 0.2 M pH 6.0 added.

The gels were removed from the tube using running water passed through a fine syringe needle and were incubated in individual tubes containing substrate solution. At the end of the incubation period (1 hr. at 37°C), the substrate solution was decanted and an aqueous solution containing fast garnet GB (1 mg./ml.) was added. Maximum colour development occurred after 1 hr. The gels were washed once with distilled water prior to scanning. Scanning of the enzyme zones and protein fractions was made using a Chromoscan in absorption mode at 520 nm.

The isoenzymes of γ-glutamyl transpeptidase were found to diffuse both into the surrounding medium and into the gel, thereby making direct scanning of the gel impossible. To overcome this problem the gels

were removed from their running tubes and cut transversely into 3 mm slices. Each sliced portion was then incubated in 0.5 ml substrate (6 μM of L-γ-glutamyl-B-naphthylamide, 10μM magnesium chloride, 20μM of glycyl-glycine per ml. of 0.1M phosphate buffer, pH 7.4) and incubated at 37°C for 18 hrs. 0.2 ml of fast garnet GBC (100 mg. per 100 ml., water) solution was added and the solution read colorimetrically at 520 nm. The enzyme activity of each tube was calculated and expressed as a percentage of the total serum enzyme activity.

The total serum enzyme activity was estimated by electrophoresis of a duplicate serum sample on polyacrylamide gel. The gel was cut as above and all the slices combined in a test tube containing 6 ml. of substrate which was incubated at 37°C for 18 hrs. 2.4 ml. of fast garnet solution was then added and the solution read at 520 nm.

Crushing of the gel sections was not found necessary since the enzyme completely diffused out from the gel during 18 hours incubation. Total enzyme activity of ten duplicate analysis was 11.7 - 12.5 I.U. per L. ($X = 12.1$, $S = 0.3$). Table 3 lists the mean values and standard deviations of replicate assays of the isoenzymes found in normal serum.

3.7. 3 Gradient polyacrylamide electrophoresis. The isoenzymes of both n-acetyl-β-glucosaminidase and β-glucoronidase were investigated by this technique. The localization of the isoenzymes were made as described by Huddleston et al. (1971).

The substrate for n-acetyl-β-glucosaminidase contained 10 mg. of naphthol AS-BI-β-D-glucosaminide dissolved in 1.5 ml. of ethylene glycol monomethyl ether and mixed with 50 ml. of citrate buffer, 0.5 M, pH 4.5. Ten mg. of fast garnet GBC salt was then added.

B-Glucuronidase substrate contained 15 mg. of naphthol AS-BI-β-D-glucosiduronic acid dissolved in 1 ml. of 0.05 M. sodium bicarbonate. 50 ml of acetate buffer, 0.5 M, pH 4.25 and 10 mg of fast garnet GBC salt was then added.

Table 3. Mean and standard deviation of ten duplicate isoenzyme assays in normal serum.

Isoenzyme Zone	1	2	3	4	5
Mean ($\bar{x}$)	3.0	0.8	1.8	2.0	4.3
Standard Deviation (SD)	0.32	0.24	0.26	0.26	0.51

The gels were removed from their glass cells and incubated in their respective substrate for 16 hours at 37°C. At the end of the incubation period the substrate was decanted and the gels washed in running tap water for 30 mins. Eight samples were normally run on one gel slab in which case prior to scanning each sample run was cut with a scalpel. Scanning of the gels was made using the Chromoscan in absorption mode at 540 nm.

3.8 Heat stability, activators and inhibitors of isoenzymes.

A number of activators and inhibitors were investigated in an attempt to isolate, differentiate or to specifically estimate the isoenzymes. The heat stability of some enzymes was also used as a guide in isolating the isoenzyme of placental origin.

3.8. 1. Heat stability of alkaline phosphatase isoenzymes. Serum or tissue extracts with alkaline phosphatase activity of approximately 15 K.A. units per 100 ml. were diluted one in two with distilled water and heated for 5, 10, 15, 20, 25 and 30 mins. at 5°C intervals between 37°C and 70°C. The diluted sera were immediately cooled and the enzyme estimated by the King Armstrong method. Each heated serum was further subjected to electrophoresis as described in 3.7.1. above.

3.8. 2. Activators and inhibitors.

Neuraminidase, - 0.5 mg. neuraminidase type V. from Cl. perfringens was added to 1.0 ml of tissue extract. For serum 50 µl of a 10 mg. per 5 ml. solution of neuraminidase was added to 0.2 ml of serum. Both tissues and sera were incubated for 2 hours at 37°C prior to electrophoresis. Neuraminidase was used to investigate the terminal neuraminic acid of each isoenzyme.

Triton-x-100, - was added to both tissue extracts and sera to give a final concentration of 0.5%. Coutinho et al. (1966) found it to increase mobility of isoenzymes.

L-Phenyl-alanine, - final concentration of 5 mM was used prior to electrophoresis.

L-methionine, - a final concentration of 50 mM in substrate was used during the estimation of leucine and alanine aminopeptidase isoenzymes. Smith and Rutenburg (1966) found inhibition of certain naphthylamidases by L-methionine.

Urea, - 0.05 M in substrate used in the estimation of γ-glutamyl transpeptidase.

Cysteine, glutathione and mercaptoethanol were used to investigate the sulphydryl groups of the enzymes. Cysteine and glutathione were added to sera or tissue extracts at final concentrations of 2 mg. per ml. Three concentrations of mercaptaethanol were added to both sera and homogenates. Three dilutions of mercaptoethanol 1/100, 1/50 and 1/10 were made and equal volumes of each dilution added to tissue homogenates and serum samples.

NADP - 32 mg. dissolved in 10 ml. of phosphate buffer 0.1M pH 7.6. Normally 0.2 ml of extract or serum was added to 0.1 ml. of NADP solution. This solution was used to protect the enzyme molecule from oxidation of - SH groups.

Oestriol was added to serum and tissue extracts so that a final concentration similar to that found in pregnancy serum at 38 weeks of gestation (12 µg./100 ml.) was obtained.

For differentiation of 5'-nucleotidase, glucose-6-phosphatase and alkaline phosphatase magnesium chloride (10 mM), manganese sulphate (1mM), nickel chloride (10mM) and berylium sulphate (1.4 mM) were employed as either activators or inhibitors. The concentrations given are final concentration in the reaction vessels.

3.9 Histochemical methods.

Fresh placenta were obtained and a portion 1 cm x 4 cm taken through the cross section of the organ. The portion was immediately, quenched with carbon dioxide, transferred to a cryostant and sections cut 8 mµ thick. Slides were pre-cooled and immediately taken for staining. After staining the sections were mounted in water and examined microscopically.

3.10 Sub-cellular investigations.

Tissue extracts were prepared in 0.9% saline and 2 ml volumes superimposed on a suchrose gradient ranging from 10% to 65%. The whole was centrifuged in an M.S.E. superspeed 50 at 25,000 r.p.m. and a centrifugal force of 69,000g for 6 hours. Temperature was maintained at 4°C. throughout the run. The sucrose gradient consisted of 2 ml. volumes of 10, 20, 30, 40, 50 and 65% suchrose.

The bottom of one plastic tube was pierced and 2 ml. fractions taken, the second tube was pierced and 1.0 ml taken for the first fraction followed by 2 ml. volumes for all other fractions. The second tube gave fractions where the gradient interfaces were complete.

A slide was made of each fraction taken and stained with haematoxilyn and eosin, so that each fraction could be identified as far as possible.

3.11 Iso-enzyme purification.

The iso-enzymes of n-acetyl-β-glucosaminidase and β-glucuronidase were purified in two stages. Stage I consisted of purification by preparative electrophoresis and a stage 2 by gel filtration.

3.11 1. Preparative polyacrylamide electrophoresis. The Gradipore electrophoresis system was employed. The gradient gel slabs approximately 70 x 70 x 3 mm were electrophoresed in tris buffer for 1 hour to remove the sodium azide preservative. The buffer was discarded and fresh buffer used. Approximately 2 ml. of tissue extracts were added to each gel slab. During the electrophoresis at 80 mA for seven hours the buffer was cooled to 5°C by circulation through a refridgerated cooling bath.

The localization of the enzyme zones was made by slicing a portion of the gel approximately 1 cm. wide in the direction of the gradient and the slice incubated with substrate as described under

3.7.3 above. The gels were stored at 4°C until the zones of enzyme activity were detected. The boundaries of enzyme activity were marked with a scalpel, cut and homogenized in 0.9% saline. The suspension of gel in saline was centrifuged at 3000 r.p.m. for ten minutes and the supernatent removed for gel filtration.

3.11. 2. Sephadex gel filtration. Further purification of the isoenzymes was made by Sephadex G-200 gel filtration. The column 2.5 x 90 cm. was equilibrated with 0.01 M Tris-HCl buffer, pH 7.4 containing 0.14M NaCl. The eluates were collected in 3 ml. volumes and aliquots of each eluate used to estimate both the total enzyme activity and protein concentration. The total enzyme activity of the eluates was estimated fluorimetrically using the method described by Woollen and Walker (1965a). Total protein was estimated turbidometrically by precipitation with 3% sulphosalicylic acid. The tubes of highest enzyme activity were pooled. Sufficient enzyme protein was obtained by pooling sufficient fractions to yield a solution containing 1 mg. of enzyme protein per 1ml.

3.12 Molecular weight determinations.

A jacketed glass column measuring 2.5 x 90 cm. was packed with sephadex G-200 (particle size 40-120μ) equilibriated in 0.01M Tris-HCl buffer, pH 7.4 containing 0.14M NaCl. This buffer was also used as eluant for the enzymes, and 5 ml. eluates were collected. During the experiment the column was cooled with running water.

To calibrate the column four enzymes, leucine aminopeptidose lactic dehydrogenase, malic dehydrogenase and muramidase were gel filtrated and their elution volume calculated. All enzymes were obtained from Miles-Seravac Laboratories Ltd., Stoke Court, Stoke Poges, Slough. The molecular weights of leucine aminopeptidase was taken as 300,000 lactic dehydrogenase 135,000 malic dehydrogenase 70,000 and muraminidase 14,000.

2 mg. of each enzyme were dissolved in 10 ml. of eluting buffer and 1 ml. added to the sephadex column. An aliquot of each eluate was assayed for all four enzymes. The leucine amino-peptidase was assayed as previously described for serum in 3.4.8. Lactic dehydrogenase activity was assayed by the method of Reeves and Finoguari (1963); muramidase by the method of Shugar (1952) and malic dehydrogenase by the method of Mehler et al. (1948).

1 ml. of each isoenzyme of n-acetyl-β-glucosaminidase purified as described in 3.11 above were added to the sephadex column and the eluate volume calculated.

3.13 Antienzyme production.

Antisera were made in rabbits in response to placental and foetal liver isoenzymes of n-acetyl-β-glurosaminidase and β-glucuro-nidase. All the isoenzymes were purified by preparative polyacryl-amide electrophoresis and by sephadex filtration as described in 3.11.1 and 3.11.2.

Eight Geaut de Boufcat rabbits were each immunized intramuscu-larly at multiple sites. Antiserum was raised against the placental and foetal liver isoenzymes A and B of n-acetyl-β-glucosaminidase and of β-glucuronidase. Each immunization contained 1.0 mg. of enzyme protein in 1 ml. of buffer emulsified with 1.0 ml. of complete Freunds adjuvant. Booster immunizations were given at two week intervals. Antibody activity was evaluated at 6 weeks and the animals were bled from a ear vein when antibody was present in a satisfactory titer. The antiserum was collected and stored at -20°C.

3.14 Immunodiffusion.

Gel diffusion, immunoelectrophoresis and the tube inactivation method were employed to immunologically investigate the isoenzymes.

3.14. 1 Gel diffusion. The Ouchterlony (1962) technique was employed using plastic petri dishes containing 1% agar in 0.1M barbitone buffer

pH 8.6. The wells were 4 mm in diameter and 8 mm distant. 30µl volumes of antiserum and tissue extracts were employed. The gels were incubated for 24 hours at room temperature in a moist chamber and washed in 0.9% saline for 5 hours. The enzyme activity of the immunoprecipitate was estimated histochemically as described by Huddlestone et al. (1971). The optimum concentration of antigen and antibody for n-acetyl-β-glucosaminidase and glucuronidase was performed by the double immunodiffusion in two series, one with a fixed amount of antigen diffusing against a series of antibody dil utions ranging from 1/2 to 1/40 and the other with a fixed amount of antibody with dilutions of antigen.

3.14.2 Immunoelectrophoresis. The Shandon immunoelectrophoresis system was employed, using the Wieme technique (1959). Glass slides coated with 1% agar in barbitone buffer at pH 6.8 was employed. Eight slides with 10 µl. of sera and tissue extracts were electrophoresed at 150V for three hours. The trough was filled with antiserum and diffusion was allowed to proceed for 18 hours. The slides were washed in 0.9% saline for 5 hours. The enzyme immunoprecipitate was detected histochemically as described for the Ouchterlony method described in 3.12.1.

3.14.3 Tube inactivation of pregnancy serum. 1.0 ml of a serum pool obtained from ten patients at 38 weeks of pregnancy was mixed with 0.1 ml. of each rabbit antiserum and as a control 1.0 ml of pregnancy serum was mixed with 0.1 ml of 0.9% saline. The samples were incubated at 37°C for one hour and then placed at 4°C for two days. Each tube was then centrifuged at 3,000 r.p.m. for fifteen minutes and the supernatant assayed for n-acetyl-β-glucosaminidase and β-glucuronidase. The immunological inactivation of the serum enzyme was expressed as a percentage of the enzyme activity in the control tube.

4. RESULTS

4.1. 1 Enzyme activities of pregnancy serum.

To obtain the normal values of enzymes found in serum during pregnancy the enzymes carboxypeptidase, dipeptidase, tripeptidase, leucine aminopeptidase, alanine aminopeptidase, cystine aminopeptidase, ɣ-glutamyl transpeptidase, alkaline phosphatase, n-acetyl-β-glucosaminidase, n-acetyl-β-galactosaminidase and β-glucuronidase were estimated in sera from pregnant women attending the ante-natal clinic. The patients were unselected with regard to age and parity.

After delivery the consultant obstetrician examined the clinical data in retrospect, to ascertain which patients were found to have had normal pregnancies. The results found for normal pregnancies are illustrated graphically in Figs. 7 to 17.

No progressive increase in dipeptidase or tripeptidase activity was found during pregnancy and therefore no further investigations were made in regard to these enzymes. All other enzymes investigated were found to increase between the 16th week of pregnancy and term. Each scattergraph of the results also illustrates the mean value and the 95% confidence limits for each week of pregnancy.

Tables 4, 5 and 6 list the mean, standard deviation and the predicted y (regression line figures) for each enzyme activity between the 14th week of pregnancy and term. Both the quadratic term and the test for linearity were estimated for each enzyme and the results listed in Table 7. Carboxypeptidase, ɣ-glutamyl transpeptidase, alkaline phosphatase, alanine aminopeptidase, glucose-6-phosphatase and 5́-nucleotidase were found to increase linearly, the activity approximately doubling during each trimester.

Cystine aminopeptidase, leucine aminopeptidase and n-acetyl-β-glucosaminidase progressively increased but the increase was non-linear. The quadratic term and the test for non-linearity were both significant at the 5% level for β-glucuronidase.

Figs. 7-17 also illustrate the enzyme activities found in eight patients 48 hr. after delivery. In each case the enzyme activity had decreased by more then 50% of the mean value obtained during the 38th week of pregnancy.

Between the 14th and 18th week of pregnancy all enzyme activities were within the normal found in non-pregnant females. After about the 18th week of pregnancy until term a progressive increase in enzyme activity was detected. Serial serum enzyme estimations on the same patient gave increasing enzyme activity during gestation.

A wide scatter of results was found during each week of pregnancy and furthermore the scatter increased as pregnancy advanced. Some of the wide scatter could be attributed to the inherent error of each method and this error would increase with increasing enzyme values. Later during this work a number of the enzymes were automated so that precision could be improved.

4.1. 2 Automated methods of enzyme assay. During the course of this work it was decided to automate certain enzymes with the object of both improving precision and to increase the number of sera assayed during the working day. The enzymes automated fell into two groups, colorimetric and fluorimetric. The colorimetric method was used for leucine aminopeptidase, and the fluorimetric methods for n-acetyl-β-glucosaminidase, n-acetyl-β-galactosaminidase and β-glucuronidase.

The automation of serum leucine aminopeptidase.

Typical standard and test runs for leucine aminopeptidase are shown in Fig. 18. The standard curve for p-nitroaniline was linear to a nitroaniline concentration of 3mM per litre. Ten replicate determinations carried out on a serum of mean activity of 433 I.U. per litre gave a standard deviation of 1.5. This indicated between sample carry over to be negligible. In addition when sera were alternately preceded by sera having high or low activity, nearly identical values were obtained, again indicating negligible sample interaction. Duplicate determinations, interaction patterns and steady state recordings obtained with a pooled pregnancy serum are shown in Fig. 19.

Within batch precision, determined from results of duplicate determinations on 48 sera with activities between 162 and 546 I.U. per litre showed a coefficient of variation of 2.1% (mean 346, standard deviation 2.4). The results were compared with values obtained by a standard

manual method and the correlation was excellent (r = 0.976) between the two procedures (Fig. 20). The scattergraph of the results obtained for the enzyme activity in normal pregnancies is shown in Fig. 21. The mean, standard deviation and the regression line values are listed in Table 8. A linear increase in serum enzyme activity was found similar to that found while using leucyl-β-naphthylamide as substrate.

The automation of serum n-acetyl-β-glucosaminidase (AGA), n-acetyl-β-galactosaminidase (A Gal A) and β-glucuronidase (GLUC).

Peak height was linearly related to concentration from 0.1 to 4 mg./100 ml of methylumbelliferone, which corresponds to enzyme activity of between 0.5 and 15.4 I.U. per litre.

The concentration of the standards found appropriate for each enzyme was 3.0 to 15.4 I.U. per litre for AGA, 1.0 to 6.0 I.U. per litre for A Gal A and 0.5 to 2.5 I.U. per litre for β-glucuronidase. Figs. 22, 23 and 24 show typical calibration curves and test runs for each enzyme.

Interaction between a high serum enzyme activity to a low sample was negligible and nearly identical values were obtained in each case. The within batch precision of the automated method was excellent with means and standard deviation of 12.5 and 0.07 for AGA 5.1 and 0.03 for A Gal A and 1.4 and 0.04 for GLUC.

Figs. 25, 26 and 27 show duplicate determinations, interaction patterns and steady state recordings for AGA, A Gal A and GLUC respectively.

Comparisons were made between the autoanalyzer and the manual methods with excellent correlation AGA, r = 0.983; A Gal A, r = 0.978; GLUC, r = 0.980. The comparisons are illustrated graphically in Figs. 28, 29 and 30.

Scattergraphs of the serum enzyme results obtained with each of the enzymes are shown in Figs. 31, 32 and 33. The scattergraph for AGA and GLUC were similar to that found for the manual techniques with no decrease in the scatter during pregnancy. Tables 9, 10 and 11 list the means, standard deviations and regression values for AGA, A Gal A, and GLUC respectively.

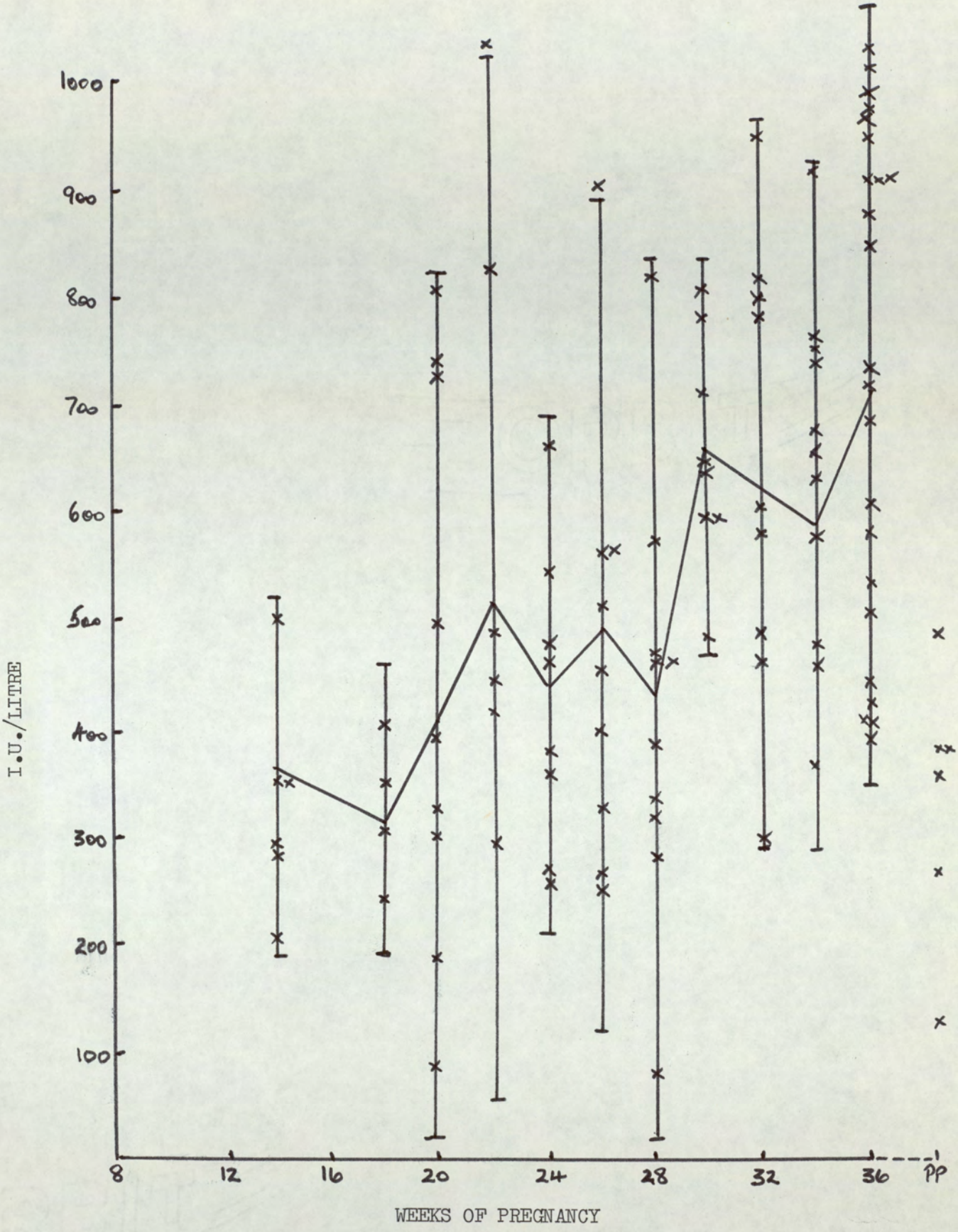


Fig. 7. Serum carboxypeptidase B activities during pregnancy and 2 days post partum (P.P.).

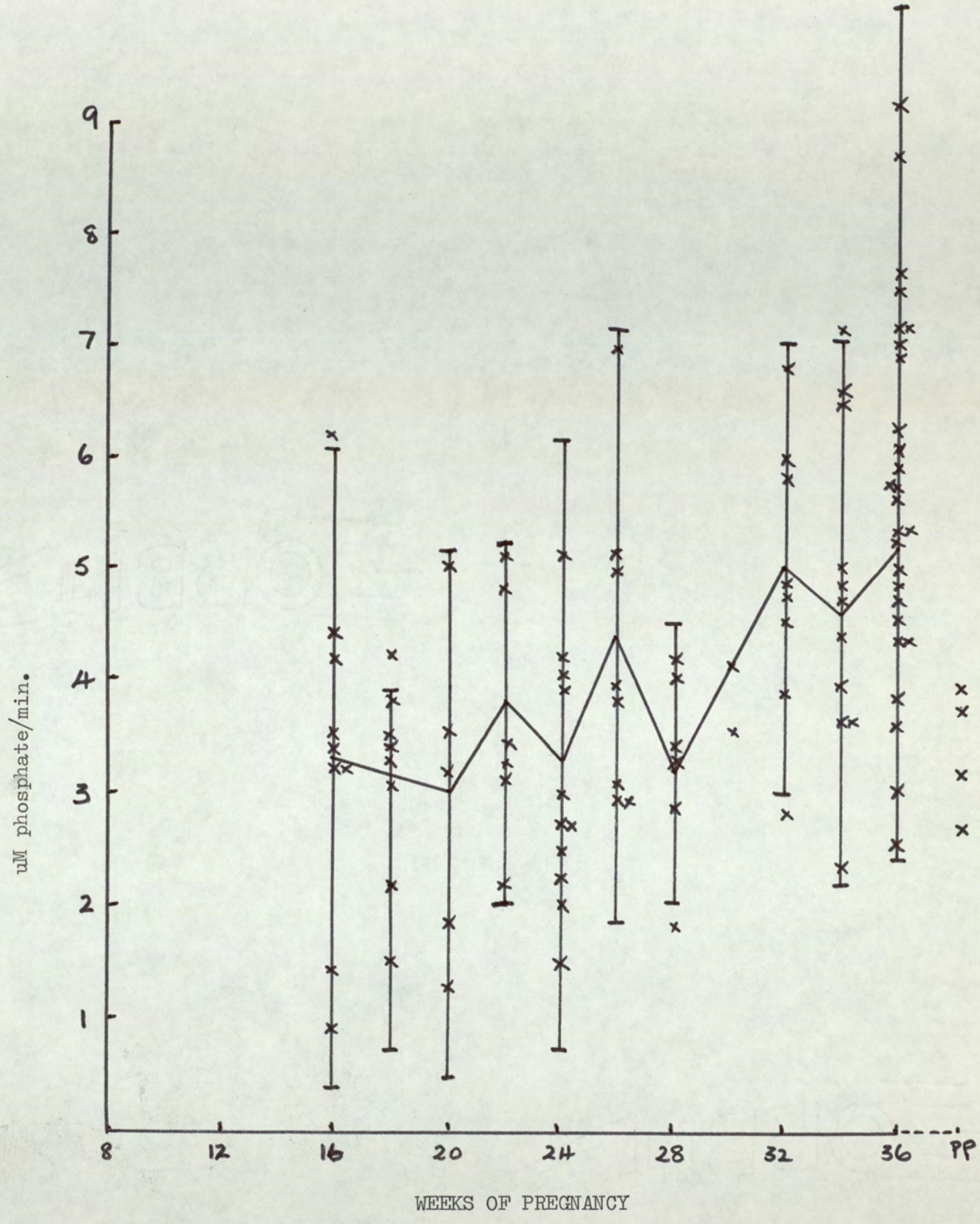


Fig. 8. Serum 5′-nucleotidase levels during pregnancy and 2 days post partum (PP).

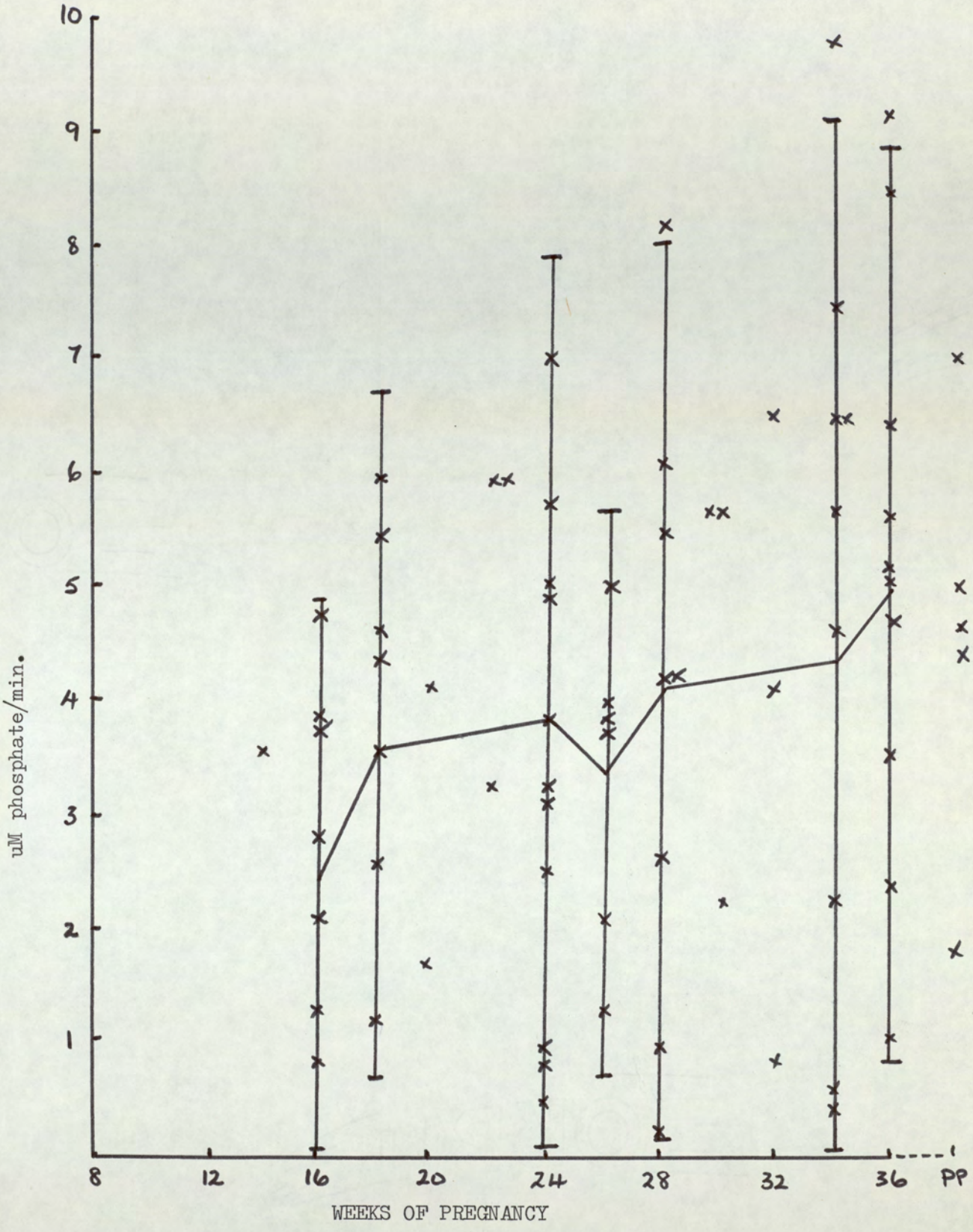


Fig. 9. Serum glucose-6-phosphatase levels during gestation and 2 days post partum (PP).

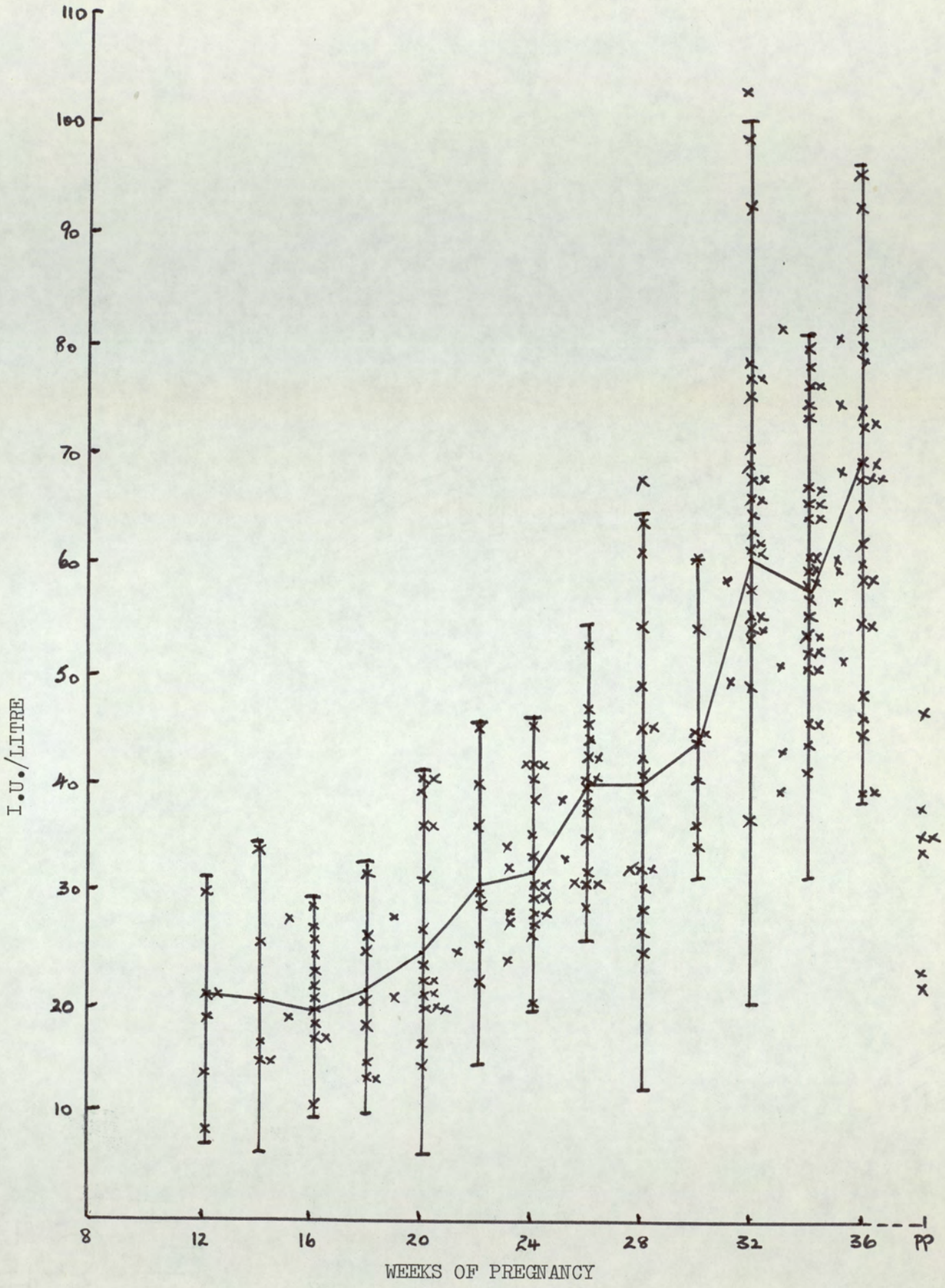


Fig. 10. Scattergraph of pregnancy serum leucine amino peptidase activity during normal pregnancy and two days post partum (PP).

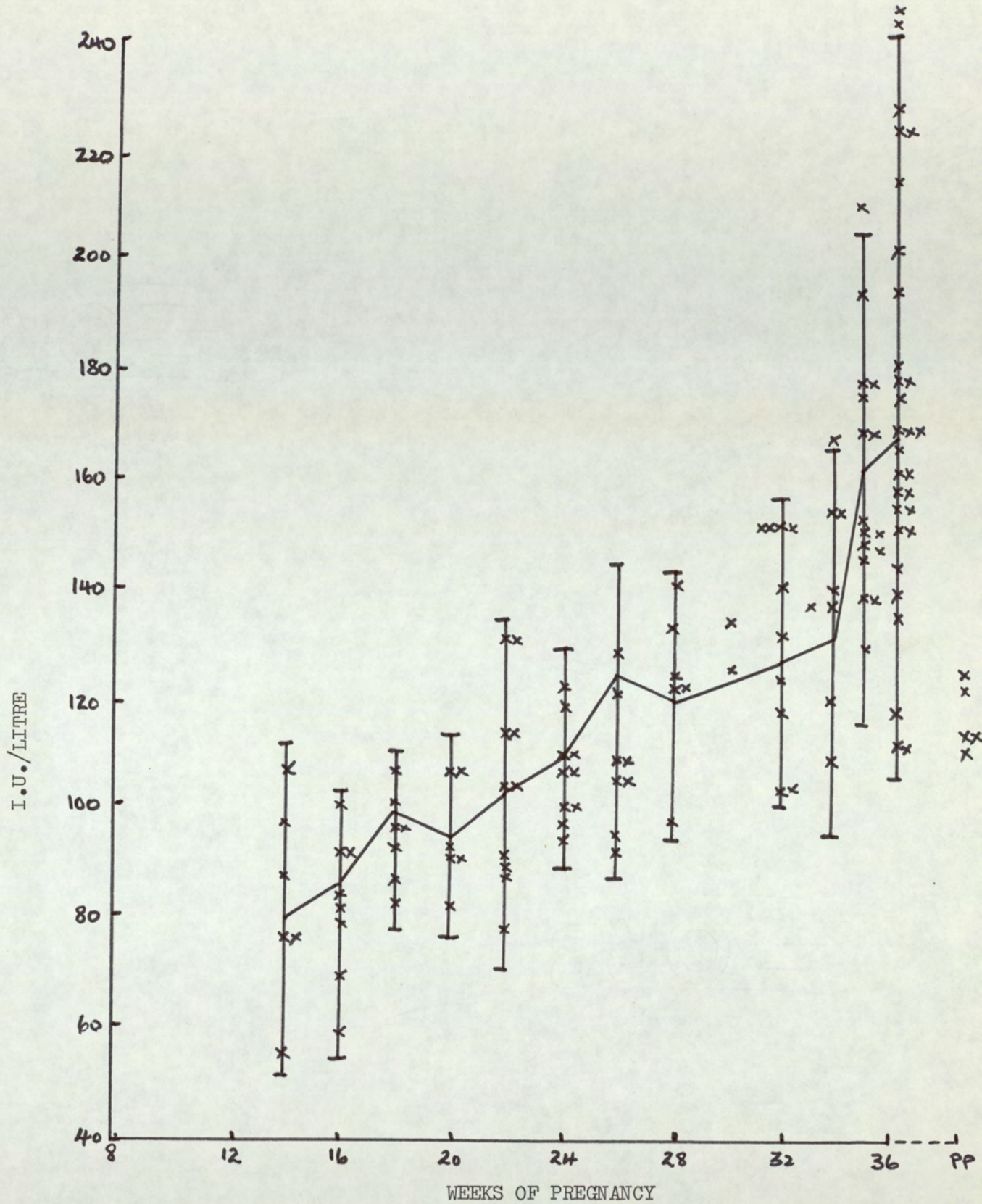


Fig. 11. Pregnancy Serum alanine aminopeptidase during gestation.

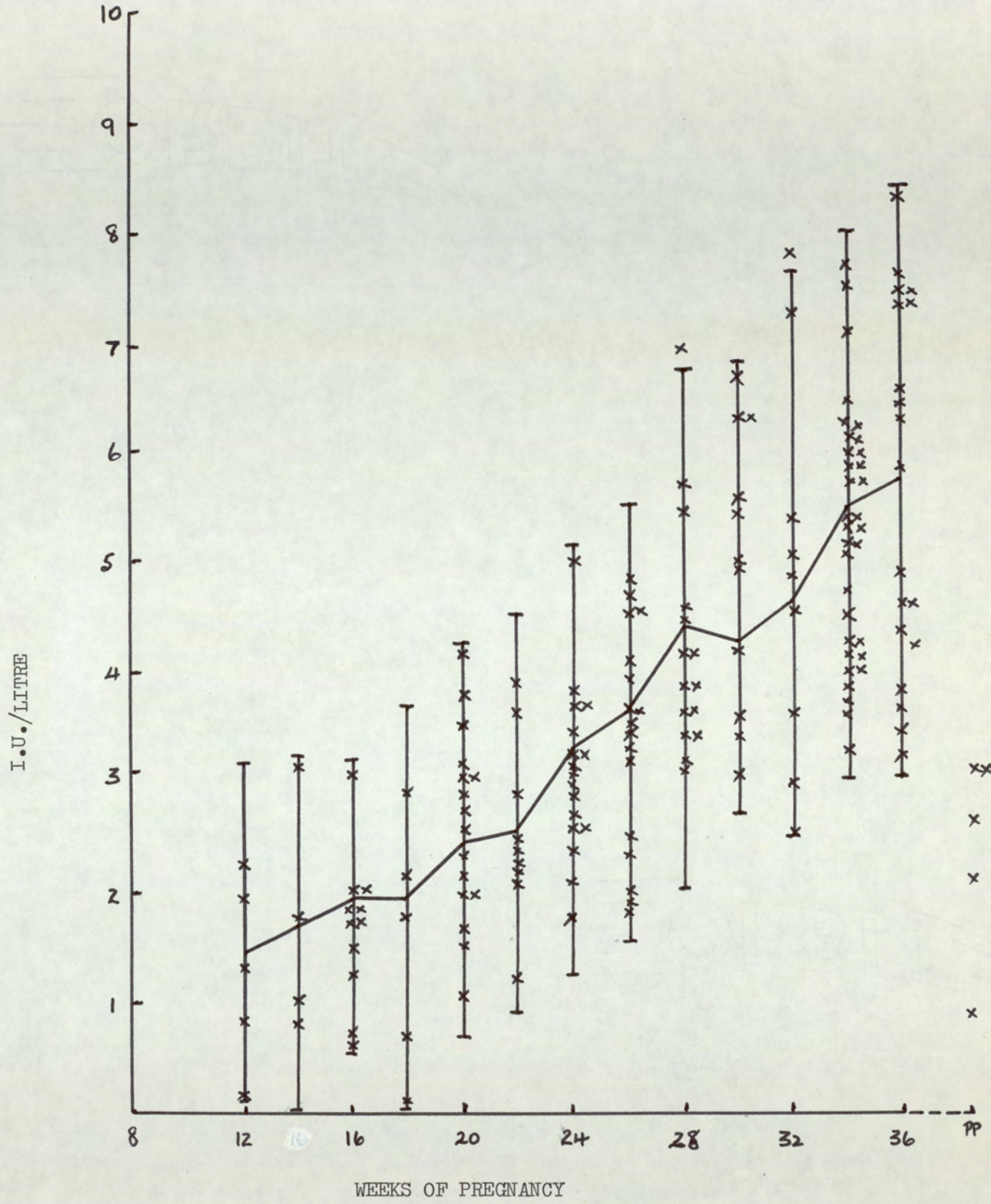


Fig. 12. Pregnancy serum cystine aminopeptidase activities during normal pregnancy and 2 days post partum (PP).

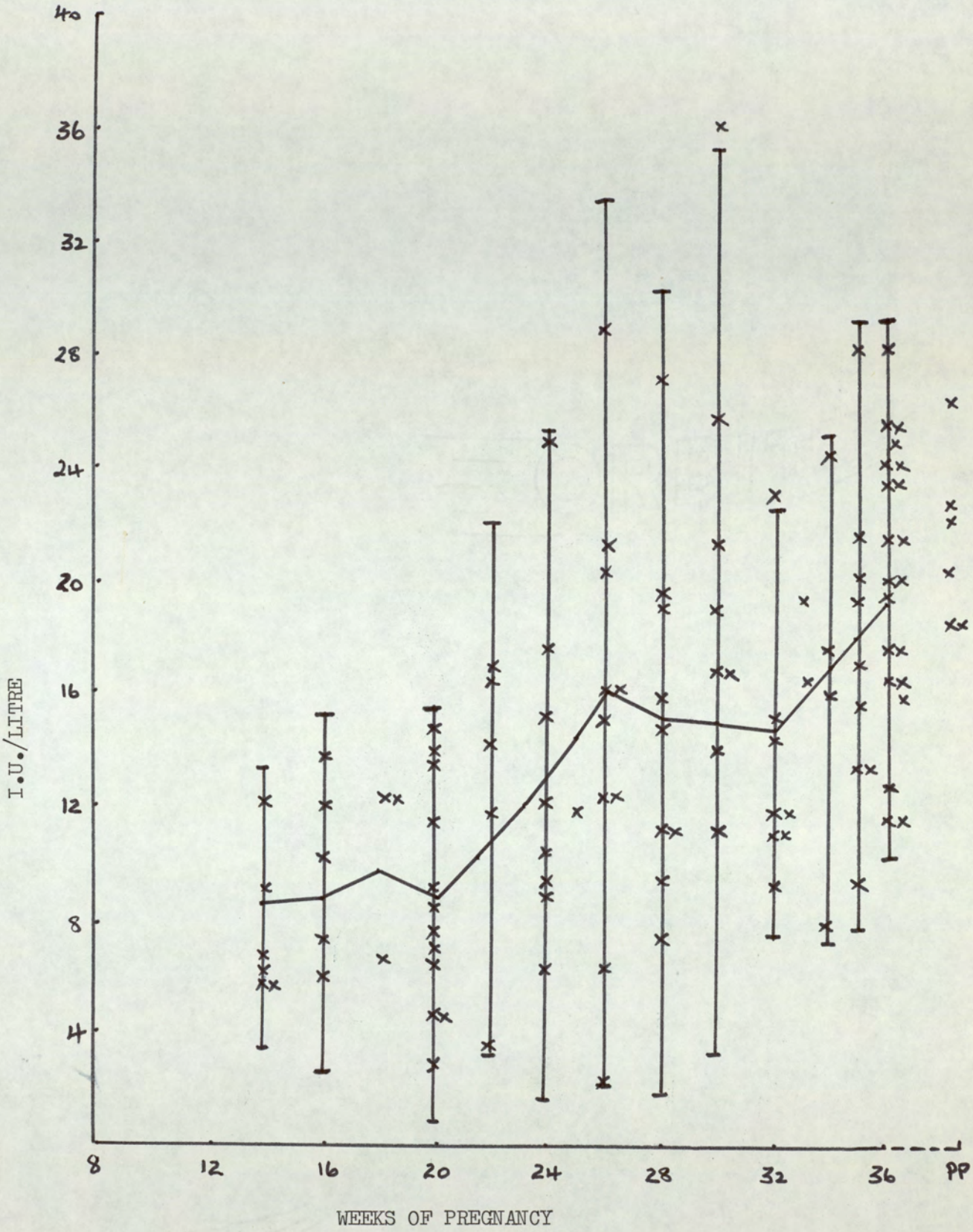


Fig. 13. Scattergraph of the serum δ-glutamyl transpeptidase activities in pregnancy and 2 weeks post partum (PP).

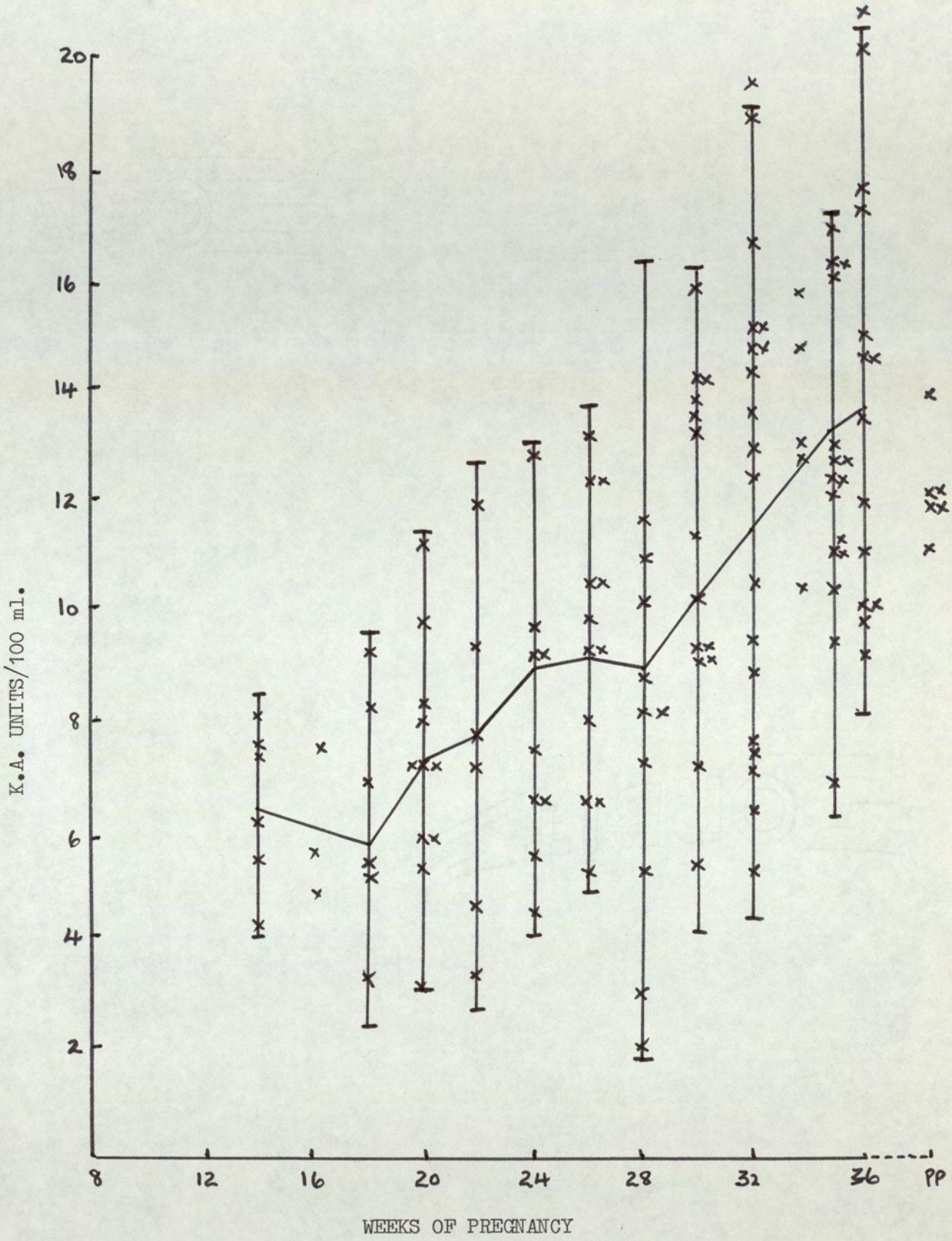


Fig. 14. Total serum alkaline phosphatase during pregnancy and 2 days post partum (PP).

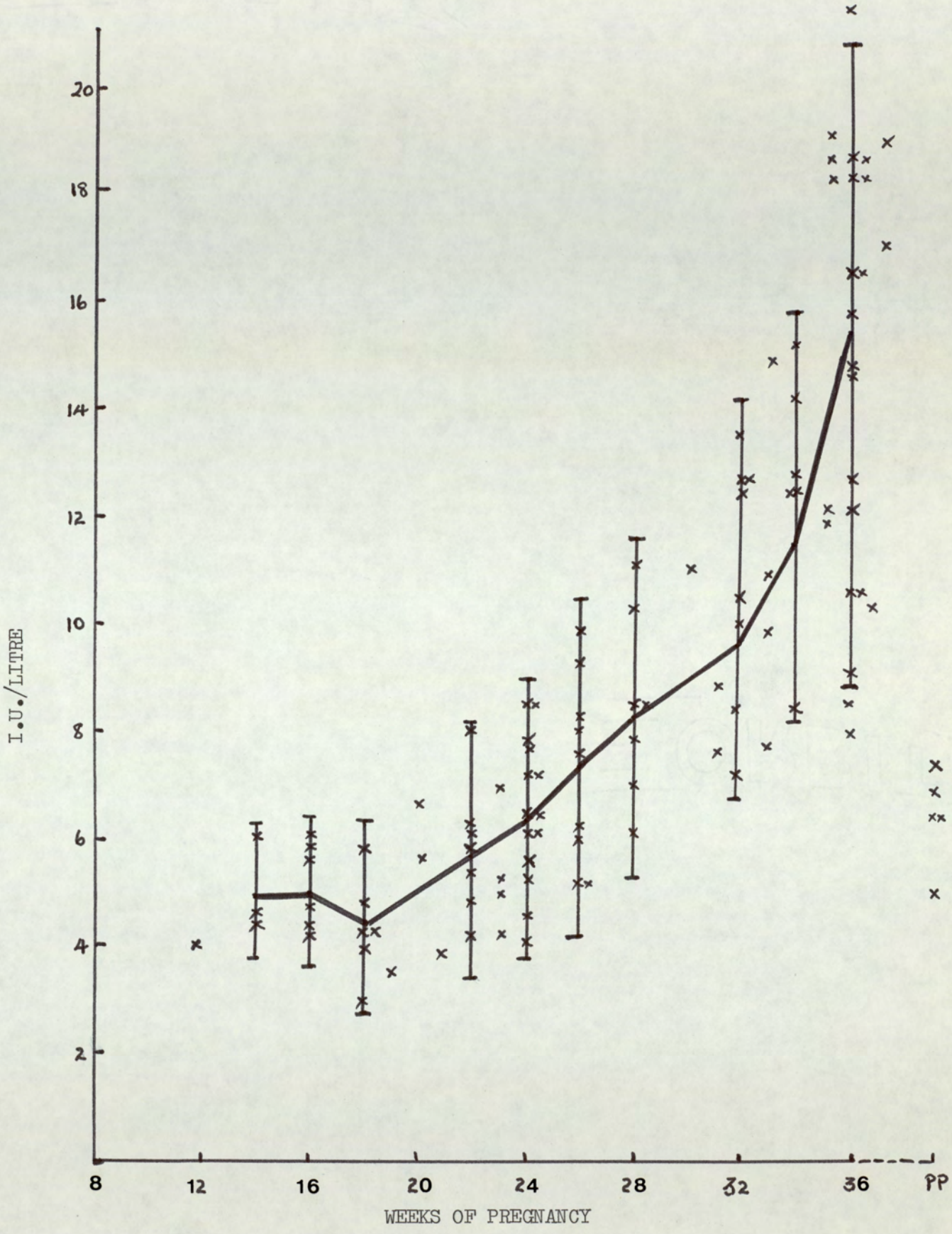


Fig. 15. Scattergraph of the serum enzyme results obtained for n-acetyl-β-glucosaminidase.

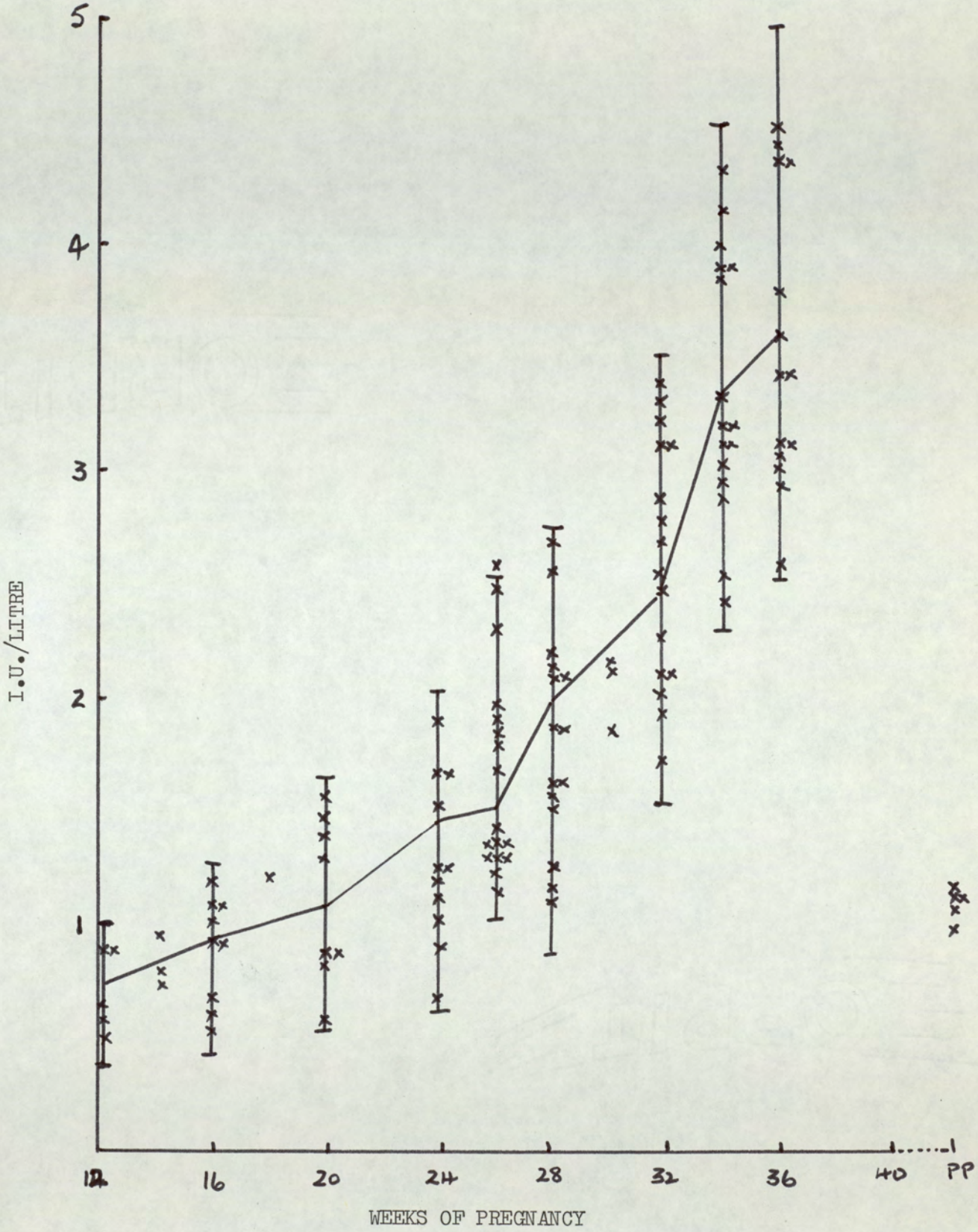


Fig. 16. Serum n-acetyl-β-galactosaminidase activities during pregnancy and 2 days post partum (PP).

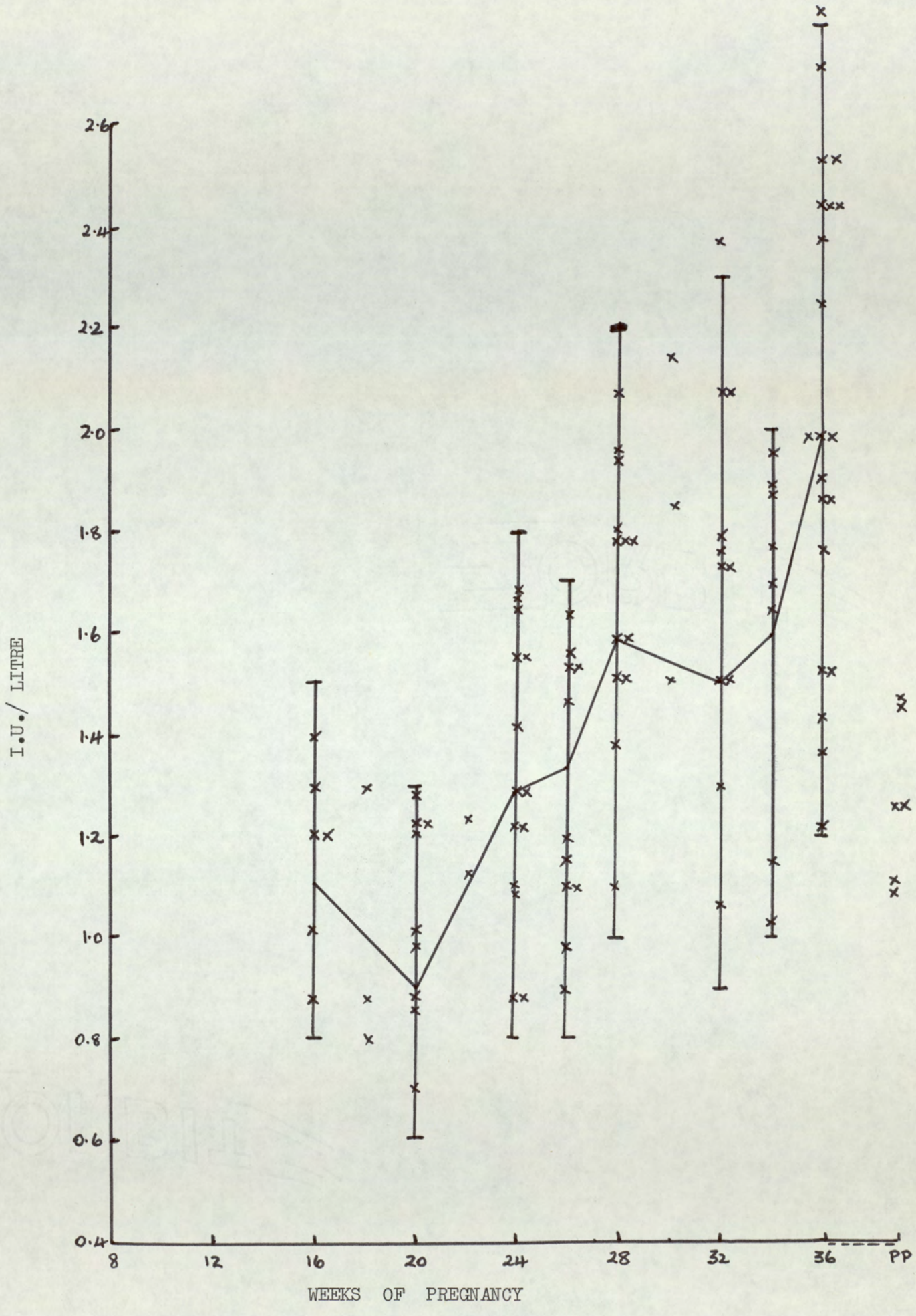


Fig. 17. Pregnancy Serum β-glucuronidase during pregnancy.

Table 4. The means ($\bar{x}$), standard deviations (s) and predicted y for ɣ-glutamyl transpeptidase (GTP), carboxypeptidase (CARB), alkaline phosphatase (TAP), and alanine aminopeptidase (AAP).

GTP				CARB			
Week of pregnancy	$\bar{x}$	s	y	Week of pregnancy	$\bar{x}$	s	y
14	8.3	2.57	7.0	14	357	85.8	303
16	8.8	3.20	8.2	18	321	67.8	372
18	9.8	7.72	9.4	20	422	211.0	407
20	8.1	3.68	10.5	22	538	244.3	442
22	11.1	5.51	11.6	24	442	119.0	476
24	12.9	6.15	12.8	26	500	189.5	511
26	16.3	8.48	14.0	28	427	212.9	546
28	15.6	7.42	15.0	30	653	90.5	581
30	19.0	8.02	16.2	32	627	167.2	616
32	14.8	3.77	17.3	34	596	161.4	650
35	18.3	5.43	19.0	36	703	182.2	685
36	19.5	4.78	19.6				
38	21.8	4.16	20.8				
	TAP				AAP		
14	6.3	1.0	4.7	14	80	15.4	73.3
18	5.9	1.8	6.2	16	85	16.7	81.0
20	7.1	2.2	6.9	18	98	3.7	88.9
22	7.6	2.5	7.7	20	95	9.2	96.7
24	8.8	4.4	8.4	22	101	16.6	104.5
26	9.0	2.3	9.2	24	107	10.2	112.3
28	8.8	3.8	9.9	26	123	20.4	120.1
30	10.0	3.0	10.7	28	118	12.2	127.9
32	11.3	3.6	11.5	32	126	15.3	143.5
34	8.0	2.5	12.2	34	129	18.2	151.3
35	11.4	2.8	12.6	35	159	22.2	155.2
36	14.3	3.2	13.0	36	168	40.9	159.1
				38	166	13.8	166.9

Table 5. The means, standard deviation and the predicted y for n-acetyl-glucosaminidase (AGA), β-glucuronidase(GLUC), cystine aminopeptidase (CAP) and leucine aminopeptidase (LAP).

Week of pregnancy	AGA $\bar{x}$	s	y	Week of pregnancy	GLUC $\bar{x}$	s	y
14	5.0	0.6	2.5	16	1.2	0.2	0.9
16	5.0	0.7	3.5	20	0.9	0.2	1.1
18	4.5	0.9	4.6	24	1.3	0.3	1.3
22	5.8	1.2	6.7	26	1.3	0.2	1.4
24	6.4	1.4	7.7	28	1.6	0.3	1.5
26	7.3	1.7	8.7	32	1.5	0.4	1.7
28	8.4	1.6	9.8	34	1.6	0.2	1.8
32	9.7	2.3	11.9	36	2.0	0.4	1.8
34	11.7	2.1	12.9				
36	15.8	4.8	13.9				
	CAP				LAP		
12	1.4	1.2	0.8	12	20.7	6.5	5.9
14	1.7	1.1	1.3	14	20.0	7.3	11.0
15	1.8	0.7	1.5	16	19.6	5.7	16.2
16	1.9	0.7	1.7	18	21.8	6.1	21.3
18	1.8	0.9	2.1	20	24.3	9.7	26.4
20	2.5	0.9	2.5	22	31.2	7.0	31.6
22	2.5	0.9	2.9	24	32.2	7.2	36.8
23	2.0	0.4	3.1	26	39.9	7.6	41.9
24	3.3	1.0	3.3	28	39.5	14.2	47.0
26	3.4	1.0	3.7	30	46.1	7.9	52.2
28	4.4	1.2	4.2	32	59.7	20.6	57.3
29	4.3	0.9	4.4	33	59.5	5.8	59.9
30	4.5	0.4	4.6	34	57.4	12.0	62.4
32	4.6	0.9	5.0	35	61.2	10.7	65.0
33	4.8	0.9	5.2	36	69.8	19.7	67.6
34	5.5	1.3	5.4	37	76.7	10.9	70.2
35	6.0	0.9	5.6	38	85.0	12.1	72.8
36	5.7	1.3	5.8	40	72.2	19.4	71.9
37	6.1	2.3	6.0				
40	6.5	1.6	6.6				

Table 6. Standard deviations, mean values and predicted y values for glucose-6-phosphatase (G6P) and 5'-nucleotidase (5NT) in serum during pregnancy.

Week of pregnancy	$\bar{x}$	s	y
	G.6.P		
16	2.5	1.3	2.7
18	3.7	1.6	2.9
24	3.8	2.0	3.6
26	3.2	1.4	3.8
28	4.1	1.9	4.0
34	4.4	2.5	4.6
36	4.9	2.0	4.8
	5.N.T.		
16	1.6	0.8	1.4
18	1.1	0.4	1.5
20	1.5	0.6	1.6
22	1.9	0.4	1.7
24	1.7	0.7	1.8
26	2.2	0.7	1.9
28	1.6	0.3	2.1
32	2.5	0.5	2.3
34	2.3	0.7	2.4
35	2.3	0.6	2.4
36	2.6	0.7	2.5
37	2.5	0.7	2.6

Table 7. Results obtained for the quadratic term and for test for non-linearity (N.S. - not significant).

	CAP	LAP	GTP	CARB	TAP	AAP	AGA	GLUC	G6P	5NT
Number of assays	255	260	110	102	113	124	64	63	68	102
Quadratic term	5%	0.1%	N.S.	N.S.	N.S.	5%	0.1%	5%	N.S.	N.S.
Test for non-linearity	1%	5%	N.S.	N.S.	N.S.	N.S.	5%	5%	N.S.	N.S.

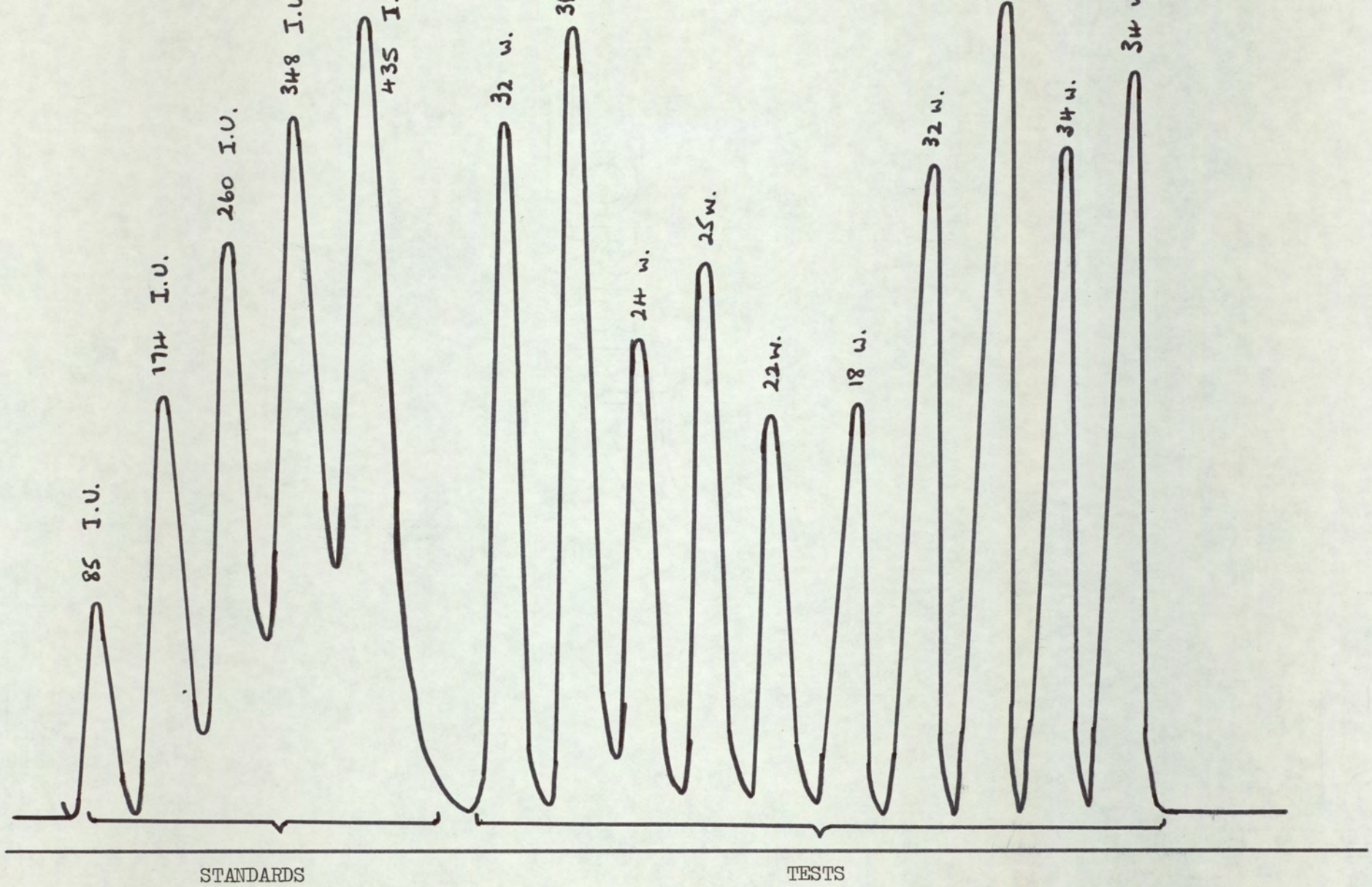


Fig. 18. Typical standards and test runs for pregnancy serum leucine aminopeptidase.

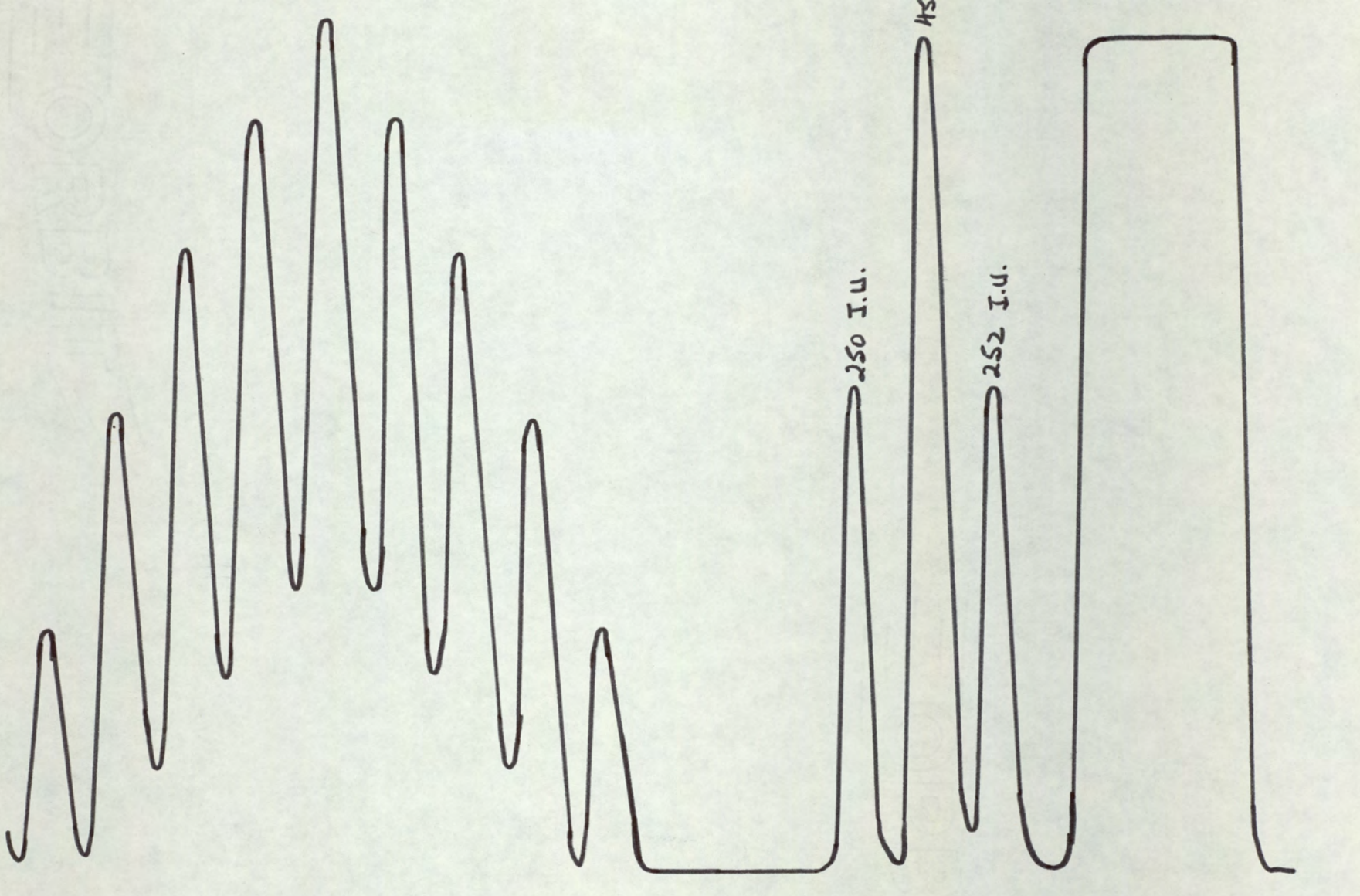


Fig. 19. Autoanalyzer tracings, showing left to right standards run in duplicate, interaction patterns and steady state recordings.

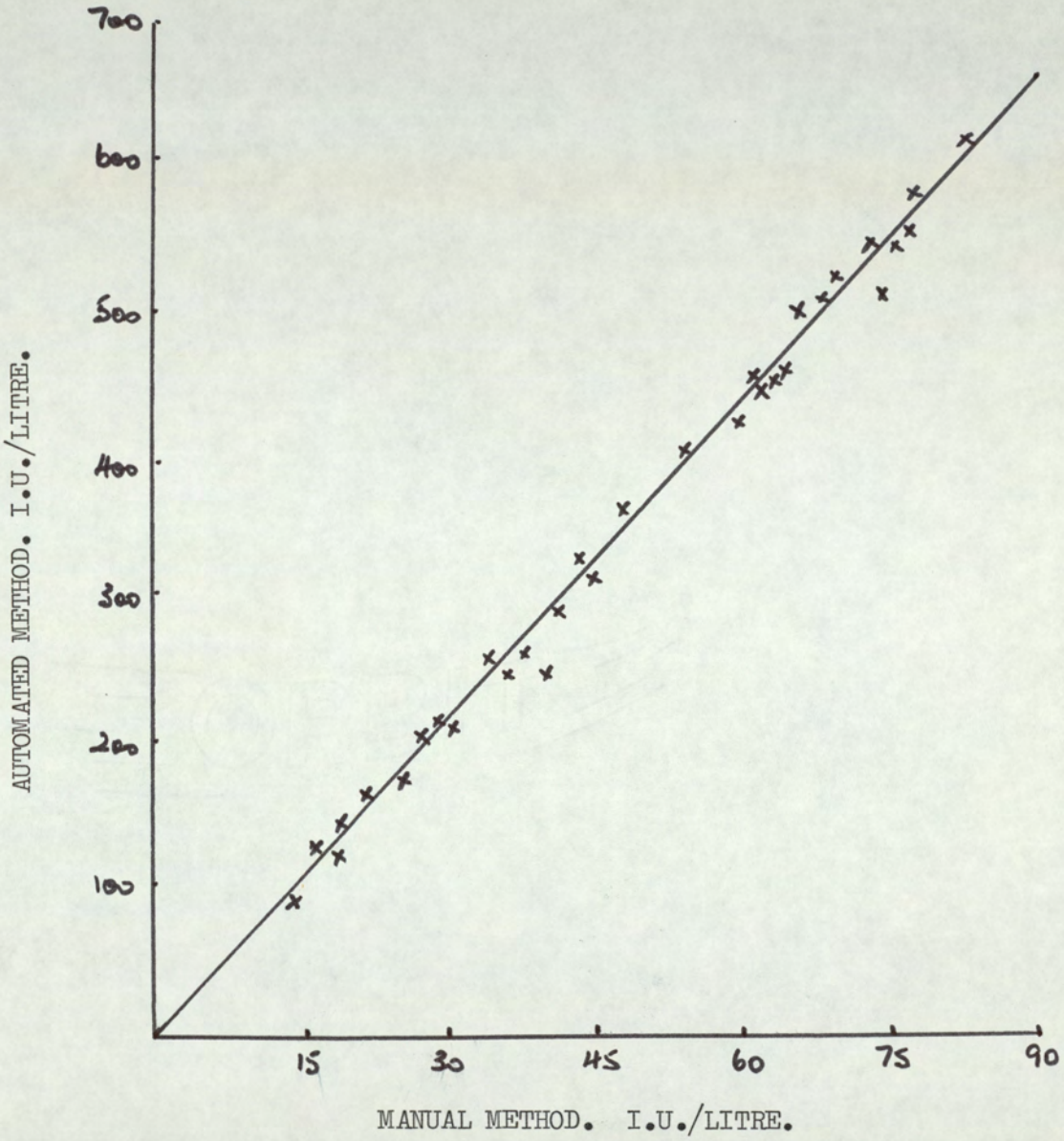


Fig. 20. Comparison of leucine aminopeptidase of thirty sera as determined by the automated and the manual method.

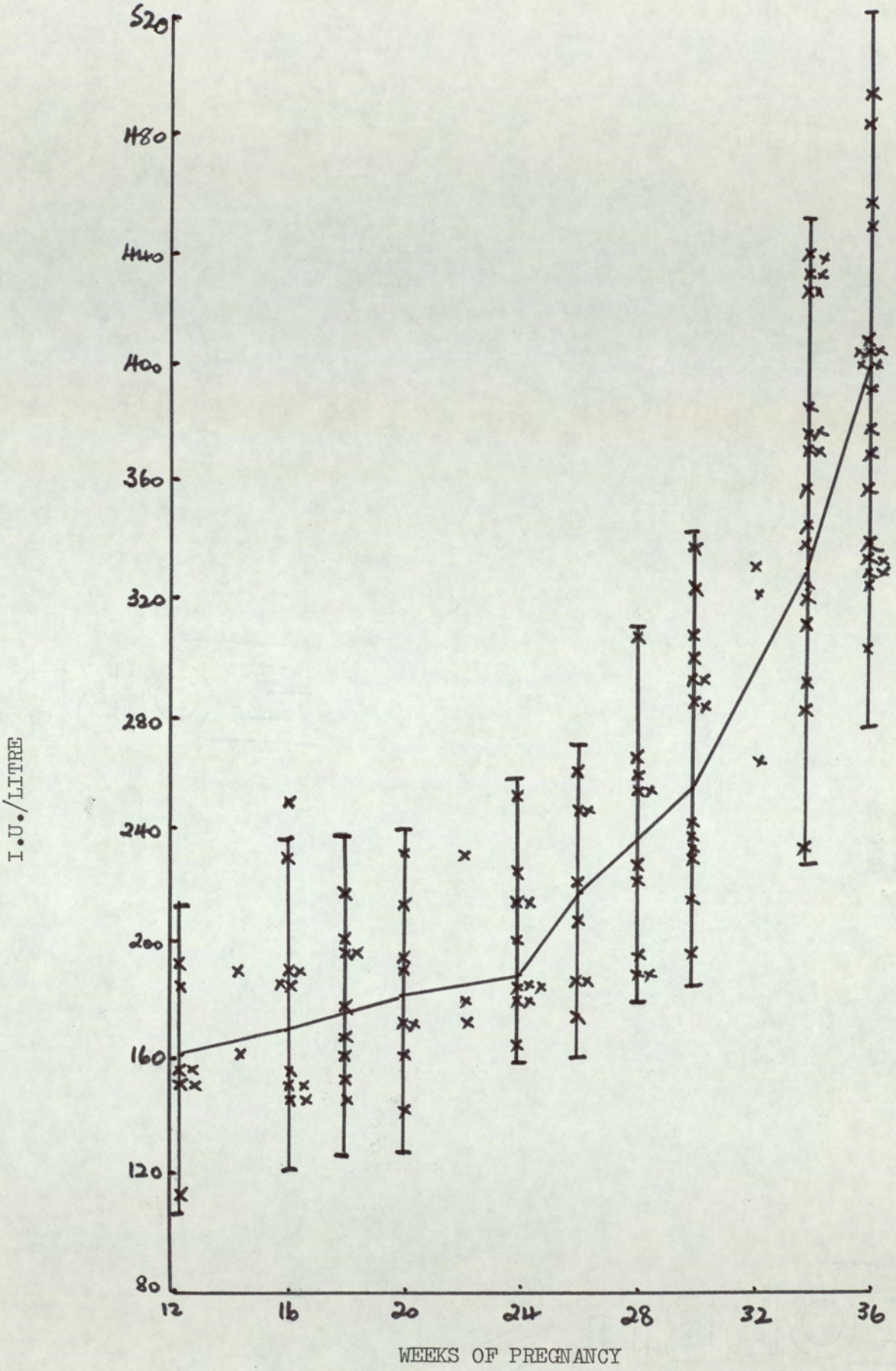


Fig. 21. Scattergraph of serum leucine aminopeptidase activities estimated by the automated method.

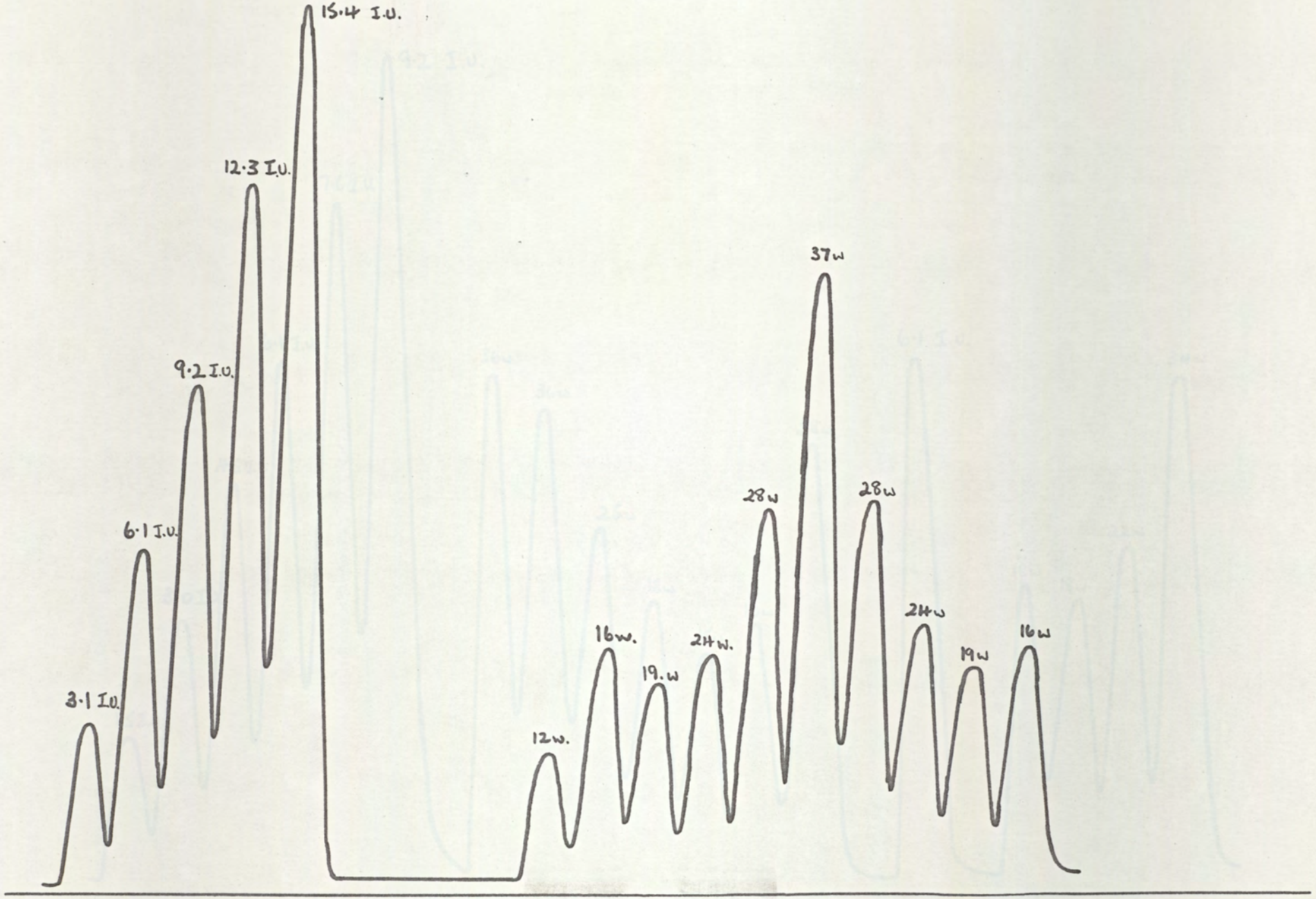


Fig. 22. Typical calibration curve and test runs for the automated method of estimating n-acetyl-β-glucosaminidase in pregnancy sera.

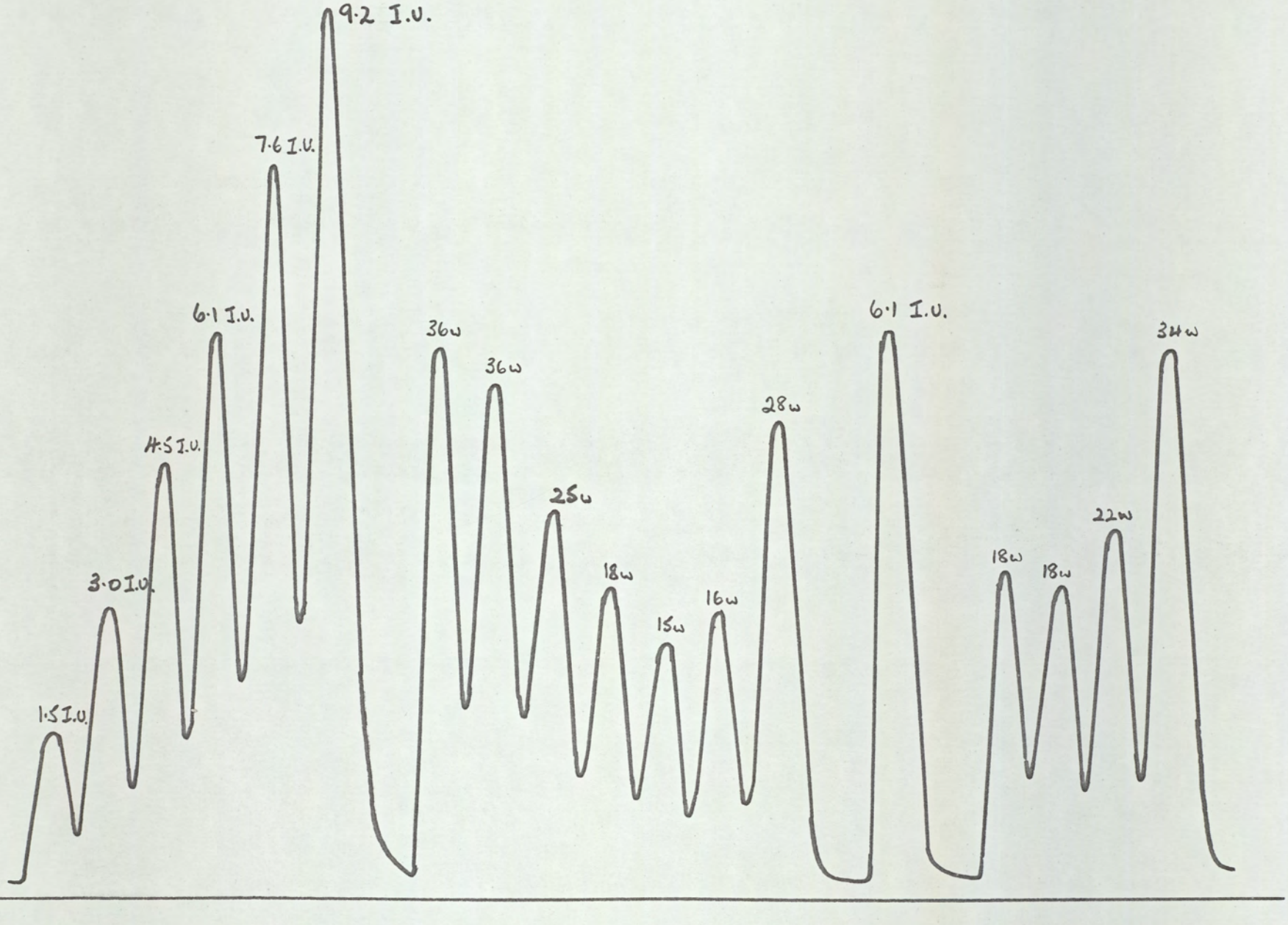


Fig. 23. Typical calibration curve and test run for the automated assay of n-acetyl-β-galactosaminidase in pregnancy serum.

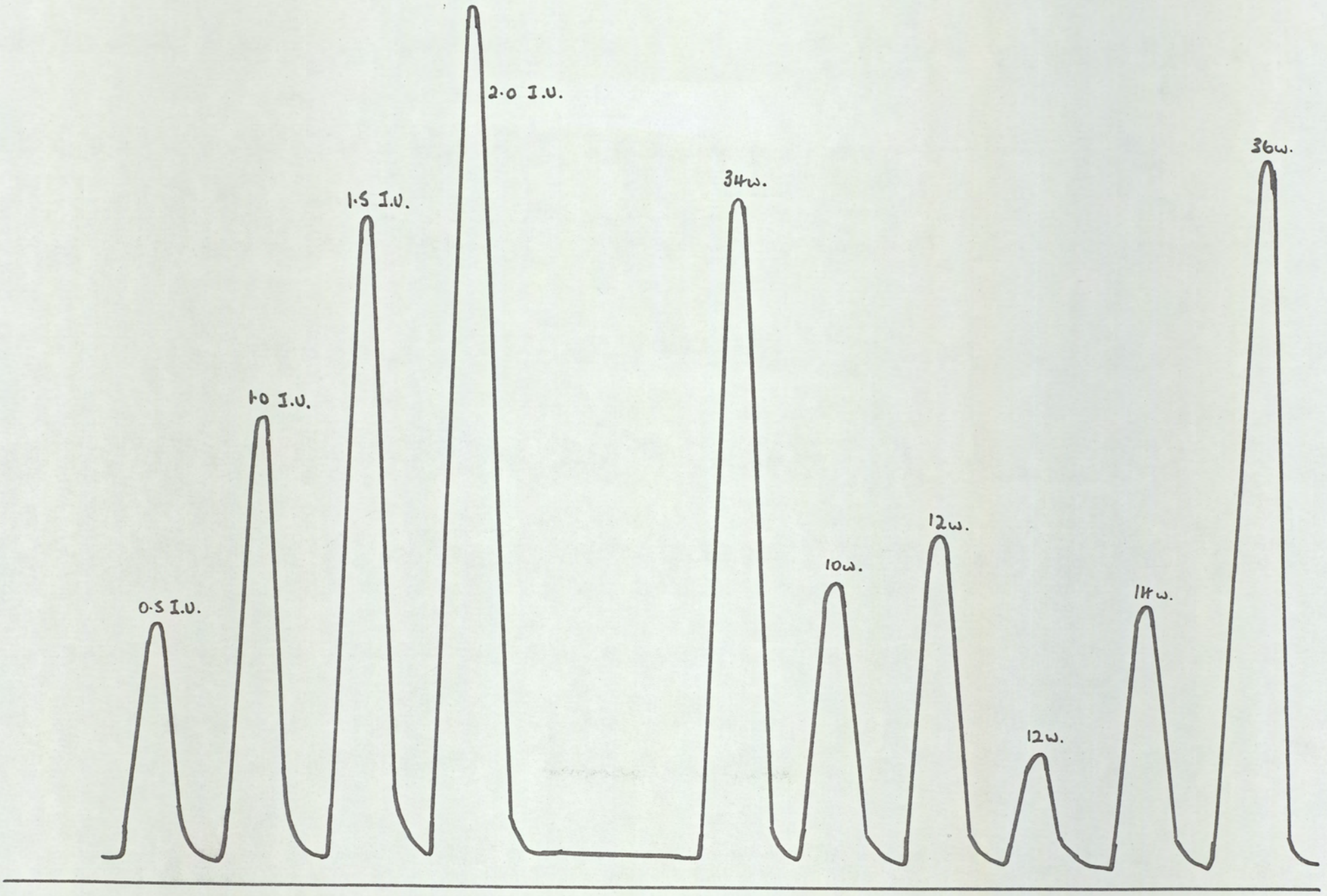


Fig. 24. Typical calibration curve and test run for the assay of serum β-glucuronidase.

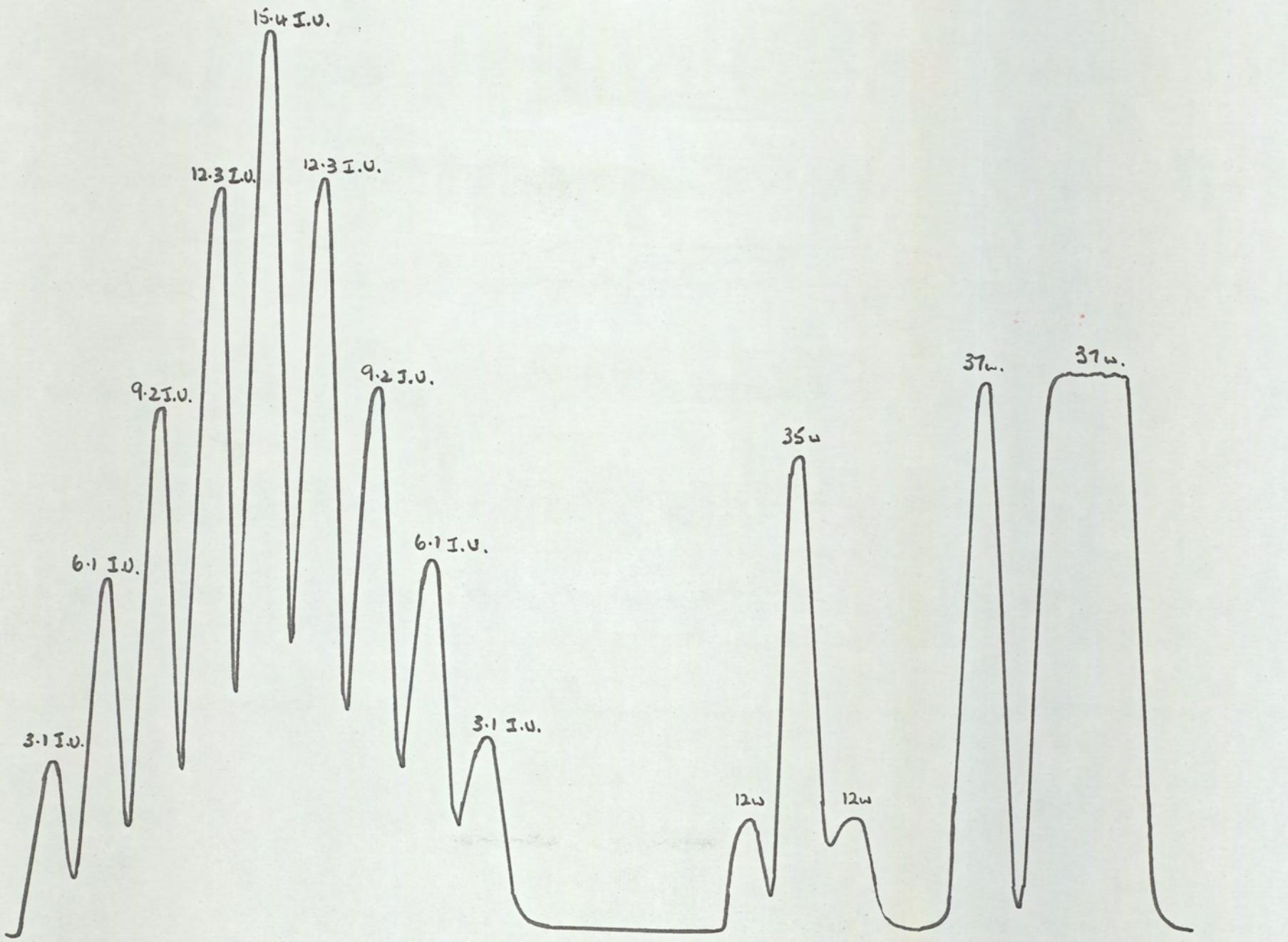


Fig. 25. Auto-analyzer tracings showing standards, interaction patterns and steady rate recordings.

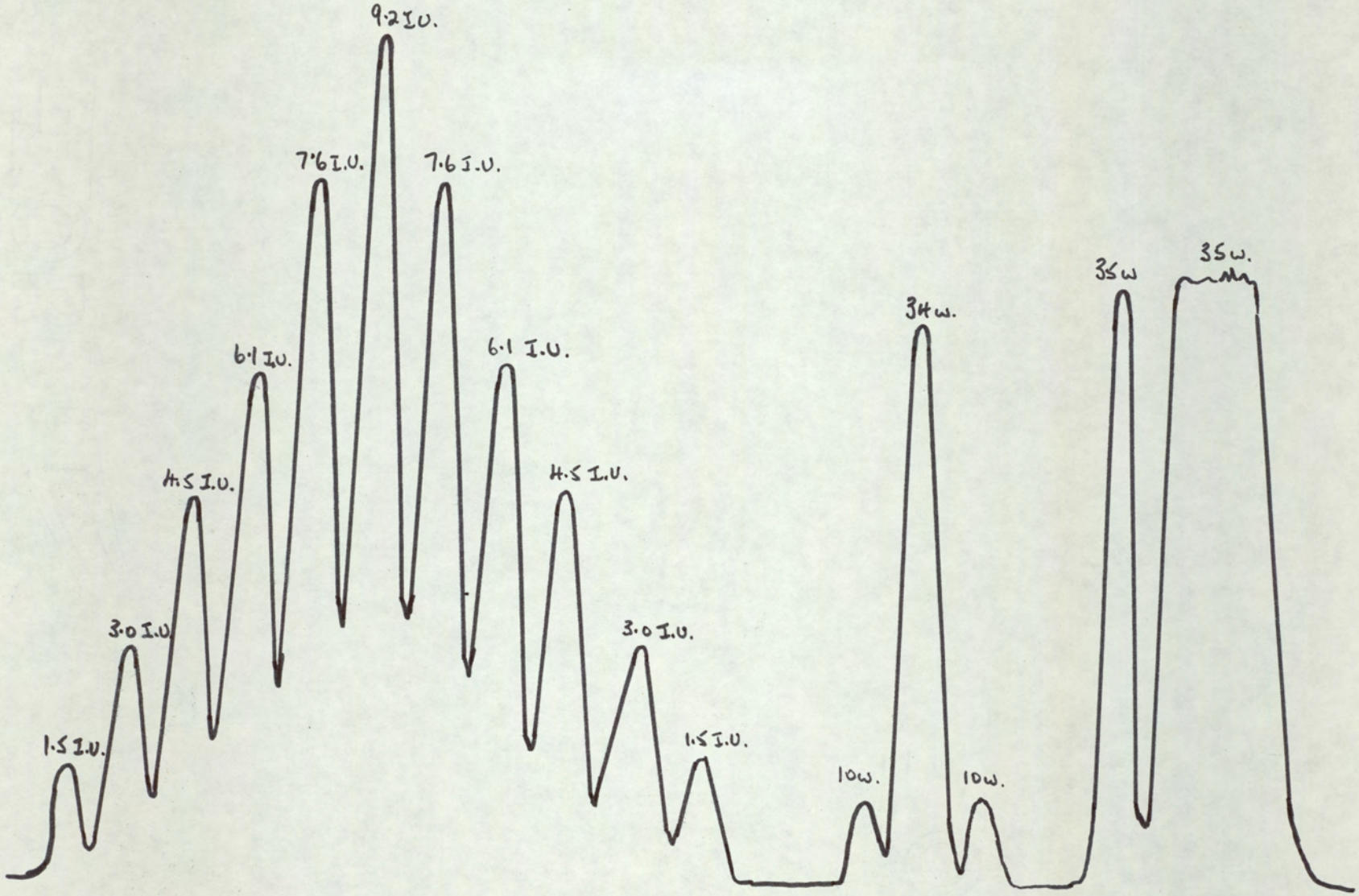


Fig. 26. Calibration curves, interaction patterns and steady state recordings for the automated assay of n-acetyl-β-galactosaminidase.

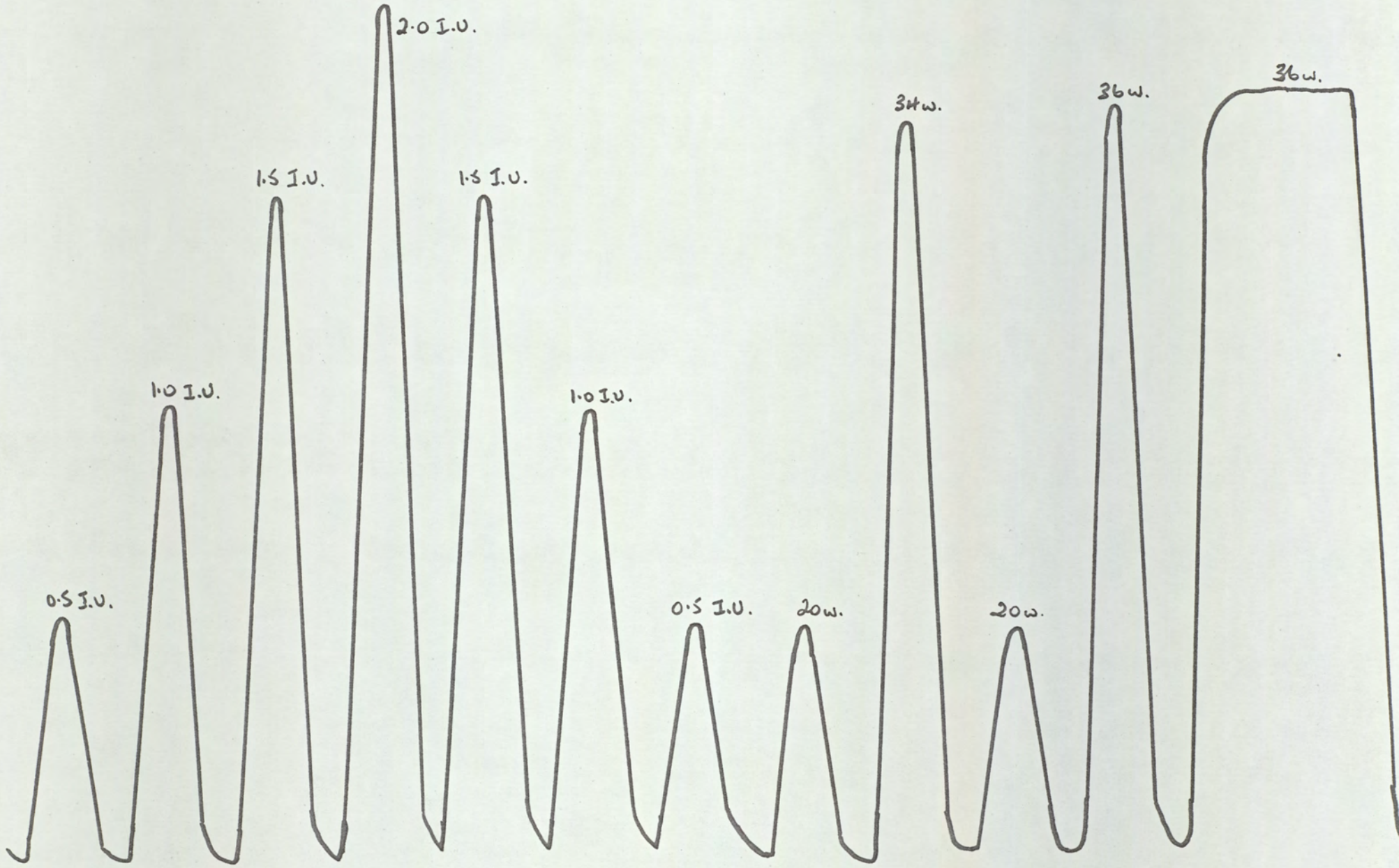


Fig. 27. Standard curve, interaction patterns and steady state recordings for the serum estimation of β-glucuronidase.

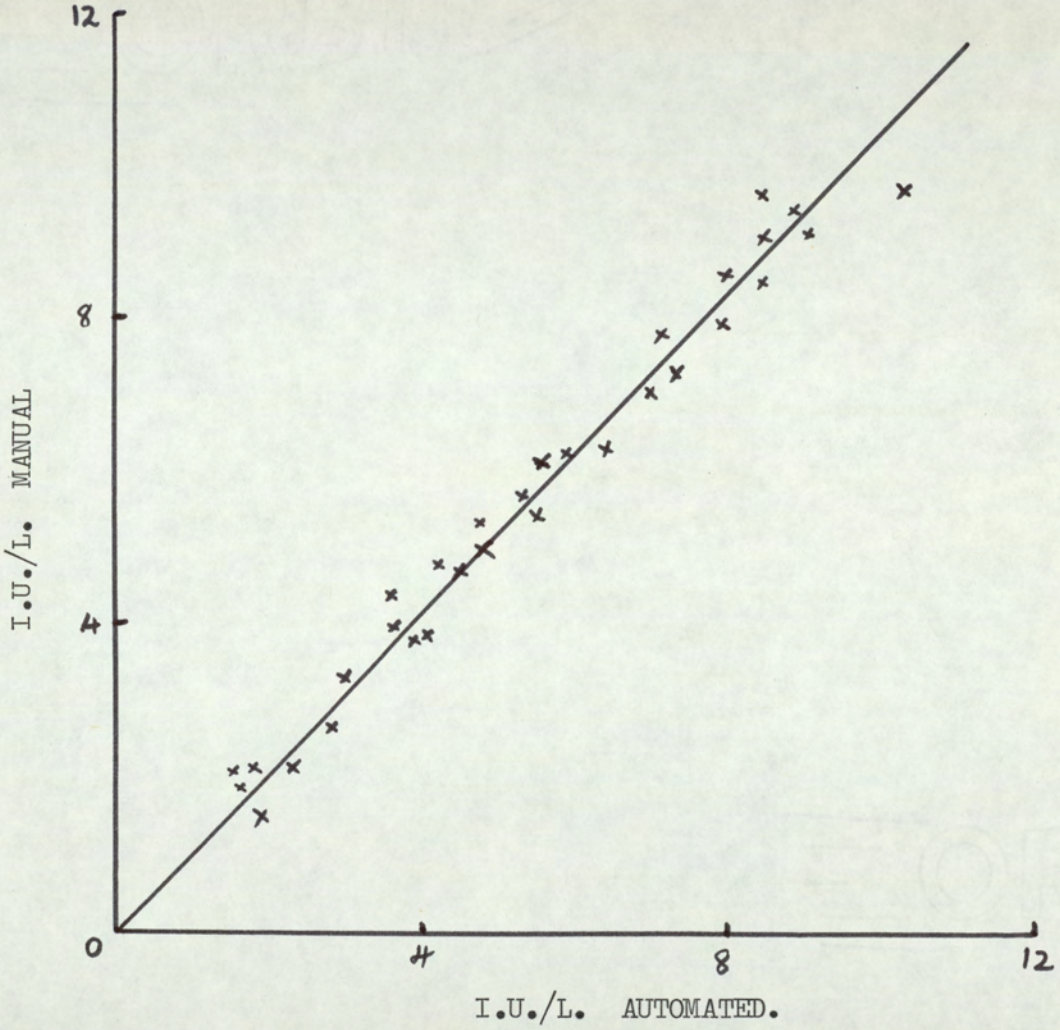


Fig. 28. Comparison of n-acetyl-β-glucosaminidase as determined by the manual and automated methods.

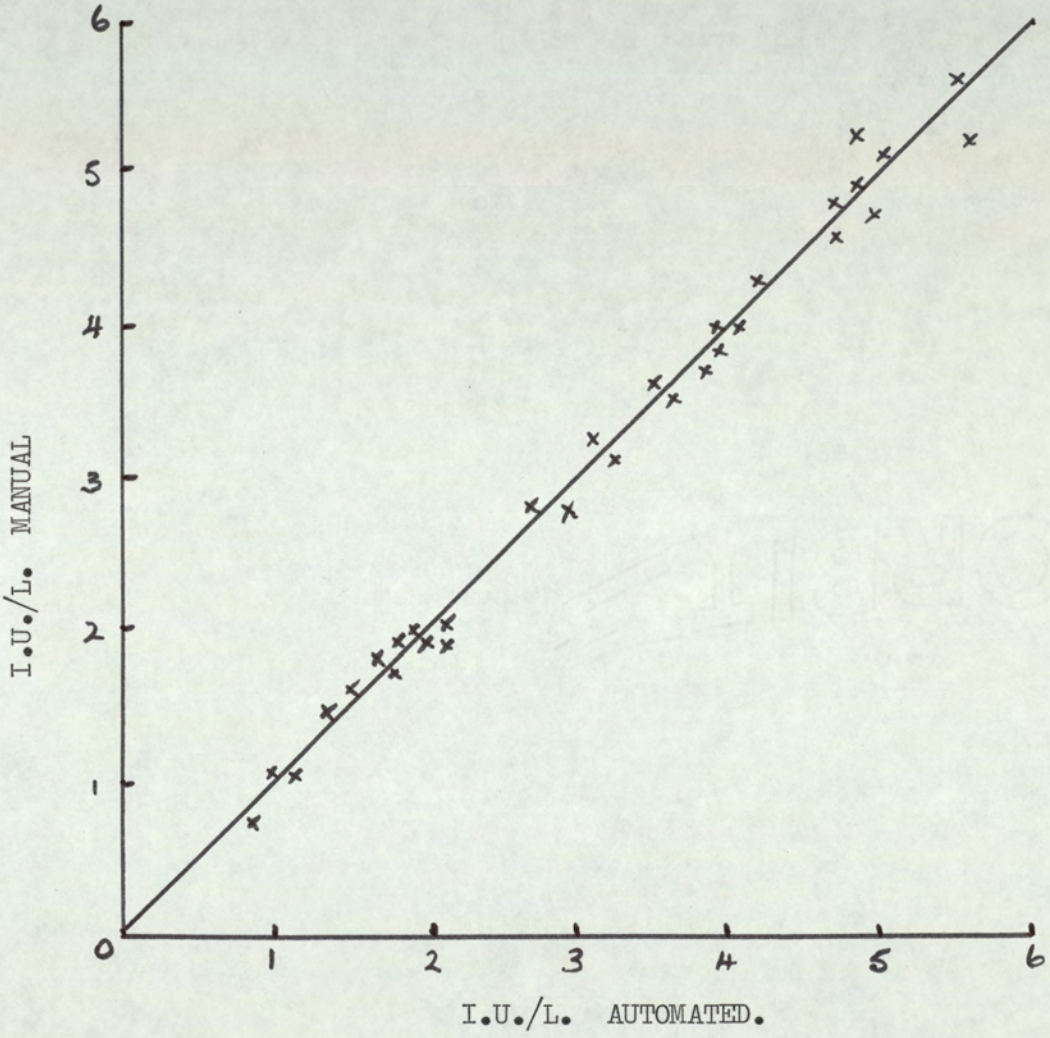


Fig. 29. Comparison of manual and automated results for the estimation of n-acetyl-β galactosaminidase.

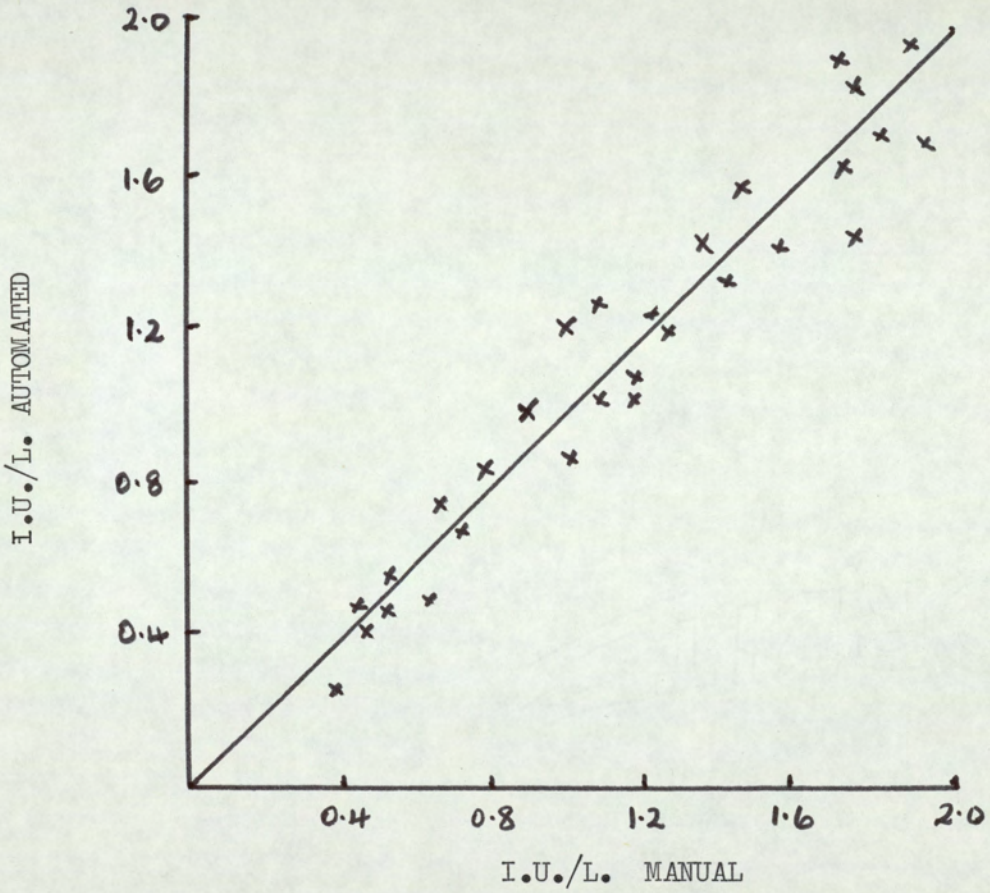


Fig. 30. Comparison between manual and automated methods for the serum estimation of β-glucuronidase.

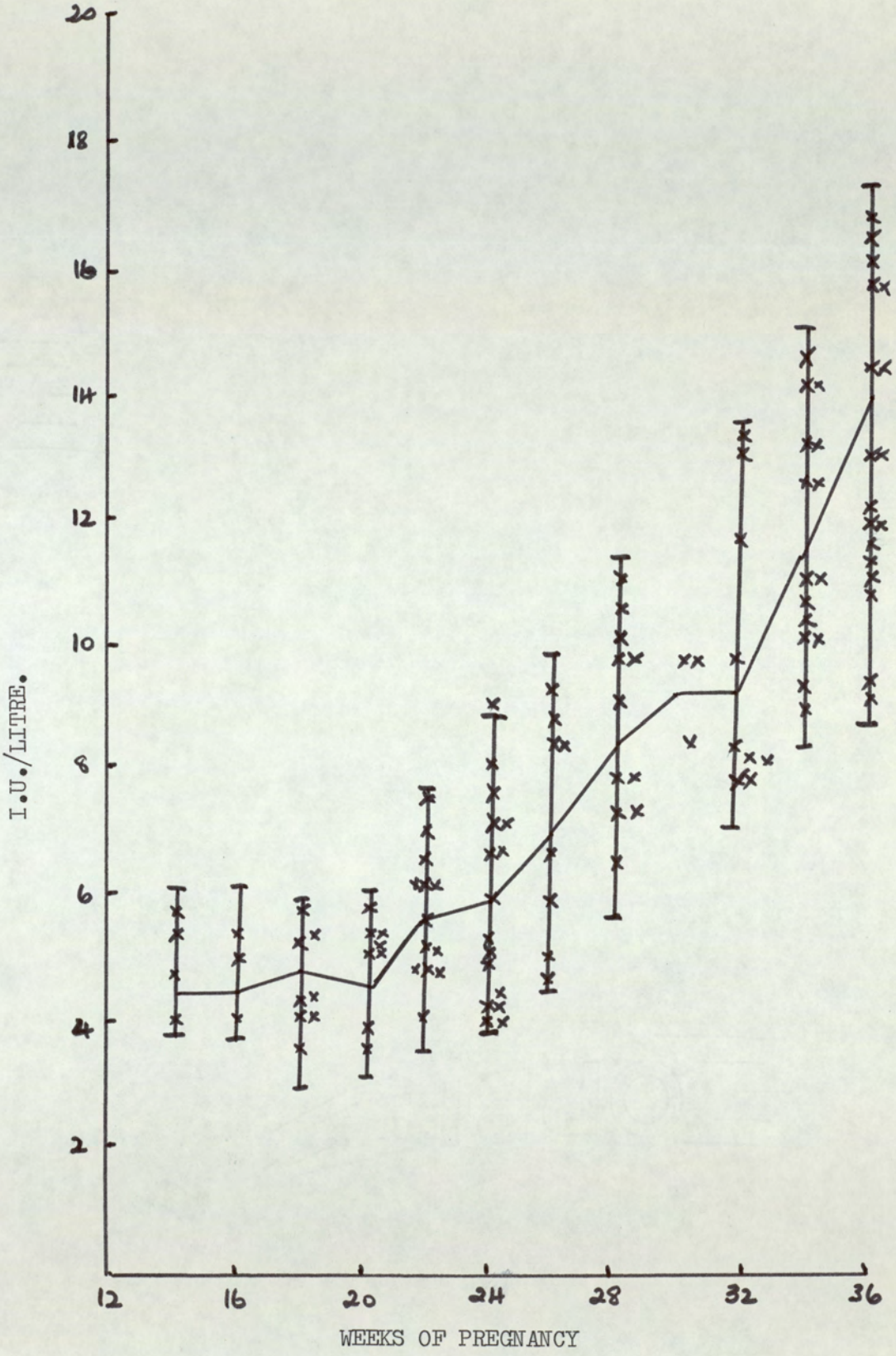


Fig. 31. Scattergraph of results for serum n-acetyl-β-glucosaminidase using the automated technique.

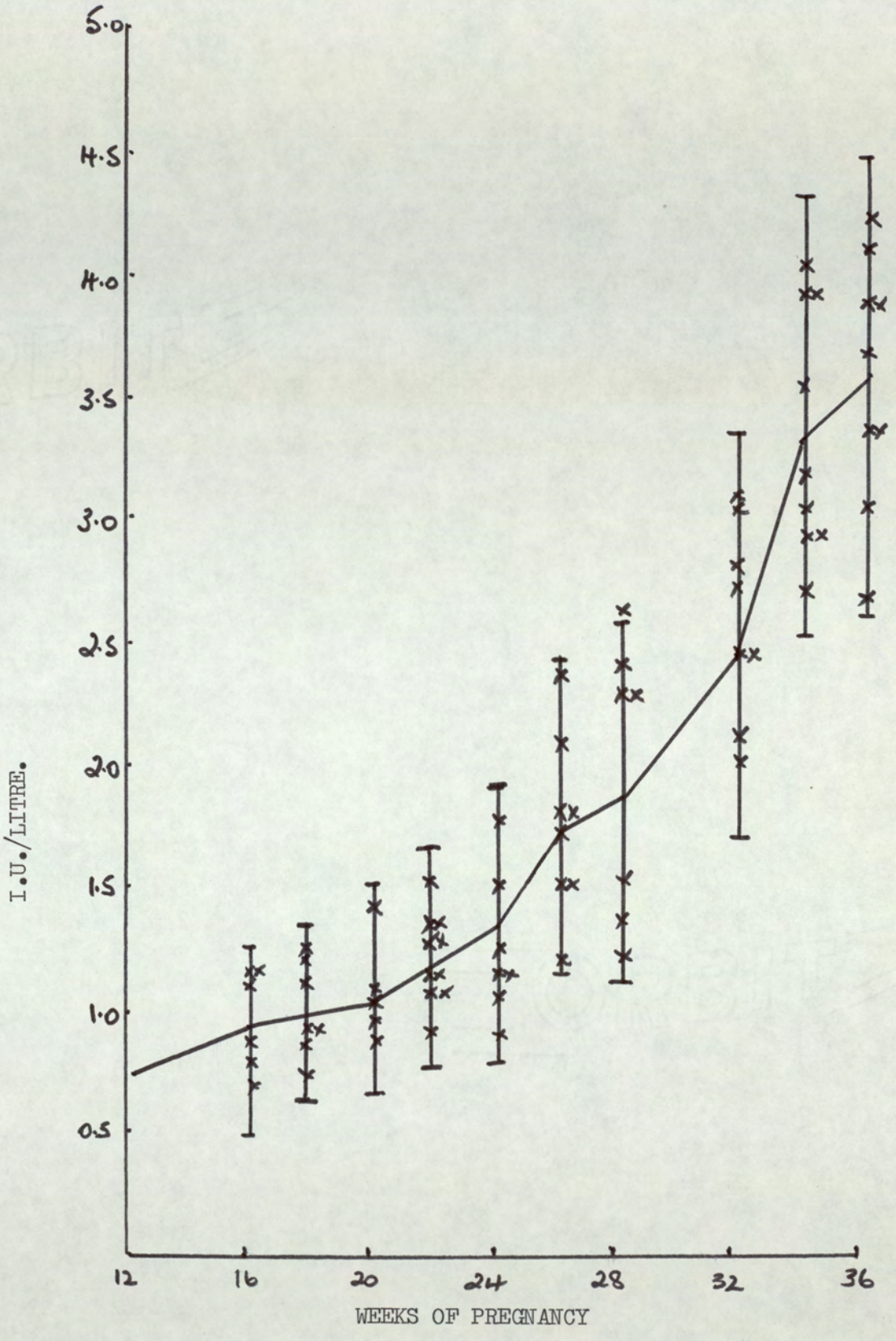


Fig. 32. Results of serum n-acetyl-β-galactosaminidase during pregnancy using the automated technique.

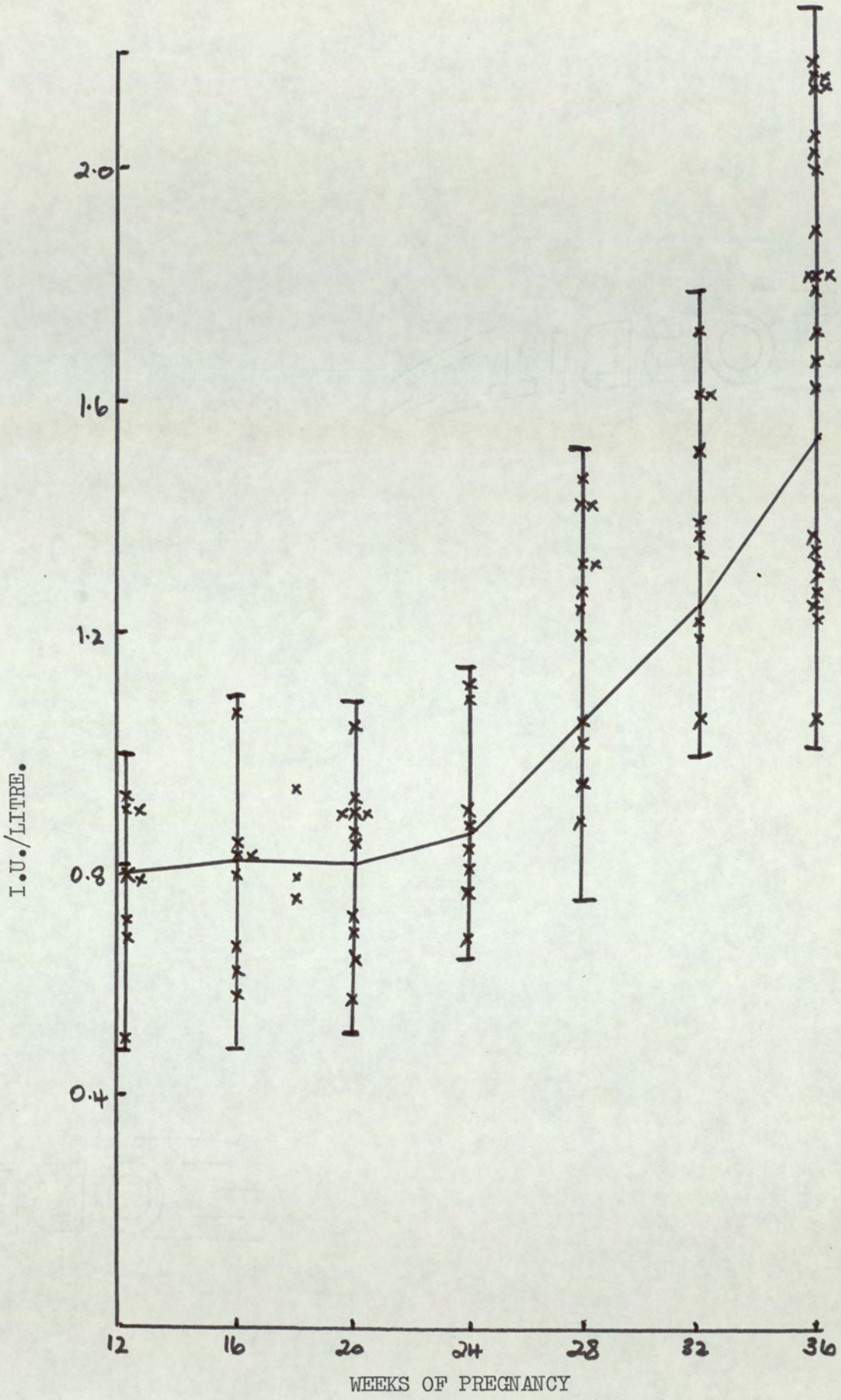


Fig. 33. Scattergraph of results of pregnancy serum β-glucuronidase using the automated method.

Table 8. Mean, standard deviation and regression figures for leucine aminopeptidase using the automated technique.

Week of pregnancy	$\bar{x}$	s	y
14	160	22	140
16	170	22	144
18	178	20	150
20	190	24	172
24	230	25	210
28	250	31	280
32	315	46	312
34	364	60	340
36	386	65	364
38	408	66	380

Table 9. Standard deviations, mean values and predicted y values for n-acetyl-glucosaminidase using the automated technique.

Week of pregnancy	$\bar{x}$	s	y
12	2.5	1.0	2.6
14	2.6	1.1	2.8
16	2.8	1.4	2.8
18	3.0	1.5	2.8
20	3.0	1.4	3.0
22	3.1	1.5	3.0
24	3.2	1.5	2.8
26	4.0	2.1	4.0
28	4.5	2.3	4.2
30	5.0	2.4	4.8
32	6.0	2.9	6.2
34	7.2	3.6	7.6
36	7.8	3.8	7.8

Table 10. Means standard deviations and regression values for the automated method of n-acetyl-β-galactosaminidase estimation.

Week of pregnancy	$\bar{x}$	s	y
12	0.8	0.21	0.5
16	1.1	0.25	1.0
18	1.2	0.2	1.3
20	1.25	0.3	1.6
24	1.5	0.4	2.2
28	2.2	0.52	2.8
32	2.8	0.8	3.3
34	3.5	0.9	3.7
36	4.0	1.1	4.0

Table 11. Means, standard deviations and regression values for β-glucuronidase using the automated method.

Week of pregnancy	$\bar{x}$	s	y
14	0.60	0.12	0.50
18	0.65	0.13	0.70
22	0.72	0.14	0.82
24	0.80	0.17	0.88
28	0.92	0.21	1.00
32	1.12	0.24	1.14
36	1.24	0.25	1.25
38	1.4	0.26	1.30

DISCUSSION

A progressive increase during pregnancy in a number of serum enzymes has been reported by Hunter et al. (1970) for alkaline phosphatase, Page et al. (1961) for cystine aminopeptidase, Walker et al. (1960) for n-acetyl-β-glucosaminidase, McDonald and Odell (1947) for β-glucuronidase and Lewis (1962) for leucine aminopeptidase. The present investigation confirms the work of these authors. The increase in total alkaline phosphatase would be due to the presence of the placental isoenzyme which has been found to be heat stable (McMaster et al., 1964).

The progressive increase in serum γ-glutamyl transpeptidase, carboxypeptidase, alanine aminopeptidase and 5'-nucleotidase have not been previously reported. The large scatter in the results for carboxypeptidase was due in part to the poor reproducability of the method. This would be expected since an optical density change of less than 0.1 was recorded for each serum assay.

With the exception of γ-glutamyl transpeptidase, the rate of disappearance of the serum enzymes following delivery parallels the rate of disappearance of other proteins peculiar to pregnancy such as chorionic gonadotrophin. The progressive increase in the serum enzymes also follows the oestrogen concentration in blood during pregnancy. Both observations would implicate the placenta as the source of origin for the enzymes with the exception of γ-glutamyl transpeptidase. The decrease found in the serum activity of γ-glutamyl transpeptidase after delivery took twice as long to return to normal than that found for the other enzyme examined.

Although Ichaliotis and Lambrinopoulos (1964) and Blunt (1971) found no correlation between foetal or placental weights with enzyme levels, it was interesting to note that the increased scatter of results as pregnancy advanced paralleled the increased scatter found by Boyd and Hamilton (1970) for placental and foetal weights.

The isoenzymes.

The wide scatter of results for each week of pregnancy would

unlikely be of any clinical value without an investigation of the isoenzyme contributing to the total enzyme activity.

To find the source of the increased serum enzymes during pregnancy would require a detailed study of their isoenzymes. Furthermore such an investigation could lead to the isolation of a tissue specific isoenzyme. It would also be likely that more than one isoenzyme from a number of organs would contribute to the total serum enzyme, which would in turn tend to increase the scatter of enzyme results during each week of pregnancy.

4.2. 1 Iso-enzymes of alkaline phosphatase.

Iso-enzyme migration in relation to the migration of protein fractions.

Fig. 34 illustrates the migration of the iso-enzymes fractions found in bone, liver, intestine, kidney and placenta and Fig. 35 illustrates the results obtained with the sera. The bone iso-enzyme was detected between the α_1 and α_2 globulins; the kidney and liver fractions between the α_2 and B globulins and the placental enzyme corresponded with the β globulin. The intestinal enzyme was located between the β and γ globulins.

Tissue isoenzymes.

Table 12 lists the per cent migration of each iso-enzyme found in tissue when the migration of albumin was taken as 100 per cent. The fastest moving iso-enzyme was found to be bone (84 per cent) followed by kidney (76 per cent) liver (73 per cent) placenta (55 per cent) and intestine (41 per cent). Neuraminidase retarded completely the mobility of the liver, bone and kidney fractions but the intestinal fraction was unaffected. The mobility of the placental enzyme was partially retarded and had decreased from 60 per cent to 35 per cent that of albumin.

The addition of L-phenyl alanine did not affect the mobility of the liver, bone, kidney or placental enzymes. The intestinal enzyme was reduced in mobility from 41 per cent to 37 per cent.

Triton-x-100 did not change the mobility of the liver, bone or kidney enzymes but the placental fraction was reduced from 55 per cent

Table 12. Percent migration of alkaline phosphatase isoenzymes after electrophoresis on cellogel. (Albumin = 100 per cent migration).

Sample	Treatment of Sample Prior to Electrophoresis					
	Untreated	Neuraminidase	L-Phenyl Alanine	Triton-x-100	Heating at 56°C	Heating at 65°C
Bone (tissue extract)	84	-	84	84	-	-
Kidney (tissue extract)	76	-	76	76	-	-
Liver (tissue extract)	73	-	73	73	-	-
Placenta (tissue extract)	55	35	55	45	55	45
Intestine (tissue extract)	41	22	37	38	-	-
Paget's Disease (serum)	33, 84	33	33, 84	33, 84	-	-
Obstructive Jaundice (serum)	44, 73	44	44, 73	44, 73	-	-
Pregnancy 36th week (serum)	55, 60	10, 55, 60	10, 55, 60	-	55	55

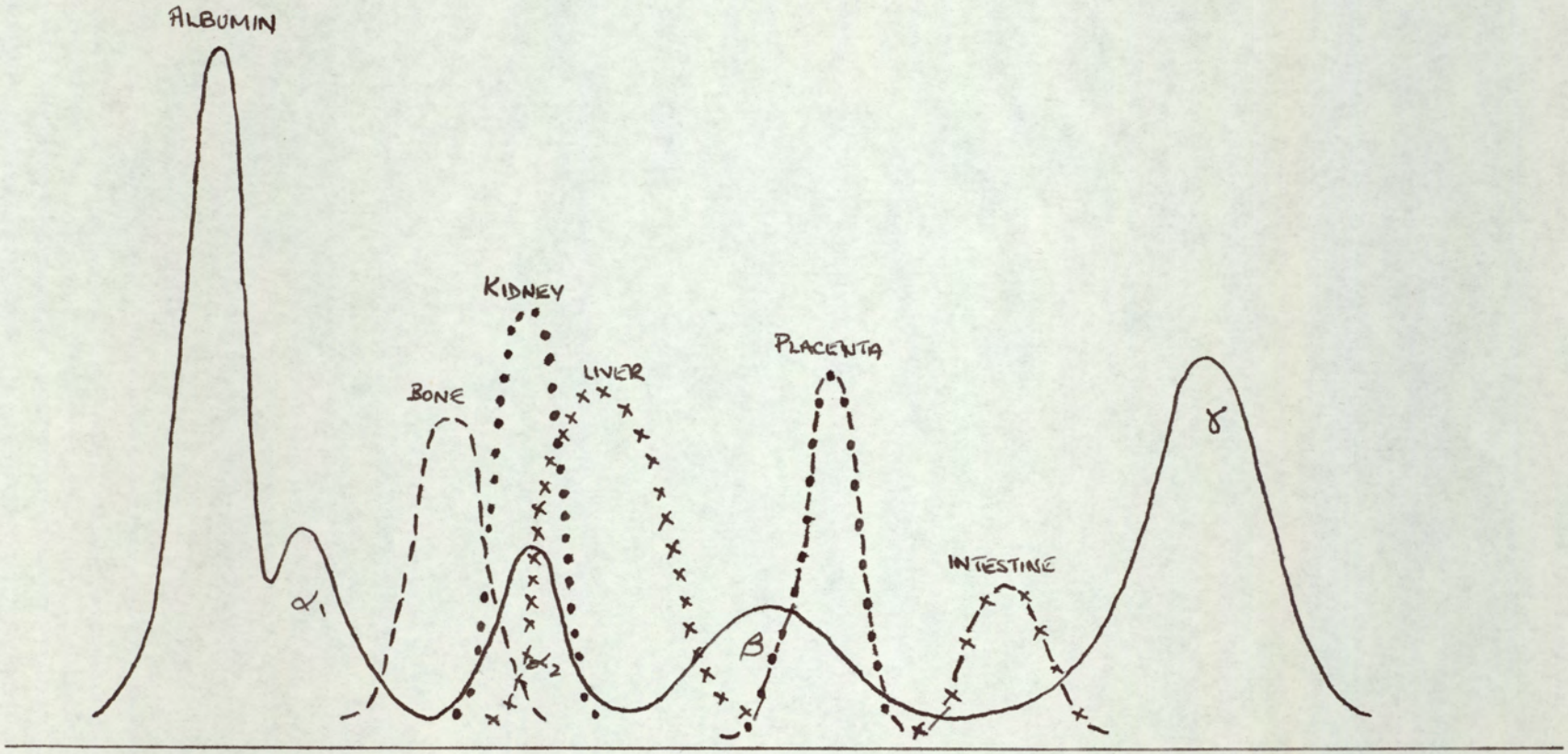


Fig. 34. The migration of tissue isoenzymes in relation to the migration of protein fractions. Supporting medium cellogel.

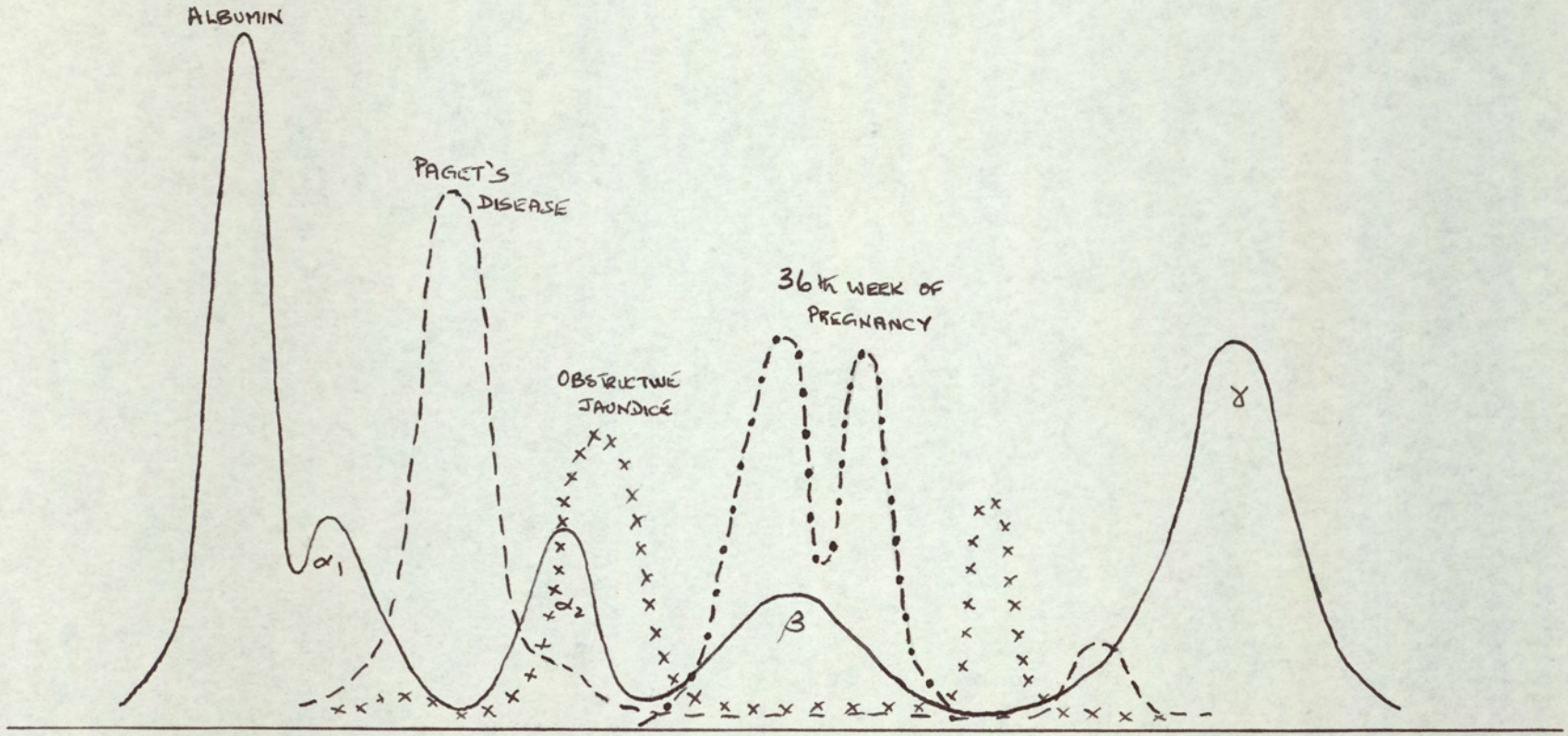


Fig. 35. Isoenzymes detected in serum from Paget's disease obstructive jaundice and maternal serum in relation to protein fractions.

to 45 per cent and the intestinal enzyme was reduced from 41 per cent to 38 per cent.

No visible iso-enzyme bands were detected after heating to 56°C and 65°C samples of liver, bone, intestine and kidney extracts. The placental extract was unaffected after heating to 56°C. Heating the placental extract to 65°C reduced the mobility from 55 per cent to 45 per cent furthermore the concentration was reduced to 62 per cent of the original fraction.

Serum isoenzymes.

The results of the iso-enzyme fractions found in Paget's disease, obstructive jaundice and during pregnancy are illustrated in Fig. 36. Two distinct bands were found in all three samples. In Paget's disease the migration was 84 per cent and 33 per cent; obstructive jaundice 73 per cent and 44 per cent and during the 36th week of pregnancy 60 per cent and 55 per cent that of albumin.

The neuraminidase treated samples from both the obstructive jaundice and Paget's disease resulted in the fastest hand in each case being completely retarded. The fastest moving band in the pregnancy serum was only partially retarded the mobility was reduced from 55 per cent to 10 per cent and the concentration reduced to 12 per cent of the original concentration.

L-Phenyl alanine did not affect the mobility or concentration of the fractions obtained from the Paget's disease or the obstructive jaundice sera. The mobility of the fastest moving iso-enzyme during pregnancy had not changed but was reduced in concentration and a third fraction appeared with a mobility of 10 per cent that of albumin. The concentration of this third fraction corresponded with the decrease in concentration found for the fastest moving fraction.

The sera treated with Triton-x-100 resulted in the fractions being reduced in concentration by approximately 12 per cent. The pregnancy iso-enzymes did not migrate.

No iso-enzymes were detected in the Paget's and obstructive jaundice sera after heating to 56°C or 65°C. The fastest moving iso-enzyme found in pregnancy serum did not migrate after heating to 56°C; the slowest iso-

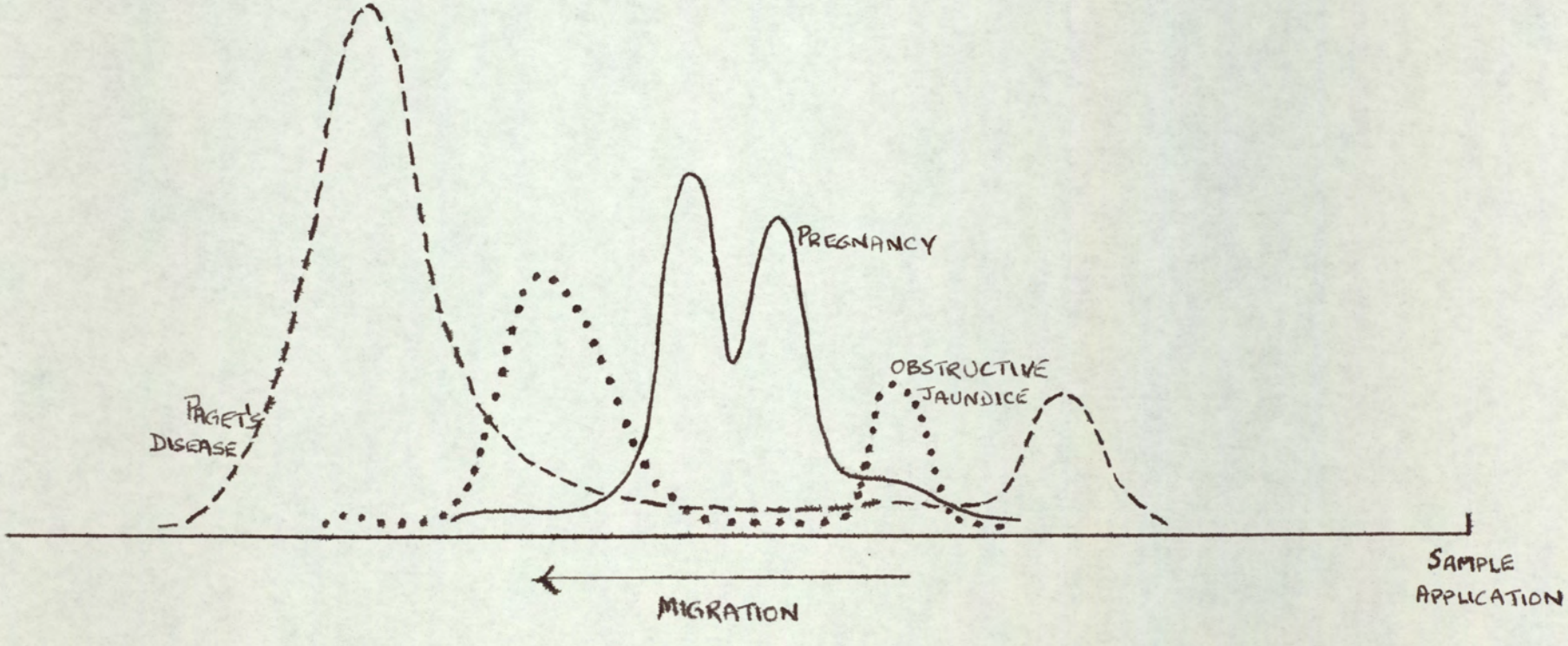


Fig. 36. Isoenzyme scans of sera from Paget's disease, obstructive jaundice and during 36th weeks of pregnancy.

enzyme was unchanged in mobility or concentration. Heating to 65°C not only eliminated the fastest moving enzyme but also reduced the concentration of the placental iso-enzyme by 43 per cent. Furthermore a third fraction was obtained with a mobility of 45 per cent that of albumin. This fraction had a phosphatase concentration equal to the decrease in the placental iso-enzyme.

Heat stable alkaline phosphatase (65°C for 30 mins).

Fig. 37 illustrates the heat stable alkaline phosphatase of pregnancy serum between the 14th week of pregnancy and term. The progressive increase in the isoenzyme was linear. Table 13 lists the mean, standard deviation and the predicted regression line. The scatter of results during each week of pregnancy was less than that found while estimating the total alkaline phosphatase activity. During the 36th week of pregnancy the range of activity was 8.0 to 21.0 K.A. units/100 ml and the range for heat stable alkaline phosphatase was 2.0 to 9.0 K.A. units/100 ml.

DISCUSSION

The difference in mobility between the alkaline phosphatase iso-enzymes found in bone, liver, intestine and kidney with that found in placenta and maternal serum was sufficiently large to provide a basis for the separation and estimation of the iso-enzymes found in pregnancy.

Neuraminidase reduced the mobility of the faster moving iso-enzymes while the slowest (intestinal) was unaffected. This would suggest that the bone, liver and kidney fractions possess a terminal sialic acid group, the removal of which would reduce the negative charge on the enzyme molecule. This result would confirm the work of Robinson and Pierce (1964), Posen et al. (1967) and Moss et al. (1967). Since the placental enzyme was partially retarded it would suggest that this iso-enzyme did not contain as much sialic acid as that found for the faster moving fractions of bone, liver, and kidney.

The fastest moving bands found in Paget's disease and in obstructive jaundice sera were completely inhibited which suggest that these bands were of bone and liver origin respectively. The slower moving

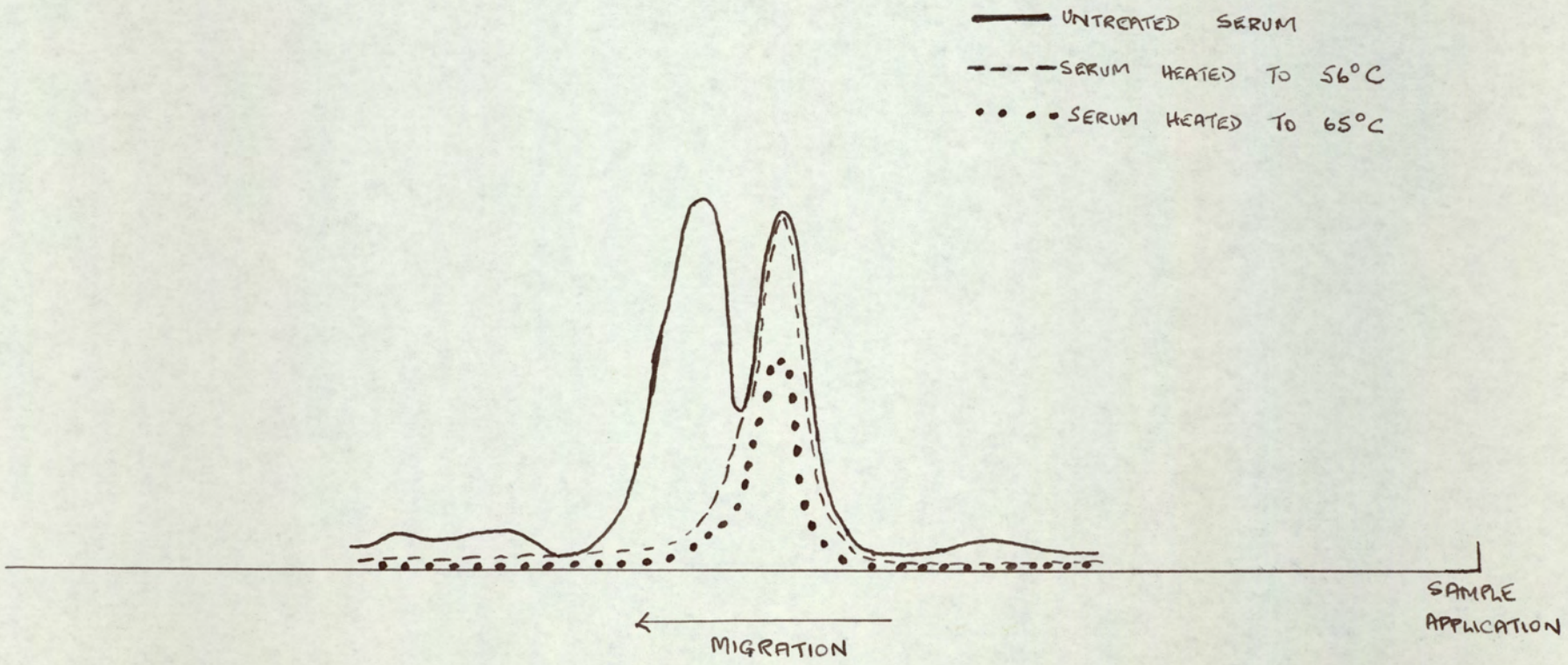


Fig. 37. Serum isoenzymes during the 36th week of pregnancy and after heating at 56°C and 65°C.

Table 13. The mean ($\bar{x}$), standard deviation (s) and predicted y for heat stable alkaline phosphatase.

Week of pregnancy	$\bar{x}$	s	y
14	0.72	0.81	0.61
18	1.15	0.75	1.43
20	2.28	1.16	1.84
22	2.04	1.05	2.25
24	2.50	1.88	2.66
26	3.11	1.01	3.07
28	3.55	1.41	3.48
30	3.49	0.94	3.89
32	3.96	1.2	4.3
34	2.78	1.3	4.71
35	4.77	1.5	4.92
36	5.57	1.7	5.12

bands were not removed after neuraminidase treatment and their mobilities did not coincide with that found for the intestinal fraction. Fishman et al. (1968) obtained similar results but suggested that the slower moving bands were of intestinal origin.

An inhibitory effect by L-phenyl alanine on the intestinal enzyme has been found by many workers (Moss, 1965: Newton, 1966: Warnock, 1966) and similar results were found during the present study. The fastest moving band found during pregnancy was decreased in activity while the slower moving band was unaffected. This stereospecific inhibition by L-phenyl alanine was found by Fishman (1968) to be pH dependant and to be of the uncompetitive type.

Coutinho et al. (1966) suggested the use of triton-x-100 (a non-ionic detergent) to increase the electrophoretic mobility of enzymes and this effect was investigated in the present work. A reduction in mobility rather than an increased mobility was found for all the iso-enzymes. Both iso-enzymes in pregnancy sera were completely removed, the triton-x-100 having acted as an inhibitor.

Placental alkaline phosphatase has been found to be heat stable and this property has had some clinical applications. A heating temperature of 56°C was suggested by Neale et al. (1965) and a higher temperature of 65°C was suggested by Hunter (1969) to obtain an index of the placental iso-enzyme in maternal serum. The clinical implications of such an estimation have been investigated by both workers. The present work brings into question whether heating at either temperature gives a true indication of the iso-enzyme in pregnancy serum. Heating at 56°C removed the fastest moving fraction and heating at 65°C reduced the concentration of the slower moving fraction. A trace of the results obtained are seen in Fig. 37. Both iso-enzymes in maternal serum are specific to pregnancy, their mobilities did not correspond to that found for other tissues and sera. These results could in part explain the wide normal scatter obtained by Hunter (1969) and Neale et al. (1965) while estimating the placental enzyme in pregnancy serum during

the last trimester of pregnancy.

4.2. 2 Isoenzymes of leucine aminopeptidase. The trivial name leucine aminopeptidase is used for the enzyme arylamidase (aminoacylnaphthylamide E.C. 3.4.1.1).

Tissue isoenzymes.

Ten extracts of pancreas, placenta, kidney, bone, liver and intestine were found to contain one or more zones after polyacryla ide electrophoresis. Fig. 38 illustrates the results obtained. The protein fractionation of a normal serum is seen in Fig. 39. Placenta, kidney and bone extracts contained a fast moving band (zone 1) which migrated in the $F\alpha$ globulin region of serum. In placenta a second slower moving fraction (zone 2) was detected and found to correspond to the β_2 globulin of serum. Pancreas, kidney and intestinal extracts were found to contain one zone each which migrated with the fastest moving haptoglobin (zone 3). Kidney extract contained a zone 3 enzyme together with a slower moving zone with migration similar to that found for the slower moving haptoglobin fraction (zone 4). Liver and kidney extracts contained a zone which migrated to zone 4. Placenta and kidney extracts contained an enzyme fraction which did not migrate and was located near the $S\beta$ globulin in serum (zone 5).

Leucine aminopeptidase fractions in serum.

One fraction (zone 1) was detected in sera obtained from ten healthy males and six females. Forty pregnancy sera were investigated for leucine aminopeptidase fractions. During the 20th week of pregnancy, a zone 2 appeared with a migration rate similar to that found for the slower moving fraction (zone 2) from placental extract. A zone 3 enzyme was detected after the 30th week of pregnancy with electrophoretic migration similar to that found for pancreas and intestinal extracts. Towards the end of pregnancy between the 34th week and term, a zone 5 appeared with migration pattern similar to that found in kidney and placental extracts.

The concentration of zones 1, 2, 3 and 4 in maternal serum

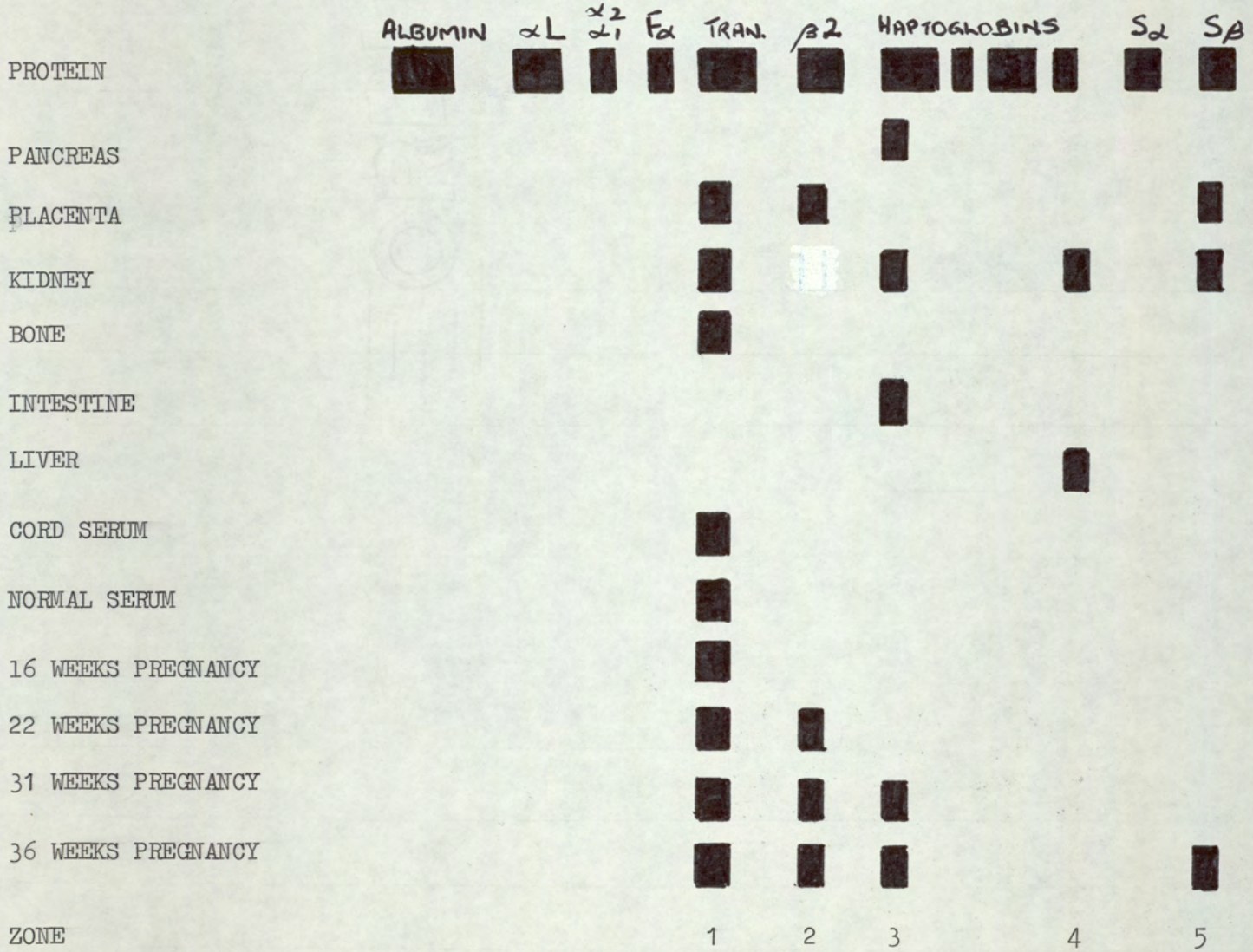


Fig. 38. The migration of leucine aminopeptidase zones in relation to the migration of protein fractions after polyacrylamide electrophoresis.

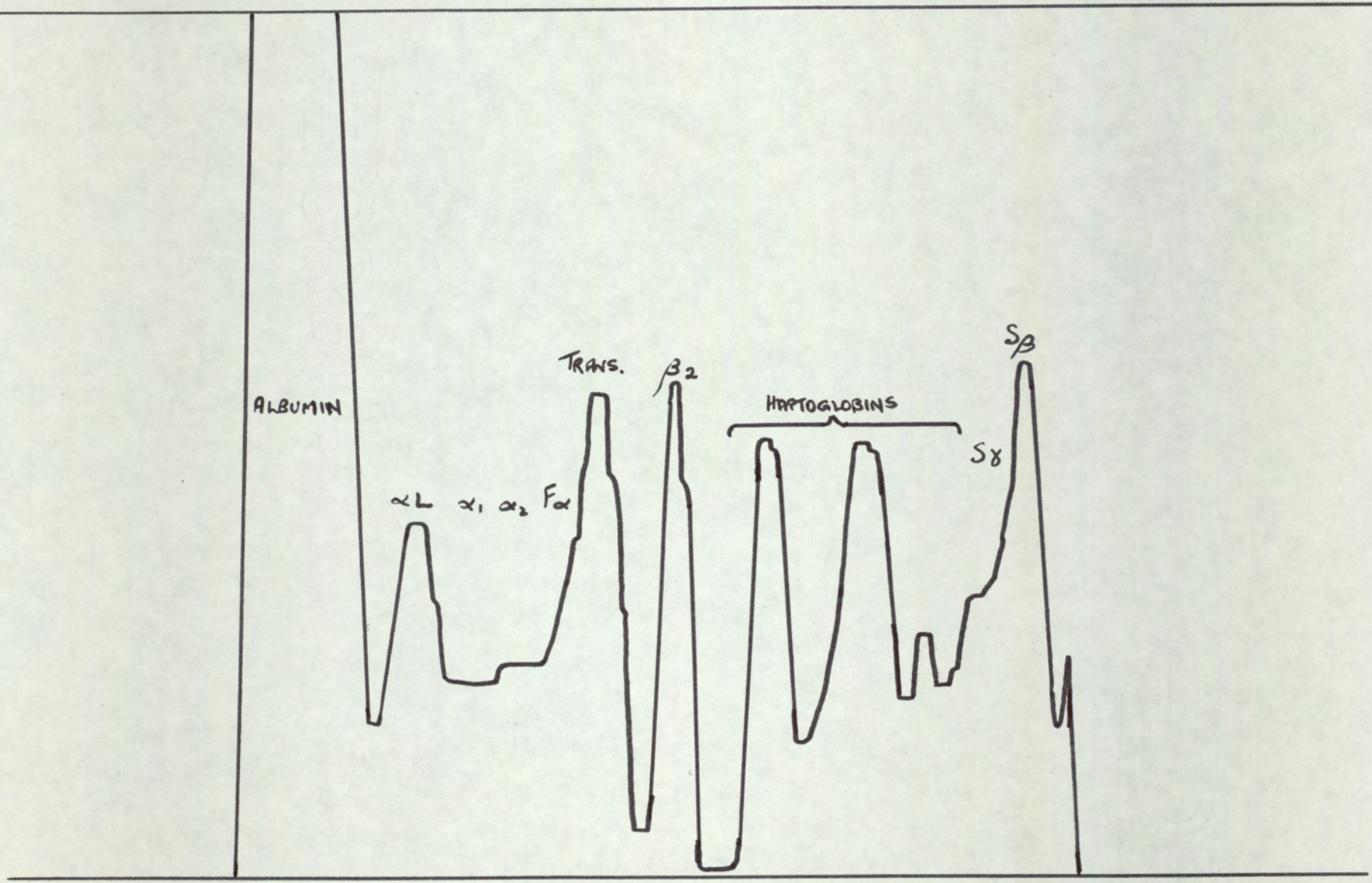


Fig. 39. Scan of serum protein fractionation by disc polyacrylamide electrophoresis.

progressively increased during gestation. Fig. 40 illustrates a typical increase in the enzyme fractions found in 3 pregnancy sera between the 16th week and term.

Cord serum was found to contain a zone 1 enzyme similar to that found in normal and maternal serum.

Effects of neuraminidase.

The enzyme fractions observed in tissue extracts and sera were all attacked by neuraminidase. Faint zones of activity with a decreased mobility were found in pancreatic and intestinal extracts and between the 36th week of pregnancy and term. The kidney and bone extracts together with the zones found in normal and cord sera were completely eliminated. Incubations in the absence of neuraminidase did not result in the loss of enzyme zones. The neuraminidase used did not hydrolyse the substrate L-leucyl-β-naphthylamide when incubated at 37° under the conditions used for the test sera.

Effect of L-Methionine.

The enzyme zones detected in the extracts of pancreas, intestine and placenta were unaltered by the addition of L-methionine to the substrate. The kidney, liver and bone enzymes were completely inhibited. Zone 2 enzyme of maternal serum was unaffected by L-methionine but zone 1 was reduced in concentration. Zone 1 of cord serum and normal adult serum were also inhibited.

Discussion

The data presented here suggests that the serum enzyme hydrolysing L-leucyl-B-naphthylamide rises appreciably and progressively during pregnancy. It is therefore likely that this increase in part reflects placental function. During the last trimester of pregnancy, maternal serum contains four zones of leucine aminopeptidase (zones 1, 2, 3 and 5). Between the 16th week of pregnancy and term a significant increase was found to occur in the concentration of all the isoenzymes in maternal serum. The increase in the non-placental fractions in pregnancy serum could in part explain the wide normal scatter found by us while estimating the total leucine aminopeptidase

concentrations during pregnancy.

Zones 1 and 2 correspond to that found for placenta, zone 3 to intestine and zone 5 to placenta and kidney. Zone 2 was found to be entirely of placental origin and zones 1 and 5 to contain in part placental fractions.

The source of origin of the individual enzyme fractions in maternal serum were investigated by comparing the electrophoretic mobility of the fractions before and after neuraminidase treatment with that found in placenta, pancreas, kidney, bone, intestine and liver extracts. In addition the degree of inhibition by methionine of each fraction further clarified the source of origin. L-methionine may inhibit by a feedback mechanism.

Beckman (1966) reported two fractions in pregnancy serum with four isoenzymes in placental extract. In the present study three zones only were detected in placental extract and serum at 22 weeks of pregnancy. A zone 3 and a zone 5 appeared in maternal serum between the 30th week and term. (Fig. 40).

After neuraminidase treatment all the enzyme fractions both in maternal serum and tissue extracts were reduced in electrophoretic mobility and, therefore, all possess a terminal neuraminic acid group. Using starch gel electrophoresis it has been reported Beckman (1966) that the fastest moving zone of placenta, liver, intestine and kidney to be unaffected by neuraminidase treatment.

L-methionine competitively inhibits hydrolysis of L-leucyl-B-naphthylamide. The kidney and bone enzymes were inhibited but no such inhibition was found with the placental extracts or in pregnancy sera. The inhibition by L-methionine can therefore be used to advantage in estimating zones of placental origin in maternal serum. Furthermore the enzyme found in normal serum was also inhibited.

Zone 2 detected in both placenta and pregnancy serum was found to be activated by L-methionine which was further evidence that this zone was of placental origin. Activation of muscle leucine aminopeptidase has been reported Fleisher, et al. (1964) but no such

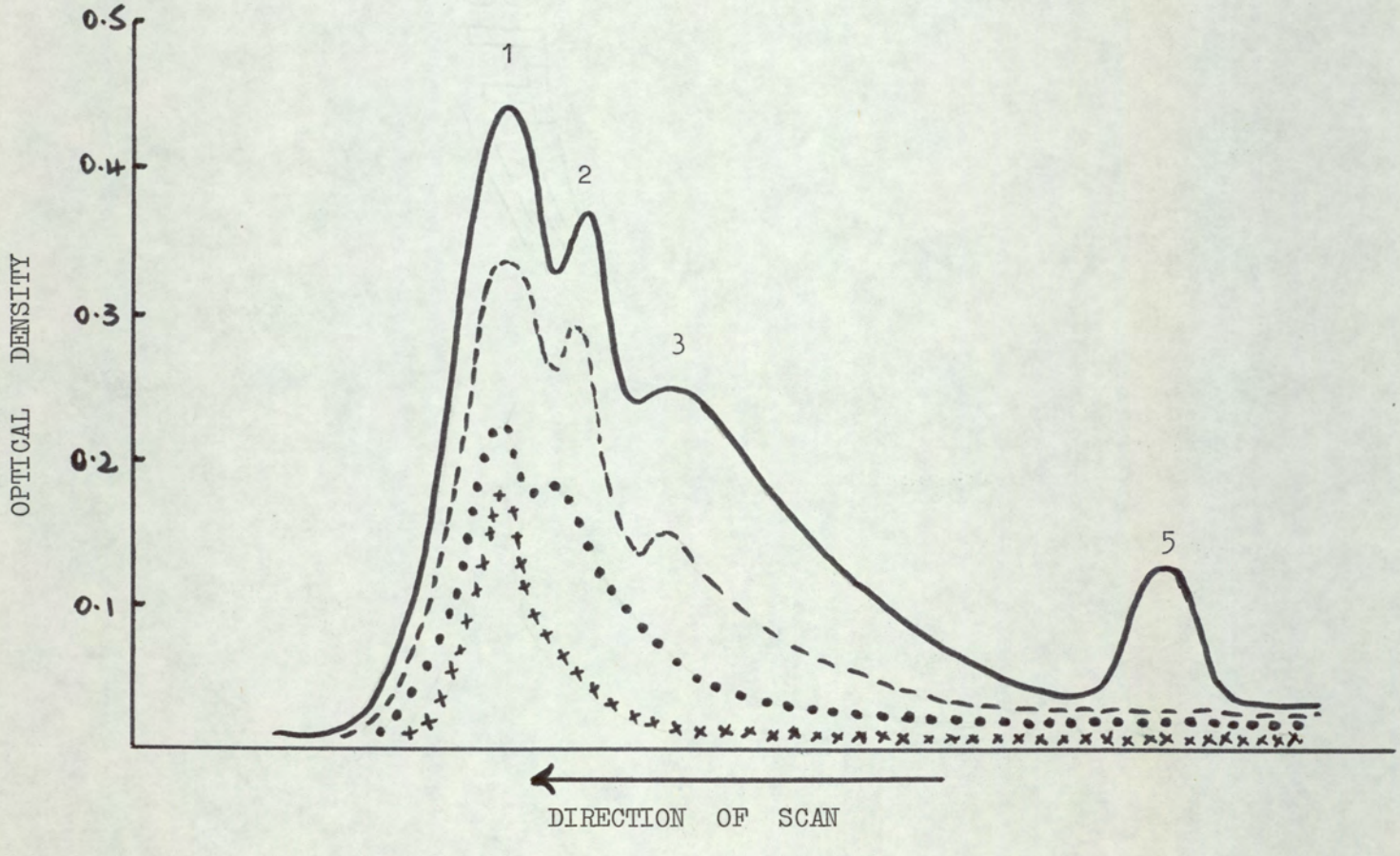


Fig. 40. Zones of leucine aminopeptidase activity in pregnancy serum after polyacrylamide electrophoresis. Scans are shown of the progressive increase in zones 1, 2, 3 and 5 during gestation. 36 weeks_____; 31 weeks _ _ _ _ ; 22 weeks.....; 16 weeksxxxxxx.

activation has been reported for the placental zones. These results also indicate the heterogeneity of the leucine aminopeptidase zones, although these data are not sufficient to class the enzymes as iso-enzymes, it does serve to establish the existence of biochemical differences suggestive of isoenzymes.

Placental leucine aminopeptidase isoenzymes of pregnancy serum.

The leucine aminopeptidase activity of pregnancy sera obtained between the 14th week of pregnancy and term were estimated in the presence of L-methionine so that the normal range for each week of pregnancy could be obtained. Fig. 41 illustrates the scattergraph obtained. At 38 weeks of pregnancy the values for leucine amino-peptidase estimated in the presence of L-methionine was twice the activity of that found for the enzyme without L-methionine. At 14 weeks of pregnancy the increased activity was 1.5. Such a result would be expected because as pregnancy advances the total activity of the placental isoenzyme in the serum would also increase in quantity, which in turn would increase the L-methionine fraction.

The scatter of results was similar to that found for total leucine aminopeptidase estimated in the absence of L-methionine. Table 14 lists the mean, standard deviation and the regression results obtained.

4.2. 3 Iso-enzymes of γ-Glutamyl transpeptidase.

Mobility of tissue isoenzymes. After polyacrylamide electrophoresis the extracts of pancreas, placenta, kidney, bone, liver and intestine contained two or more isoenzymes of different electrophoretic mobilities. (Fig. 42.) Bone and liver contained a zone 2 and zone 5 isoenzyme. A zone 4 and a zone 5 of enzyme activity was detected in the intes-tinal extract. Placenta contained three isoenzymes, zones 1, 3 and 5 and kidney contained four isoenzymes zones 1, 3, 4 and 5. Pancreatic extract was found to contain zones 1, 3 and 5 and further-more an enzyme fraction was detected in zone 1(a) which was not detected in the other tissues examined. The zone 1(a) of pancreas

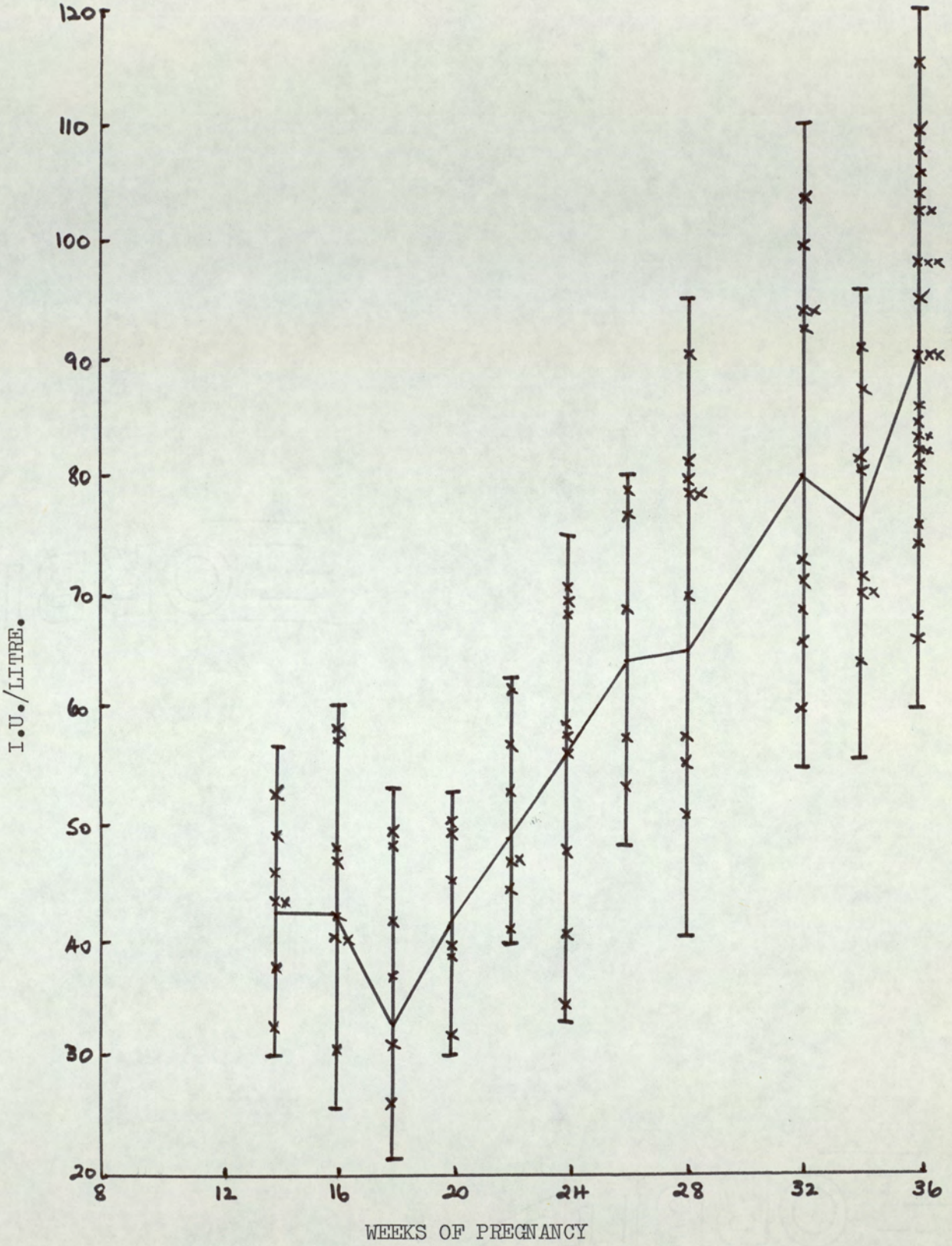


Fig. 41. Scattergraph for the serum activities of alanine aminopeptidase in the presence of L-methionine.

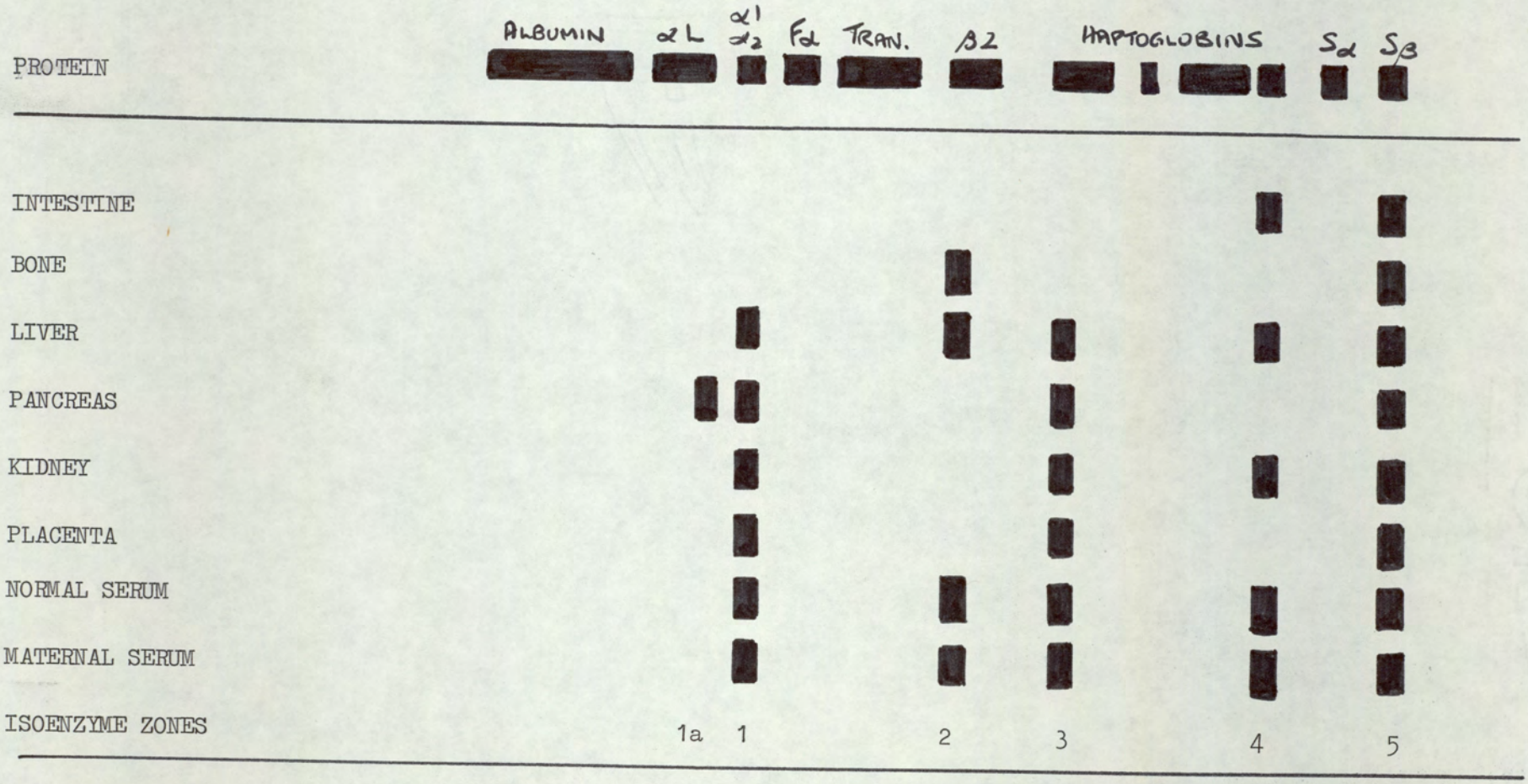


Fig. 42. The migration of γ-glutamyl transpeptidase isoenzymes in relation to migration of protein fractions by polyacrylamide gel electrophoresis.

Table 14. Mean, standard deviation and predicted y values for leucine aminopeptidase estimated in the presence of L-methionine.

Week of pregnancy	$\bar{x}$	s	y
12	33.2	15.9	4.3
16	37.8	10.5	25.1
18	38.4	11.3	35.5
20	44.6	13.4	45.9
22	50.0	8.3	56.3
24	49.5	14.9	66.7
26	70.7	31.6	77.0
28	71.0	17.6	87.5
32	79.8	17.3	108.3
34	111.6	23.9	118.6
35	121.0	20.0	123.9
36	136.1	28.5	129.0
38	160.1	37.3	139.4

Table 15. Per cent enzyme activity of each isoenzyme. The increase in activity with triton-x-100 is seen in brackets.

Isoenzyme zone	a	1	2	3	4	5
Kidney	-	4.9	-	5.2	15.9	74(x 3)
Bone	-	-	20.8	-	-	79.2
Pancreas	51.1(x 1.1)	21.0(x 4.2)	-	24.0(x 3.6)	-	3.9(x 10)
Intestine	-	-	-	-	22.0	78.0
Liver	-	10.8	21.6	3.6	4.0	60.0(x 1.1)
Placenta	-	29.9	-	3.7	-	66.4(x 1.5)
Normal Serum	-	25.4(x 1.7)	6.7	15.2	16.9	35.8
Pregnancy Serum	-	24.9(x 2.2)	9.0	16.1	18.0	32.0

corresponded in electrophoretic migration to that of the α_L protein fraction and zone 1 to that of the α_1 fraction. Zone 5 of kidney, placenta, bone, intestine and liver contained 74%, 66%, 79%, 78% and 60% respectively of the total enzyme activity. Table 14 lists the concentration of the isoenzymes.

Mobility of serum isoenzymes. Zones 1, 2, 3, 4 and 5 were detected in both normal and maternal serum. Zone 2 of the maternal serum and normal serum contained the least activity with 9% and 6.7% activity. The highest activity of both maternal serum and normal serum was found in zone 5 with enzyme activities of 32% and 35.8% respectively (Table 14). The concentration of zone 1 enzyme in maternal and normal sera were similar but an increased isoenzyme concentration was detected in zones 2, 3 and 4 of maternal serum.

Enzyme estimations. Total enzyme activity of ten duplicate analyses was 11.7 - 12.5 I.U. (x 12.1, SD 0.3). The mean values and SD of replicate assays of the isoenzymes in normal serum are seen in Table 15. Direct estimation of zone 5 of each sample was not possible since all the enzyme was not capable of penetrating the gel. Zone 5 isoenzyme was calculated from the difference between the total enzyme activity and the sum of the other isoenzymes present.

Effect of L-methionine. Zone 2 isoenzyme of bone extract, maternal serum and normal were activated by L-methionine. All other zones of enzyme activity were unaffected.

Effect of urea. Urea inhibited the enzyme activity of zone 5 of liver extract and decreased the concentration of zone 5 found in normal serum and maternal serum. The enzyme activities of all the other tissue extracts and serum were unaffected.

Effect of triton - x - 100. The isoenzyme activity of zone 5 of kidney, liver and placenta and zones 1 of normal serum were increased by the addition of triton - x - 100. All four isoenzymes of pancreas were activated. Table 14 lists the increases in the activation of the isoenzymes. The addition of triton - x - 100 did not increase the electrophoretic mobility of the isoenzymes of GTP.

DISCUSSION

The method described makes it possible to detect electrophoretic fractions of GTP. Furthermore, the technique can be used for quantitative determinations of isoenzymes 1, 2, 3 and 4. Zone 5 could not be estimated with accuracy since the enzyme fraction was found to have an insignificant electrophoretic mobility.

Zone 2 of normal serum probably originates from bone since L-methionine activates both zone 2 of bone extract and normal serum. Zone 5 of liver extract, maternal serum and normal serum were inhibited by urea suggesting that zone 5 found in serum originated in part from liver. Triton - x - 100 activated zones 1 of serum and pancreas which would suggest that zone 1 of serum was of pancreatic origin.

Bone, placenta, liver, kidney, normal and maternal serum contained a GTP fraction - zone 5 which was probably an 'insoluble' form first demonstrated by Szewczuk (1966). Since the insoluble form of GTP failed to move significantly through the gel, triton - x - 100 a non-ionic detergent was used to release the enzyme bound to cell structures. Tissue extracts and serum containing triton - x - 100 exhibited an increased enzyme activity which was probably due to activation of the enzyme rather than an increased solubility of the enzyme during extraction from tissue.

The present results confirms the work of Naftalin et al. (1969) who suggested the pancreas as a possible source of origin of GTP furthermore a fast moving isoenzyme was found in pancreatic extract which was not detected in the other tissue extracts examined.

The enzyme zones 2, 3, 4 and 5 corresponded in electrophoretic migration to that found for leucine aminopeptidase isoenzymes Jones et al. (1972). Zone 1 of leucine aminopeptidase did not correspond to that of zones 1 or 1(a) of GTP. The present results suggest there are at least five different molecular forms of GTP present in normal and maternal serum and which are probably distinct molecular species differing in their action to urea, L-methionine and triton - x - 100.

The increase in total GTP during gestation was contributed to by zones 2, 3, 4 and 5. Zone 2 originated from bone, zone 3

from kidney, pancreas and liver, zone 4 from kidney, intestine and liver and zone 5 from liver alone. Urea completely inhibited zone 5 of liver and maternal serum, but not zone 5 of placenta it is therefore unlikely that any of the GTP isoenzymes of maternal serum originate in the placenta. The estimation of GTP or its isoenzymes would not therefore be an aid to assessing placental function.

4.2. 4 Iso-enzymes of alanine aminopeptidase.

Tissue isoenzymes.

Ten extracts of pancreas, placenta, kidney, bone, liver and intestine were found to contain one or more zones of alanine aminopeptidase activity. Fig. 43 illustrates the migration of each isoenzyme in relation to the migration of protein fractions. Bone and liver contained a fast moving isoenzyme (zone 1) which migrated in the transferrin region of serum. Kidney and placental extracts contained an isoenzyme which migrated to the β_2 globulin region (zone 2). Two slower migrating isoenzymes found in kidney extract were detected in the fast haptoglobin region (zone 3) and in the Sβ globulin region (zone 5). Two zones of activity were detected in intestinal extract (zones 3 and 4) and one zone in pancreatic extract which migrated to zone 3.

Table 16 lists the isoenzyme activity found for each fraction as a percentage of the total enzymic activity. Liver, intestine, pancreas and placenta each contained one isoenzyme with a concentration greater than 80% of the total alanine aminopeptidase activity. Kidney extract contained zones 2, 3 and 5 with activity of 26%. 44% and 30% respectively.

Serum isoenzymes.

Two isoenzymes (zones 1 and 4) were detected in normal and pregnancy sera. Between the 20th and 28th week of pregnancy, an additional zone of enzyme activity appeared with similar electrophoretic migration to that found for zone 2 of placenta. This additional isoenzyme progressively increased in concentration as pregnancy advanced. Zone 1 of normal serum contained 95% of the total serum enzymic

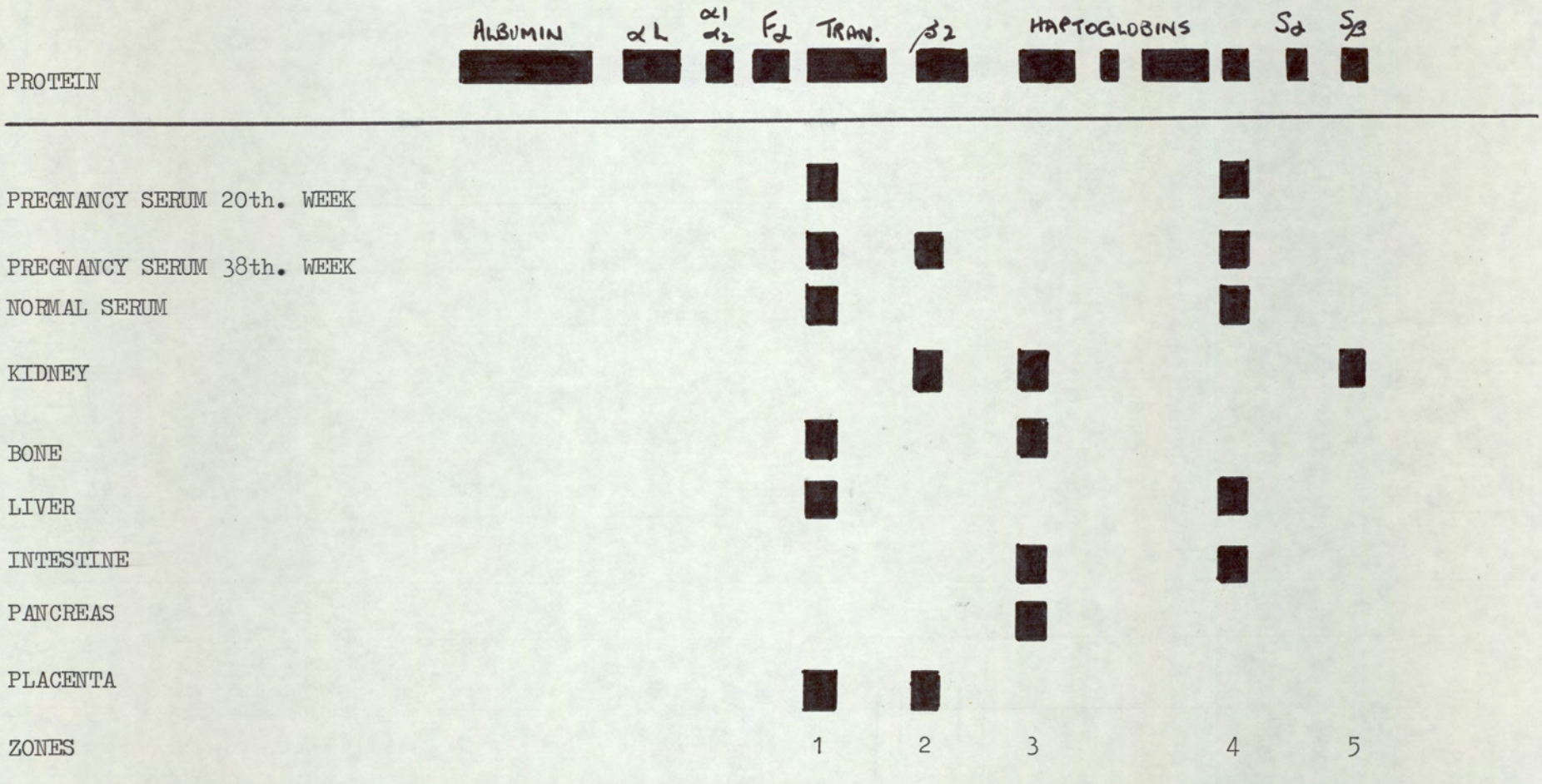


Fig. 43. The electrophoretic migration of alanine aminopeptidase isoenzymes in relation to the migration of other protein fractions.

Table 16. Mean and standard deviation of ten duplicate isoenzyme assays in normal serum.

Isoenzyme zone	1	2	3	4	5
Mean ($\bar{x}$)	3.0	0.8	1.8	2.0	4.3
Standard Deviation (SD)	0.32	0.24	0.26	0.26	0.51

Table 17. Percent concentration of each isoenzyme relative to the total alanine aminopeptidase activity.

Isoenzyme zones	1	2	3	4	5
Normal serum	94.6			5.4	
Serum during 20th week of pregnancy	92.5			7.5	
Serum during 38th week of pregnancy	47.0	48.4		4.6	
Kidney		26.0	44.0		30.0
Bone	38.0		63.4		
Liver	84.8			15.2	
Intestine			12.3	88.0	
Pancreas			100.0		
Placenta	10.0	90.0			

activity. During the 38th week of pregnancy zone 1 contained 47% and zone 2, 48% of the total serum activity. Fig. 44 illustrates the results obtained with isoenzymes in placental extracts, normal serum, and pregnancy serum.

Effect of L-methionine.

Zone 1 enzyme of bone and zone 2 of kidney extracts were completely inhibited by the addition of 50 mM L-methionine. The degree of inhibition of zone 1 found in normal and pregnancy serum ranged between 62% and 83%. Zone 2 of placental extract and pregnancy serum was activated by 50 mM L-methionine, which doubled their respective activities. Fig. 45 illustrates the inhibition and activation of the pregnancy serum isoenzyme together with the placental fraction.

DISCUSSION

Five zones of alanine aminopeptidase activity were demonstrated in tissue extracts and two zones of activity in normal serum. Placenta, pancreas, intestine and liver extracts each contained two isoenzymes one of which was greater than 80% of the total enzyme activity. The isoenzymes with activities below 15% found in each tissue may have originated from blood contamination of the tissue. The isoenzymes found in bone and kidney extracts were unlikely to have originated from blood contamination because each isoenzyme activity was greater than 26% of the total.

By excluding enzyme zones with concentrations of less than 15% which were probably due to blood contamination it follows that zone 1 of normal and pregnancy serum was derived from bone and or liver and zone 4 from intestine. The isoenzymes of kidney and pancreas were not detected in normal serum and would therefore be unlikely to be found in pregnancy serum. Isoenzyme 2 of pregnancy serum would be likely to originate in the placenta and during pregnancy it was found to progressively increase in concentration. L-methionine activated both the isoenzyme in placental extract and the pregnancy serum isoenzyme, which was further evidence that the pregnancy serum

isoenzyme was of placental origin. The kidney isoenzyme (zone 2) was completely inhibited by L-methionine thus allowing quantitation of the placental isoenzyme. The activation of placental alanine aminopeptidase was further demonstrated histochemically on frozen sections of placenta.

The fastest moving zone of normal and pregnancy serum (zone 1) was reduced in concentration in the presence of L-methionine due to the inhibition of the bone fraction.

Serum isoenzymes of non-placental origin capable of hydrolysing DL-leucine-β-naphthylamide have been shown to increase during gestation Jones (1972), but no similar increase has been found with the isoenzymes of alanine aminopeptidase.

The specific activation of the placental isoenzyme in maternal serum by L-methionine may have clinical importance in assessing placental function.

Placental alanine aminopeptidase isoenzyme during gestation.

Table 18 lists the mean and standard deviation of the serum enzyme activities estimated in the presence of L-methionine. The mean serum enzyme value found at 38 weeks of pregnancy was approximately 85 I.U. and when estimating total enzyme activity the value found was approximately 170 I.U. The decreased value was due to the inhibition by L-methionine of all isoenzymes of alanine aminopeptidase with the exception of the placental fraction. When the total enzyme values were halved and compared to that found for the enzyme estimated in the presence of L-methionine the scatter of results were similar. The progressive increase in both the total enzyme and when estimated with L-methionine gave linear increases from the 16th week of pregnancy until term.

The values found for the serum iso-enzyme were found to decrease more rapidly, than that found for the total enzyme. This would be further evidence showing the increase in isoenzymes of non-placental origin during pregnancy.

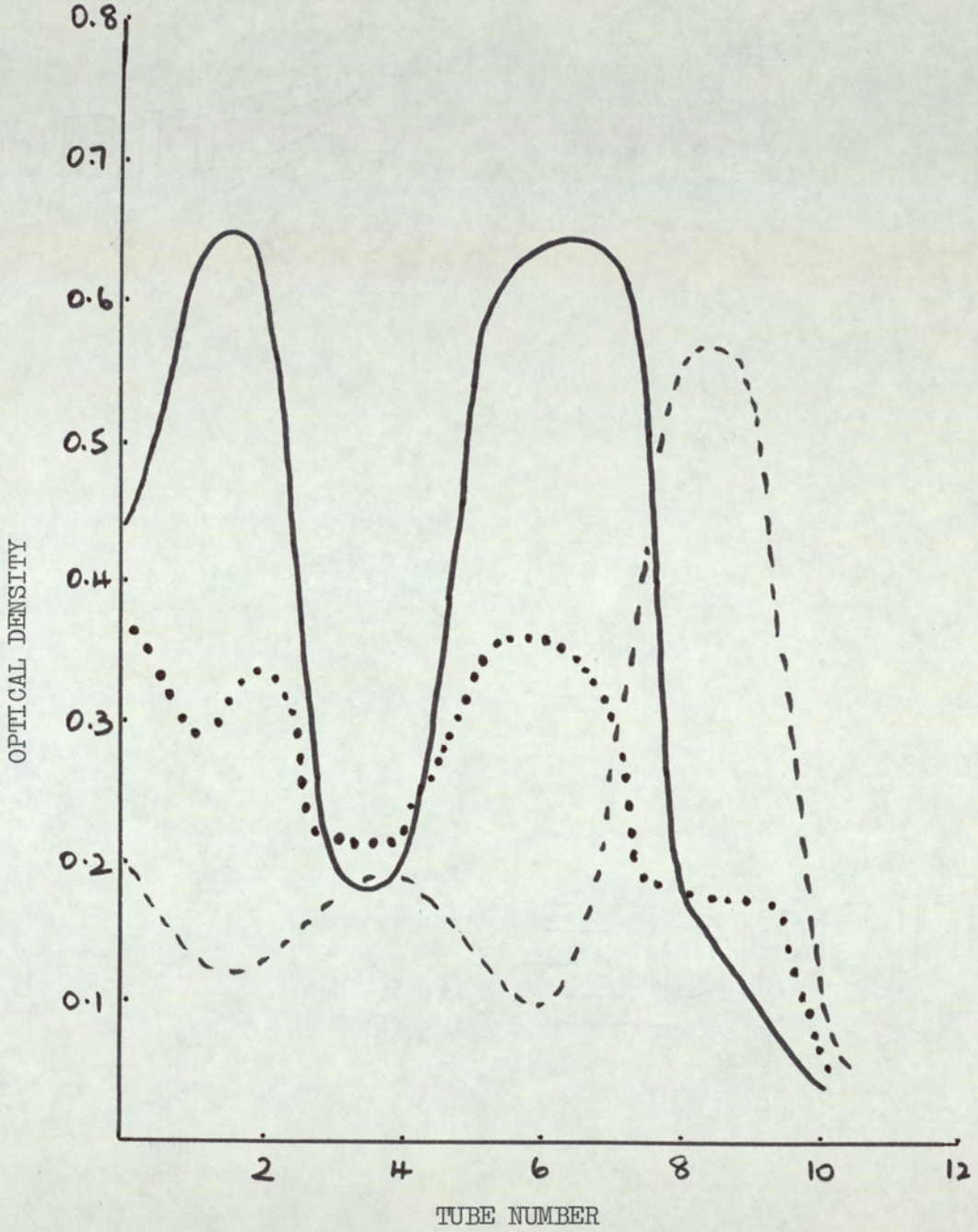


Fig. 44. Alanine aminopeptidase isoenzymes. Serum at 38 weeks of pregnancy ——— ; placental extract ••••••; normal serum - - - - .

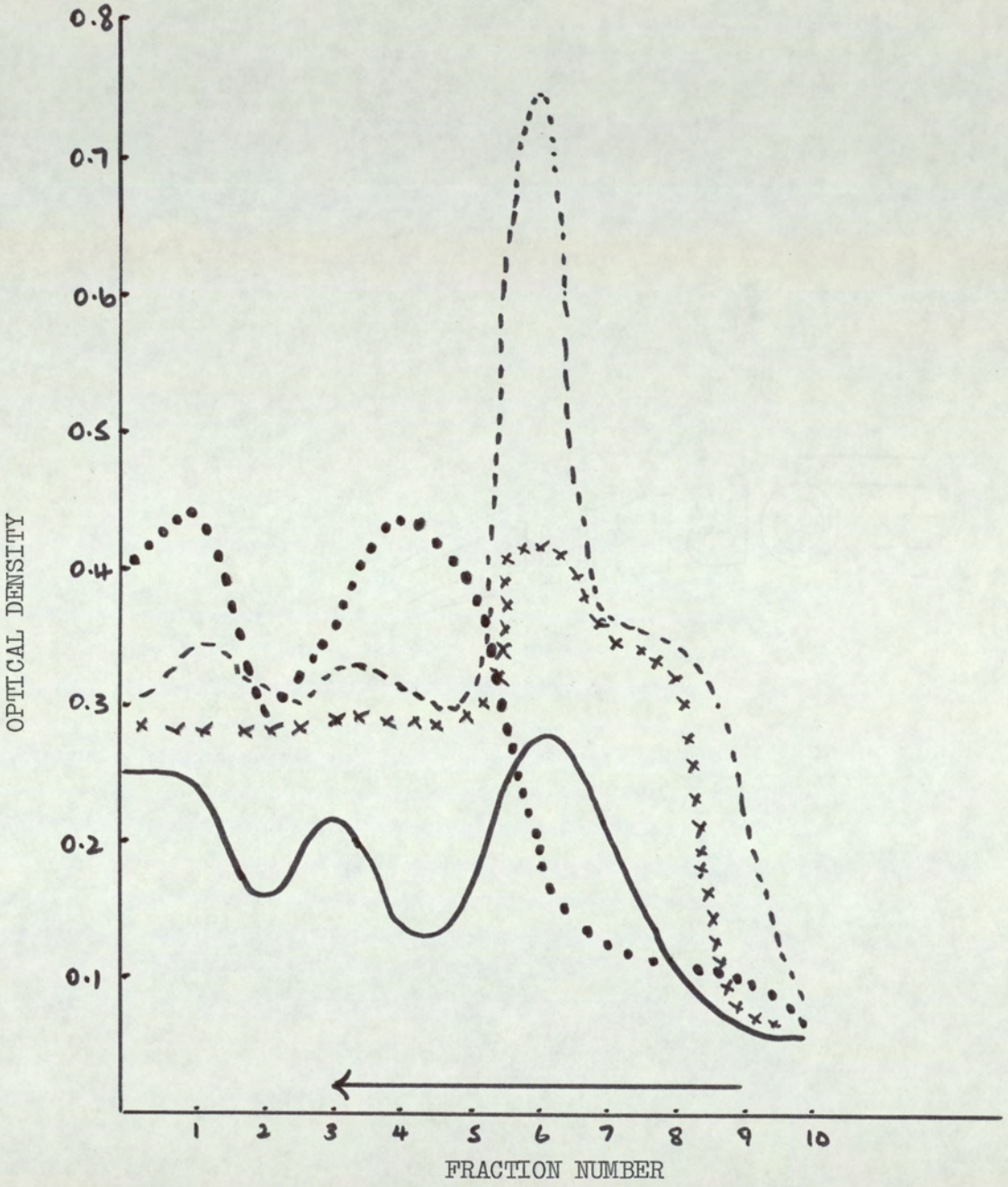


Fig. 45. The inhibition and activation of alanine aminopeptidase isoenzymes. Pregnancy serum 37 week———; normal serum •••••••; placental extract ××××××; placental extract plus methionine— — — —.

Table 18. The mean, standard deviation and y values for serum alanine aminopeptidase activities estimated in the presence of L-methionine.

Week of pregnancy	$\bar{x}$	s	y
14	43.5	8.7	33.3
16	43.4	10.7	38.2
18	33.8	12.4	43.2
20	41.9	6.1	48.2
24	56.0	13.3	58.1
26	64.6	8.2	62.9
28	65.0	17.9	67.9
32	80.5	21.8	77.8
34	76.2	10.6	82.7
35	80.0	8.4	85.1
36	91.1	20.3	87.6
38	92.0	15.1	92.5

4.2. 5 Iso-enzymes of n-acetyl-β-glucosaminidase.

Isoenzymes were fractionated using gradient polyacrylamide gel.

Tissue isoenzymes. Two isoenzymes were detected in tissue extracts, a fast moving isoenzyme A which migrated in the transferrin region and a slower B isoenzyme which migrated to the macroglobulin region. Fig. 46 illustrated the results obtained. Liver, kidney, bone and spleen extracts contained only an A isoenzyme.

Placental and foetal extracts contained both A and B isoenzymes.

Serum isoenzymes. Normal male and non-pregnant female serum contained an A isoenzyme only. Both cord serum and pregnancy serum contained the A and P isoenzyme which migrated to the immunoglobulin G and A region of serum protein. No other tissue or body fluid examined contained a P isoenzyme. During gestation the P isoenzyme increased in activity while the A isoenzyme remained relevantly constant. Fig. 47 illustrates the increase during pregnancy and typical scans of normal serum and placental extract.

Source of the pregnancy serum, isoenzyme P - biochemical investigation.

Attempts to detect the isoenzyme P in foetal and maternal tissues were unsuccessful and the origin of the enzyme was in doubt for this reason. Numerous attempts were made to produce new electrophoretic forms corresponding to the isoenzyme P. The addition of pregnancy serum to foetal and placental tissue extracts did not change the electrophoretic pattern.

(a) Addition of compounds for - SH groups. Mercaptoethanol glutathione and cysteine added to pregnancy serum or placental extracts did not change the isoenzyme patterns. The addition of these compounds in high concentrations to foetal liver extracts (extract diluted 1/2 in mercaptoethanol) did not produce an isoenzyme intermediate in migration to the P and B isoenzymes. Other foetal tissue extracts reacted in a similar manner.

(b) Effect of steroids. During pregnancy elevated circulating steroids are found and to investigate the possible effect of such steroids on the isoenzymes, sera were obtained from patients on oral contraceptives

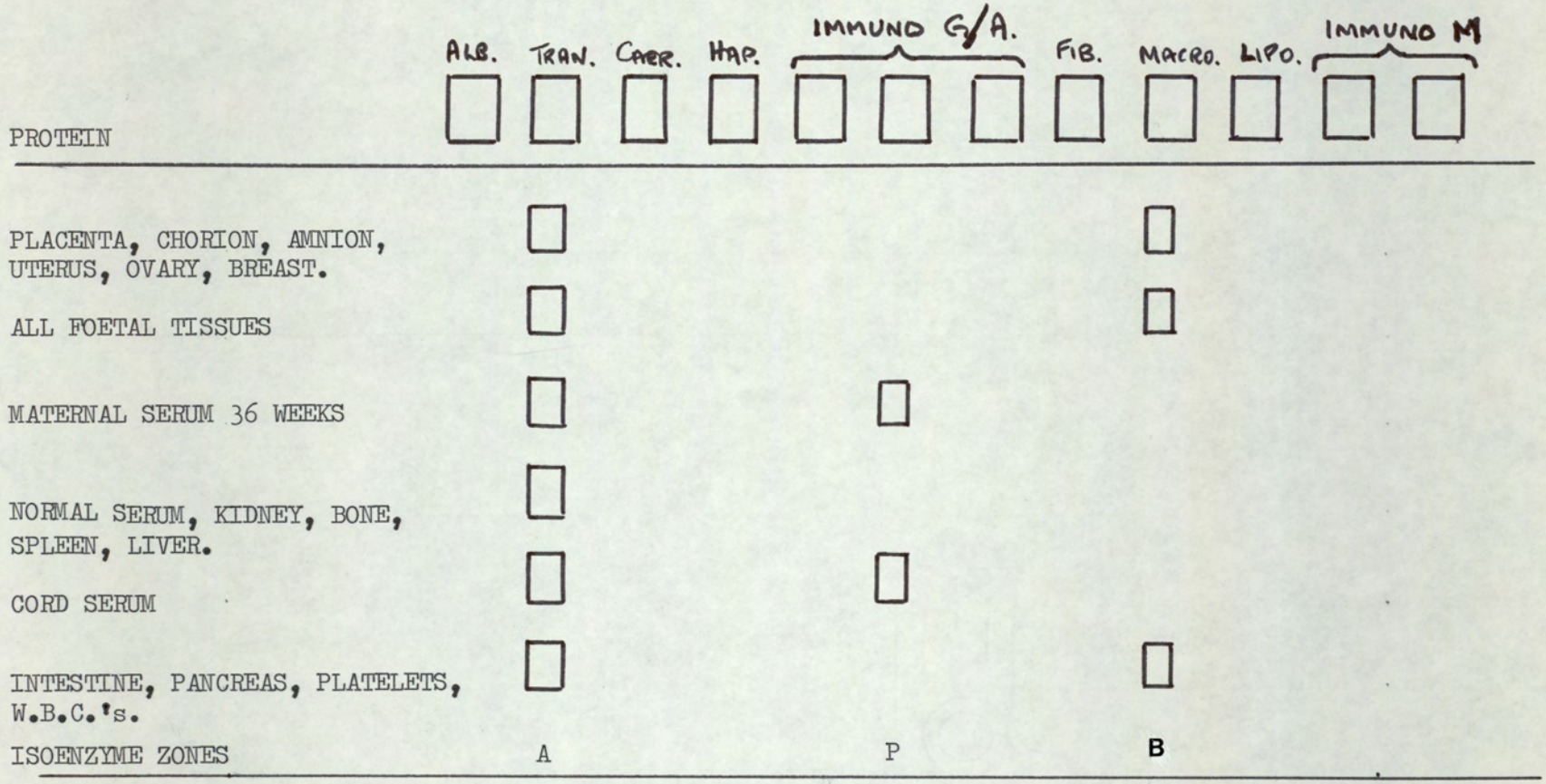


Fig. 46. The migration of n-acetyl-β-glucosaminidase isoenzymes in relation to the migration of protein fractions on gradient polyacrylamide gel slabs.

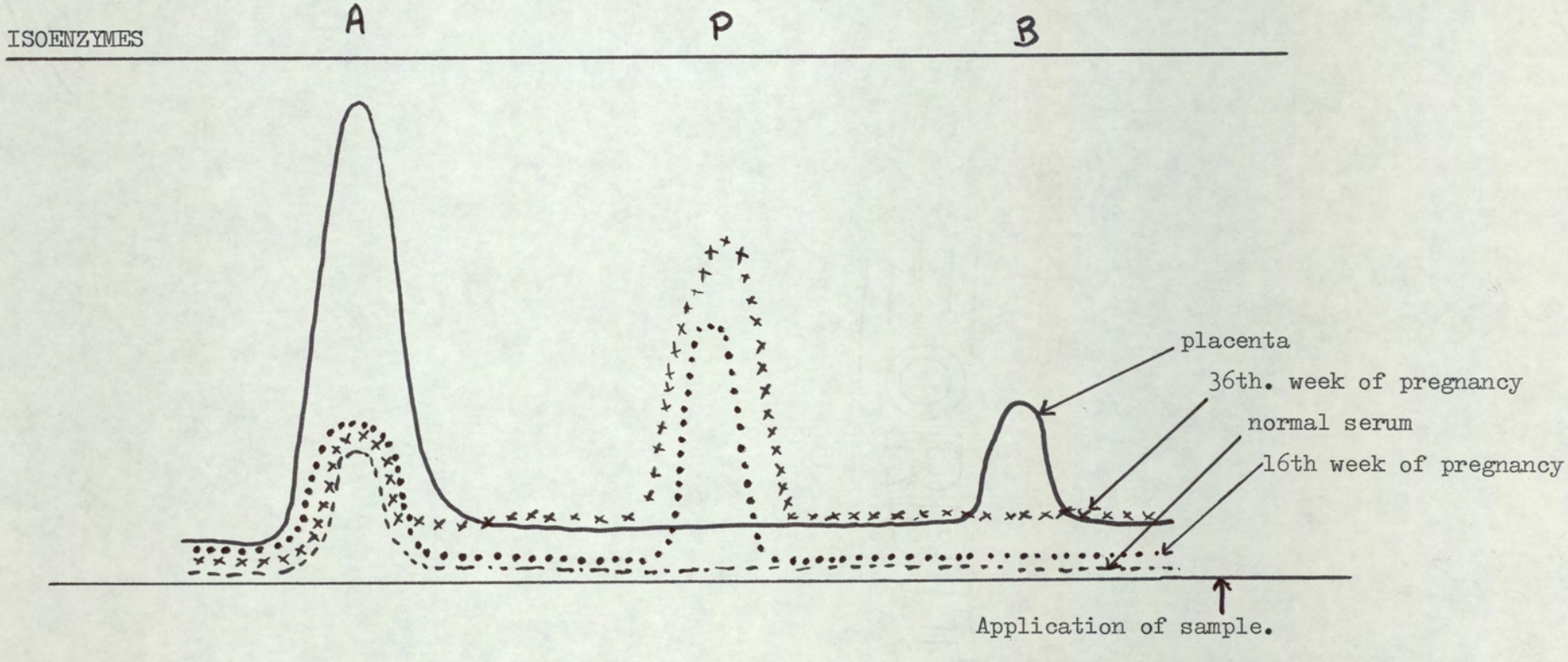


Fig. 47. Typical isoenzyme scans of n-acetyl-β-glucosaminidase. Placental extract ——— ; serum at 36 weeks pregnancy ×××××; serum at 16 weeks pregnancy ·····; normal serum – – – – –.

and from patients receiving high doses of steroid during treatment for other diseases. None of these sera contained isoenzymes electrophoretically similar to the P isoenzyme, and therefore the isoenzyme is unlikely to be oestrogen responsive.

The addition of oestriol at concentrations found in plasma during pregnancy and diethyl ether extracts of pregnancy urine added to foetal and placental extracts did not produce the P isoenzyme.

(c) Effect of neuraminidase. A possibility would be that the P isoenzyme would contain fewer terminal sialic groups and to investigate this possibility neuraminidase was added to both tissue extracts and pregnancy serum. No changes were detected.

(d) Other diseases. Increased plasma enzyme activity, has been found in liver disease, myocardial infarction and in diabetes by Woollen and Turner (1965). Therefore sera were obtained from four of these cases to investigate whether the increased activities was due to a P isoenzyme. No P isoenzyme was detected. The increase in each case was in the concentration of the A isoenzymes.

(e) Animal isoenzymes. Serum was obtained from both a pregnant and a non-pregnant rabbit. One isoenzyme only was detected which migrated to a position between the A and P isoenzyme of human serum. A four fold increase in the concentration of this isoenzyme was found during pregnancy.

The effect of NADP on the isoenzymes of n-acetyl-β-glucosaminidase.

During the investigation into the source of the P iso-enzyme described above it was found that prior addition of NADP protected the isoenzyme molecules from the action of such thiol groups as mercaptoethanol, cysteine and glutathione. In the presence of NADP there was no change in the migration of the isoenzymes found in foetal tissues after the addition of thiol groups.

Such enzyme protection by NADP has been noted by Datta (1971), with homoserine dehydrogenase, Reeves and Brehmeyer (1969) isocitrate dehydrogenase and by Prochazka (1973) with lactic acid dehydrogenase.

This phenomenon suggests that one of the sites on the native protein which is affected by thiol agents is involved with NADP binding and that thiol groups cause a partial disruption of the tertiary structure of the enzyme resulting in the exposure and subsequent oxidation of essential - SH groups. In the presence of NADP the oxidation of these - SH groups is protected. It would also be possible therefore, that a conformational change in the enzyme molecule could account for the change in electrophoretic mobility.

DISCUSSION

The results of the work described above confirm the work of Stirling (1972) who fractionated the isoenzymes A and P by their characteristic behaviour on DEAE cellulose. Attempts to detect n-acetyl-β-glucosaminidase P form in foetal or maternal tissues have been unsuccessful and the origin of the enzyme is in doubt for this reason. Attempts to produce new electrophoretic forms were unsuccessful. However it is possible that the P isoenzyme may arise from the A or B isoenzymes or by a serum component which arises in significant amounts during pregnancy.

Price and Dance (1972) described two forms designated I_1 and I_2 which were intermediate in electrophoretic mobility between the A and B forms. They found that the I_2 form was indistinguishable from the pregnancy serum P form and appeared in serum during storage. Furthermore they found that conversion of the A to I_1 and I_2 was accelerated by heat treatment. Although similar results were obtained during the course of this work it would appear that the new forms produced were denatured products of the A form. A serum with bacterial contamination and which was stored at 22°C for 3 days also produced intermediate forms. Such drastic denaturation of the enzyme molecule would be unlikely to occur in vivo. It has been suggested that the thermal denaturation of form A is not a simple process in that the enzyme may be converted to forms of intermediate activity and stability (Dance et al. 1970).

The source of the isoenzyme P was subsequently investigated immunologically the results of which are described later.

4.2. 6 Iso-enzymes of β-glucuronidase. Enzyme fractions were obtained using gradient polyacrylamide gel slabs.

Tissue isoenzymes. Four zones of enzyme activity A, B, C and D were detected in tissue extracts. The fastest moving isoenzyme (Zone A) migrated to the immunoglobulin G and A region of serum protein. The slowest moving isoenzyme zone D migrated to the β lipoprotein region of protein. Fig. 48 illustrates the migration of each isoenzyme in relation to protein migration. More than 90% of enzyme activity in each tissue extract was found in one isoenzyme. Spleen and placental extracts contained a D isoenzyme and bone and kidney a B isoenzyme. Pancreas, liver and intestine contained the C isoenzymes.

Serum isoenzymes. Fig. 48 illustrates the migration of the isoenzymes found in cord, nonpregnant female and pregnancy serum. Serum from non-pregnant females contained trace quantities of the A and C isoenzymes. During pregnancy there was a progressive increase in the serum isoenzyme A but the isoenzyme C did not increase. Cord serum contained isoenzymes A and C the concentration of which were approximately similar to that found in pregnancy serum during the last trimester of pregnancy. Fig. 49 illustrates typical serum isoenzyme patterns found during pregnancy.

Source of the pregnancy serum A isoenzyme. No tissue extract examined contained an isoenzyme with similar electrophoretic migration to that found in pregnancy serum, but similar results were obtained with the isoenzymes of n-acetyl-β-glucosaminidase. With the object of producing new electrophoretic forms of β-glucuronidase similar parameters were investigated to those used with n-acetyl-β-glucosaminidase.

The addition of steroids, oestrogens and neuraminidase to tissue extracts failed to produce new electrophoretic forms. The addition of mercaptoethanol, glutathione and cystine did reduce the mobility

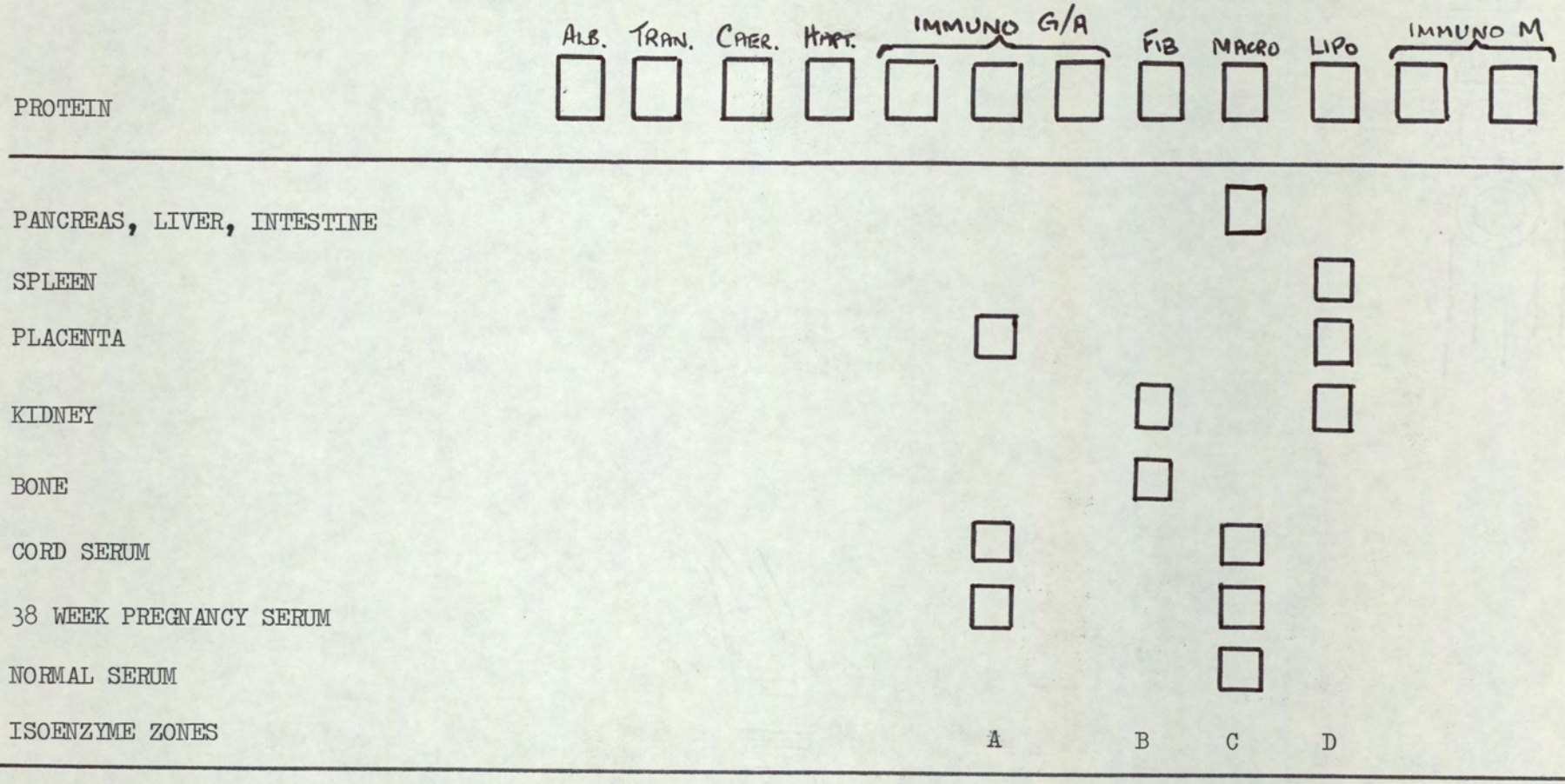


Fig. 48. Migration of β-glucuronidase isoenzymes in relation to protein migration using gradient polyacrylamide gel slabs as supporting medium.

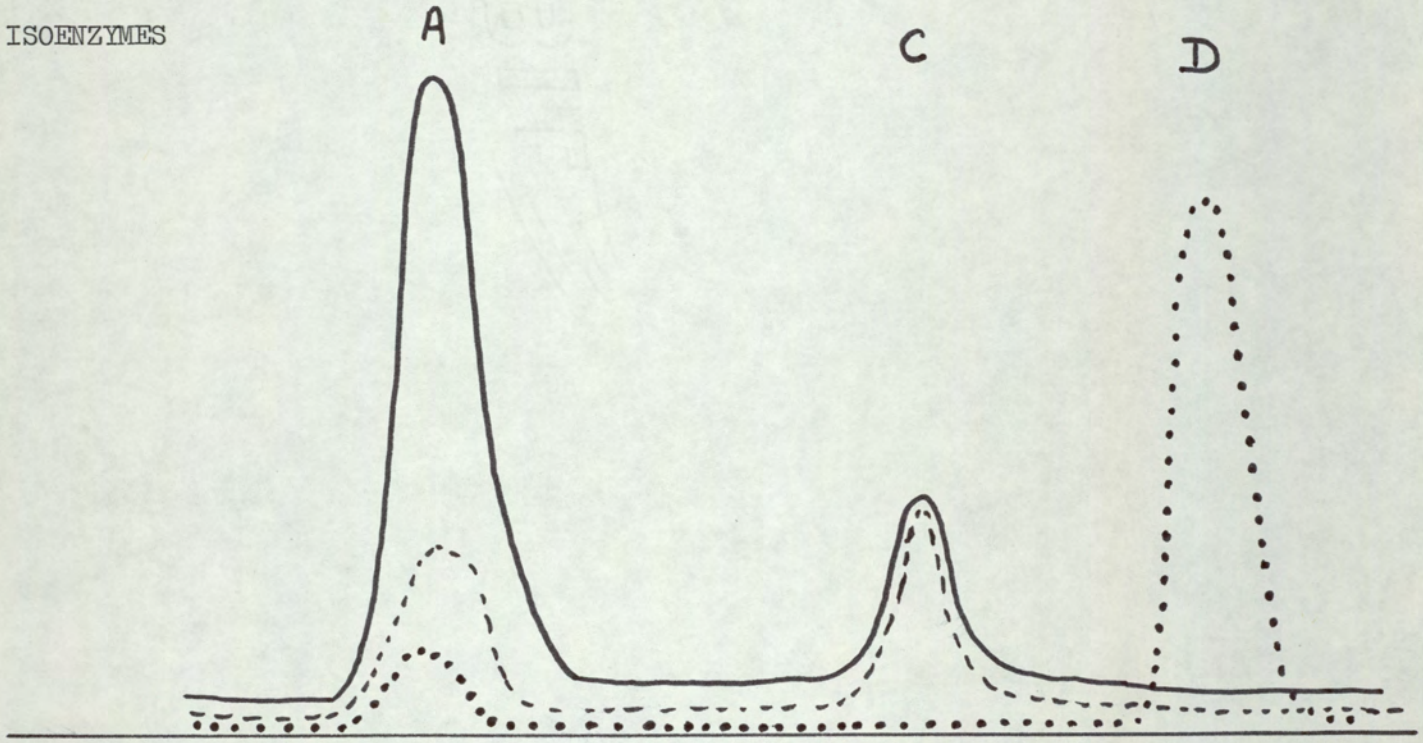


Fig. 49. Isoenzymes scans of β-glucuronidase. Placental extract ·····; pregnancy serum ——; normal serum – – – –.

of all the isoenzymes which suggests the enzyme has terminal sialic acid groups.

In bone and liver disease the increase was in the B and C isoenzymes rather than in the A isoenzyme.

Serum obtained from a pregnant and non-pregnant rabbit did not contain an A, B, C or D isoenzyme of B gluc ronidase. The isoenzymes of rabbit serum migrated faster than the A of human serum. A further two isoenzymes of rabbit serum migrated between the A and B isoenzymes of human pregnancy serum. The faster migrating isoenzyme of rabbit pregnancy serum was found to be five times the concentration of that found in non-pregnant rabbit serum. Further investigation of rabbit isoenzymes would not therefore elucidate the site of origin of the human pregnancy serum A isoenzyme.

Sera obtained from women using combination type low-dosage oral contraceptives contained an increased concentration of the placental A isoenzyme. Serum from non-pregnant women not taking oral contraceptives did not show an increase.

DISCUSSION

It seems likely that each tissue contains only one isoenzyme of β-glucuronidase since the concentration of other isoenzymes were less than 10% of the total enzyme activity, and which could be attributed to contamination of the organ with blood.

The source of origin of the isoenzyme A of pregnancy serum was not possible to find using electrophoretic mobilities as a criterion which was similar to that found for n-acetyl-β-glucosaminidase. The results confirm those found by Huddleston et al. (1971) who found two isoenzyme present in placental extracts. Fishman (1967) received several reports of elevated β-glucuronidase during pregnancy and to account for the increase, suggested that the placenta may contribute the enzyme to the maternal circulation. Huddleston et al. (1971), on the other hand, suggested that the source of the increased serum levels must be other than the placenta because of the difference in isoenzyme mobility during electrophoresis.

Since an increased isoenzyme A activity was found in women taking oral contraceptives it is likely that the increase would be due to the response of some tissue to an augmented oestrogen concentration either from pregnancy or from exogeneous administration. Later during this project the source of the isoenzyme was investigated immunologically the results of which will be described later.

4.2.7 Other enzymes. The enzyme hydrolysing hippuryl-L-lysine has been named by Erdos et al. (1965) as carboxypeptidase N rather than carboxypeptidase A or B which hydrolyse the polypeptide bradykinin. Human blood may or may not contain several carboxypeptidases which cleave basic C-terminal amino acids. Techniques are not at present available to locate isoenzymes of carboxypeptidase and therefore no further investigations were made. The total enzyme estimated in pregnancy serum has been termed carboxypeptidase N and may not be an isoenzyme.

Two isoenzymes of cystine aminopeptidase were detected in pregnancy serum. After electrophoresis on gradient polyacrylamide electrophoresis. The fastest moving isoenzyme (zone A) migrated to the transferrin region of protein and the slowest moving isoenzyme (zone B) migrated to the immunoglobulin G and A region. These results are similar to that found by Page et al. (1961). Placental extracts were found to contain both isoenzymes with approximately 85% of the enzyme activity in zone B. Extracts of chorion and amnion were found to contain the A zone only. No further foetal or adult tissue extracts contained zones of cystine aminopeptidase activity. Serum from males and non-pregnant women were found not to contain cystine aminopeptidase.

The results suggest that the isoenzyme A of pregnancy serum could contain the isoenzymes from placenta, chorion and/or amnion. The isoenzyme B of pregnancy serum would most like ly be of placental origin. Furthermore the enzyme has never been observed in a non-pregnant woman and only plasma and placental extracts

have shown significant inhibition of enzyme activity when oxytocin is added (Page et al. 1961). The particular cells of origin as observed histologically were the syncytium (Semm 1961).

Since it would seem almost certain that the placenta and foetal membranes were the source of origin of both isoenzymes of cystine aminopeptidase no further investigations were made.

The results obtained with the isoenzymes of n-acetyl-β-galactosaminidase were identical to that found for n-acetyl-β-glucosaminidase. Two zones A and B were found after polyacrylamide gel electrophoresis of placental tissue extracts. No similar isoenzyme to that found in pregnancy serum was detected in maternal or foetal tissue extracts. The results obtained after the addition of mercaptoethan, steroids and neuraminidase were identical to that found for n-acetyl-β-glucosaminidase. The enzyme activities using methylumbelliferyl n-acetyl-β-galactosaminide was lower than that found using methylumbelliferyl n-acetyl-β-glucosaminide suggesting that the glucosaminide salt was the preferred substrate. Walker et al. (1961) and Woollen et al. (1961) were unsuccessful in separating the two enzyme activities from rat-kidney and ram testis extracts and concluded that both activities were associated with the same enzyme protein. Furthermore these workers while examining a variety of enzyme preparations found no enzyme source which had one activity but not the other.

The present results also suggest that the two activities were due to one and the same enzyme protein.

4. 3. Serum Activity of Glucose-6-Phosphatase and 5́ Nucleotidase During Human Pregnancy.

Since there is a striking similarity in the increases in maternal serum 5́-nucleotidase, and glucose-6-phosphatase, and alkaline phosphatase, and also the substrates used for their determinations are related, the possibility was considered that alkaline phosphatase was responsible for all three measured enzyme activities. The aim was

to investigate whether 5́-nucleotidase and glucose-6-phosphatase activity in serum of pregnant women was due to the action of alkaline phosphatase.

The results of the alkaline phosphatase, 5́-nucleotidase and glucose-6-phosphatase assays of a pooled serum are seen in Fig. 50. No peak of enzyme activity was obtained with either enzyme between a pH of 6 and 8. Maximum hydrolysis of each substrate occurred at pH 10.0. The addition of magnesium ions activated the hydrolysis of all three substrates. At pH 10.0 the alkaline phosphatase was activated by 28%, 5́-nucleotidase by 25% and glucose-6-phosphatase by 5%. Fig. 51 illustrates the similar results obtained with manganese which was found to activate alkaline phosphatase substrate by 17% at pH 10.0 and the 5-nucleotidase by approximately 22% throughout the pH range. The activation of glucose-6-phosphate was similar to that found for alkaline phosphatase, Fig. 51.

At a pH of 10.0 nickel ions were found to activate the three enzymes, alkaline phosphatase, 5́-nucleotidase and glucose-6-phosphatase, by 24%, 48% and 38% respectively. In each instance the nickel inhibited the hydrolysis between pH 6 and 9 as illustrated in Fig. 52. Beryllium sulphate Fig. 53, was found to inhibit the three enzymes between a pH of 5 and 10.

Heating the pooled serum to 65^{o}C for 30 minutes was found to inactivate the enzymes between pH 6 and 8. None of the above experiments showed a peak of enzyme activity between pH 6.0 and 8.0.

The activities of 5́-nucleotidase, glucose-6-phosphatase and alkaline phosphatase were estimated in thirty individual samples of serum taken between the fourteenth week of pregnancy and term. The results for alkaline phosphatase were plotted against both 5-nucleotidase and glucose-6-phosphatase. In each case a straight line was obtained, Fig. 54. Similar results were obtained by plotting the figures against the heat stable alkaline phosphatase results, Fig. 55.

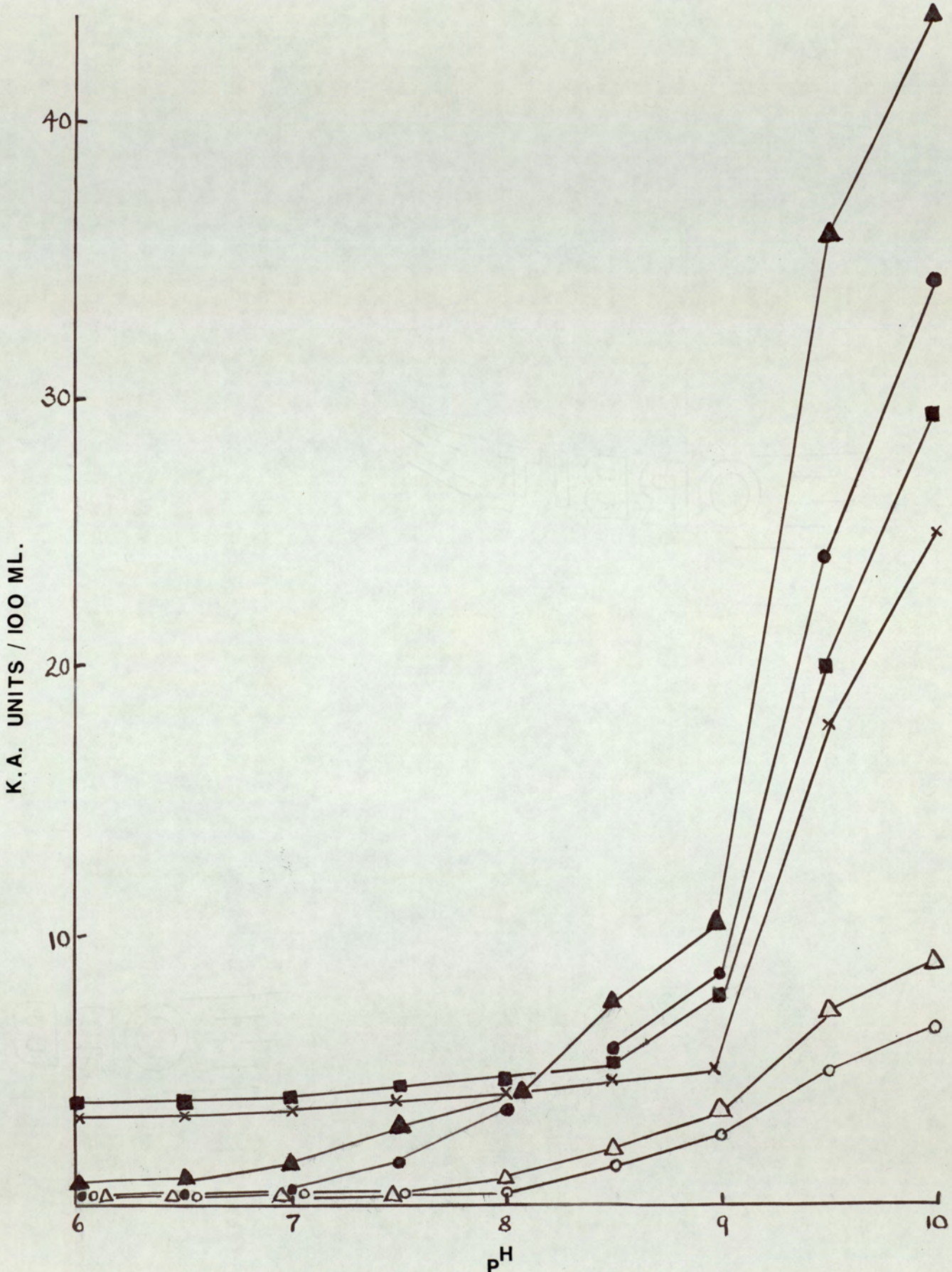


Fig. 50. The action of magnesium ions on the estimation of glucose-6-phosphatase, 5́-nucleotidase and alkaline phosphatase. Glucose-6-phosphatase **O**, with magnesium △; 5́-nucleotidase **X**, with magnesium ■; alkaline phosphatase ● with magnesium ▲.

Fig. 51. Glucose-6-phosphatase, 5́-nucleotidase and alkaline phosphatase activity in the presence of manganous ions. Glucose-6-phosphatase O, with manganous ions △ ; 5́-nucleotidase X, with manganous ions ■ ; alkaline phosphatase ● , with manganous ions ▲ .

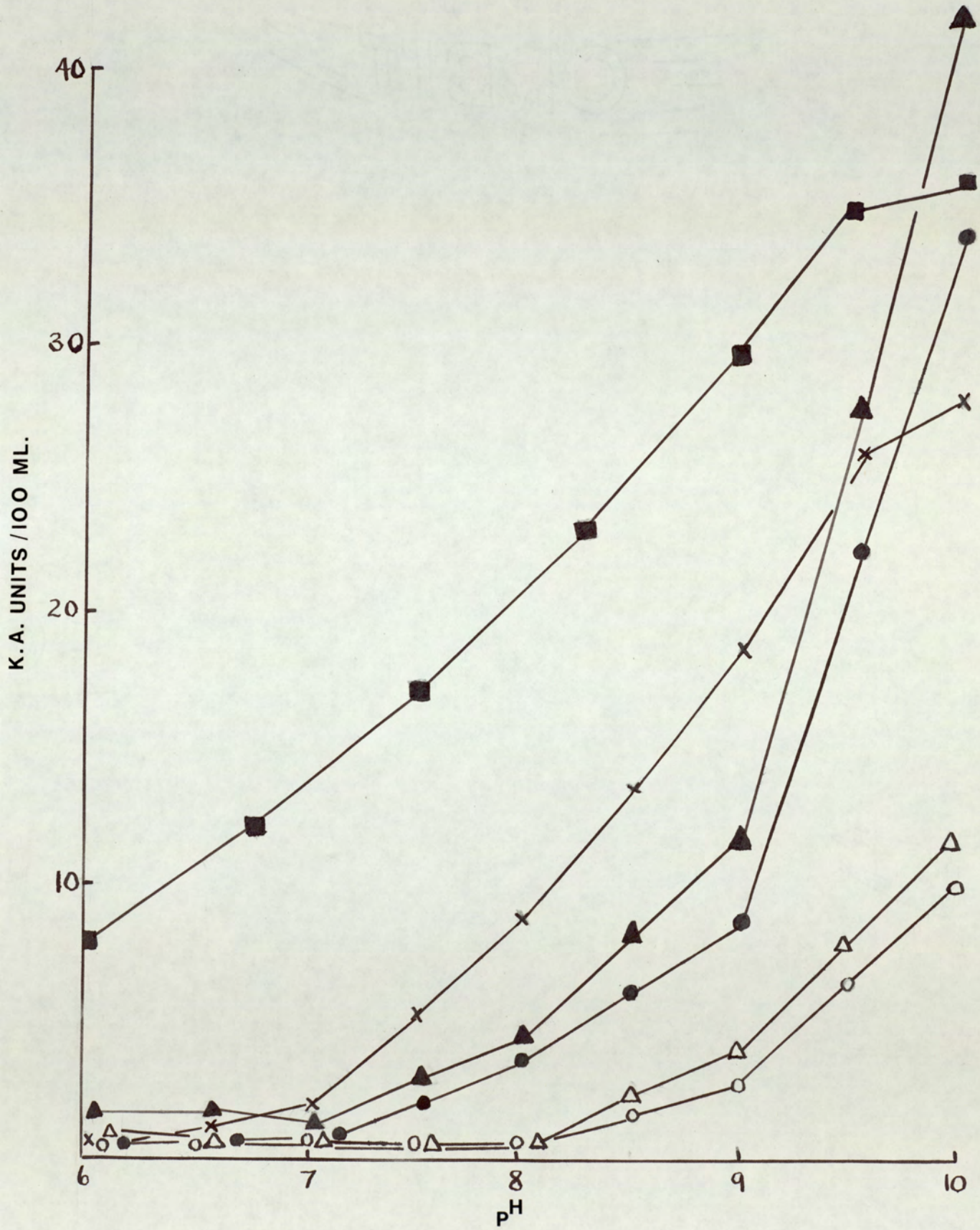


Fig. 52. The effect of nickel ions on the estimation of glucose-6-phosphatase, 5-nucleotidase and alkaline phosphatase. Glucose-6-phosphatase O, with nickel ions △ ; alkaline phosphatase ● , with nickel ions ▲ ; 5-nucleotidase X, and with nickel ions ■ .

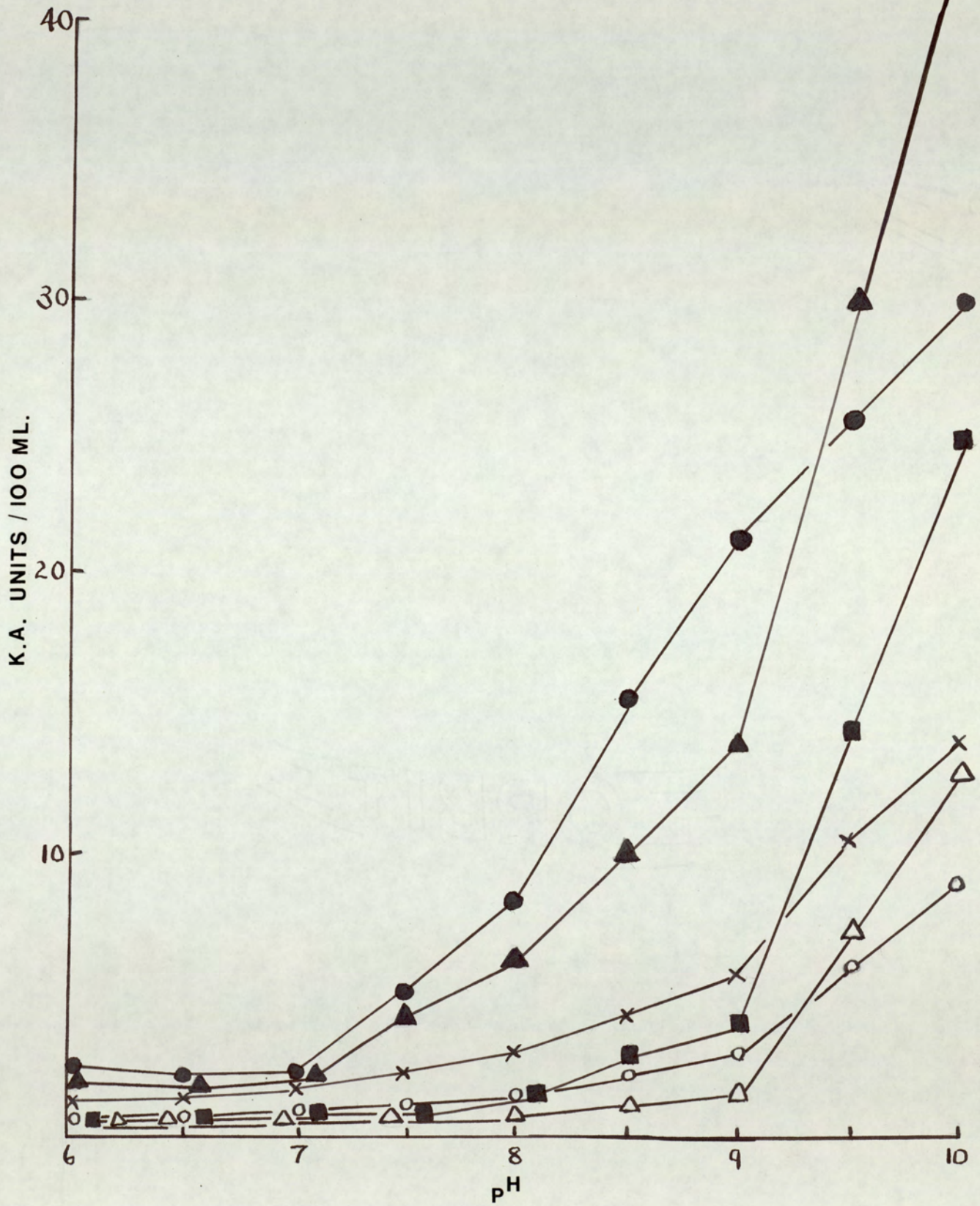
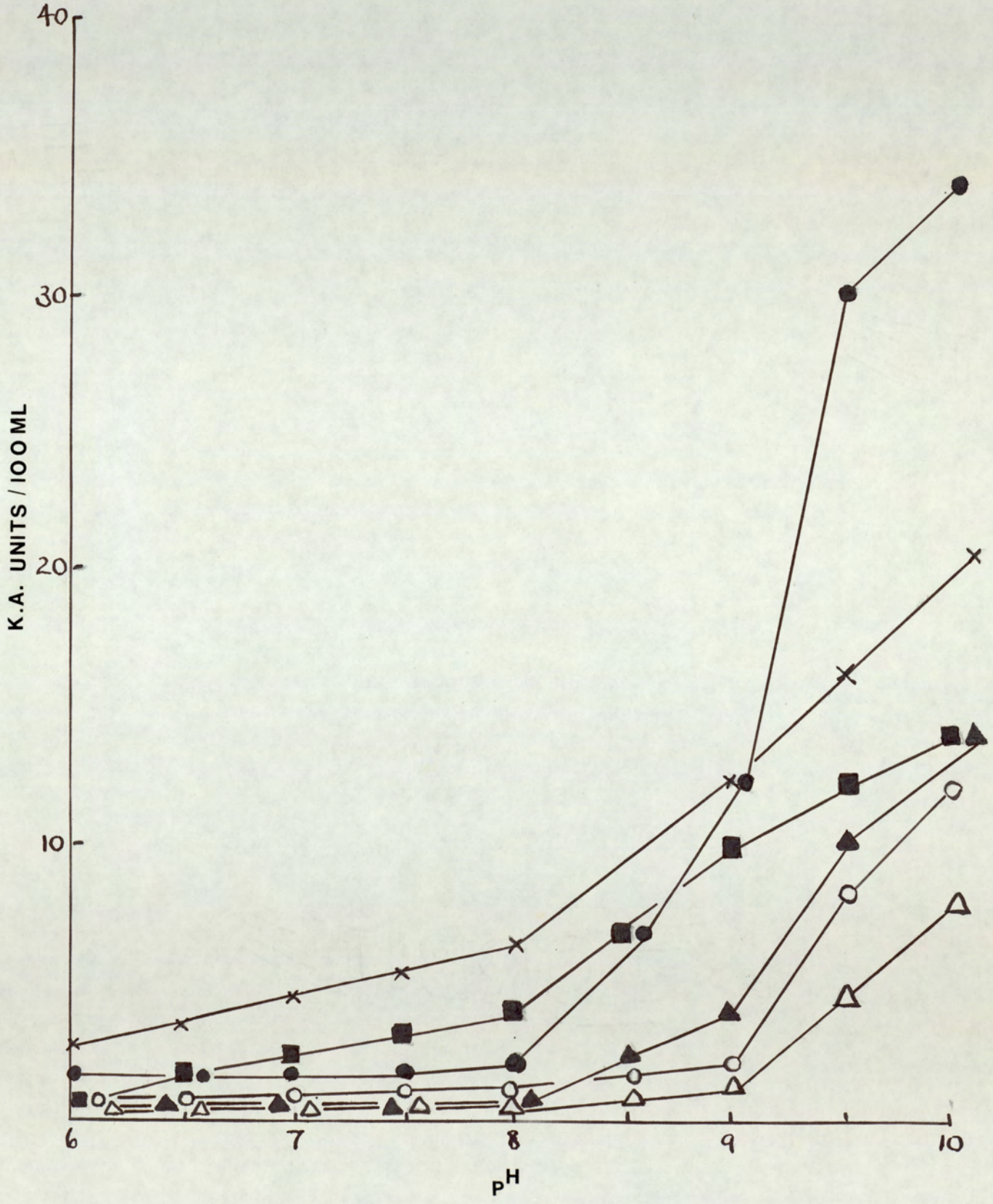


Fig. 53. The effect of beryllium ions on the estimation of glucose-6-phosphatase, 5-nucleotidase and alkaline phosphatase in pregnancy serum. Alkaline phosphatase ●, with beryllium ▲; glucose-6-phosphatase O, with beryllium △; 5-nucleotidase X, with beryllium ■.



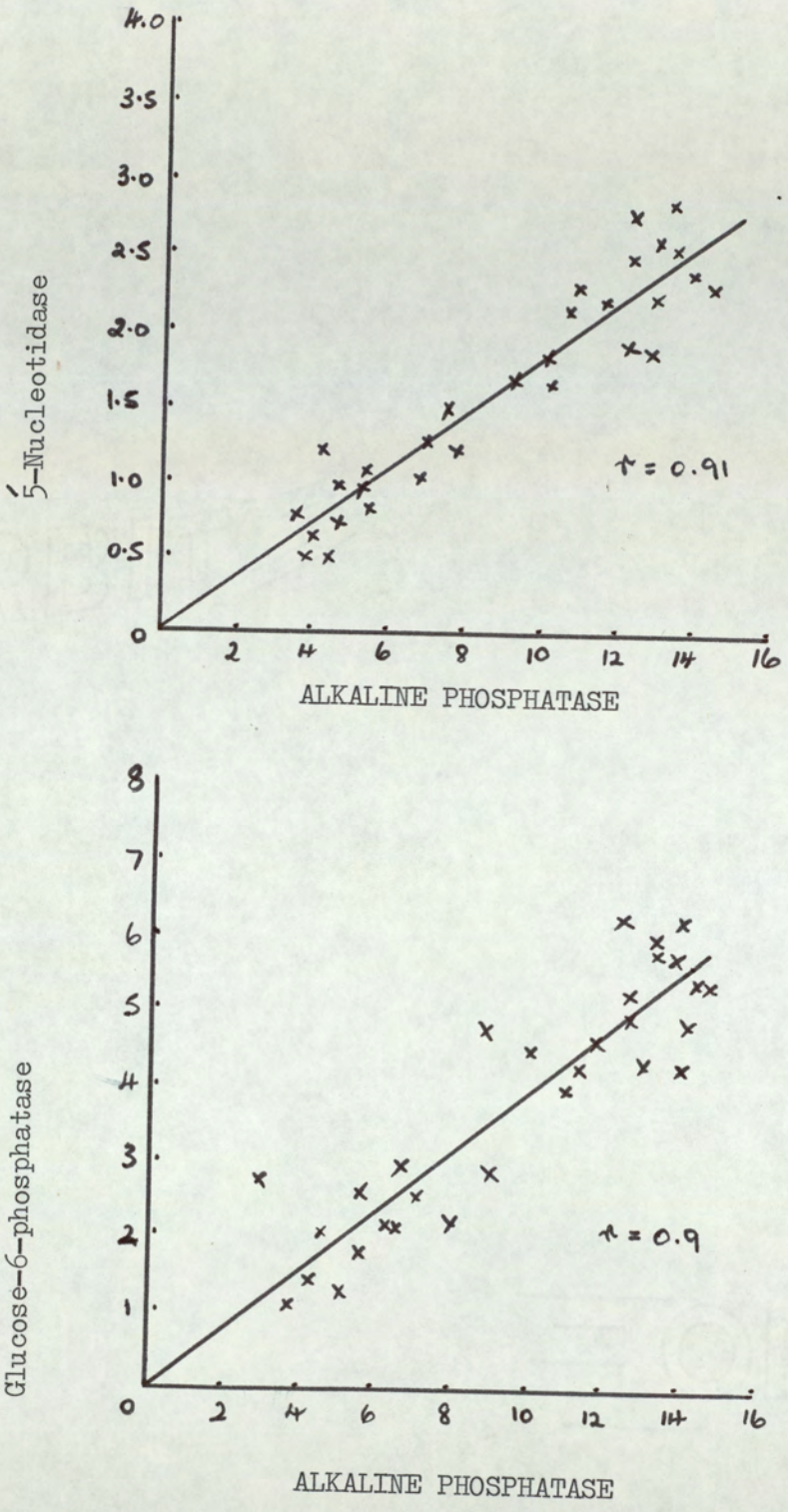
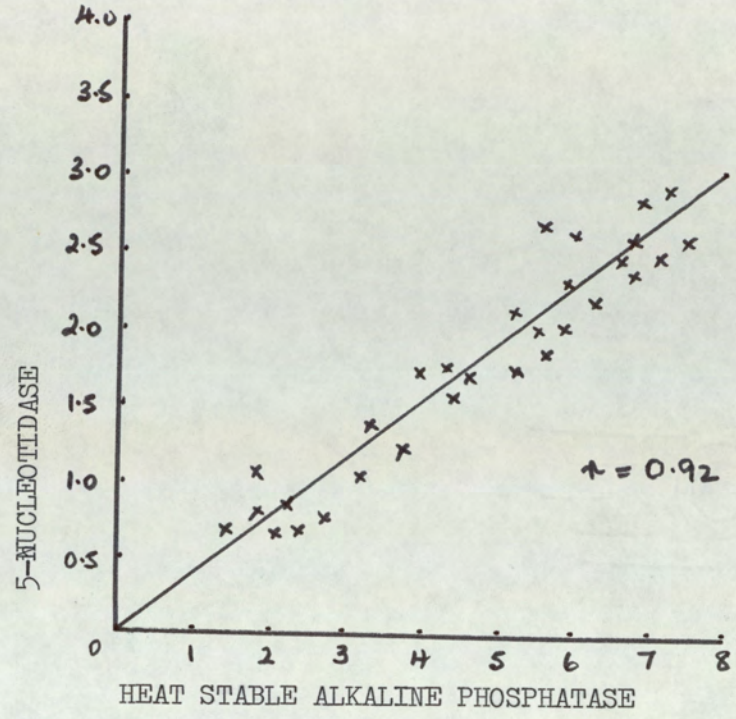


Fig. 54. Comparison of 5'-nucleotidase, glucose-6-phosphatase and alkaline phosphatase activities in individual patients.



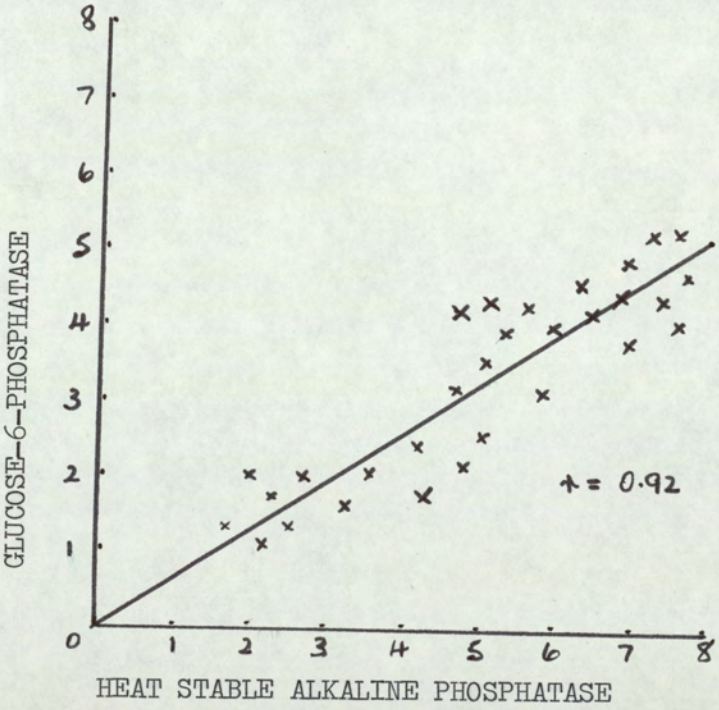


Fig. 55. Correlation between heat stable alkaline phosphatase with 5-nucleotidase and glucose-6-phosphatase.

5′-nucleotidase and glucose-6-phosphatase could be demonstrated in liver extracts. Placental extract contained 5′-nucleotidase with optimum activity at pH 7.0 but no glucose-6-phosphatase was detected (Fig. 56).

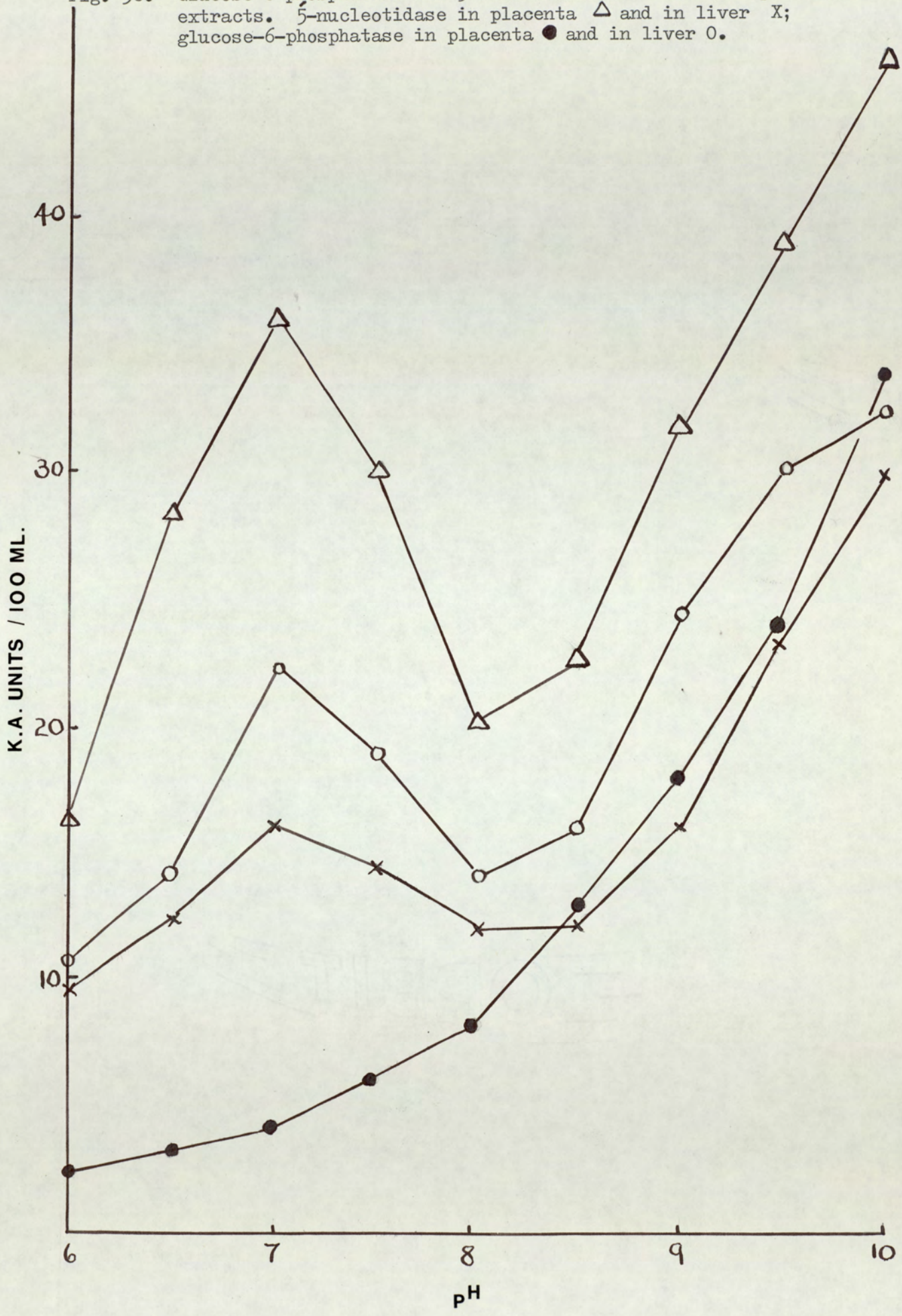
DISCUSSION

Mistilis (1968) has reported that serum 5′-nucleotidase may reach or exceed the normal upper limit of activity during the third trimester of pregnancy. We have also found similar results with glucose-6-phosphatase. A number of workers Bellfield and Goldberg (1969), Campbell (1962), Elder et al. (1972) and Fog (1967) have described activators and inhibitors to estimate the 'true' 5′-nucleotidase and glucose-6-phosphatase in serum. During pregnancy where a progressive increase is found in serum alkaline phosphatase the estimation of 5′-nucleotidase and glucose-6-phosphatase can present difficulties due to the 'tailing' of the alkaline phosphatase activity at less than the optimum pH of 9.7.

The addition of magnesium and manganese, both activators of 5′-nucleotidase, did not produce a peak of enzyme activity in the pH range of 6 to 8. The addition of beryllium to inhibit the alkaline phosphatase did not isolate enzyme activity between pH 6 and 8. Elder (1973) using beryllium ions found no progressive increase in serum glucose-6-phosphatase during pregnancy. Since beryllium ions have been found to inhibit alkaline phosphatase and the hydrolysis of glucose-6-phosphate was due to non-specific alkaline phosphatase then no increase would be expected in the serum glucose-6-phosphatase levels.

Nickel which is known to inhibit 5′-nucleotidase (Campbell, 1962) was found to inhibit the hydrolysis of all three substrates between pH 6 and 9 and to activate at a pH of 10.0. These results suggest the absence of 5′-nucleotidase in pregnancy serum. It has been suggested by Seitaniditis and Moss (1969) that 5′-nucleotidase has a larger molecular weight than alkaline phosphatase and therefore diffusion out of the placenta into the maternal circulation may be

Fig. 56. Glucose-6-phosphatase and 5́-nucleotidase in liver and placental extracts. 5́-nucleotidase in placenta △ and in liver X; glucose-6-phosphatase in placenta ● and in liver 0.



limited.

Elder et al. (1972) found the presence of glucose-6-phosphatase like activity in serum using beryllium as inhibitor of alkaline phosphatase but the hydrolysis of the three substrates in the presence of beryllium was found by us to be similar. Although a glucose-6-phosphatase like activity has been demonstrated histochemically in placenta by Carter and Weber (1966) it would be unlikely that the enzyme which is microsome bound would be liberated into the maternal circulation.

It is interesting to note that a straight line was obtained when plotting the activity of 30 serum alkaline phosphatase against 5′-nucleotidase and glucose-6-phosphatase. A straight line was also obtained while plotting the results against the heat stable alkaline phoaphatase. Although this in itself would not be sufficient evidence to implicate the non-specific alkaline phosphatase for hydrolysing glucose-6-phosphate and adenosine monophosphate it does serve as further evidence.

Alkaline phosphatase was found to be active over a wide pH range and the hydrolysis of adenosine-5′-monophosphate and glucose-6-phosphate was due to non-specific alkaline phosphatase. It appears that the glucose-6-phosphatase and 5′-nucleotidase reported to be found in pregnancy serum would be entirely due to the non-specific action of alkaline phosphatase.

4. 4 Molecular weight of n-acetyl-β-glucosaminidase and β-glucuronidase isoenzymes.

As a further guide in the search for the source of origin of β-glucuronidase and n-acetyl-β-glucosaminidase the approximate molecular weight of the isoenzymes was estimated.

One possibility would be that during the passage of the isoenzyme from the foeto-placental unit into the maternal circulation a change could occur in its molecular weight with subsequent change in electrophoretic mobility.

The sephadex column was calibrated with enzymes ranging in molecular weight between 14,000 and 300,000. Fig. 59 illustrates the results obtained with leucine aminopeptidase, lactic dehydrogenase, malic dehydrogenase and lysozyme. The placental A and B isoenzymes and the pregnancy serum isoenzymes were subjected to gel filtration and the elution volume calculated. Each isoenzyme of β-glucuronidase, and each isoenzyme of n-acetyl-β-glucuronidase were found to have similar elution volumes and hence molecular weight. Fig. 60 illustrates the results obtained for the isoenzymes. The molecular weight of n-acetyl-B-glucosaminidase was calculated as 115,000 and B-glucuronidase 300,000.

Robinson et al. (1967) found the molecular weight of rat kidney n-acetyl-β-glucosaminidase as between 150-160,000, and Plapp and Cole (1967) for bovine liver β-glucuronidase found a molecular weight of 280,000.

These results suggest that the difference in electrophoretic mobility of both enzymes were not due to differences in molecular weight but rather to a conformational change in the enzyme molecule.

4. 5 Isoenzymes of n-Acetyl-β-glucosaminidase and β-glucuronidase: An Immunological investigation.

Before the serum estimation of the isoenzymes of β-glucuronidase or n-acetyl-β-glucosaminidase can be investigated as a possible index of foeto placental function it is necessary to locate the tissue of origin. To this end an immunological study was made of the isoenzymes of placental and foetal liver extracts in relation to the circulating maternal serum isoenzymes.

4.5. 1 N-Acetyl-β-Glucosaminidase. Antibodies to the isoenzymes A and B found in placental extract and foetal liver extracts were raised in rabbits and the rabbit serum used untreated.

By adding the antibodies against both A and B isoenzymes to four pools of serum taken during the 38th week of pregnancy the

total activity of N-acetyl-β-glucosaminidase was reduced in concentration to that found during the 14th week of pregnancy. Table 19 lists the inactivation of the total enzyme activity after the addition of the antisera. There was no significant inactivation after one hours incubation with the antisera. However after forty eight hours the antiserum to placental B and foetal B isoenzymes had inactivated the maternal serum enzyme by 71.9% and 75% respectively.

The total activity of N-acetyl-β-glucosaminidase during the 38th week of pregnancy was reduced to the concentration found in maternal serum during the 14th week of pregnancy after the addition of antiserum to placental B and foetal B isoenzymes.

Using gel diffusion an enzyme immunoprecipitate formed when the four antisera were assayed against the purified isoenzyme extracts. The antiserum to the isoenzyme A of placenta and foetal liver (Fig. 61 and 62) did not react with pregnancy serum. The antiserum to the B isoenzymes of placenta and foetal liver formed an immunoprecipitate with maternal serum. Fig. 63 and 64 illustrates the progressive increase in the immunoprecipitate of pregnancy serum between twelve and thirty eight weeks of pregnancy. Both antisera of the foetal liver isoenzymes A and B reacted with extracts of liver, spleen, pancreas, intestine and kidney but the placental antisera did not react with the tissues tested (Fig. 65). The titre of all four antisera were found to be 1/32 when the concentration of the antigen was 1 mg/1 ml enzyme protein.

DISCUSSION

The antigenicity of N-acetyl-β-glucosiminidase molecule was observed by the production of the antibody after a course of only three injections. The foetal liver A and B and the placental A and B isoenzymes reacted with their corresponding antisera. All four isoenzymes also cross reacted with the four antisera indicating that the four isoenzymes were antigenically similar. The isoenzyme P of pregnancy serum reacted only with antiserum of foetal liver B and

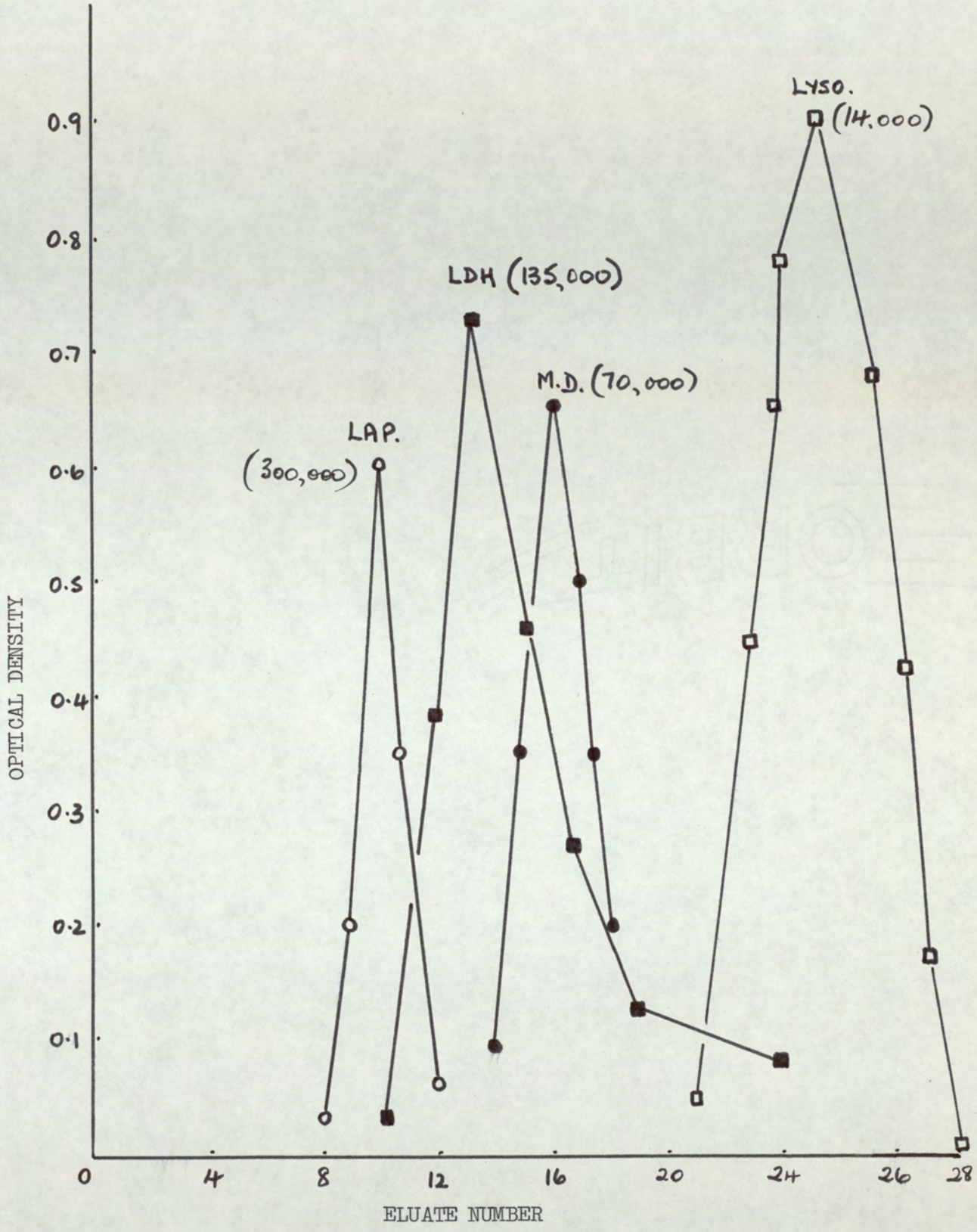


Fig. 59. Enzyme calibration of Sephadex Column prior to molecular weight determinations. LAP - leucine aminopeptidase; LDH - lactic dehydrogenase; MD - malic dehydrogenase; LYSO - muraminidase (lysozyme).

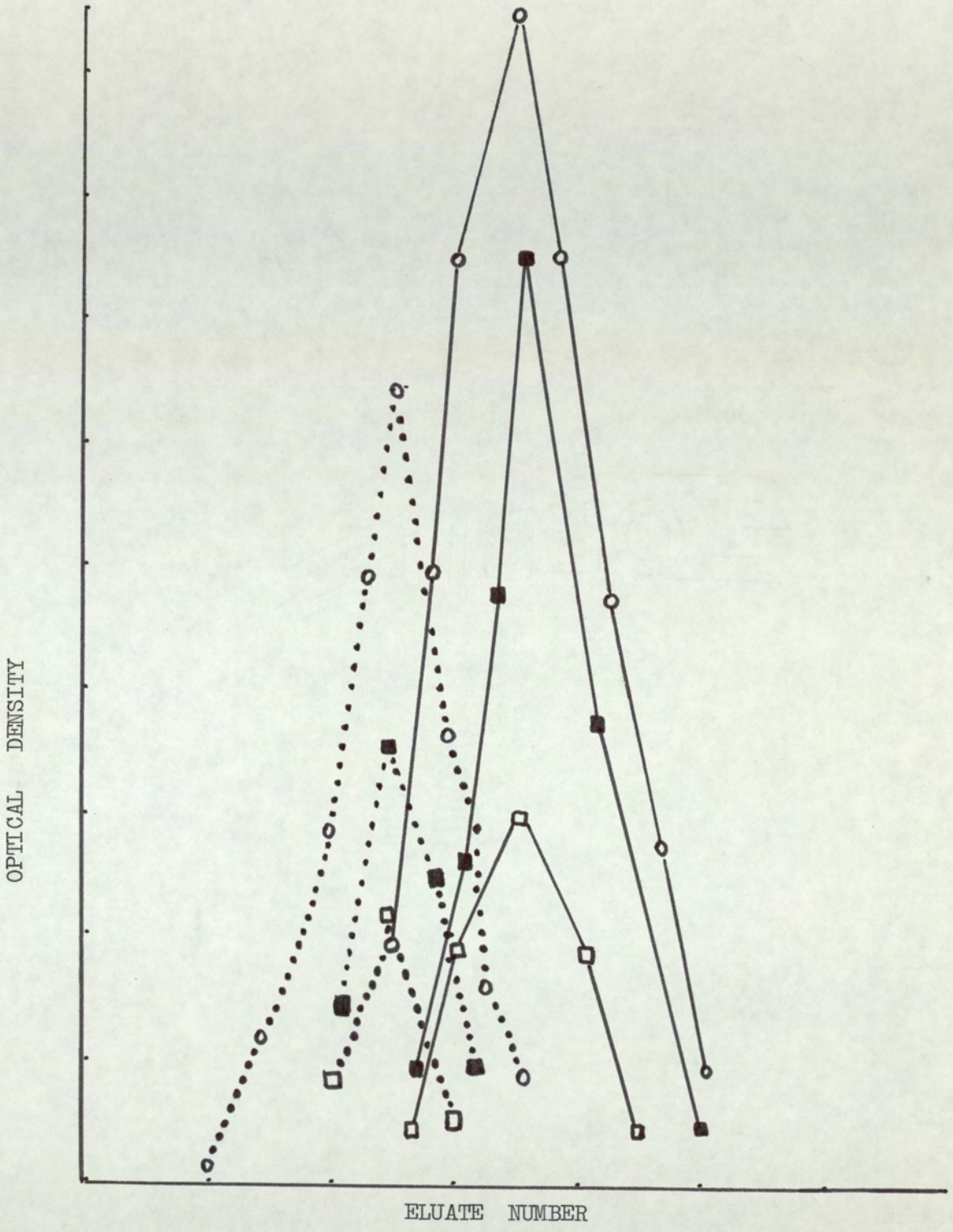


Fig. 60. Molecular weight of n-acetyl-β-glucosaminidase isoenzymes (———), and β-glucuronidase (.....) isoenzymes from placenta and pregnancy serum.
Pregnancy serum, □ ; Placental A, ■ ; Placental B, O .

Table 19. The inactivation of pregnancy serum N-acetyl-β-glucosaminidase by antisera raised against placental and foetal liver isoenzymes A and B.

Antiserum		Pregnancy serum N-acetyl-glucosaminidase μmole/min/L		% inactivation
		Untreated	Treated	
Placental A isoenzyme	1 hr.	8.2	8.0	2.4
	2 days	7.9	7.6	3.7
Placental B isoenzyme	1 hr.	8.3	8.0	3.6
	2 days	8.2	2.3	71.9
Foetal A isoenzyme	1 hr.	8.3	8.2	1.2
	2 days	8.1	7.8	3.7
Foetal B isoenzyme	1 hr.	8.1	7.8	3.7
	2 days	8.0	2.0	75.0

placental B which would establish that the pregnancy serum P isoenzyme was either of foetal or placental origin.

Both antisera to foetal liver A and B reacted with all adult tissue and were therefore antigenically indistinct although the structures may or may not be the same. On the other hand the placental A and B antisera did not react with other adult tissues and were therefore antigenically indistinct and have different structures.

During the passage of the isoenzyme from placenta to the maternal circulation it would be likely that a change in structure such as unfolding of the chain or a conformational change may occur in the enzyme molecule which may produce the P isoenzyme found in pregnancy serum.

The antisera to foetal A and placental A did not react with the pregnancy enzyme of maternal serum which suggests different enzyme structure. It would be reasonable to expect the antiserum to the foetal B isoenzyme to react with the pregnancy serum enzyme since this antiserum was found to react with all adult and foetal tissue enzymes.

The concentration of the P isoenzyme in cord serum was found to be approximately one eighth to that found in maternal serum taken during the 38th week of pregnancy, it would therefore be unlikely that the pregnancy serum P isoenzyme would arise in the foetus. Since the antiserum to the placental B isoenzyme reacts with pregnancy serum it would be reasonable to speculate that the pregnancy serum enzyme is derived entirely from the placenta. Furthermore the antibodies raised against the placental B and foetal liver B isoenzymes neutralized the major portion of the maternal serum N-acetyl-β-glucosiminidase taken during the 38th week of pregnancy, to an activity found during the 14th week of pregnancy. A conformational change in the placental B isoenzyme may give rise to the placental A isoenzyme although antigenically the two enzymes were similar.

The foetal and adult tissue isoenzymes A and B can be assumed

Fig. 61. Immunodiffusion of enzyme antiserum to foetal isoenzyme A of n-acetyl-β-glucosaminidase against pregnancy serum taken between 12 weeks and 38 weeks of pregnancy.

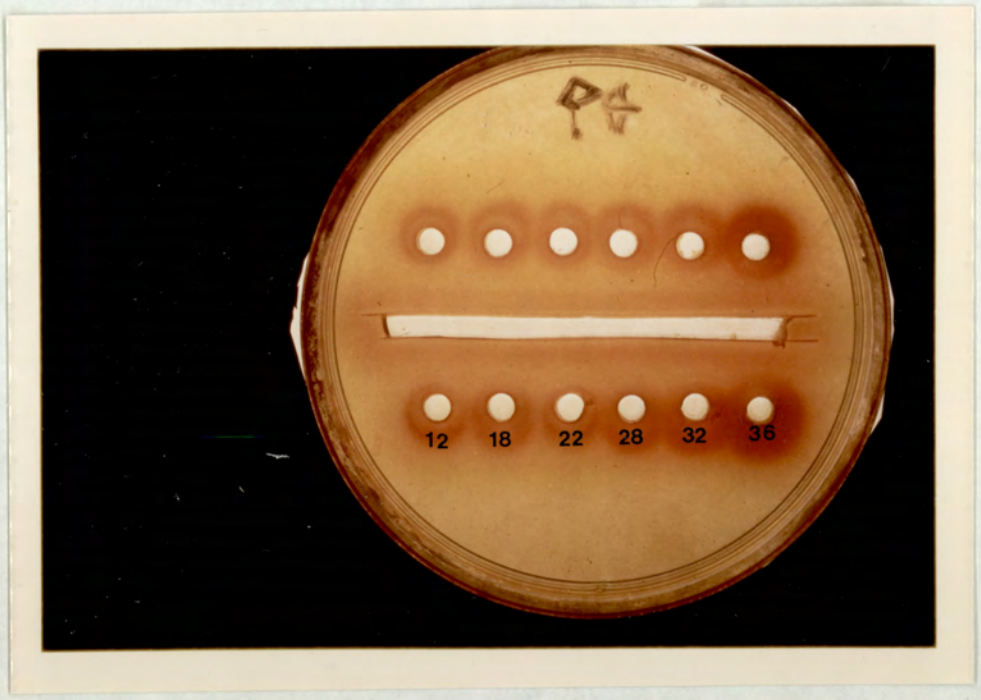


Fig. 62. Immunodiffusion as in Fig. 61 but using enzyme antiserum to the placental isoenzyme A.

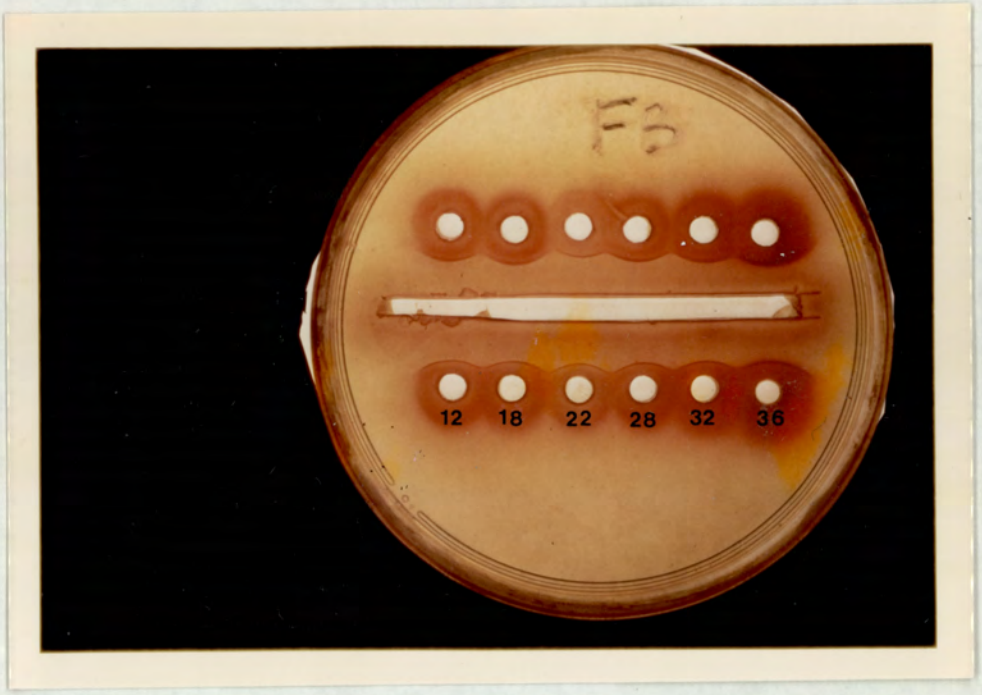


Fig. 63. Immunodiffusion of enzyme antiserum to foetal isoenzyme B of n-acetyl-β-glucosaminidase against pregnancy serum taken between 12 weeks and 38 weeks of pregnancy. An enzyme immunoprecipitate is seen to progressively increase.

Fig. 64. Immunodiffusion as in Fig. 63 but using antiserum to the placental B isoenzyme. Immunoprecipitate is seen to progressively increase.

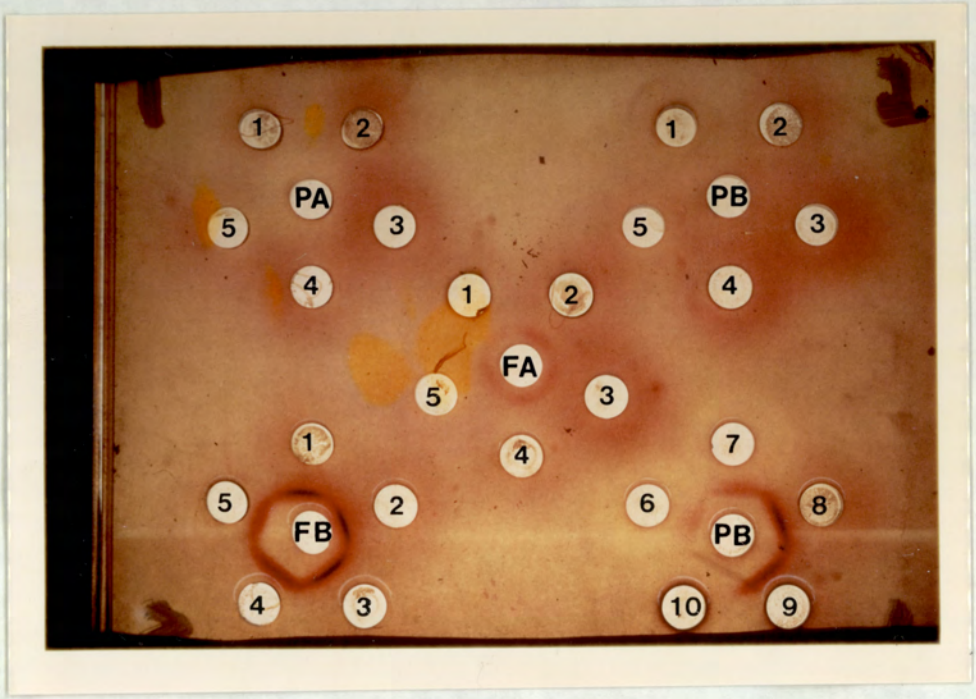


Fig. 65. Immunodiffusion of tissue extracts and pregnancy sera against antiserum raised against the foetal liver isoenzymes A and B and the placental isoenzymes A and B.

(1) Pancreas (2) Intestine (3) Spleen (4) Kidney
(5) Liver (6) 12 week serum (7) 38 week serum
(8) Foetal liver extract (9) Placental extract
(10) Normal male serum.

to be heterogenous but the placental A and B were found to be organ specific although cross reactions were found with the foetal liver isoenzymes.

The difficulties hitherto in finding the source of origin of the pregnancy isoenzyme have been in the main due to the fact that the properties measured by electrophoresis and chromatography on DEAE cellulose would be unaltered by molecular substitutions.; Such substitutions would not affect the size or charge but could be changed by molecular binding to other components in biological fluids.

From these data it is concluded that the pregnancy serum P isoenzyme is of placental origin and that the foetal isoenzymes are antigenically similar to that found in adult tissues. The progressive increase in the pregnancy serum during gestation can be estimated immunologically using a placental specific antiserum.

4.5. 2 β-Glucuronidase. No inactivation of the serum isoenzymes occurred when antibodies against both A and D isoenzymes of β-glucuronidase were added to pools of pregnancy serum taken during the 38th week of pregnancy. The decrease in enzyme activity of control tubes without antisera was similar to that found for sera with antisera added.

Using gel diffusion an immunoprecipitate formed when the four antisera were assayed against the purified isoenzyme extracts and also with the crude placenta and foetal liver homogenates. Cross reaction did occur between the four antisera and the purified isoenzyme extracts.

Both the foetal A and foetal D antiserum formed enzyme immunoprecipitates with the adult tissue extracts of liver, bone, kidney, pancreas and intestine. The antisera to placental A and D isoenzymes did not react with adult tissue. Fig. 66 illustrates the results obtained.

No immunoprecipitate was formed when the antisera were tested

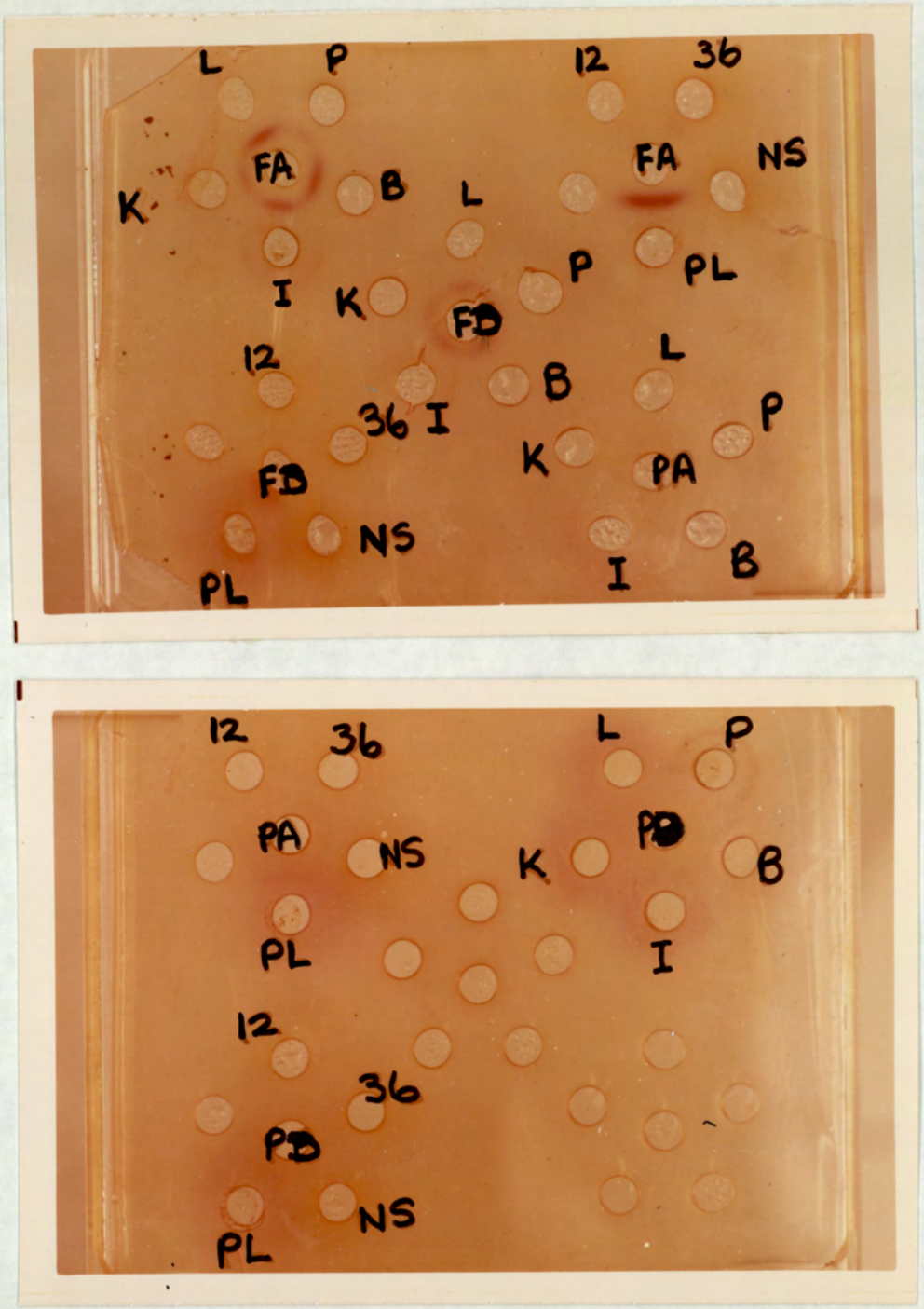


Fig. 66. Immunodiffusion of tissue extracts, normal serum and pregnancy serum against β-glucuronidase isoenzymes A and D antisera of placental and foetal liver. FA and FD are foetal liver A and D antisera and PA and PD antisera to the placental isoenzymes. L — LIVER ; P — PANCREAS ; PL — PLACENTA ; B — BONE ; I — INTESTINE ; K — KIDNEY ; NS — NORMAL SERUM ; 36 AND 12-WEEKS OF PREGNANCY.

against pregnancy serum, cord serum or normal serum.

The titre of the placental A and placental D antisera was found to be 1/64 and 1/16 respectively and for the foetal A and foetal D, 1/64 and 1/32 respectively. The concentration of the antigen in each case was 1 mg./1 ml. enzyme protein.

DISCUSSION

All four antisera cross reacted with the four purified isoenzymes and were therefore antigenically similar. The foetal liver antisera were antigenically indistinct from adult tissues whereas the placental isoenzymes were antigenically distinct. None of the antisera reacted with the isoenzyme of pregnancy serum and therefore the source of origin could not be located immunologically.

The passage of an isoenzyme from placenta to the maternal circulation may cause a change in structure such as unfolding of the chain or a comformational change in the enzyme molecule such as probably occurs with n-acetyl-β-glucosaminidase. In the case of β-glucuronidase the enzyme molecule may have changed one step further and therefore no antigenic similarity would then be found between the serum and tissue enzymes.

An increased isoenzyme A activity has been found in women taking oral contraceptives and the possibility exists that the increase would be due to a response of some tissue to an increased oestrogen concentration.

The placental antisera reacted with adult tissue extracts in a similar manner to that found for n-acetyl-β-glucosaminidase. Furthermore, the foetal isoenzymes were found to be antigenically similar to adult tissue isoenzymes which were also similar to those found for n-acetyl-β-glucosaminidase.

It would be likely that both β-glucuronidase and n-acetyl-β-glucosaminidase arise in the same organ since it has been shown by Linker et al.(1955) that oligosaccharides formed from hyaluronic

acid by hyaluronidase are further degraded by both enzymes.

4.6 Histochemistry.

L-methionine was found to activate both leucine aminopeptidase and alanine aminopeptidase found in placental tissue extracts. Further confirmation was obtained by histochemical reactions with and without L-methionine as activator.

A marked activation of both enzymes was found when 0.5M L-methionine was added to the substrate. Figs. 67 and 68 illustrate the activation found. Wachstein et al. (1963), Kraver and Ludwig (1968), Fox and Kharkongor (1969) and Curzen (1967) found that leucine aminopeptidase was in the connective tissue stroma of the villi and in the walls of blood vessels. The Hofbauer cells could be distinguished from the fibroblasts by their high content of leucine aminopeptidase (Fox and Kharkongor, 1969). A moderate reaction has also been noted in cytotrophoblastic cells and in general stronger reactions were found in decidual cells, (Wachstein et al., 1963).

The distribution of the enzymes leucine and alanine aminopeptidase was found to be similar to that found by these workers.

4.7 Sub-cellular localization of the placental enzymes.

Sub-cellular investigations were made with the object of identifying whether a particular cell fraction would contain a specific isoenzyme which in turn may give some indication of the enzyme or isoenzyme function.

The sub-cellular fractions obtained by ultracentrifugation in a suchrose gradient were filmed and stained by haemotoxilyn and eosin. The results of which gave an indication together with the theoretical position of the sub-cellular fractions at the sucrose gradient interfaces. Table 20 lists the cellular fractions located at each sucrose interface.

Three parameters were investigated:- total enzyme assay, isoenzyme fractionation and in the case of n-acetyl-β-glucosaminidase and β-

Table 20. Identification of cellular fractions from placental extract after ultracentrifugation in a sucrose gradient.

Fraction	Sucrose concentration	Cellular fraction
1	65%	None
2	65/50%	Microsomes, mitochondria and trace of nuclei
3	50/40%	Nuclei and mitochondria
4	40/30%	Membranes
5	30/20%	None
6	20/10%	Some cytoplasm
7	10/ 0%	Cytoplasm, lysosomes and soluble
8	Nil	Lysosomes and soluble enzymes

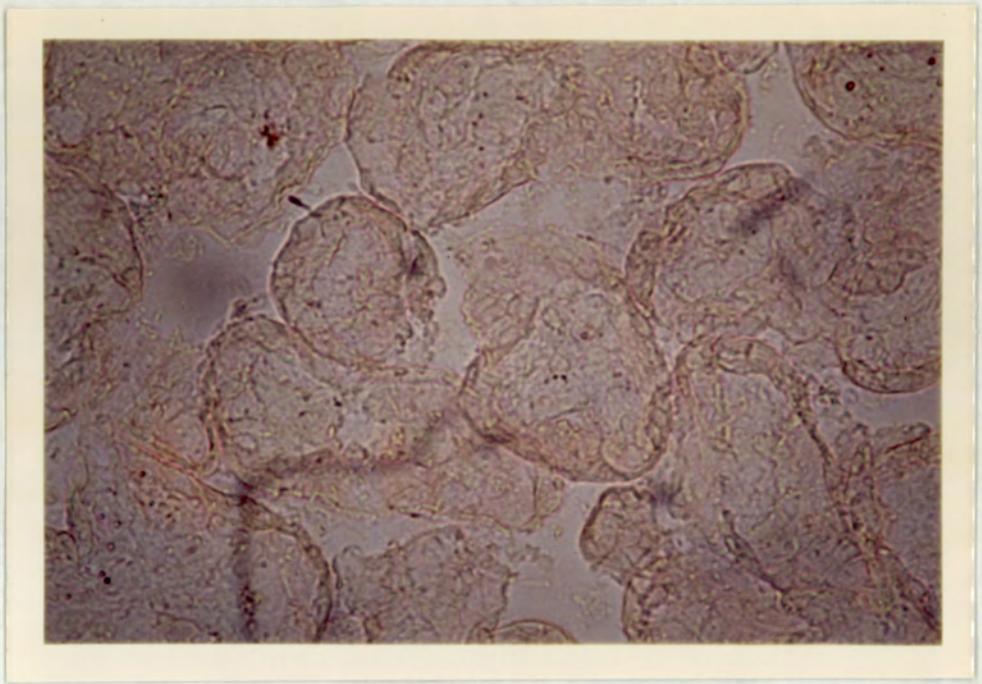


Fig. 67 (a) Histochemical localization of leucine aminopeptidase in a frozen section of placental tissue.

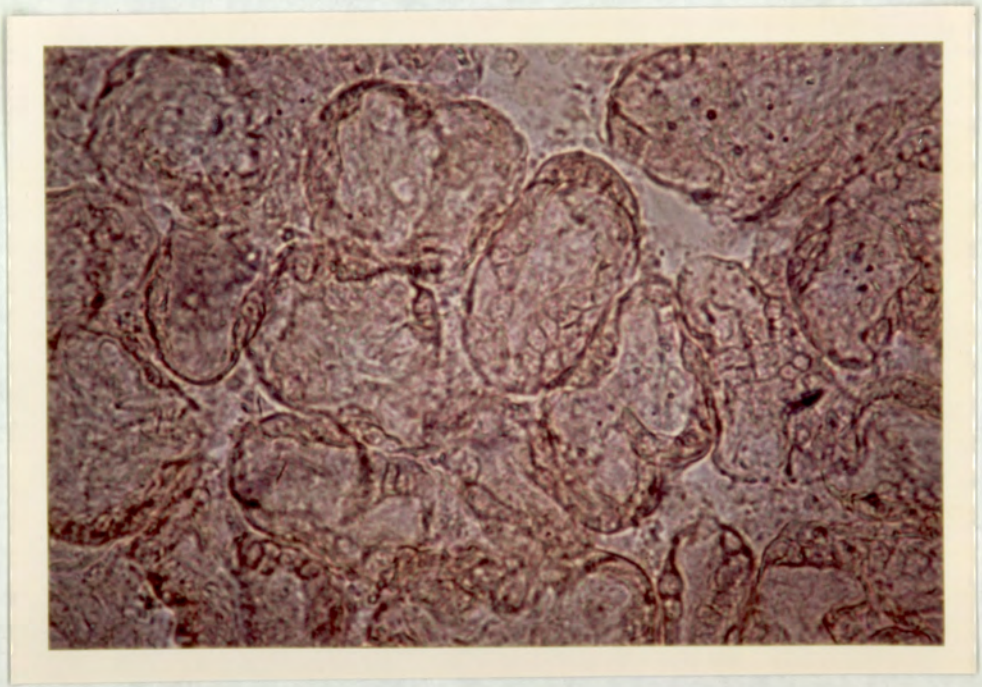


Fig. 67 (b) Leucine aminopeptidase localized in the presence of L-methionine. Section as in Fig. 58 (a) above.

Fig. 68 a. The localization of alanine aminopeptidase in a frozen section of placental tissue.

Fig. 68 b. Localization of alanine aminopeptidase in the presence of L-methionine. Section of placental tissue as in Fig. 57 a above.

glucuronidase an immunological investigation.

4.7. 1 Total enzyme assays. The greatest cystine aminopeptidase activity (Fig. 69) was detected in the fraction containing membranes with 10% to 15% of total activity in each adjoining fraction. 5% and 8% activity was detected in the cytoplasm and aqueous fractions respectively.

Fig. 70 illustrates the results obtained with leucine aminopeptidase with the greatest enzyme activity in the aqueous layer. Twelve per cent of the total enzyme activity was found in the membrane fraction.

Alanine aminopeptidase activity was only found in the aqeous and cytoplasm fractions (Fig. 71). No enzyme was detected in the membrane fraction.

The results for total alkaline phosphatase activity were similar to that found for leucine aminopeptidase with greatest activity in the aqeous layer followed by the membrane fraction (Fig. 72).

The pattern of results for n-acetyl-β-glucosaminidase and β-glucuronidase (Figs. 73 and 74) were similar with greatest enzyme activity in the cytoplasm and aqeous fractions. The membrane fractions contained between 5 and 9.5% activity in each instance.

4.7. 2 Iso-enzyme assays. The isoenzyme location methods were not capable of detecting enzyme activity values of 10% and under (each placental extract had been diluted to an enzyme concentration found in serum). Therefore with the exception of cystine aminopeptidase the placental isoenzymes of each enzyme was or were detected in the lysosome and aqeous fractions. Cystine aminopeptidase on the other hand had greatest enzyme activity in the membrane section, where the isoenzymes were also detected. n-Acetyl-β-glucosaminidase isoenzymes A and B were found in approximately equal concentration in the lysosome and aqeous fractions but the B isoenzyme was twice the concentration of the

A fraction in the membrane fraction. The isoenzymes were of greatest activity in the lysosomal and aqueous fractions. Fig. 75 illustrates the results obtained.

β-glucuronidase isoenzymes A and D were also in approximately equal and greatest concentrations in the lysosomal and aqueous fractions. The membrane fractions contained twice the concentration of the B isoenzyme (Fig. 76).

4.7. 3 Immunological investigations. This technique was the most sensitive employed. Fig. 77 to 80 illustrate the enzyme precipitin lines obtained with antisera to n-acetyl-β-glucosaminadase A and D isoenzymes of placenta and foetal liver. The results in general followed that found with the isoenzymes except that fractions with lower enzyme concentrations were found to contain trace quantities of isoenzymes.

The membrane, lysosomal and aqueous layers were found to contain the isoenzymes. Since there were cross reactions between the antisera of foetal liver A and B with the isoenzymes A and B of placenta there was no means of differentiating the foetal from the placental extracts.

The antisera to β-glucuronidase reacted in a similar manner to n-acetyl-β-glucosaminidase, in general following the pattern of the isoenzymes, with zones of activity in the membrane, cytoplasm and aqueous fractions.

4.7. 4 The aqueous fraction. The aqueous fraction described above could contain both lysosomes and soluble enzymes. Therefore the aqueous layer was recentrifuged with a button of 65% sucrose. The interface between the aqueous layer and the button would then contain the lysosomes. No increase in enzyme activity was detected at this interface over that found for the aqueous layer.

DISCUSSION

With the exception of alanine aminopeptidase all the enzymes investigated were localized both in membrane and in lysosomes.

The greatest activity was found in the soluble fraction.

Very little information is available regarding the intracellular distribution of arylamidase (enzymes hydrolysing amino acid arylamides such as leucyl-β-naphthylamide). Mahedevan and Tappel (1967) found arylamidase activity of rat liver and kidney to occur in lysosomes. Kowlessar et al. (1961) found activity in the microsomal and supernatant fractions of rat kidney. From histochemical studies however, Sylven and Bois (1964) and Sylven and Lippi (1965) have suggested that arylamidases belong to the lysosomal class of hydrolases. Although some enzyme activity has been found in membranes the greatest amount has been found in the lysosomal and soluble fractions.

Contractor and Shane (1972) found B-glucuronidase activity in lysosomes with the remainder in endoplasmic reticulum. Their homogenization technique using the nitrogen bomb was gentler than the method used here which would give larger fragments of endoplasmic reticulum. Fishman (1967) found the origin of β-glucuronidase as the lysosome.

n-Acetyl-β-glucosaminidase and β-glucuronidase have become one of more than a dozen acid hydrolases which inhabit the lysosome organelle in a latent condition until the cell dies at which time the lytic enzymes are released into the cytoplasm (Fishman, 1967). The isoenzyme B of n-acetyl-glucosaminidase was found to be in higher concentration than the A form in membranes which would suggest that form A represents a form present in membranes and the specificity of such an isoenzyme would offer a possible recognition site for transport of N-acetyl-glycosamine containing substance or some other membrane reaction. Similar results have been found by Robinson and Stirling (1968) with human spleen. Fishman et al. (1967) have suggested a similar location for β-glucuronidase although it would be clear that both forms could occur in a single cell type.

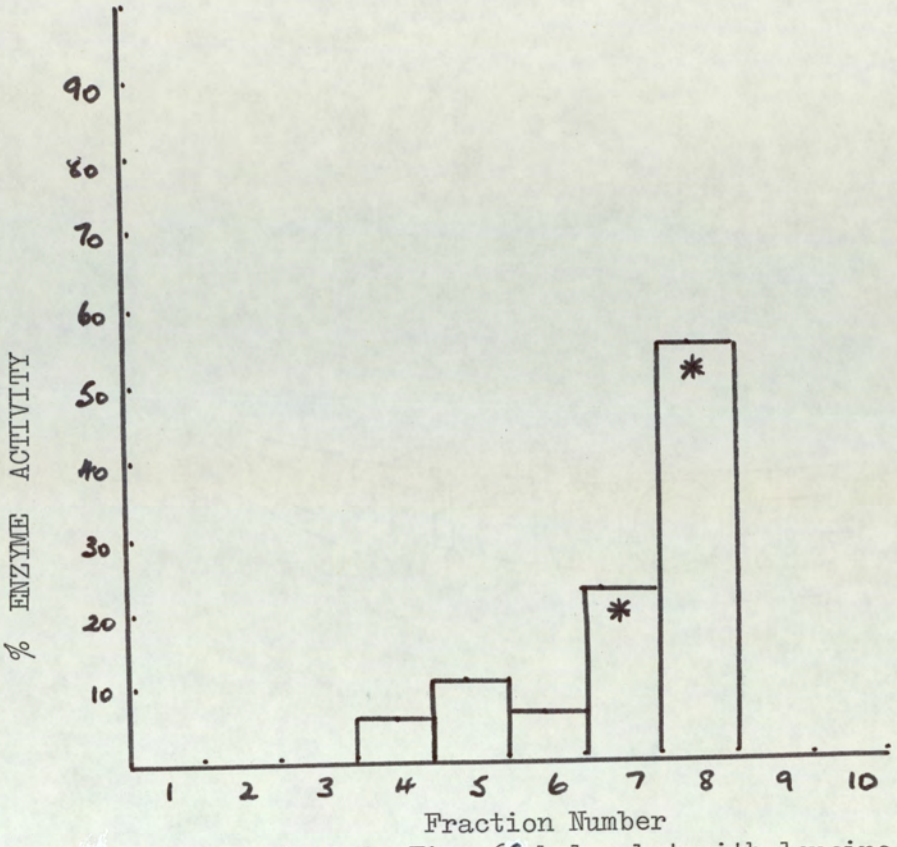


Fig. 70. As described in Fig. 69 below but with leucine aminopeptidase activity.

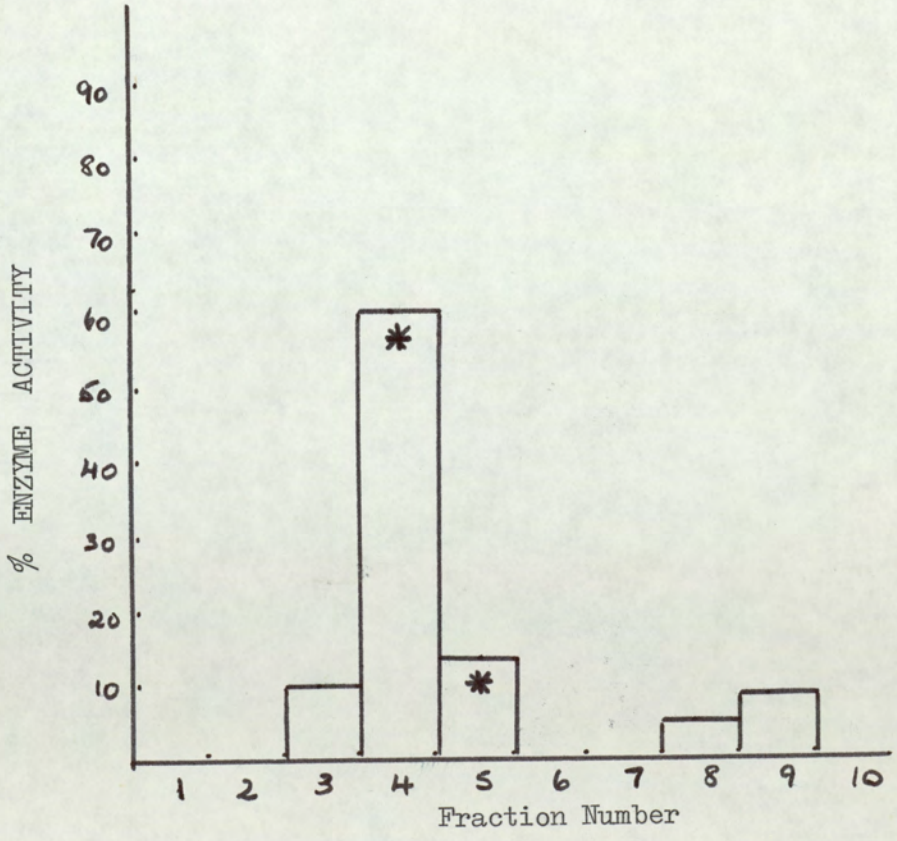


Fig. 69. Total cystine aminopeptidase activity after ultracentrifugation of placental extract in a suchrose gradient. Isoenzymes detected by electrophoresis.

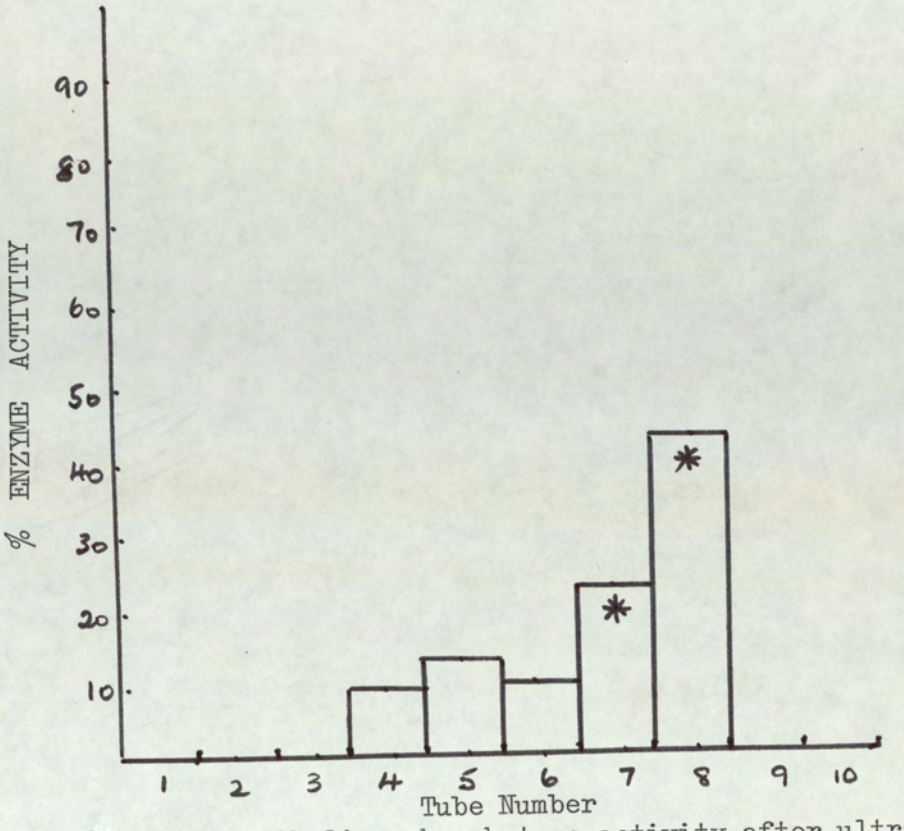


Fig. 72. Total alkaline phosphatase activity after ultra-centrifugation as in Fig. 71 below.

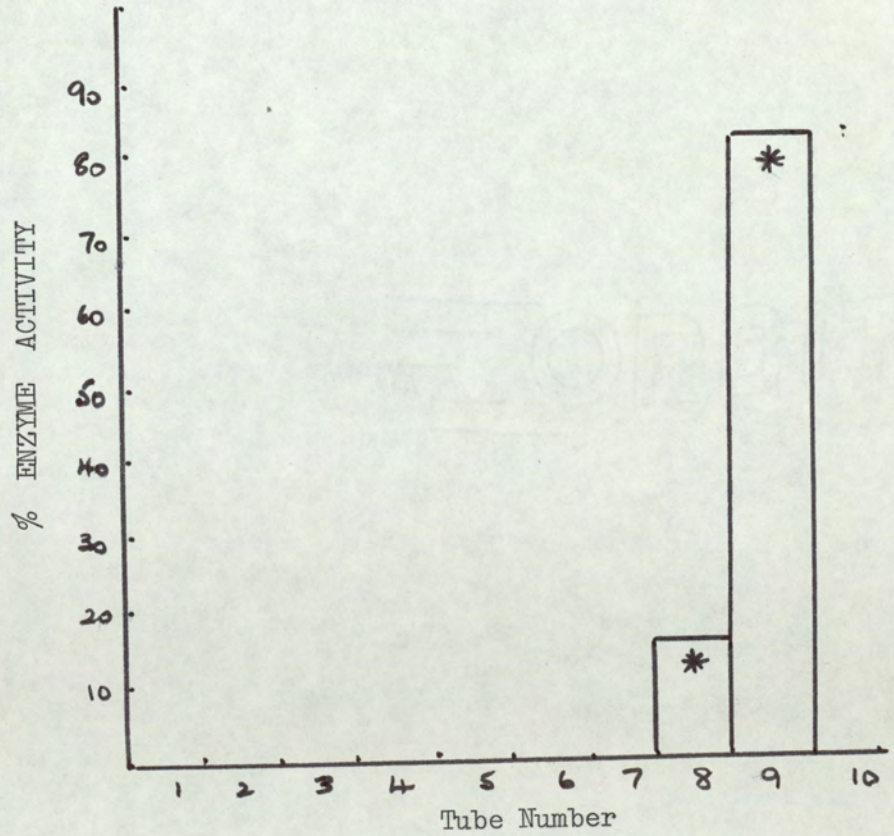


Fig. 71. Total alanine aminopeptidase activity after ultra-centrifugation of placental extract in a suchrose gradient. Isoenzymes detected by electrophoresis.

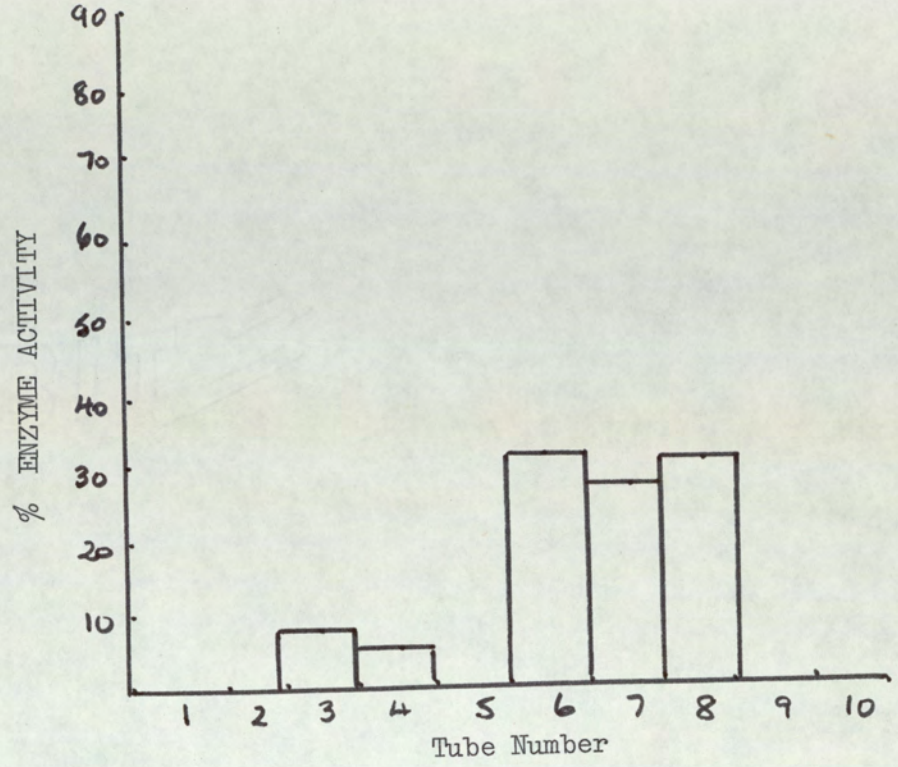


Fig. 74. As described in Fig. 73 below but with β-glucuronidase activity.

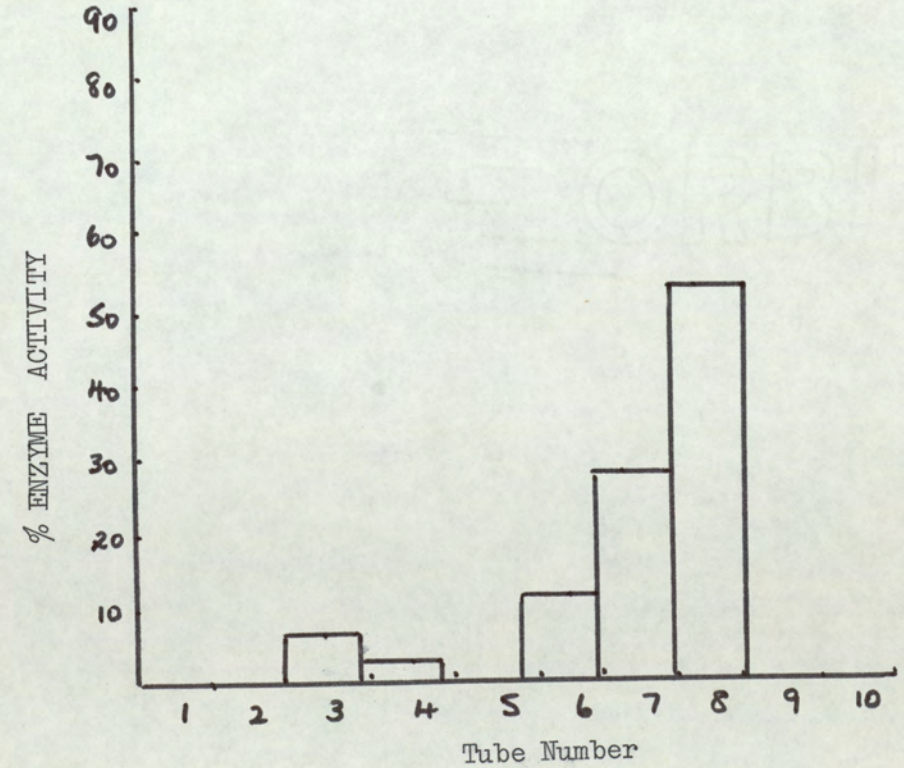


Fig. 73. Total n-acetyl-β-glucosaminidase activity after ultracentrifugation of placental extract in a suchrose gradient.

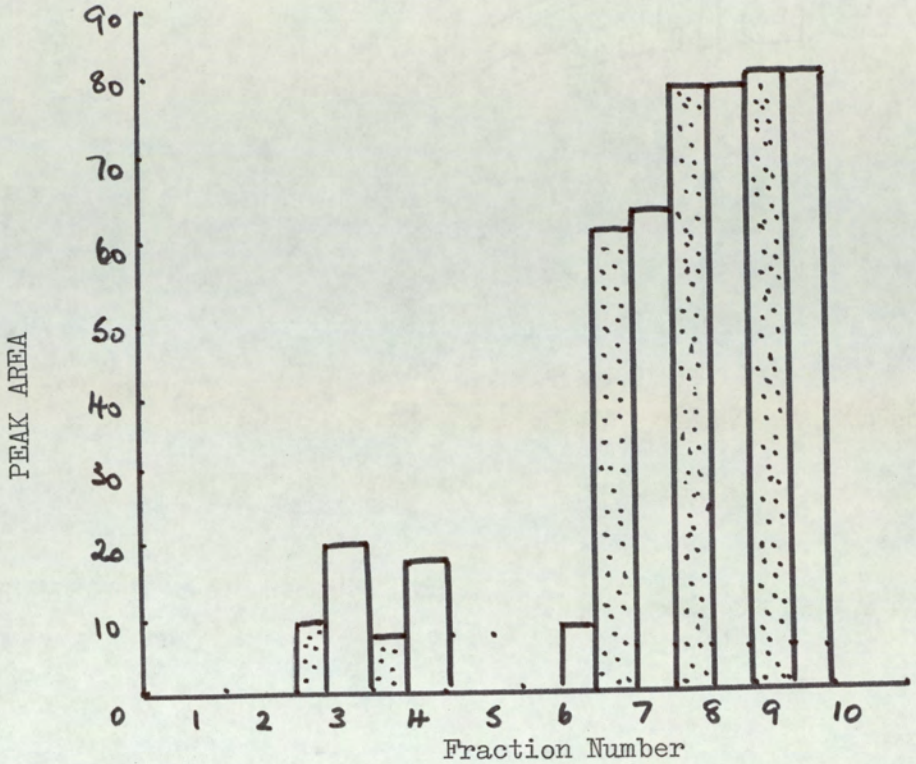


Fig. 74. Isoenzymes of β -glucuronidase after ultracentrifugation of placental extract. Isoenzyme A, ⊡ ; isoenzyme B, □ .

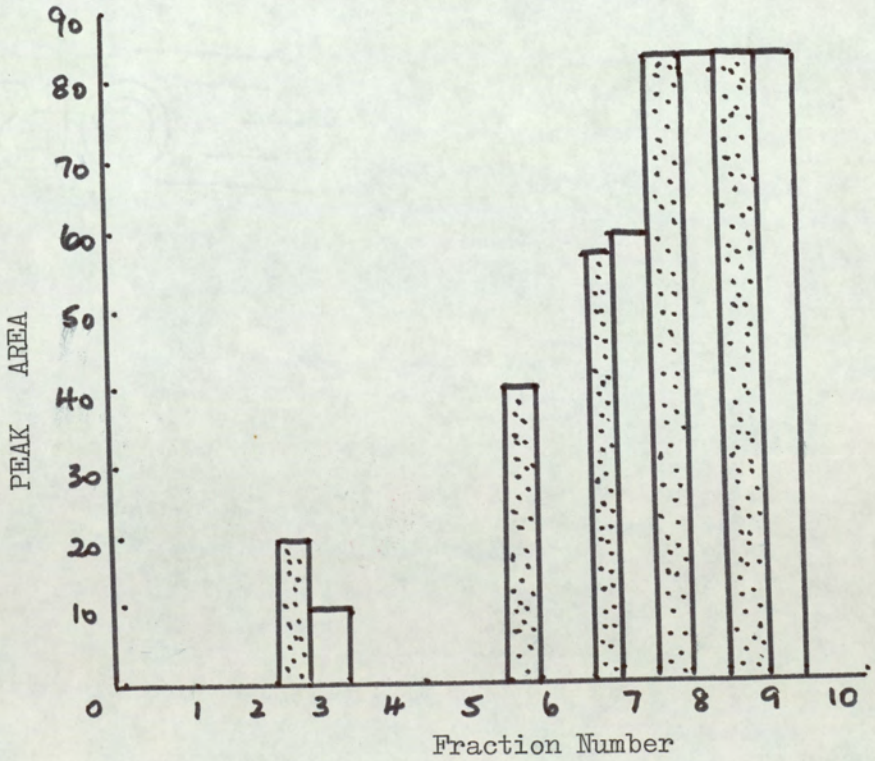


Fig. 75. Isoenzymes of n-acetyl-β-glucosaminidase after ultracentrifugation of placental extract in a suchrose gradient. Isoenzyme A, ⊡ ; Isoenzyme B, □ .

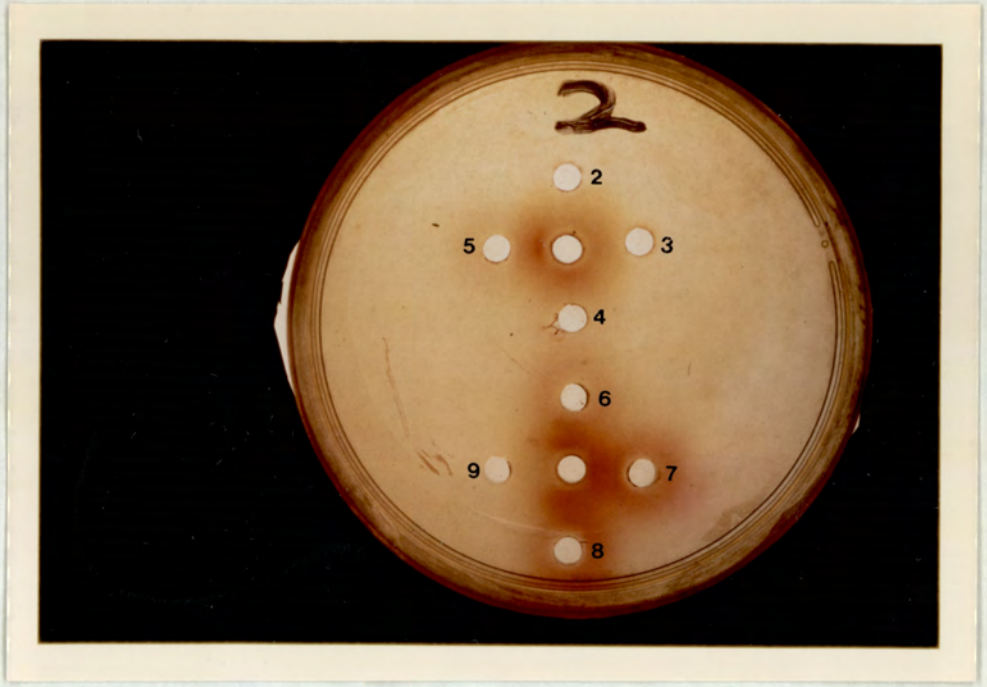


Fig. 78. Immunodiffusion as in Fig. 77 below but with antiserum to placental B isoenzyme of n-acetyl-β-glucosaminidase.

Fig. 77. Immunodiffusion of subcellular fractions 2 to 9 against antiserum to n-acetyl-β-glucosaminidase of placental A isoenzyme.

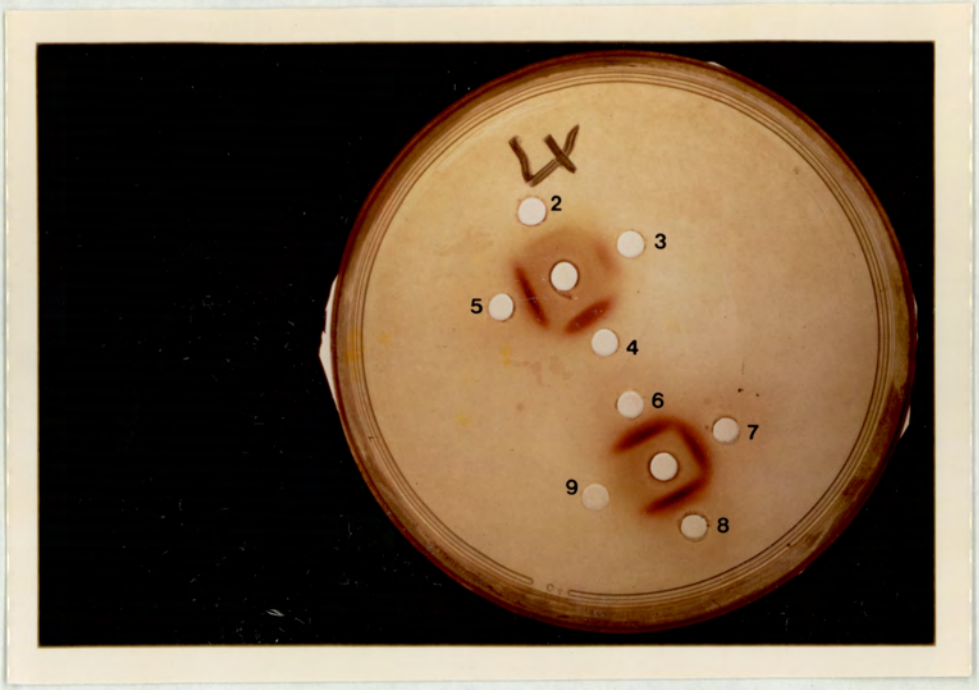


Fig. 80. Immunodiffusion as in Fig. 79 below but with antiserum to foetal B isoenzyme.

Fig. 79. Immunodiffusion of subcellular fractions 2 - 9 against antiserum to foetal n-acetyl-β-glucosaminidase A isoenzyme.

5. GENERAL DISCUSSION

DISCUSSION

It is abundantly evident from the results of this investigation that certain maternal serum enzymes increase progressively during pregnancy. Furthermore the increase in each enzyme can be attributed to a placental isoenzyme appearing in maternal serum.

Maternal serum was found to contain isoenzymes of alkaline phosphatase, leucine and alanine aminopeptidase of non-placental origin. In the main enzymes of non-placental origin were found to originate in the liver. The progressive increase in the total enzyme activity was therefore contributed to by isoenzymes of placenta and liver. A wide scatter of results was found during each week of pregnancy and although the estimations were automated to better the reproducability the scattergraphs were not improved upon. The greatest advantage of the automated methods was that one technician could estimate up to 120 assays per day which could be a major consideration if all patients attending ante-natal clinics would be screened.

It is also clear that the placental isoenzymes of maternal serum should be estimated rather than the total enzyme. Total enzyme assay may give an index of the overall state of the mother during pregnancy but is unlikely to be of value in assessing foeto-placental function. Results of serum enzyme assays have not to date been favoured by obstetricians as good indexes of foeto-placental function and a contributory factor could well be that in the past only total enzyme assays have been used. The one exception to this has been alkaline phosphatase where the heat stable alkaline phosphatase fraction has been utilized. Assays of heat stable alkaline phosphatase have varied tremendously in technique with differences in heating temperatures, time, pH and substrate concentrations, all of which would not improve clinical evaluation.

Isoenzymes of foetal origin have not been detected throughout this investigation and therefore the maternal serum enzyme assays would be an index of placental rather than foeto-placental function, although

foetal function could be secondary to that of placenta. The results of serial determinations of maternal serum enzyme activities were found to increase progressively and serial estimations to detect any decrease in an individual may well be of clinical value as has been reported by Hunter (1969) in respect of heat stable alkaline phosphatase. Similar situations exist at present with other tests of foeto-placental function, for example urinary oestrogens and serum placental lactogen, where serial estimations have been found to be of more value than an isolated estimation.

The scattergraphs of the results have been found to fall into two categories. The first group include those which linearly increase, carboxypeptidase, alanine aminopeptidase and alkaline phosphatase. The second group include those where the increase was progressive until the 36th week of pregnancy and thereafter until term remain more or less unchanged - cystine aminopeptidase leucine aminopeptidase and n-acetyl-β-glucosaminidase fall into this category.

A linear increase in enzyme activity would be expected in a major organ such as the placenta, where exchange of material between mother and foetus would be necessary for the maintenance of pregnancy. The production of increasing amounts of enzymes depends on the functional status of the placental cellular elements, which in turn would reflect the functional capacity of the placenta. The scattergraphs of the serum enzymes are similar to the scattergraphs found by Boyd and Hamilton (1970) while plotting the crown-rump length of the foetus and the weight of the placenta. This would suggest a relationship between foetal growth and serum enzyme activities and parallel with foetal growth one would expect increased metabolic activity.

The scattergraphs found for the enzymes compared to Boyd and Hamilton's scattergraphs of foetal and placental weight were very similar. The enzyme concentrations would be more likely to follow foetal or placental weights which would parallel cell mass rather

than diameter.

Scattergraphs showing a non-linear increase in maternal serum enzymes with little if any enzyme increase during the last few weeks of pregnancy may be explained by the foetus having taken over the role of these enzymes and by the ageing of the placenta. Both explanations are inter-related in that degenerative changes are accompanied by changes in metabolic processes. Wislocki and Dempsey (1946) investigated degenerative changes in the syncytiotrophoblast and found by the end of pregnancy that the cells contained much less fat and there was no glycogen in the syncytium. The number of mitochondria (phospholipids) and the degree of basophilism declined while the quantity of phosphatase increased. The basophilism was shown by Dempsey and Wislocki (1945) to be due to the disappearance of the nucleoproteins and their gradual replacement by phosphates. Such examples of changes in the placenta are sufficient to illustrate why alkaline phosphatase on the one hand would increase linearly while other enzymes involved in glycogen and phospholipid metabolism may cease to increase towards the end of pregnancy. Benirschke and Driscoll (1967) point out that placental ageing in a general way are indicative of its gradual maturation rather than its degeneration.

The enzymes that cease to increase towards the end of pregnancy could also be related to the four 'indices of maturity' as described by Becker (1962). The indices are the decrease in the villous diameter, increase in the foetal vascular spaces in the villi, diminution of the peripheral intravillous connective tissue and a marked increase in the connective tissue sheaths of the vessels in the trunci chorii. Although no evidence has been found in the present investigation the possibility exists, however remote that the increase in serum enzymes could be purely an index of placental degeneration. Placental degeneration would involve the retraction and autolysis of cells with the liberation of their enzyme contents to the maternal circulation.

The maternal serum enzymes increase from the eighteenth week of pregnancy which coincides with considerable placental growth and differentiation (Boyd and Hamilton (1970). Prior to this time it is likely that the uterus would be supplying the energy requirements of the foetus. In addition to the lysosomes the enzymes have been found both in the membrane and soluble fractions, and since maternal blood bathes the cellular membranes the placental isoenzyme is likely to seep into the maternal circulation.

All the maternal serum enzymes found to progressively increase during pregnancy are all classed as hydrolases which catalyse the hydrolysis of one compound or a series of related compounds. None of these enzymes are involved in energy supplying reactions but do produce substances essential to foetal nutrition. These products fall into Page's (1957) second group of compounds that cross the placental barrier e.g. glucose amino acids, calcium and phosphate. The transport of these substances may include active processes.

The hydrolases investigated included the peptide hydrolases, which catalyse the hydrolytic cleavage of peptide bonds, the glycosidases which hydrolyse glycosidic bonds and the ester hydrolases which hydrolyse ester linkages. The hydrolases are likely to increase to satisfy the increasing requitements of the foetus in carbohydrates, fats and proteins.

In placental tissue the ester hydrolase alkaline phosphatase has been located at the surface of the trophoblastic syncytium (Wachstein et al., 1963) and is found in abundance in those tissues concerned with transport of nutrients. It is often present in secretory organs and developing tissues. Although it is not possible to assign a precise function for alkaline phosphatase, undoubtedly the enzyme is concerned in the localized production of high concentrations of phosphate in the placenta which would be utilized by the developing embryo.

It is also of significance that the enzyme is localized at the absorptive surface, suggesting a direct role in the transport of

nutrients across the epithelial membrane. Fuchs and Fuchs (1956) found that the guinea pig foetal plasma contained twice as much inorganic phosphate as the maternal plasma.

The specific glucose-6-phosphatase activity in placental extracts and maternal serum has not been characterized during this investigation. The phosphatase activity demonstrated in vitro upon glucose-6-phosphate by Walker et al. (1968) can be attributed to the action of alkaline phosphatase. The possibility is that glucose may be formed by the terminal action of a non-specific phosphatase in the placenta. The same workers found hydrolysis of fructose 1, 6 diphosphate which could also be due to the non-specific action of alkaline phosphatase. Hagerman et al. (1959) has suggested that this could account for the formation of the small amounts of fructose formed by human placental tissue.

It is likely that the non-specific action of alkaline phosphatase can exert control over glucose formation from glycogen and gluconeogenic precursors in placental tissue.

Another possible role for the increased alkaline phosphatase is that of a pyrophosphatase. Patients with a deficiency of cytoplasmic alkaline phosphatase in certain cells (hypophosphatasia) have pyrophosphaturia, suggesting that this inborn error of metabolism is associated with an impaired metabolism of inorganic pyrophosphate (Russell, 1965). It is therefore possible that the physiological role of alkaline phosphatase is that of an inorganic pyrophosphatase.

Alkaline phosphatase is known to be present in nuclei, Danielli, (1953), and could play a role in nucleic acid synthesis by hydrolysing pyrophosphate and in the phosphorylation and dephosphorylation of nuclear proteins.

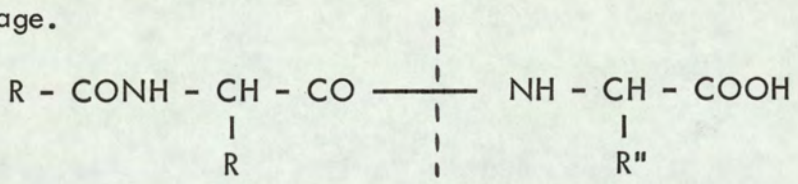
A progressive increase in the activity of maternal serum peptidases has been found. These enzymes would be essential for the hydrolysis of proteins to amino acids for ease of placental transfer to the foetus.

One of the peptidases found to increase has been carboxypeptidase which is active on a wide range of polypeptides and proteins. From

synthetic studies no restriction has been found on the length of peptide from which the carboxyl-terminal amino acid is removed.

From the viewpoint of specificity and mode of action Newath (1960) has suggested that the resistance of a protein to the action of carboxypeptidase would be more significant than its sensitivity to the enzyme. A protein substrate will obviously be refractory if it is devoid of a carboxyl terminal group such as a cyclic polypeptide chain or if the terminal group is an α-amide as in the case of oxytocin or vasopressin. Other configurations resistant to the action of carboxypeptidase would include those where the carboxyl terminal group does not conform to the specificity requirements of the enzyme, such as carboxyl-terminal arginine or lysine residues.

All known substrates of carboxypeptidase are hydrolysed at the peptide bond adjacent to the free carboxyl group. The most sensitive known peptide substrates are acylated dipeptides of the type illustrated below in which the dotted line indicates the point of cleavage.



The terminal free carboxyl group is essential for the action of the enzyme. Smith (1951) found that carboxypeptidase in contrast to dipeptidases which require both a free amino group and a free carboxyl group adjacent to the sensitive peptide linkage, can dispense with the amino group. Typical dipeptides such as glycyl-L-tyrosine and L-tyrosyl-L-tyrosine were found to be hydrolysed extremely slowly.

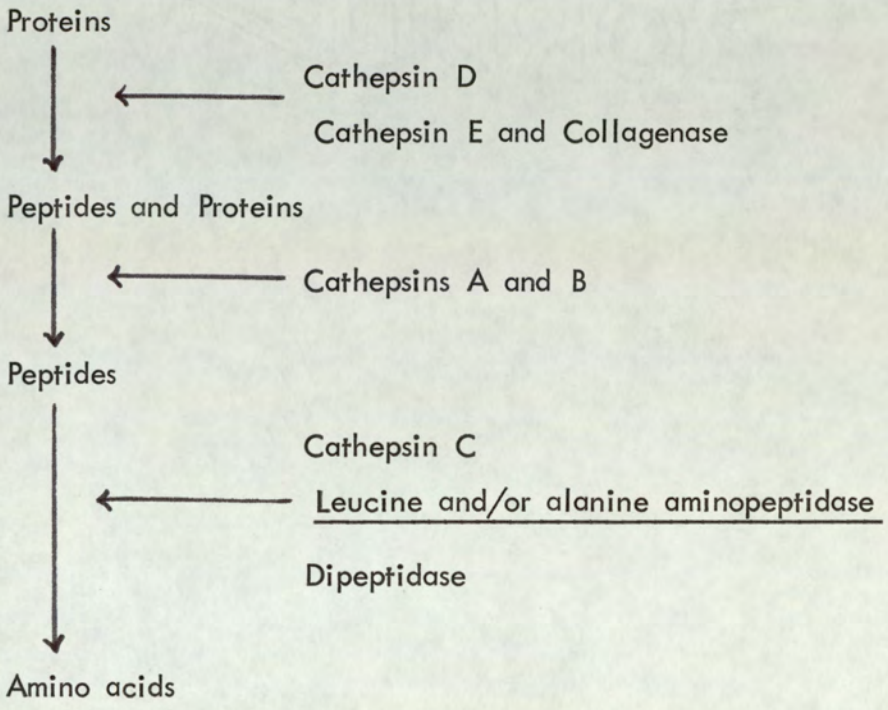
During recent years confusion has arisen regarding what names to assign enzymes which hydrolyze B-naphthylamide substrates. It would therefore be pertinent at this stage before discussing the role of leucine cystine and alanine aminopeptidase to briefly clarify the enzyme nomenclature. The commonly used term 'leucine amino-

peptidase' hydrolyzes unsubstituted amides and peptides which possess a free α amino group and are of the L-configuration. The nature of the R' group on the peptide or amide nitrogen influences the rate of hydrolysis Smith et al. (1960). In L-leucyl-β-naphthylamide the R' group consists of the β-naphthyl ring, as the nitrogen bonded to the amino acid is connected directly to a ring carbon. In contrast the peptide substrates containing an aromatic ring, a CH_2 group intervenes between the ring and the peptide nitrogen. Nachlas et al. (1962) suggested that it was unrealistic to characterize the enzyme by the amino acid and suggested the term 'aryl aminopeptidase'. Other workers have used the term 'leucylnaphthylamide hydrolases' (Thompson and Schwartz, 1959 and O'Connell and Winzler (1963). Other workers Novikoff et al. (1960) have used the terms 'amino acid naphthylamides' or more generally 'arylamidases.'

With respect to the enzyme cystine aminopeptidase the term 'oxytocinase' has been used by a number of workers including Tuppy (1968). Other workers Wintersberger et al. (1960) and Tuppy et al. (1963) used the name oxytocinase to describe enzymes hydrolysing L-leucyl-β-naphthylamide. Throughout this work the peptidases described are the enzymes hydrolysing salts of β-naphthylamine.

Since the physiological role of both leucine aminopeptidase and alanine aminopeptidase are similar, they are best discussed together. Both enzymes were found to exist in membrane, lysosomes and in the soluble fraction. De Duve and Wattiaux (1966), Weissmann (1965) and Weissmann (1964) have provided histological studies of apparent protein hydrolysis in lysosomes. More detailed studies of protein degradation by lysosomal enzymes have been provided by Iodice et al. (1966) and by Coffey (1968). Proteins are hydrolysed primarily by cathepsin D and also by cathepsin E and collagenase (EC 3.4.4.19). The resulting peptides and also proteins can be further hydrolyzed by cathepsins A and B. Further action by the more specific peptidases, cathepsin C, arylamidase and the dipeptidases will result in the hydrolysis of some peptides. This concerted

action of the lysosomal proteases and peptidases results in the major hydrolysis of proteins to amino acids in the placenta. A schematic pathway of hydrolysis is shown below.



It is likely that in the placenta as in other tissues that the hydrolysis does not follow a linear pathway because of the involvement of multiple enzymes and enzymes of broad specificity. To maintain a sufficient amino acid pool for the growing foetus peptidase activity would need to increase progressively. Raekallio and Makinen (1971) have demonstrated histochemically and biochemically an increase in leucine aminopeptidase in wound tissue. The increase was to maintain an amino acid pool for essential protein synthesis (Schmidt, 1968).

Cystine aminopeptidase probably has an unique role from other peptidases in that it destroys oxytocin but the enzyme has not been found to be specific but will attack many peptide substrates, other than oxytocin. The present results suggest that the enzyme leucine aminopeptidase may have a non-specific effect upon oxytocin and that cystine aminopeptidase is rather more specific than has hitherto been recorded. Both enzymes have been found to be distinct by electrophoresis and furthermore all placental extracts were found to contain

leucine aminopeptidase but not all contained cystine aminopeptidase. Tuppy et al. (1963) and Sjoholm and Yman (1966) have described the use of L-leucine-β-naphthylamide as chromogenic substrate for the location of isoenzymes of oxytocinase and found a number of isoenzymes in pregnancy serum. During this investigation the enzyme 'oxytocinase' was found to have two isoenzymes when cystine-β-naphthylamide was used as substrate and their electrophoretic migration was totally distinct from the migration found for enzymes hydrolysing leucyl-β-naphthylamide. Our results confirm the work of Page et al. (1961) where two isoenzymes were detected in pregnancy serum.

Croxatto et al. (1953) assumed that the same enzyme was involved in the inactivation of both hormones, oxytocin and vassopressin. This assumption has been proved correct by the work of Tuppy and Wintersberger (1960), Stoklaska and Wintersberger (1959) and Glendening et al. (1965). The two forms of cystine aminopeptidase after separation on starch gel electrophoresis has been found to destroy oxytocin and vasopressin at similar rates by Glendening et al. (1965). There seems to be no doubt that the function of the enzyme cystine aminopeptidase is to hydrolyse the cyclopeptide hormones.

During the later months of pregnancy nearly the entire serum aminopeptidase activity as determined by means of L-cystine-di-β-naphthylamide as substrate has been found by Ryden (1966) to be due to 'oxytocinase'. Klimek (1966) points out that the oxytocin in pregnancy blood represents the difference between endogenous oxytocin and that inactivated by oxytocinase. Klimek (1966) has also pointed out that oxytocin and oxytocinase form a biochemical system which is dependant on both the foetus and the mother. Positive correlation exists between serum cystine aminopeptidase and the weight of the foetus and the placenta, and intrauterine death of the foetus is quickly followed by a decrease in the level of oxytocinasaemia. Klimek (1963) and Klimek and Krol (1964) have found that oxytocinasaemia is raised by exogenous and

endogenous oxytocin, a phenomenon described by these authors as an 'enzymatic block' which maintained pregnancy. As a result of the exhaustion of this block towards the end of pregnancy at a time when synthesis of oxytocinase is maximal minimal additional amounts of oxytocin (e.g. doses used in the intravenous test) are sufficient to disrupt the oxytocinasaemia towards the side of the hormone eliciting contractions of the uterus. Indication of labour by oxytocin 'titration' has been described by Turnbull and Anderson (1968a) and (1968b), and an automatic oxytocin infusion equipment has been described by Francis et al. (1970). The basis of the oxytocin 'tritration' may well be due to a shift in the enzyme/hormone equilibrium which would in turn induce labour.

The isoenzymes of cystine aminopeptidase have been found in the foetal membranes which confirms the work of Melander (1965), and it would be possible that the membranes may contribute to the inactivation of oxytocin.

γ-Glutamyl transpeptidase was found to increase in maternal serum but was found to originate in the liver. The increase in the non-placental enzyme is similar to that found for the isoenzymes of leucine aminopeptidase and alanine aminopeptidase which originate in liver. During pregnancy it is likely that the liver would be required to increase its metabolic output so as to maintain the mothers and foetal well being. Other peptidases found to increase have all been found to have a hydrolytic role only, whereas γ-glutamyl transpeptidase has both hydrolytic and synthetic properties. The peptides formed would be by the interaction between free amino acids and the tripeptide glutathione.

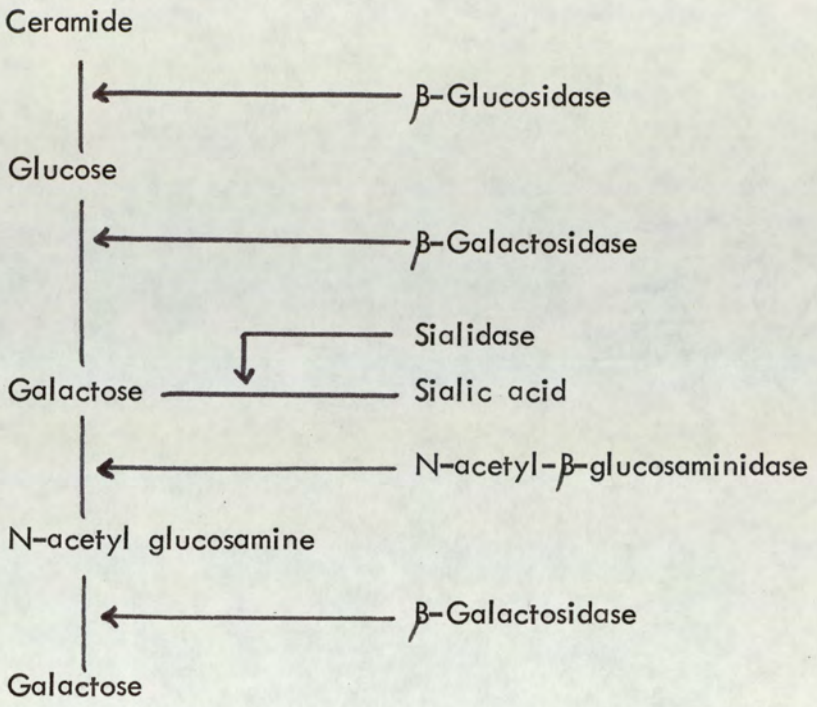
As further evidence that γ-glutamyl-transpeptidase was of liver origin can be seen by the maternal activity taking twice as long as other enzymes to decrease to the non-pregnancy values. Since the enzyme was found to originate in liver its estimation would be of no value as a test for foeto-placental function.

The glycosidases n-acetyl-β-glucosaminidase and β-glucuronidase

in mammalian tissues is by no means clear but since their distribution is similar to that of B-glucuronidase, it might be expected that the group should be involved in similar pathways.

It is generally assumed that the function of N-acetyl-β-glucosaminidase is to contribute to the hydrolytic degradation of glycoproteins, mucopolysaccharides or glycolipids. Natural substances may include erythrocyte-membrane glycoproteins, blood group substances and y-globulins all of which contain significant amounts of N-acetylhexosamine. Both β-glucuronidase and n-acetyl-β-glucosaminidase and n-acetyl-β-galactosaminidase are constituents of crude hyaluronidase complex acting on some of the oligosaccharides produced during the breakdown of hyaluronic acid and chondroitin (Halin 1947; Meyer et al., 1951; Linker et al., 1955). Since the enzymes B-glucuronidase, n-acetyl-B-glucosaminidase and n-acetyl-B-galactosaminidase are probably involved in the same pathways then the functions of all three in the placenta will be considered together. All three enzymes were located in membranes, lysosomes and the soluble fraction and the lysosomal enzymes are known to degrade a variety of mucopolysaccharides (Hutterer, 1966), but the pathways are not known in detail.

Gibian et al. (1966) has described the enzymes degrading hyaluronic acid, chondroitin 4-sulphate, chondroitin-6-sulphate, heparin and keratin sulphate. The structure of a typical mucopolysaccharide-protein complex such as chondroitin sulphate B (galactose-xylose-serine peptide) suggests that glycosidases and proteolytic enzymes are involved in their degradation. The glycosidases could hydrolyze the connecting sugars and proteolytic enzymes could hydrolyze the protein. The hydrolysis of glycolipids involves many of the glycosidases and the hydrolysis of a typical monosialoganglioside is schematically shown overleaf.



In another context a link between n-acetyl-β-glucosaminidase and β-glucuronidase was provided by the work of Linker et al. (1955) on the breakdown of hyaluronic acid. Hyaluronic acid composed of chains of alternating glycosidically linked residues of glucuronic acid and n-acetylglucosamine forms oligosaccharides by the random hydrolysis of β-glucosaminide linkages within the chain. These are further degraded to monosaccharides by the successive actions of β-glucuronidase and n-acetyl-β-glucosaminidase which removes terminal non-reducing sugar residues. Therefore changes in activity of both enzymes during pregnancy may be associated with alterations in the metabolism of mucopolysaccharides and of the connective tissue cells from which ground substances are derived.

Another possible function of β-glucuronidase is that it is concerned with the conjugation of oestrogenic hormones and thus limiting interference by maternal oestrogens in the genital development of the foetus (Bulmer, 1962). It is probably more reasonable to suppose that β-glucuronidase is concerned with the hydrolysis of glucuronides.

Substrates of physiological interest include a variety of steroid β-D-glucosiduronic acids and the lower oligosaccharides of hyaluronic acid hydrolysates (Linker, 1955). β-Glucuronidase has also been given a hydrolytic role in the glucuronic acid cycle of glucose

metabolism (Burns, 1959 and Hollmann and Touster, 1962), in order to explain the origin of free glucuronic acid, which is the obligate precursor of ascorbic acid and of xylulose.

It is likely that all or some of the above pathways may occur at one and the same time, and may involve B-glucuronidase and n-acetyl-B-glucosaminidase or both.

The biological significance of finding multiple forms of B-glucuronidase is unknown but it is possible that such multiplicity is a peculiar characteristic of lysosomal enzymes. Beaufay and De Duve (1959) have suggested that lysosomes have a lipoprotein membrane surrounding the enzymes which may remain bound to the enzymes when the lysosome is ruptured.

Plapp and Cole (1967) have suggested that lysosomal enzymes may have bound carbohydrates or fatty acids or other material to protect them from the catheptic action and the isoenzymes represent various amounts of protective material. The present results do not suggest that the multiple forms have different physiological functions but they may be a clue to the in vivo state of the enzyme.

With respect to maternal serum enzymes a pattern of its enzymatic content has been established, which in turn reflects the enzymatic capabilities of the placenta. Intrauterine foetal welfare would be in part dependant upon the quantity of the hydrolytic products such as phosphate, sugars and amino acids that would be transferred to the foetus. Page (1957) has listed a number of parameters which may be involved in the transport mechanism of the placenta; and includes the molecular weights, shape, electrical charge, concentration gradients, diffusion and active transport. For many substances the concentration of the material in foetal plasma exceeds that in maternal plasma and hence the tissues of the placenta actively transport the material from one blood stream to another (Snell, 1959). Villee (1953) and Page (1954) point out that the processes of active transport require energy which is obtained by energy-yielding reactions such as those of the glycol ytic cycle

and the tricarboxylic acid cycle coupled with oxidative phosphorylation.

The concentration of amino acids, inorganic phosphate, sugars and other substances are higher in foetal blood than in maternal blood. The advantage to the foetus of having these substrates for growth processes are obvious but the mechanisms which provide the higher concentrations in foetal blood are not clear. Christensen and Riggs (1956) have documented the results of experiments showing the accumulation of amino acids within cells, and also they were the first to show that the human placenta had a similar ability.

It is now well established that the amino acids are transferred by active transport. Page et al. (1961) demonstrated that naturally occurring L-histidine crosses the placenta more rapidly than does the D-isomer which only crosses at a rate consistent with diffusion. The concentrations of amino acids is lower in the plasma of pregnant women than in non-pregnant women. Bonsnes (1947) and Ghadimi and Pecora (1964) found the concentrations to decrease near term.

As further evidence that the enzymes hydrolyze protein to amino acids in the placenta can be seen when the concentrations of amino acids in the blood of both monozygotic and dizygotic twins may be quite different. Clemetson (1966) has suggested that this would reflect differences in the transfer mechanism of the placentas that nourish them such as occurs in the parabiotic syndrome.

As described earlier alkaline phosphatase is probably involved in carbohydrate metabolism, phosphate metabolism and in the placental transfer of both. Originally it was thought that glucose crossed the placenta by passive diffusion, but Widdas (1952) had demonstrated that facilitated diffusion would be required to account for the observed transfer rates. Additional evidence can be provided by the work of Dancis et al. (1958) who found D-xylose to be transferred more rapidly than the L-isomer, and that glucose an aldohexose is transported more rapidly than fructose a ketohexose. The maternal glucose concentration is higher than the foetal concentration and it has been suggested by Dancis (1955) that placental metabolism accounts

for part of the maternal-to-foetal glucose concentration difference. Villee (1953) has shown that placental glycogen is maximal at 8 weeks gestation and thereafter declines throughout the course of gestation. The decrease in placental glycogen is accompanied by a reciprocal increase in the foetal liver glycogen. Dancis (1955) points out that there is a continuous exchange between maternal glucose, placental glycogen and foetal glucose.

During this work a number of isoenzymes originating in liver were found to increase during pregnancy which suggests other metabolism caused by the conceptus have been listed by Freinkel (1965). They include the capacity of the conceptus to elaborate hormones which alter maternal metabolism and which are not subject to the usual feedback mechanisms; the addition of a site for the irreversible degradation of maternal hormones particularly insulin; and the continuous and unremitting drain upon maternal substrates. All three properties of the conceptus would involve the maternal liver in increased metabolic activity.

One placental hormone in particular alters maternal metabolic processes is placental lactogen (Josimovick and MacLaren, 1962). According to Grumbach et al. (1968) placental lactogen has the capacity to increase the mobilization of maternal fat stores and conserves the utilization of maternal protein.

It is therefore apparent that the increase in maternal serum enzymes during pregnancy is in part due to the increase in placental specific isoenzymes which may be quantitatively assayed. Any one or a combination of more than one isoenzyme of alanine aminopeptidase, leucine aminopeptidase, n-acetyl-β-glucosaminidase and β-glucuronidase may be of value as placental function tests. Each placental isoenzyme has been found to be tissue specific and furthermore a change in placental metabolism should be directly reflected by a change in maternal serum isoenzyme pattern. The measurement of each isoenzyme is technically simple and the assays have been

automated so that rapid results are available to the clinician, and all pregnant women attending ante-natal clinics could be screened at each visit.

Future work would include a full clinical evaluation of the concentration and isoenzyme patterns in serial serum samples obtained from abnormal pregnancies. Other enzymes that are not found or do not increase in maternal serum may appear or change in abnormal pregnancy. These enzymes could be better indications of primary impairment of placental function.

Further advances would require a more extensive study which would be in part dependant upon a prior more complete understanding of the basic cellular mechanisms which could last a whole lifetime.

6. APPENDIX A

Appendix A. Actual enzyme results used for reproducability calculations.

Duplicate Number	Cystine	Aminopeptidase	Carboxypeptidase		Dipeptidase		Tripeptidase	
1	8.8	8.2	600	580	3.6	3.4	34.6	35.1
2	8.8	8.4	640	620	3.8	3.7	34.0	34.1
3	8.4	8.4	650	650	4.0	3.4	34.2	35.0
4	8.4	8.6	605	650	3.8	3.4	35.1	34.0
5	8.9	8.6	480	650	3.4	3.8	34.0	34.0
6	8.6	8.8	660	575	4.0	3.6	34.6	34.0
7	8.6	8.4	560	490	3.7	3.8	34.0	35.0
8	8.6	8.7	780	610	3.4	3.8	35.2	34.6
9	8.8	8.7	600	640	3.8	3.8	34.1	34.7
10	8.2	8.5	660	650	3.9	4.0	35.0	34.2

Appendix A continued.

Duplicate Number	Alkaline Phosphatase		n-Acetyl-B-glucosaminidase		n-Acetyl-B-galactosaminidase		B-Glucoronidase	
1	12.5	13.4	12.6	12.6	4.6	4.8	1.8	1.6
2	13.0	14.1	13.2	13.0	4.8	4.5	1.7	1.7
3	12.8	14.0	12.7	13.0	4.8	4.8	1.7	1.7
4	12.5	12.0	12.6	12.8	5.0	5.1	1.7	1.6
5	13.4	14.0	13.0	13.1	4.7	4.8	1.9	1.6
6	13.7	13.4	12.8	13.0	5.2	4.7	1.7	1.6
7	13.0	12.7	13.0	12.6	5.1	4.8	1.6	1.9
8	12.7	12.9	13.2	12.8	4.6	4.8	1.7	1.6
9	14.0	14.1	12.7	12.7	4.9	5.0	1.7	1.7
10	14.0	13.7	12.7	12.8	4.5	5.0	1.7	1.7

Appendix A. Continued.

Duplicate Number	-Glutamyl Transpeptidase		Leucine aminopeptidase		Alanine aminopeptidase		Glucose-6-phosphatase		5-Nucleotidase	
1	20.4	20.2	65.0	65.4	160	157	0.6	0.7	3.1	2.7
2	20.0	20.8	65.2	65.0	156	159	0.8	0.5	3.0	2.7
3	20.0	20.7	65.3	65.0	164	164	0.7	0.5	3.0	2.8
4	20.0	20.0	65.0	65.0	164	160	0.7	0.7	3.2	2.9
5	20.6	19.8	64.8	64.5	160	160	0.7	0.7	3.1	2.9
6	20.5	20.0	64.7	64.7	160	155	0.7	0.6	3.2	3.3
7	20.4	20.0	64.8	65.4	160	155	0.8	0.8	3.4	3.4
8	19.8	20.2	66.1	65.2	164	160	0.8	0.5	3.5	3.5
9	20.8	20.2	65.4	65.2	157	161	0.7	0.6	3.6	3.6
10	20.0	20.6	65.4	64.5	157	162	0.5	0.6	3.6	3.5

7. REFERENCES

REFERENCES

1. Ahmed, Z. and King, E. J., Biochem. Biophys. Acta, 1959, 34, 313.
2. Babuna, C. and Yenen, E., Am. J. Obstet. Gynecol., 1966(a), 94, 868.
3. Babuna, C. and Yenen, E., Am. J. Obstet. Gynecol., 1966(b), 95, 925.
4. Batstone, G. F., Blair, A. W. and Slater, J. M., Ed's., A Handbook of Pre-natal Paediatrics, 1971(a), Medical and Technical Publishing Co. Ltd., Aylesbury, p. 34.
5. Batstone, G. F., Blair, A. W. and Slater, J. M., Ed's., A Handbook of Pre-natal Paediatrics, 1971(b), Medical and Technical Publishing Co. Ltd., Aylesbury, p. 104.
6. Beaufay, H. and De Duve, C., Biochem. J., 1959, 73, 604.
7. Benirschke, K. and Driscoll, S. G., The Pathology of the Human Placenta, 1967, Springer, Berlin.
8. Beckman, L., Isozyme Variations in Man, 1966, Pub. Carlger. p.43.
9. Beckman, L., Bjorling, G. and Christodoulou, C., Acta genet., Basel, 1966, 16, 223.
10. Blunt, A., Aust. N. Z. J. Obstet. Gynaecol., 1971, 11, 37.
11. Bodansky, A., J. Biol. Chem., 1934, 104, 473.
12. Bonsnes, R. W., J. Biol. Chem., 1947, 168, 348.
13. Boyd, J. D. and Hamilton, W. J., The Human Placenta, 1970, Pub. W. Heffer, Cambridge.
14. Bradshaw, T. E. T. and Jessop, W. J. E., J. Endocrinol, 1953, 9, 427.
15. Brown, J. B., Lancet, 1956, I, 704.
16. British Medical Journal, 1972, Leading article.
17. Bulmer, D., Nature (Lond.), 1962, 40, 805.
18. Burns, J. J., Am. J. Med., 1959, 26, 740.
19. Cerletti, P. and Zichella, L., Clin. Chim. Acta, 1960, 5, 748.
20. Christensen, H. N. and Riggs, T. R., J. Biol. Chem., 1956, 220, 265.
21. Clemetson, C. A. B., J. Obstet. Gynaecol. Br. Commonw., 1956, 63, 15.
22. Clemetson, C. A. B., and Churchman, J., J. Obstet. Gynaecol. Br. Commonw., 1954, 61, 364.

23. Coffey, J. W. and DeDuve, C., J. Biol. Chem., 1968, 243, 3255.

24. Contractor, S. F. and Shane, B., Biochem. J., 1972, 128, 11.

25. Coutinho, H. B., Katchlwrian, E. and Pearse, A. G. E., J. Clin. Pathol., 1966, 19, 617.

26. Coyle, M. G. and Brown, J. B., J. Obstet. Gynaecol. Br. Commonw., 1963, 70, 225.

27. Croxatto, H., Vera, C. and Barnafi, L., Proc. Soc. Exp. Biol. (N.Y.), 1953, 83, 784.

28. Curzen, P., J. Obstet. Gynaecol. Br. Commonw., 1964, 71, 388.

29. Curzen, P., J. Obstet. Gynaecol. Br. Commonw., 1967, 74, 385.

30. Curzen, P., Enzyme Assays in Medicine, Association of Clinical Pathologist Symposium, 1970, 90.

31. Curzen, P. and Morris, I., J. Obstet. Gynaecol. Br. Commonw., 1966, 73, 640.

32. Curzen, P. and Morris, I., J. Obstet. Gynaecol. Br. Commonw., 1968, 75, 151.

33. D'Agostino, B. A., Riv. Med. Vet. Zootecn., 1952, 4, 19.

34. Dance, N., Price, R. G. and Robinson, D., Biochem. J., 1969, III, 749.

35. Dancis, J., Olsen, G. and Folkart, G., Am. J. Physiol., 1958, 194, 44.

36. Danielli, J. F., in Cytochemistry: A Critical Approach, p. 48, John Wiley, New York.

37. Datta, P., Biochemistry, 1971, 10, 402.

38. Davies, J., Am. J. Physiol., 1955, 181, 532.

39. DeDuve, C. and Wattiaux, R., Annu. Rev. Physiol., 1966, 28, 435.

40. Dempsey, E. W. and Wislocki, G. B., Am. J. Anat., 1945, 76, 277.

41. Erdös, E. G. and Sloane, E. M., Biochem. Pharmacol., 1962, II, 585.

42. Erdös, E. G., Wohler, I. M., Levine, M. I. and Westerman, M. P., Clin. Chim. Acta., 1965, II, 39.

43. Farr. W., Rehfold, N., Reichelt, D. and Haschen, J., Z. Med. Labortechnik, 1968, 9, 78.

44. Fishman, W. H., In Methods of Biochemical Analysis, Glick, D. Ed., Vol. 15, Interscience, New York.

45. Fishman, W. H. and Ghosh, N. K., Adv. Clin. Chem. 1967, 10, 255.

46. Fishman, W. H., Inglis, N. R., and Ghosh, N. K., Clin. Chem. Acta., 1968, 19. 71.

47. Fishman, W. H., Inglis, N. R., Green, S, Austiss, C. L., Ghosh, N. K., Reif, A. E., Rustigan, R., Krant, M. J. and Stolbach, L. L., Nature (Lond.), 1968, 219, 697.

48. Fishman, W. H., Goldman, S. S. and DeLellis, R., Nature, (Lond.), 1967, 213, 457.

49. Fiske, C. H. and Subbarrow, H., J. Biol. Chem., 1925, 66, 375.

50. Fox, H., J. Obstet. Gynaecol. Br. Commonw., 1964, 71, 749.

51. Francis, J. G., Turnbull, A. G. and Thomas, F.F., J. Obstet. Gynaecol. Br. Commonw., 1970, 77, 594.

52. Frandsen, V. A. and Stakemann, G., Acta Endocr. Kbh., 1963, 44, 183.

53. Freinkel, N., Excerpta Med. Intermat. Congress, Series No. 84, Chap. 49, (1965).

54. Garrow, J. S. and Douglas, C. P., J. Obstet. Gynaecol. Br. Commonw., 1968, 75, 1034.

55. Gaull, G., Hagerman, D. D. and Villee, C. A., Biochem. Biophys. Acta, 1960, 40, 552.

56. Geelhoed, G. W. and Vander, A. J., J. Clin. Endocrinol, 1968, 28, 412.

57. Genazzani, A. R., Cocola, F., Casoli, M., Mello, G., Scarselli, G., Neri, P. and Fioretti, P., J. Obstet. Gynaecol, Br. Commonw., 1971, 78, 577.

58. Ghadini, H. and Pecora, P., Paediatrics, 1964, 33, 500.

59. Ghosh, N. K. and Fishman, W. H., Biochem. J., 1968, 108, 779.

60. Gibian, H., Balazs, E. A., Jeanloz, R. W. (Eds.), The Amino Sugars, Vol. 2B, Academic press, New York, 1966, p. 181.

61. Glendenning, M. B., Titus, M. A., Schroeder, S. A., Mohun, G. and Page, E. W., Am. J. Obstet. Gynaecol., 1965, 92, 814.

62. Green, J. W., Duhring, J. L. and Smith, K., Am. J. Obstet. Gynecol., 1965, 92, 1031.

63. Greene, J. W. and Touchstone, J. C., Am. J. Obstet. Gynaecol., 1963, 85, 1.

64. Grumbach, M. M., Kaplan, S. L. and Sciarra, J. J., Ann. N.Y. Acad. Sci., 1968, 148, 501.

65. Hagerman, D. D., Fed. Proc., 1964, 23, 785.

66. Hagerman, D. D., Roux, J. and Villee, C. A., J. Physiol., 1959, 146, 98.

67. Hahn, L., Biochem. Z., 1947, 318, 138.

68. Henry, G. R., Brit. J. Hosp. Med., 1970, 3, 516.

69. Hensleigh, P. A. and Krantz, K. E., Am. J. Obstet. Gynaecol., 1970, 107, 1233.

70. Hertig, A. T., Adams, E. C., McKay, D. G., Rock, J., Mulligan, W. J. and Menkin, M. F., Am. J. Obstet. Gynecol., 1958, 76, 1025.

71. Hollmann, S. and Tonster, O., Biochim. Biophys. Acta, 1962, 62, 338.

72. Hopkirk, R., Blahley, P. R., Alfheim, A., Raeside, J. I. and Joron, G. E., J. Clin. Endocrinol., 1960, 20, 805.

73. Huddleston, J. F., Lee, G. and Robinson, J. C., Am. J. Obstet. Gynecol., (1971), 109, 1017.

74. Hunter, R. J., J. Obstet. Gynaecol. Br. Commonw., 1969, 76, 1057.

75. Hunter, R. J., Pinkerton, J. H. M. and Johnston, H., Obstet. Gynecol., 1970, 36, 144.

76. Hutterer, F., Biochim. Biophys. Acta, 1966, 115, 312.

77. Ichaliotis, S. and Lambrinopaulous, T. C., Obstet. Gynecol., 1965, 25, 270.

78. Ichaliotis, S. and Lambrinopoulous, T. C., Gynecol. Obstet., 1964, 63, 543.

79. Iodice, A. A., Leong, V. and Weinstock, I. M., Arch. Biochem. Biophys., 1966, 117, 477.

80. Jeacock, M. K., Morris, N. F. and Plester, J. A., J. Obstet. Gynaecol. Brit. Commonw., 1963, 70, 267.

81. Jones, D. D., Williams, G. F. and Prochazka, B., Enzymologia, 1972, 43, 325.

82. Jösch, W. and Dubach, U. C., Clin. Chim. Acta, 1967, 15, 325.

83. Josinovich, J. B. and Maclaren, J. A., Endocrinology, 1962, 71, 209.

84. Kind, P. R. N. and King, F. J., J. Clin. Path., 1954, 7, 322.

85. Kleiner, H. (1969), In the Foeto-Placental Unit, Ed. A. Pecile, Excerpta Medica Foundation, Amsterdam, p. 363.

86. Kleiner, H., Bronet-Yager, M. and Graff, G., J. Obstet. Gynaecol. Brit. Commonw., 1969, 76, 127.

87. Klimek, R., Biochem. Pharmacol., 1963, 12, Suppl. 227.

88. Klimek, R., (1966), I'm 'Intra Uterine Dangers to the Foetus', Ed's. Horsky, A. and Steinbera, S. Excerpta Medica Foundation.

89. Klimek, R. and Bieniasz, A., Am. J. Obstet, Gynaecol., 1969, 104, 959.

90. Klimek, R. and Król, W., Polish Endocrinological Society, Crawcow, 1964.

91. Klopper, A. and Billewicz, W., J. Obstet. Gynaecol. Brit. Commonw., 1963, 70,1024.

92. Klopper, A. and Diczfalusy, E., Ed's. In Foetus and Placenta, 1969, Blackwell Scientific Publications, Oxford.

93. Koide, H. and Oda, T., Clin. Chim. Acta, 1959, 4, 554.

94. Kowlessar, O. D., Haeffner, L. J. and Riley, E. M., N.Y. Acad. Sci., 1961, 94, 836.

95. Kukard, R. F. P. and Freeman, M. E., Am. J. Obstet. Gynaecol. Brit. Commonw., 1973, 80, 433.

96. Lambrinopoulous, T. C., Obstet. Gynaecol., 1964, 23, 780.

97. Letchworth, A. T., Boardman, R., Bristow, C., Landon, J. and Chard, T., J. Obstet. Gynaecol. Brit. Commonw., 1971, 78, 535.

98. Letchworth, A. T. and Chard, T., Lancet, 1972, i., 704.

99. Lewis, J., Am. J. Obstet. Gynaec., 1962, 84, 1407.

100. Linker, A., Meyer, K. and Weissman, B., J. Biol. Chem., 1955, 213, 237.

101. Liggins, G. C., 1972. In: 'Human Reproductive Physiology', Ed. R. P. Shearman, Blackwell Scientific, London.

102. Lobel, B. L., Deane, H. W. and Romney, S. L., Am. J. Obstet. Gynaecol., 1962, 83, 295.

103. Mahaderan, S. and Tappel, A. L., J. Biol. Chem., 1967, 242, 2369.

104. McDonald, T. and Odell, S., J. Clin. Endocrinol., 1947, 7, 535.

105. McMaster, Y., Tennant, R., Clubb, J. S. and Neale, F. C., J. Obstet. Gynaecol. Brit. Commonw., 1964, 71, 735.

106. Mehler, A. H., Kornberg, A., Grisolia, S. and Ochoa, S., J. Biol. Chem., 1948, 179, 961.

107. Melander, S., Acta Endocr. (Kbh), 1965, 49, Suppl. 96.

108. Meyer, K. , Linker, A. and Rapport, M. M., J. Biol. Chem., 1951, 192, 275.

109. Miller, R. A., Arch. Dis. Childh., 1941, 16, 22.

110. Moss, D. W., Biochem. J., 1965, 94, 458.

111. Moss, D. W., Eaton, R. H., Smith, J. K. and Whitby, L. G., Biochem. J., 1967, 102, 53.

112. Nachlas, M. M., Crawford, D. T. and Seligman, A. M., J. Histochem. Cytochem., 1957, 5, 264.

113. Nachlas, M. M., Goldstein, T. P. and Seligman, A. M., Arch. Biochem. Biophys., 1962, 97, 223.

114. Naftalin, L., Child, V. J. and Morley, D. A., Clin. Chim. Acta., 1969, 26, 297.

115. Neale, F. C., Chubb, J. S., Hotchkiss, D. and Posen, S., J. Clin. Path., 1965, 18, 359.

116. Nemeth, A. M., J. Biol. Chem., 1954, 208, 773.

117. Nemeth, A. M., J. Biol. Chem., 1959, 234, 2921.

118. Neurath, H., 1970., In The Enzymes, Ed's. P. A. Boyer, H. A. Lardy, and K. Myrback, Vol. 3. Academic Press, N.Y.

119. Newton, M. A., J. Clin. Path., 1966, 19, 491.

120. Novikoff, A. B., Shin, W. Y. and Drucker, J., J. Histochem. Cytochem., 1960, 8, 37.

121. Oakey, R. E., Bradshaw, L. R. A., Eccles, S. S., Stitch, S. R. and Heys, R. F., Clin. Chim. Acta, 1967, 15, 35.

122. O'Connell, W. and Winzler, R. J., Cancer Research, 1963, 23, 78.

123. Okada, S. and O'Brien, J. S., Science, 1969, 165, 698.

124. Ouchterlony, Diffusion-in-gel Methods for Immunological Analysis, 1962, II, Progr. Allergy VI.

125. Oudhensden, A. P. M., Clin. Chim. Acta, 1971, 32, 140.

126. Page, E. W., Am. J. Obstet. Gynaec., 1946, 52, 1014.

127. Page, E. W., In: Transactions of the First Conference on Gestation, Josiah Macy Jr. Foundation, 1954, p. 225.

128. Page, E. W., Am. J. Obstet. Gynaec., 1957, 74, 705.

129. Page, E. W., Am. J. Obstet. Gynaec., 1969, 104, 378.

130. Page, E. W. and Glendenning, M. B., In: Gestation, L. B. Flexner Ed., Josiah Macy Jn'r. Foundation, N.Y.

131. Page, E. W., Glendenning, M. B., Margolis, A. and Harper, H. A., Am. J. Obstet. Gynaecol., 1957, 73, 857.

132. Page, E. W., Titus, M. A., Mohun, G. and Glendenning, M. B., Am. J. Obstet. Gynaec., 1961, 82, 1090.

133. Placer, Z. and Horký, J., Clin. Chim. Acta., 1962, 7, 190.

134. Plapp, V. and Cole, D., Biochemistry, 1967, 6, 3676.

135. Platt, D. and Platt, M., Klin. Wschr., 1968, 46, 768.

136. Pöch, G., Martinez, A. L., Holzer, E. and Hohlweg, W., Arch. Gynäk., 1970, 208, 416.

137. Posen, S., Neale, F. C., Birkett, D. J. and Brindene - Woods, J., Am. J. Clin. Path., 1967, 48, 81.

138. Prochazka, B., University of Aston, Birmingham, Unpublished Observations, Personal communication.

139. Price, R. G. and Dance, N., Biochim. Biophys. Acta, 1972, 271, 145.

140. Pulkkinen, M. O. and William, K., Acta Obstet. Gynec. Scand., 1968, 47, 273.

141. Raekallio, J. and Mäkinen, Y., Experientia, 1971, 27, 1276.

142. Reeves, H. C. and Brehmeyer, B. A., Biochim. Biophys. Acta, 1969, 191, 722.

143. Reeves, W. J. and Funognari, G. M., J. Biol. Chem., 1963, 238, 3853.

144. Rimbach, E. and Schreiner, R., Med. Welt (Stuttg.), 1967, 51, 3118.

145. Robinson, D., Price, R. G. and Dance, N., Biochem. J., 1967, 102, 525.

146. Robinson, D. and Stirling, J. L., Biochem. J., 1968, 107, 321.

147. Robinson, J. C. and Pierce, J. E., Nature, (Lond.), 1964, 204, 472.

148. Rockerbie, R. A. and Rasmussen, K. L., Clin. Chim. Acta, 1967, 18, 183.

149. Russell, R. G. G., Lancet, 1965, ii, 461.

150. Ryden, G., Acta Obstet. Gynaec. Scand., 1966, 44, Suppl. 3.

151. Saling, E., Geburtsh, u. Frauenh., 1962, 22, 830.

152. Schmidt, A. J., In: Cellular Biology of Vertebrate Regeneration and Repair, University of Chicago Press, Chicago, 1968.

153. Schreiner, W. E., Schweiz. Med. Wschr., 1965, 95, 1571.

154. Semm, K., Personal communication to Page, et al. 1961.

155. Shugar, D., Biochim. Biophys. Acta, 1952, 8, 302.

156. Sjöholm, I. and Yman, L., Acta Pharm. Suecica., 1967, 4, 65.

157. Smith, E. L., J. Biol. Chem., 1948, 173, 571.

158. Smith, E. L., In: The Enzymes, Ed's. J. B. Sumner, and K. Myrbäck, Vol. 1. Part 2, p. 463, Academic Press, N.Y. 1960.

159. Smith, E. E. and Rutenburg, A. M., Science, 1966, 152, 1256.

160. Snell, F. M., Proceedings of the 32nd Ross Conference on Paediatric Research, 1959, p. 61.

161. Southren, A. L., Kobayashi, Y., Carmody, N. C. and Weingold, A. B., Am. J. Obstet. Gynaec., 1966, 95, 615.

162. Southren, A. L., Kobayashi, Y., Weingold, A. B. and Carmody, N.C., Am. J. Obstet. Gynaec., 1966, 96, 502.

163. Spellacy, W. N., Teoh, E. S., Buhi, W. C., Birk, S. A. and McCreary, S. A., Am. J. Obstet. Gynaec., 1971, 109, 588.

164. Stirling, J. L. Biochim. Biophys. Acta, 1972, 271, 154.

165. Stoklaska, E. and Wintersberger, E., Wien, Klin. Wschr., 1962, 74, 944.

166. Swinnen, J., Clin. Chim. Acta, 1967, 17, 255.

167. Sylven, B. and Bois, I., Histochemie, 1964, 4, 135.

168. Sylven, B. and Lippi, U., Exp. Cell Res., 1965, 40, 145.

169. Szewcznk, A., Clin. Chim. Acta, 1966, 14, 608.

170. Teoh, E. S., Spellacy, W. N. and Buhi, W. C., J. Obstet. Gynaecol. Brit. Commonw., 1971, 78, 673.

171. Thomsen, K., Arch. Gynäk., 1955a, 187, 1.

172. Thomsen, K., Arch Gynäk., 1955b, 187, 264.

173. Thomsen, K. and Hiersche, H. D., In: Foetus and Placenta, Ed. A. Klopper and E. Diczfalusy, Blackwell Scientific Publications, Oxford. 1971.

174. Thomsen, K. and Netz, L., Arch. Gynäk., 1955, 185, 794.

175. Thompson, D. P. and Schwartz, R. B., Clin. Research., 1963, 23, 78.

176. Titus, M. A., Reynolds, D. R., Glendenning, M. B. and Page, E. W., Am. J. Obstet. Gynaec., 1960, 80, 1124.

177. Torok, E. E., Brewer, J. I. and Dolkart, R. E., J. Clin. Endocrinol., 1970, 30, 59.

178. Tuppy, H., In: Neurohypophysial Hormones and Similar Polypeptides, Ed. B. Berde, Heidelberg, N.Y., 1968.

179. Tuppy, H., Wiesbaner, U. and Wintersberger, E., Mh. Chem., 1963, 94, 321.

180. Tuppy, H. and Wintersberger, E., Mh. Chem., 1960, 91, 1001.

181. Turnbull, A. C. and Anderson, A. B., J. Obstet. Gynaecol. Brit. Commonw., 1968a, 75, 32.

182. Turnbull, A. C. and Anderson, A. B., J. Obstet. Gynaecol. Brit. Commonw., 1968b, 75, 24.

183. Van der Crabben, H., Proceedings of Second European Congress of Perinatal Medicine, London.

184. Verlado, J. T. and Rosa, C. G., In: Handbuch der Histochemie Ed's. Graumann and Neumann, 7/3, p. 1, 1963, Fischer, Stuttgart.

185. Villee, C. A., J. Appl. Physiol., 1953, 5, 437.

186. Wachstein, M., Meagher, J. G. and Ortiz, J., Am. J. Obstet. Gynaecol., 1963, 87, 13.

187. Walker, D. G., Lea, M. A., Rossiter, G. and Addison, M. E. B., Arch. Biochem. Biophys., 1967, 120, 646.

188. Walker, P. G., Woollen, M. E. and Pugh, D., J. Clin. Path., 1960, 13, 353.

189. Warnock, M. L., Clin. Chim. Acta, 1966, 14, 156.

190. Watson, D., Siddiqui, S. A., Stafford, J. E. H., Gibbard, S. and Hewitt, V., J. Clin. Path., 1973, 26, 294.

191. Weissmann, G., Blood, 1964, 24, 594.

192. Weissmann, G., New. Eng. J. Med., 1965, 273, 1084.

193. Widdas, W. F., J. Physiol. (Lond.), 1952, 118, 23.

194. Widmer, W., Schweiz. Med. Wschr., 1948, 78, 439.

195. Wielenga, G. and Willighagen, R. G. J., Am. J. Obstet. Gynaecol., 1962, 84, 1059.

196. Wieme, R. J., Rev. Belge Pathol. Med. Exptl., 1957, 25, 62.

197. Wintersberger, E., Müller-Hartburg, W. and Tuppy, H., Clin. Chim. Acta., 1966, 14, 786.

198. Wintersberger, E. and Tuppy, H., Proc. Sec. Interm. Pharmacol. Meet., Prague, 1963, p. 143, Pergamon Press.

199. Wislocki, G. B. and Dempsey, E. W., Endocrinology, 1946, 38, 90.

200. Wislocki, G. B. and Dempsey, E. W., Am. J. Anat., 1948, 83, 1.

201. Woollen, J. W. and Turner, P., Clin. Chim. Acta, 1965, 12, 671.

202. Woollen, J. W. and Walker, P. G., Clin. Chim. Acta, 1965a, 12, 647.

203. Woollen, J. W. and Walker, P. G., Clin. Chim. Acta, 1965b, 12, 659.

204. Wray, P. M. and Russell, C. S., J. Obstet. Gynaecol. Brit. Commonw., 1964, 71, 97.

PUBLICATION (I)

Enzymologia, 45, 325, (1972).

Multiple molecular forms of enzymes hydrolysing L-leucyl-β-naphthylamide during gestation.

Enzymologia, vol. 43, 5 pag. 325–332, 1972

MULTIPLE MOLECULAR FORMS OF ENZYMES HYDROLYSING L-LEUCYL-β-NAPHTHYLAMIDE DURING GESTATION

BY

D. D. JONES, G. WILLIAMS AND B. PROCHAZKA

(Bronglais Gen. Hosp., Aberystwyth and Dept. of Biol. Sciences, Univ. of Aston, Gosta Green, Birmingham)

(Received: 30th March, 1972)*

Introduction

Arylamidase (aminoacylnaphthylamide amidohydrolase E.C. 3.4.1.1.), formerly known as leucine aminopeptidase, has been investigated by electrophoresis on starch gel and a single zone of activity has been found in normal human serum[1, 2, 3]. In electropherograms of maternal serum, two zones of activity have been detected[4, 5], and a similar pattern has been found in cord blood by MEADE and ROSALKI[5]. Chromatography using Sephadex G-200 has yielded two zones of arylamidase activity in maternal serum[6], one of which hydrolysed leucine-β-naphthylamide and the other hydrolysed cystine-di-β-naphthylamide. The present investigation deals with the arylamidase hydrolysing leucyl-β-naphthylamide and will be referred to as leucine aminopeptidase.

Characteristic isoenzyme patterns have been found in various tissues by BECKMAN, BJORLING and CHRISTODOULOU[7], with four different molecular forms in human placentae, three in kidney and two in heart. Also an increased leucine aminopeptidase activity has been reported in maternal serum as pregnancy advances[8]. LEWIS[9], has also suggested that the enzyme was of value in differentiating hydatiform mole (primary tumour of the trophoblast) in the 2nd trimester of pregnancy.

It is likely that the site of origin of the leucine aminopeptidase fractions found in maternal serum is the placenta. In the present study, polyacrylamide gel electrophoresis was employed to identify the source of origin of the leucine aminopeptidase zones found in maternal serum.

The action of neuraminidase (N-acetyl-neuraminate glycohydrolase, EC 3.2.1.18) in removing a terminal neuraminic acid group

* Accepted for Publication: 12th May, 1972.

which would reduce the negative charge on the enzyme molecule and the inhibition by L-methionine were both used to further identify the isoenzymes of leucine aminopeptidase.

Materials and methods

Sera

Bloods were taken without anticoagulant between 16 weeks and term. Estimations of leucine aminopeptidase activity were made on the fresh sera or on frozen sera (—20°) stored for not more than one week. Cord bloods were treated in a similar manner.

Tissue extracts

Liver, intestine, kidney, pancreas and bone were obtained from fresh post mortem material. Approximately 0.5 g. of tissue was homogenized with 10 ml. of 0.9% saline and centrifuged for 10 min. to remove cellular debris. All tissues were homogenized in a tissue grinder. Placentae were washed with pre-cooled 0.9% saline (4°) and approximately 0.5 g. of tissue taken for extraction.

The bone extract was obtained by crushing 0.5 g. of sternum in a pestle and mortar containing 10 ml. of saline.

Buffers

A Tris (hydroxymethyl) methylamine was prepared by dissolving 45.5 g. of Tris in 900 ml. of water and 1.2 ml. of N, N^1-tetramethyl-ethylenediamine* was titrated to pH 9.7 with boric acid and the whole diluted to 1 l. This buffer was used to prepare the gel.

The buffer for use in both electrode vessels was prepared as above but omitting the TEMED. For use, this buffer was diluted 1 in 4.

Preparation of gels

Gel monomer, – Acrylamide (19 g.) and N, N^1 – Methylenebis-acrylamide (1 g.) were dissolved in 100 ml. of distilled water.

To 15 ml. of Tris-TEMED buffer was added 15 ml. of gel monomer and 10 ml. of 0.4% persulphate. The mixture was immediately added to the gel tubes and allowed to stand for 1 h for polymerisation. Gel tube size was 6 mm. × 65 mm. More consistent results were obtained when the gels were used after standing for 3 h or overnight at 4°. After polymerisation the gels were covered with 40 µl. of water.

---

* Abbreviations N, N′-tetramethylethylenediamine – TEMED.

Disc electrophoresis

The water layer over each gel tube was discarded and 5 µl. of tissue extracts of serum was applied to a filter paper disc, the diameter of which was slightly less than the diameter of the gel tube. A solution containing 2.5% polyacrylamide was used to fill the tube prior to electrophoresis. Current was adjusted to 5 mA and 30 V per tube. Gels were run at 4° and the experiment was terminated when the albumin of a control serum containing bromo-phenol blue had reached the bottom of the gel tube and required approximately 45–60 min. to complete. The bromophenol blue stained serum sample was subsequently used to stain the protein fractions with ponceau S.

Isoenzyme location procedure

Substrate, – 0.68 mM L-leucyl-β-naphthylamide hydrochloride in 0.1 M. phosphate buffer pH 7.0.

The gels were removed from the tube using running water passed through a fine syringe needle and were incubated in individual tubes containing substrate solution. At the end of the incubation period (1 h at 37°), the substrate solution was decanted and an aqueous solution containing Fast Garnet GBC (1 mg./ml.) was added. Maximum colour development occurred after 1 h. The gels were washed once with distilled water prior to scanning. Scanning of the enzyme zones and protein fractions was made using a Chromoscan obtained from Joyce Loeble Ltd., Gateshead.

Neuraminidase

Neuraminidase (0.5 mg.) (Sigma) Type V. from *Cl. perfringens* was added to 1 ml. of tissue extract prior to electrophoresis. 50 µl of a 0.2 g./100 ml. aqueous solution of neuraminidase was added to 0.2 ml. of serum.

L-methionine

A final concentration of 50 mM of substrate.

Total leucine aminopeptidase estimations

The hydrolysis of L-leucyl-β-naphthylamide was determined according to the method of ROCKERBIE and RASMUSSEN[10], where the naphthylamide liberated was measured using a fluorimeter with a 365 nm. primary filter and a 405 nm. secondary filter.

Results

Tissue isoenzymes

Ten extracts of pancreas, placenta, kidney, bone, liver and intestine were found to contain one or more zones after polyacrylamide electrophoresis. Fig. 1. illustrates the results obtained together with the protein fractionation of a normal serum. Placenta, kidney and bone extracts contained a fast moving band (zone 1) which migrated in the Fα globulin region of serum. In placenta a second slower moving fraction (zone 2) was detected and found to correspond to the β_2 globulin of serum. Pancreas, kidney and intestinal extracts were found to contain one zone each which migrated with the fastest moving haptoglobin (zone 3). Kidney extract contained a zone 3 enzyme together with a slower moving zone with migration similar to that found for the slower moving haptoglobin fraction (zone 4). Liver and kidney extracts contained a zone which migrated to zone 4. Placenta and kidney extracts contained an enzyme fraction which did not migrate and was located near the Sβ globulin in serum (zone 5).

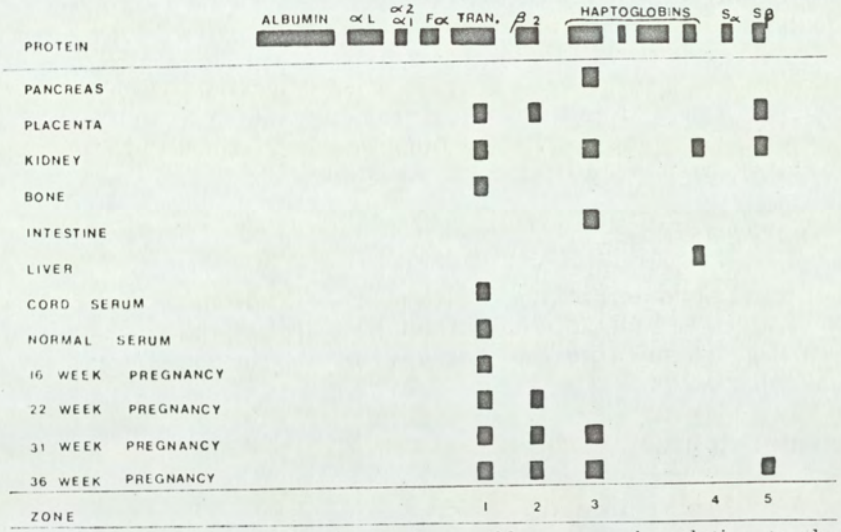


Fig. 1. The migration of leucine aminopeptidase zones in relation to the migration of protein fractions after polyacrylamide electrophoresis.

Leucine aminopeptidase fractions in serum

One fraction (zone 1) was detected in sera obtained from ten healthy males and six females. Forty pregnancy sera were investigated for leucine aminopeptidase fractions. During the 20th week of

pregnancy, a zone 2 enzyme appeared with a migration rate similar to that found for the slower moving fraction (zone 2) from placental extract. A zone 3 enzyme was detected after the 30th week of pregnancy with electrophoretic migration similar to that found for pancreas and intestinal extracts. Towards the end of pregnancy between the 34th week and term, a zone 5 appeared with migration pattern similar to that found in kidney and placental extracts.

The concentration of zones 1, 2, 3 and 4 in maternal serum progressively increased during gestation. Fig. 2 illustrates a typical increase in the enzyme fractions found in 40 pregnancy sera between the 16th week and term.

Cord serum was found to contain a zone 1 enzyme similar to that found in normal and maternal serum.

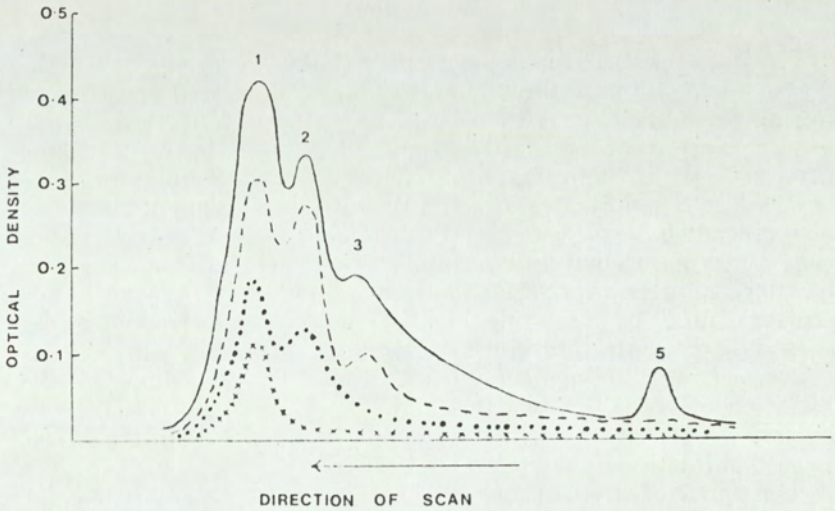


Fig. 2. Zones of leucine aminopeptidase activity in pregnancy serum after polyacrylamide electrophoresis. Scans are shown of the progressive increase in zones 1, 2, 3 and 5 during gestation. 36 weeks ————; 31 weeks – – – –; 22 weeks . . . . .; 16 weeks x x x x x.

Effect of neuraminidase

The enzyme fractions observed in tissue extracts and sera were all attacked by neuraminidase. Faint zones of activity with a decreased mobility were found in pancreatic and intestinal extracts and between the 36th week of pregnancy and term. The kidney and bone extracts together with the zones found in normal and cord sera were completely eliminated. Incubations in the absence of neuraminidase did not result in the loss of enzyme zones. The

neuraminidase used did not hydrolyse the substrate L-leucyl-β-naphthylamide when incubated at 37° under the conditions used for the test sera.

Effect of L-Methionine

The enzyme zones detected in the extracts of pancreas, intestine and placenta were unaltered by the addition of L-methionine to the substrate. The kidney, liver and bone enzymes were completely inhibited. Zone 2 enzyme of maternal serum was unaffected by L-methionine but zone 1 was reduced in concentration. Zone 1 of cord serum and normal adult serum were also inhibited.

Discussion

The data presented here suggests that the serum enzyme hydrolysing L-leucyl-β-naphthylamide rises appreciably and progressively during pregnancy. It is therefore likely that this increase in part reflects placental function. During the last trimester of pregnancy, maternal serum contains four zones of leucine aminopeptidase (zones 1, 2, 3 and 5). Between the 16th week of pregnancy and term a significant increase was found to occur in the concentration of all the isoenzymes in maternal serum. The increase in the non-placental fractions in pregnancy serum could in part explain the wide normal scatter found by us while estimating the total leucine aminopeptidase concentrations during pregnancy.

Zones 1 and 2 correspond to that found for placenta, zone 3 to intestine and zone 5 to placenta and kidney. Zone 2 was found to be entirely of placental origin and zones 1 and 5 to contain in part placental fractions.

The source of origin of the individual enzyme fractions in maternal serum were investigated by comparing the electrophoretic mobility of the fractions before and after neuraminidase treatment with that found in placenta, pancreas, kidney, bone, intestine and liver extracts. In addition the degree of inhibition by methionine of each fraction further clarified the source of origin.

BECKMAN[11] reported two fractions in pregnancy serum with four isoenzymes in placental extract. In the present study three zones only were detected in placental extract and in serum at 22 weeks of pregnancy. A zone 3 and a zone 5 appeared in maternal serum between the 30th week and term. (Fig. 2)

After neuraminidase treatment all the enzyme fractions both in maternal serum and tissue extracts were reduced in electrophoretic mobility and, therefore, all possess a terminal neuraminic acid group. Using starch gel electrophoresis it has been reported BECK-

MAN[11], that the fastest moving zone of placenta, liver, intestine and kidney to be unaffected by neuraminidase treatment using starch gel electrophoresis.

L-methionine competitively inhibits hydrolysis of L-leucyl-β-naphthylamide. The kidney and bone enzymes were inhibited but no such inhibition was found with the placental extracts or in pregnancy sera. The inhibition by L-methionine can therefore be used to advantage in estimating zones of placental origin in maternal serum. Furthermore the enzyme found in normal serum was also inhibited.

Zone 2 detected in both placenta and pregnancy serum was found to be activated by L-methionine which was further evidence that this zone was of placental origin. Activation of muscle leucine aminopeptidase has been reported FLEISHER, PANKOW, and WARMKA[12], but no such activation has been reported for the placental zones. These results also indicate the heterogeneity of the leucine aminopeptidase zones, although these date are not sufficient to class the enzymes as isoenzymes, it does serve to establish the existence of biochemical differences suggestive of isoenzymes.

Further work is in hand to establish the clinical importance of leucine aminopeptidase isoenzyme fractionation with a view to evaluating the results as an aid to assessing placental function.

Summary

Zones of leucine aminopeptidase (aminoacyl-naphthylamide amidohydrolase, E.C. 3.4.1.1.) were fractionated from extracts of placenta, kidney, bone, intestine, liver and pancreas and from normal and pregnancy serum by polyacrylamide gel electrophoresis. The migration of the fractions was examined in relation to the migration of protein fractions in serum. Five zones of activity were demonstrated in tissue extracts and four zones in maternal serum. A progressive increase was found to occur in the concentration of all the isoenzymes in maternal serum during pregnancy. All the enzyme fractions in tissue extracts and serum were attacked by neuraminidase N-acetyl-neuraminate glycohydrolase, E.C. 3.2.1.18) and therefore possess a terminal neuraminic acid group. The addition of L-methionine inhibited the activity of the kidney, bone and normal serum zones and activated the zones from placental extract and maternal serum. The inhibition by L-methionine can be used to estimate zones of leucine aminopeptidase activity of placental origin found in maternal serum.

Acknowledgements. We wish to thank DR. W. H. BEASLEY, Consultant Pathologist for his help and encouragement and to the Welsh Regional Hospital Board for a grant in support of this work.

1) C. A. Dubbs, C. Vivonia, J. M. Hilburn, *"Subfractions of Human Serum Enzymes"*, Science, N.Y., 131, *1529* (1960).
2) E. E. Smith, E. P. Pineda, A. M. Rutenberg, *"Localization of Serum Leucine Aminopeptidase Activity by Paper Electrophoresis"*, Proc. Soc. exp. Biol. Med., 110, *683* (1962).
3) E. E. Smith, A. M. Rutenberg, *"Starch-gel Electrophoresis of Human Tissue Enzymes Hydrolysing L-Leucyl-β-Naphthylamide"*, Nature, Lond. 197, *800* (1963).
4) O. D. Kowlessar, L. J. Haeffner, E. M. Riley, *"Localization of Serum Leucine Aminopeptidase, 5-Nucleotidase and Non-specific Alkaline Phosphatase by Starch Gel"*, Ann. N.Y. Acad. Sci., 94, *836* (1961).
5) B. W. Meade, S. B. Rosalki, *"Localization of Leucine Aminopeptidase Isoenzymes"*, J. clin. Path., 17, *61* (1964).
6) V. Goebelsman, F. K. Beller, *"Separation of Cystine Aminopeptidase and Leucine Aminopeptidase and their Determination in Pregnant and Non-pregnant women"*, Z. klin. Chem., 3, *49* (1965).
7) L. Beckman, G. Bjorling, C. Christodoulou, Unpublished Observations, See Reference 2 (1966).
8) H. Kleiner, P. Wilkin, J. Snoek, *"Presence of Three Isoenzymes Hydrolysing L-Leucyl-β-Naphthylamide in Normal Human Serum"*, Geburtshilfe Frauenheilkunde, 22, *986* (1962).
9) J. Lewis, *"Serum Leucine Aminopeptidase Values in Patients with Trophoblastic Tumours and in Normal Pregnancy"*, Am. J. Obst. Gynae., 84, *1407* (1962).
10) R. A. Rockerbie, K. L. Rasmussen, *"An Ultramicro Method for The Fluorometric Determination of Leucine Aminopeptidase in Serum"*, Clin., Chim. Acta., 18, *183* (1967).
11) L. Beckman, *"Isoenzyme Variations in Man"*, Monographs in Human Genetics, Vol. 1, *p. 43–75*, S. Karger, Basel (Switzerland).
12) G. A. Fleisher, M. Pankow, C. Warmka, *"Leucine Aminopeptidase in Human Serum"*, Clin. Acta, 9, *259* (1964).
13) L. Beckman, G. Bjorling, C. Christodoulou, *"Multiple Molecular Forms of Leucine Aminopeptidase in Man"*, Acta Genetics, Basel, 16, *223* (1966).

PUBLICATION 2

Enzymologia, 43, 339, (1972)

Multiple molecular forms of the enzyme hydrolysing DL-alanine-β-naphthylamide during gestation.

Enzymologia, vol. 43, 5 pag. 339–343, 1972

MULTIPLE MOLECULAR FORMS OF THE ENZYME HYDROLYSING DL-ALANINE-β-NAPHTHYLAMIDE DURING GESTATION

BY

DILWYN D. JONES, GEOFFREY F. WILLIAMS AND BOHUSLAV PROCHAZKA

(Bronglais Gen. Hosp., Aberystwyth and Dept. of Biol. Sciences, Univ. of Aston, Gosta Green, Birmingham).

(Received: 20th June, 1972)*

Introduction

Organ-specific isoenzymes of alanine aminopeptidase have been found by BEIER et al.,[1] using agar gel electrophoresis. Normal serum was found to contain one isoenzyme, which increased in concentration in extrahepatic cholestasis and an additional isoenzyme was found in pancreatic disease.

PETERS et al.,[2] have characterized the isoenzymes immunologically by agar gel precipitation techniques. The human isoenzymes were similar in immunochemical response but distinct differences were found in different species.

The present work was initiated to clarify the isoenzymes of the arylamidase hydrolysing DL-alanyl-β-naphthylamide (alanine-peptidehydrolase) and will be referred to by the trivial name alanine aminopeptidase. The isoenzymes of alanine aminopeptidase have been investigated in tissue extracts in relation to the increase in total serum alanine aminopeptidase during gestation.

Materials and Methods

Sera

Blood samples were taken, without anticoagulant, between 16 weeks of pregnancy and term. Estimations of alanine aminopeptidase activity were made on the fresh sera or on frozen sera (—20°) stored for not more than one week.

Tissue extracts

Liver, intestine, kidney, pancreas and bone were obtained from fresh post mortem material. Approximately 0.5 g of tissue was homogenized with 10 ml of 0.9% physiological saline and cen-

* Accepted for Publication; 30th June, 1972.

trifuged for 10 min to remove cellular debris. All tissues were homogenized in a tissue grinder. Placentae were washed with previously cooled 0.9% saline and approximately 0.5 g of tissue were taken for extraction. The bone extract was obtained by crushing 0.5 g of sternum in a pestle and mortar containing 10 ml of saline.

Polyacrylamide gel electrophoresis

The preparation of the gels and disc electrophoresis was performed as described previously[3].

Isoenzyme location procedure

The location of the isoenzymes and quantitative scanning were as described previously[3] except that DL-alanine-β-naphthylamide (125 mg in 100 ml of 0.1 M phosphate buffer, pH 7.0) was used as substrate.

Results

Tissue isoenzymes

Ten extracts of pancreas, placenta, kidney, bone, liver and intestine were found to contain one or more zones of alanine amino-

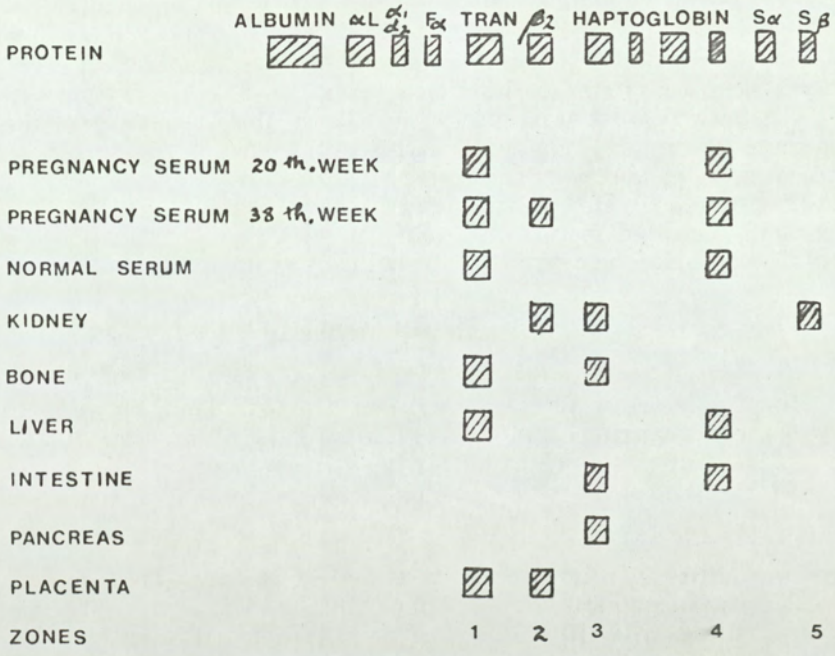


Fig. 1. The electrophoretic migration of alanine aminopeptidase isoenzymes in relation to the migration of other protein fractions.

peptidase activity. Fig. 1 illustrates the migration of each isoenzyme in relation to the migration of protein fractions. Bone and liver contained a fast moving isoenzyme (zone 1) which migrated in the transferrin region of serum. Kidney and placental extracts contained an isoenzyme which migrated to the β_2 globulin region (zone 2). Two slower migrating isoenzymes found in kidney extract were detected in the fast haptoglobin region (zone 3) and in the $S\beta$ globulin region (zone 5). Two zones of activity were detected in intestinal extract (zones 3 and 4) and one zone in pancreatic extract which migrated to zone 3.

Table I lists the isoenzyme activity found for each fraction as a percentage of the total enzymic activity. Liver, intestine, pancreas and placenta each contained one isoenzyme with a concentration greater than 80% of the total alanine aminopeptidase activity. Kidney extract contained zones 2, 3 and 5 with activity of 26%, 44% and 30% respectively.

TABLE I

Percent concentration of each isoenzyme relative to the total alanine aminopeptidase activity

Isoenzyme zones	1	2	3	4	5
Normal serum	94.6			5.4	
Serum during 20th week of pregnancy	92.5			7.5	
Serum during 38th week of pregnancy	47.0	48.4		4.6	
Kidney		26.0	44.0		30.0
Bone	38.0		63.4		
Liver	84.8			15.2	
Intestine			12.3	88.0	
Pancreas			100.0		
Placenta	10.0	90.0			

Serum isoenzymes

Two isoenzymes (zones 1 and 4) were detected in normal and pregnancy sera. Between the 20th and 28th week of pregnancy, an additional zone of enzyme activity appeared with similar electrophoretic migration to that found for zone 2 of placenta. This additional isoenzyme progressively increased in concentration as pregnancy advanced. Zone 1 of normal serum contained 95% of the total serum enzymic activity. During the 38th week of pregnancy zone 1 contained 47% and zone 2, 48% of the total serum activity.

Effect of L-methionine

Zone 1 enzyme of bone and zone 2 of kidney extracts were com-

pletely inhibited by the addition of 50 mM L-methionine. The degree of inhibition of zone 1 found in normal and pregnancy serum ranged between 62% and 83%. Zone 2 of placental extract and pregnancy serum was activated by 50 mM L-methionine, which doubled their respective activities.

Discussion

Five zones of alanine aminopeptidase activity were demonstrated in tissue extracts and two zones of activity in normal serum. Placenta, pancreas, intestine and liver extracts each contained two isoenzymes one of which was greater than 80% of the total enzyme activity. The isoenzymes with activities below 15% found in each tissue may have originated from blood contamination of the tissue. The isoenzymes found in bone and kidney extracts were unlikely to have originated from blood contamination because each isoenzyme activity was greater than 26% of the total.

By excluding enzyme zones with concentrations of less than 15% which were probably due to blood contamination it follows that zone 1 of normal and pregnancy serum was derived from bone and or liver and zone 4 from intestine. The isoenzymes of kidney and pancreas were not detected in normal serum and would therefore be unlikely to be found in pregnancy serum. Isoenzyme 2 of pregnancy serum would be likely to originate in the placenta and during pregnancy it was found to progressively increase in concentration. L-Methionine activated both the isoenzyme in placental extract and the pregnancy serum isoenzyme, which was further evidence that the pregnancy serum isoenzyme was of placental origin. The kidney isoenzyme (zone 2) was completely inhibited by L-methionine thus allowing quantitation of the placental isoenzyme. The activation of placental alanine aminopeptidase was further demonstrated histochemically on frozen sections of placenta.

The fastest moving zone of normal and pregnancy serum (zone 1) was reduced in concentration in the presence of L-methionine due to the inhibition of the bone fraction.

Serum isoenzymes of non-placental origin capable of hydrolysing DL-leucine-β-naphthylamide have been shown to increase during gestation[3] but no similar increase has been found with the isoenzymes of alanine aminopeptidase.

The specific activation of the placental isoenzyme in maternal serum by L-methionine may have clinical importance in assessing placental function.

Summary

Isoenzymes of the enzyme hydrolysing DL-alanine-β-naphthyl-

amide have been investigated by polyacrylamide gel electrophoresis. Five zones of activity were found in extracts of bone, liver, placenta, kidney, intestine and pancreas. Normal and pregnancy serum contained two isoenzymes. During the last trimester of pregnancy a third isoenzyme of placental origin was detected in maternal serum. The source of origin of the normal serum isoenzymes was bone, liver and intestine. L-methionine inhibited the bone and kidney isoenzymes and activated the isoenzyme found in placental extract and pregnancy serum. The activation of the placental isoenzyme by L-methionine can be used to advantage in estimating isoenzymes of alanine aminopeptidase activity of placental origin.

Acknowledgements. We wish to thank DR. W. H. BEASLEY, Consultant Pathologist, for his encouragement and to the Welsh Regional Hospital Board for a grant in support of this work.

1) L. Beier, I. Beier, R. J. Haschen, "*Isoenzyme der alaninaminopeptidase als spezifische parameter in der diagnostik von erkrankrungen der gallenwege und des pankreas*", Clin. Chim. Acta, 24, *405* (1969).
2) J. E. Peters, N. Rehfeld, L. Beier, R. J. Haschen, "*Immunologische charakterisierung der isoenzyme der alanin-aminopeptidase*", Clin. Chim. Acta, 19, *277* (1968).
3) D. D. Jones, G. Williams, B. Prochazka, "*Multiple Molecular Forms of Enzymes Hydrolysing L-Leucyl-β-naphthylamide during Gestation*", Enzymol., (1972) in press.

PUBLICATION 3

Molecular and Cellular Biology, in press 1973.

Multiple molecular forms of γ-glutamyl transpeptidase

In press 'Enzyme', 1973.

MULTIPLE MOLECULAR FORMS OF γ - GLUTAMYL TRANSPEPTIDASE DURING HUMAN PREGNANCY

D. D. Jones, G. Williams, and B. Prochazka,
Bronglais General Hospital, Aberystwyth and Department of Biological Siences, University of Aston, Gosta Green, Birmingham.

SUMMARY

During human pregnancy an increase was found in total serum γ-glutamyl transpeptidase (GTP). The enzyme has been fractionated into five fractions by polyacrylamide electrophoresis. Four of the five isoenzymes detected in pregnancy serum were increased, none of which were found to originate in the placenta. Intestine and bone extracts were found to contain two fractions with different electrophoretic mobilities. Four zones were detected in pancreas and kidney extracts and five zones in normal and pregnancy serum. The enzyme fractions differed in molecular form and in their action with L - methionine, urea and triton - x - 100.

INTRODUCTION

γ - Glutamyltranspeptidase (gamma- glutamyltransferase, E.C.2.3.2.1) was first described by Hanes et al[1] and the electrophoretic heterogenity was established by Kokot and Kuska[2], using paper electrophoresis. Laursen and Jacyszyn[3] determined the heterogenity of GTP by agar gel electrophoresis and the fractions were localized by a fluorescent technique. Differences were found by Kokot and Kuska[2] in both the electrical charge and in the molecular weight of GTP fractions using starch gel and sephadex filtration.

Naftalin et al[4] found GTP in the canaliculi of the liver and also the luminal border of the epithelial cells lining the fine biliary ductules. They also suggested the pancreas as a possible source of the enzyme.

Orlowski and Szczeklik[5] suggested that the determination of GTP fractions may be of clinical value in pathological states and Kokot and Kuska[2] found no distinct distribution curves specific for any disease.

During an investigation into enzymes of placental origin in human maternal serum a small progressive increase in total GTP was found as pregnancy advanced. The method used was that of Kulhanek and Dimov[6]. During the 16th week of pregnancy the range of serum GTP of 20 sera was 5 - 16 I.U. per L, and at 37 weeks of pregnancy 16-32 I.U. per L. The increase in maternal serum GTP prompted us to examine the electrophoretic mobility of GTP in tissue extracts and sera. To identify the source of origin of the GTP isoenzymes found in maternal serum, human tissue extracts and normal serum were subjected to polyacrylamide electrophoresis.

MATERIALS

Sera. Both maternal and normal bloods were taken without anti-coagulant. Ten maternal sera were taken during the 37th week of pregnancy and equal volumes of serum were pooled. Ten normal

sera were treated similarily.

Human tissue extracts. Liver, intestine, pancreas, bone and kidney were obtained from fresh post mortem material. Approximately 0.5 g. of tissue were homogenized with 10 ml. of 0.9% saline and centrifuged at 3000 r.p.m. for 10 mins. to remove cullular debris. Placentae were washed with pre-cooled 0.9% saline (4°C) and approximately 0.5 g. of tissue taken for extraction. All tissue extracts were diluted with saline to give a final enzyme activity of 30 I.U. per litre.

Substrate. 6 u moles of L - γ - glutamyl - β - napthylamide (Sigma Chemical Co., Ltd., 12, Lettice Street, London, S.W.6), 10 u moles of magnesium chloride, 20 u moles of glycyl-glycine per ml. of 0.1 M phosphate buffer, pH 7.4.

Fast Garnet GBC (Sigma Chemical Co., Ltd.,) 100 mg per 100 ml. of distilled water.

L - methionine 100 mg/100 ml. of substrate.

Urea 0.05 M in substrate.

Triton - x - 100 (Sigma Chemical Co., Ltd.,) 1% v/v of tissue extract and 1% v/v of substrate.

Electrophoresis buffers The electrode buffers and gel monomer were prepared as described by Jones et al[8].

METHODS

Disc electrophoresis The buffers and gels were stored at 2°C. 20 ul of tissue extracts or serum were applied to a filter paper disc, the diameter of which was less than the diameter of the gel tube. Current was adjusted to 5 M.A. per tube, and the electrophoresis run in a refrigerator at 2°C. The run required approximately 60 mins. to complete. The electrophoresis was terminated when the albumin of a serum containing bromo phenol blue had reached the bottom of the gel tube. This tube was subsequently used to stain the protein fractions with ponceau S, which served as a means of marking the enzyme zones Fig. 1.

Iso-enzymes location prodecure

Incubation of the whole gel in individual tubes for 18 hours resulted in the enzyme fractions diffusing both into the surrounding medium and into the gel, thereby making direct scanning of the gel impossible. Fast blue was also used as location reagent without obtaining increased sensitivity.

The gels were removed from the running tubes and cut transversely into 3 mm slices. Each sliced portion was then incubated in 0.5 ml substrate at 37°C for 18 hours. 0.2 ml of the fast garnet solution added and the solution read colormetrically at 520 m u. The enzyme activity of each tube was calculated and expressed as a percentage of the total serum enzyme activity.

The total serum enzyme activity was estimated by electrophoresis of a duplicate serum sample on polyacrylamide gel. The gel was cut as above and all the slices combined in a test tube containing 6 ml. of substrate which was incubated at 37°C for 18 hours. 2.4 ml. of fast garnet solution was then added and read at 520 m u.

Using the tube elution technique enzyme fractions were detected both in the serum samples and tissue extracts. Crushing of the gel sections was not found to be necessary since the enzyme completely diffused during the 18 hours incubation.

RESULTS

Enzyme estimations Total enzyme activity of ten duplicate analysis was 11.7 - 12.5 I.U. per L (x 12.1, SD 0.3). The mean values and SD of replicate assays of the isoenzymes in normal serum are seen in Table 1. Direct estimation of zone 5 of each sample was not possible since only a fraction of the enzyme was capable of penetrating the gel, the remainder being lost during the removal of the gel from the running tube. Zone 5 isoenzyme was calculated from the difference between the total enzyme activity and the sum of the other isoenzymes present.

Mobility of tissue isoenzymes After polyacrylamide electrophoresis the extracts of pancreas, placenta, kidney, bone, liver and intestine contained two or more isoenzymes of different electrophoretic mobilities (Fig. 1). Bone and liver contained zone 2 and zone 5 isoenzymes with smaller amount of others in liver. A zone 4 and a zone 5 of enzyme activity were detected in the intestinal extract. Placenta contained three isoenzymes, zones 1, 3 and 5, and kidney contained four isoenzymes, zones 1,3,4 and 5. Pancreatic extract was found to contain zones 1, 3 and 5 and furthermore an enzyme fraction was detected in zone 1 (a) which was not detected in the other tissues examined. The zone 1 (a) of pancreas corresponded in electrophoretic migration to that of the L protein fraction and zone 1 to that of the fraction (Fig. 1) Zone 5 of kidney, placenta, bone, intestine and liver contained 74%, 66%, 79%, 78%, and 60% respectively of the total enzyme activity. Table 2 lists the concentration of the isoenzymes.

Mobility of serum isoenzymes Zones 1,2,3,4 and 5 were detected in both normal and maternal serum. Zone 2 of the maternal serum and normal serum contained the least activity with 9% and 6.7% activity. The highest activity of both maternal serum and normal serum was found in zone 5 with enzyme activities of 32% and 35.8% respectively (Table 2). The concentration of zone 1 enzyme in maternal and normal sera were similar but an increased isoenzyme concentration was detected in zones 2, 3 and 4 of maternal serum.

Effect of L - methionine Zone 2 isoenzyme of bone extract, maternal and normal sera were activated by L - methionine. All other zones of enzyme activity were unaffected.

Effect of urea Urea inhibited the enzyme activity of zone 5 of liver extract and decreased the activity of zone 5 found in normal serum and maternal serum. The enzyme activities of all the other tissue extracts and serum were unaffected.

Effect of triton - X - 100 The isoenzyme activity of zone 5 of kidney, liver and placenta and zone 1 of normal serum were increased by the addition of triton - X - 100. All four isoenzymes of pancreas were activated. Table 2 lists the increases in the activation of the isoenzymes. The addition of triton - x - 100 did not increase the electrophoretic mobility of the isoenzymes of GTP.

DISCUSSION

The method described makes it possible to detect electrophoretic fractions of GTP, furthermore the technique can be used for quantitative determinations of isoenzymes 1,2,3 and 4. Zone 5 could not be estimated with accuracy since the enzyme fraction was found to have an insignificar electrophoretic mobility.

Zone 2 of normal serum probably originates from bone since L - methionine activates zone 2 of both bone extract and normal serum. Zone 5 of liver extract, maternal serum and normal serum were inhibited by urea suggesting that zone 5 activity found in serum originated in part from liver. Triton - x - 100 activated zones 1 of serum and pancreas which would suggest that zone 1 of serum was of pancreatic origin.

Bone, placenta, liver, kidney, normal and maternal serum contained a GTP fraction of zone 5, which was probably an 'insoluble' form first demonstrated by Szewczuk[9]. Since the insoluble form of GTP failed to move significantly through the gel, triton - x - 100, a non-ionic detergent, was used to release the enzyme bound to cell structures. Tissue extracts and serum containing triton - x - 100 exhibited an increased enzyme activity which was probably due activation of the enzyme rather than an increased solubility of the enzyme during extraction from tissue.

The present results confirm the work of Naftalin et al[4] who suggested the pancreas as a possible source of origin of GTP. Furthermore, a fast moving isoenzyme was found in pancreatic extract which was not detected in the other tissue extracts examined.

The enzyme zones 2,3,4 and 5 corresponded in electrophoretic migration to that found for leucine aminopeptidase isoenzymes by Jones et al[8]. Zone 1 of leucine aminopeptidase did not correspond to that of zones 1 or 1 (a) of GTP. The present results suggest there are at least five different molecular forms of GTP present in normal and maternal serum and which are probably distinct molecular species differing in their action to urea, L - methionine and triton - x - 100.

The increase in total GTP during gestation was contributed to by zones 2,3,4 and 5. Zone 2 originated from bone, zone 3 from kidney, pancreas and liver, zone 4 from kidney, intestine and liver and zone 5 from liver alone. Urea completely inhibited zone 5 of liver and maternal serum, but not zone 5 of placenta. It is therefore unlikely that any of the GTP isoenzymes of maternal serum originate in the placenta. The estimation of GTP or its isoenzymes would not therefore be an aid to assessing placental function.

ACKNOWLEDGEMENTS

We wish to thank Dr. W. H. Beasley, Consultant Pathologist for his help and encouragement and to the Welsh Regional Hospital Board for a grant in support of this work.

REFERENCES

1. C. S. Hanes, F. J. R. Hird and F. A. Isherwood, 'Synthesis of Peptides in Enzymic Reactions Involving Glutathione', Nature, 166, 288 - 290 (1950).

2. F. Kokot and J. Kuska, 'Heterogenity of Serum γ - Glutamyl Transpeptidase in Different Internal Diseases Studied by Starch Gel Electrophoresis and Sephadex Filtration, Enzym. biol. clin., 9, 59 - 67 (1968).

3. T. Laursen and K. Jacyszyn, 'Fluorimetric Determination of the Electrophoretic Heterogenity of γ-Glutamyl - Transpeptidase', Clin. Chim. Acta., 21 497 - 499 (1968)

4. L. Naftalin, V. J. Child and D. A. Morley, 'Observations on the Site of Origin of Serum γ - Glutamyl Transpeptidase'. Clin. Chim. Acta, 26, 297 - 300 (1969)

5. M. Orlowski and A. Szczeklik, 'Heterogeneity of Serum γ-Glutamyl Transpeptidase in Hepatobiliary Diseases', Clin. Chim. Acta, 15, 387 - 391 (1956)

6. V. Kulhanek and D. M. Dimov, 'A New Useful Modification for the Determination of γ Glutamyl - Transpeptidase Activity', Clin. Chim. Acta., 14, 619 - 623 (1966)

7. D. D. Jones, G. Williams and B. Prochazka, Unpublished Observations.

8. D. D. Jones, G. Williams and B. Prochazka, 'Multiple Molecular Forms of Enzymes Hydrolysing L - Leucyl - β - Naphthylamide During Gestation Enzymologia, 43, 325 - 332 (1972)

9. A. Szeszczuk, ' A Soluble Form of γ Glutamyl Transpeptidase in Human Tissues' Clin. Chim. Acta., 14, 608 - 612 (1966).

ISOENZYME ZONE	1a	1	2	3	4	5
KIDNEY.	-	4.9	-	5.2	15.9	74 (x3)
BONE	-	-	20.8	-	-	79.2
PANCREAS	51.1 (x1.1)	21.0 (x4.2)	-	24.0 (x3.6)	-	3.9 (x10)
INTESTINE	-	-	-	-	22.0	78.0
LIVER	-	10.8	21.6	3.6	4.0	60.0 (x1.1)
PLACENTA	-	29.9	-	3.7	-	66.4
NORMAL SERUM	-	25.4 (x1.7)	6.7	15.2	16.9	35.8
PREGNANCY SERUM	-	24.9 (x2.2)	9.0	16.1	18.0	32.0

Table 2. Per cent activity of each isoenzyme. The increase in activity with triton - x - 100 is seen in brackets. The total enzyme activity of each tissue extract was 30 I.U. per L

ISOENZYME ZONE	1	2	3	4	5
MEAN (x) I.U. per L	3.0	0.8	1.8	2.0	4.3
STANDARD DEVIATION (SD)	0.32	0.24	0.26	0.26	0.51

Table 1. Mean and standard deviation of ten duplicate isoenzyme assays in normal serum.

Fig. 1. Submitted for publication was similar to Fig. 42 found in main thesis.

PUBLICATION 4

Enzyme, in press 1973

Isoenzymes of n-acetyl-β-glucosaminidase during human pregnancy.

In press 'Enzyme', 1973.

Isoenzymes of N-acetyl-β-glucosiminidase During Human Pregnancy

D. D. Jones, G. F. Williams and B. Prochazka,

Bronglais General Hospital, Aberystwyth and

Department of Biological Sciences, University

of Aston, Gosta Green, Birmingham.

SUMMARY

During human pregnancy a progressive increase was found in maternal serum N-acetyl- -glucosaminidase. Two isoenzymes were detected in placental and foetal liver extracts. The isoenzymes were purified and their antibodies raised in rabbits. No antigenic difference was found between the foetal and adult tissue isoenzymes. Antisera to the placental isoenzymes did not react with the isoenzymes of adult tissues. The antibody raised against one placental and one foetal liver isoenzyme neutralized the N-acetyl-β-glucosaminidase activity in maternal serum at 38 weeks gestation to the value found at 12 weeks of pregnancy. Immunological evidence has been presented suggesting that the isoenzyme of N-acetyl-β-glucosaminidase found only in pregnancy serum was of placental origin.

INTRODUCTION

The levels of serum N-acetyl-β-glucosaminidase (β-2-acetamido-2-deosy-D-glucoside acetamido-deoxyglucohydrolase, EC 3.2.1.30) has been shown to progressively increase during pregnancy by Walker et. al.[1], Furiya and Fukuda[2], and by Woollen and Turner[3]. The estimation of N-acetyl-β-glucosaminidase in tissue extracts has been investigated by Pugh et. al.[4], and more recently a sensitive fluorimetric method for serum has been reported by Woollen and Walker[3].

Multiple molecular forms of the enzyme have been reported by Huddleston et. al.[5], Stirling[6] and by Price and Dance[7]. Robinson and Stirling[8] demonstrated two molecular forms A and B in extracts of human spleen. The A and B isoenzymes have subsequently been found in other human tissues Okada and O'Brien[9] and in serum O'Brien et. al[10].

During human gestation Stirling[6] found two forms of the enzyme A and P.. The A form lost 70% of its activity during heating at 50°C whereas the P form was heat stable. Both Huddleston et. al.[5] and Stirling[6] were not able to establish the source of the isoenzyme in gestational serum.

No electrophoretic similar isoenzyme was found on electrophoresis of placenta, foetal membranes or amniotic fluid. These workers used starch gel electrophoresis to fractionate the enzyme.

Our results obtained using gradient polyacrylamide electrophoresis confirmed the work of Stirling[6]. During our investigations into the value of maternal serum isoenzymes as an index of foeto-placental function it was necessary to locate the tissue of origin of the serum isoenzyme found only in pregnancy serum. To this end we investigated immunologically the isoenzymes of placental and foetal liver extracts in relation to the circulating maternal serum isoenzyme of N-acetyl-β-glucosaminidase.

MATERIALS AND METHODS

Human sera Maternal and normal blood were taken without anticoagulant and the serum prepared by 'fast' centrifuging[3].

Human tissue extracts Tissues were obtained from fresh post mortem material and approximately 0.5 g of tissue was homogenized with 10 ml of 0.9% saline and centrifuged at 3000 r.p.m. for 10 minutes to remove cellular debris. Placentae were washed with pre-cooled 0.9% saline (4°C) and extracts made as for other tissues. Foetuses were

obtained during hysterotomy and were dissected to remove the liver. The liver extract was prepared as for other tissues.

Buffers The electrophoresis buffer used was as recommended by Universal Scientific Ltd., 231 Plashet Rd., London, E.13. Tris 10.75 g, disodium E.D.T.A. 0.93 g, boric acid 5.04 g dissolved in 1 litre of distilled water. The polyacrylamide gel slabs were supplied containing sodium azide as preservative and was removed by electrophoresis for one hour at 80mA and the buffer discarded.

Polyacrylamide electrophoresis The Gradipore Electrophoresis system was used throughout this work (supplied by Universal Scientific Ltd.,). Concave gradient gels from approximately 4% to 26% were employed for all the investigations. 10 ul of tissue extracts and sera were introduced to the gel through an acrylic comb by the gel slot technique. During preparative electrophoresis 2 ml of tissue extracts were applied to the gel slabs without use of the acrylic combs. Three gel slabs approximately 70 x 70 x 3 mm were electrophoresed at 80mA for seven hours. With each batch a serum stained with bromo phenol blue was incorporated enabling the albumin fraction to be visually located during the run. During the electrophoresis the buffer was cooled

to 5°C by circulation through a cooling bath.

Isoenzyme location procedure The gels were removed from the glass cells and incubated for eighteen hours at 37°C using the substrate naphthol AS-BI- -D-glucosaminide as described by Huddleston et. al[5]. The localisation of the enzyme zones for preparative work was made by slicing a portion of the gel approximately 1 cm wide in the direction of the gradient and the slice incubated with substrate as described above. The gels were stored at 4°C until the zones of enzyme activity were detected. The boundaries of the enzyme activity were marked with a scalpel. The zones of isoenzymes in each gel slab corresponding to that of the A and B fractions in placental and foetal liver extracts were cut and homogenized in 0.9% saline. The suspension of gel in saline was then centrifuged at 3000 r.p.m. for ten minutes and the supernatant removed for gel filtration.

Gel filtration Further purification of the isoenzymes was made by Sephadex G-200 gel filtration. The column 2.5 x 90 cm was equilibriated with 0.01M Tris - Hcl buffer, pH 7.4, containing 0.14 NaCl. The eluates were collected in 3 ml volumes and aliquots of each eluate used to estimate both the total enzyme activity and protein concentration. The

total enzyme activity of the elnates were estimated fluorimetrically using the method described by Woollen and Walker[3]. Total protein was estimated turbidometrically by precipitation with 3% sulphosalicylic acid. The tubes of highest enzyme activity were pooled.

Rabbit anti-acetylglucosaminidase The purified isoenzymes containing 1 mg of enzyme protein per 1 ml were suspended in 1 ml of Freund's adjuvant and injected subcutaneously at multiple sites into albino rabbits at two week intervals. After six weeks the rabbits were bled and the antiserum was collected and stored at -20°C.

Gel diffusion The Ouchterlony technique was employed using plastic petri dishes containing 1% agar in barbitone buffer pH 8.2. The wells were 4 mm in diameter and 8 mm distant. 30 ul volumes of antiserum and tissue extracts were employed. The gels were incubated for twenty four hours at room temperature in a moist chamber, and washed in 0.9% saline for five hours. The enzyme activity of the immunoprecipitate was estimated histochemically as described by Huddleston et. al[5].

Immunoelectrophoresis Glass slides coated with 1% agar in barbitone buffer at pH 8.6 was employed. 10 ul of sera and tissue extracts were electrophoresed at 150V for three hours. Antibody diffusion was performed for eighteen hours and the slides washed in 0.9% saline for five hours. The enzyme immunoprecipitate was detected histochemically as described for the Ouchterlony method above.

Inactivation of pregnancy serum N-acetyl-β-glucosaminidase 1.0 ml of a serum pool obtained from ten patients at 38 weeks of pregnancy was mixed with 0.1 ml of each rabbit anti-serum and as a control 1.0 m of pregnancy serum was mixed with 0.1 ml of 0.9% saline. The samples were incubated at 37°C for one hour and then placed at 4°C for two days. Each tube was then centrifuged at 3000 r.p.m. for fifteen minutes and the supernatant assayed for N-acetyl-β-glucosaminidase. The immunological inactivation of the serum enzyme was expressed as a percentage of the enzyme activity in the control tube.

RESULTS

Placental and foetal liver extracts were found to contain two isoenzymes A and B, Fig. 1. Pregnancy serum contained two isoenzymes, one of which had similar migration

to that found for the A isoenzyme of placenta and foetal liver extracts. The second isoenzyme of pregnancy serum was found to migrate to the immunoglobulin G and A region and will be referred to as the P isoenzyme. The P isoenzyme of maternal serum was found to progressively increase during pregnancy but no similar increase was found for the A isoenzyme. No P isoenzyme was detected in homogenates of human tissues such as liver, spleen, bone, pancreas, intestine, uterus, breast and ovary.

Ninety three per cent of the N-acetyl-β-glucosiminidase activity in pregnancy serum at 38 weeks was contributed by the isoenzyme P and seven per cent by the A isoenzyme. Cord serum was found to contain the P isoenzyme, the activity of which was one eighth that found for pregnancy serum at term.

The P isoenzyme of maternal serum was found to progressively increase during pregnancy but no similar increase was found for the A isoenzyme.

By adding the antibodies against both A and B isoenzymes to four pools of serum taken during the 38th week of pregnancy the total activity of N-acetyl-β-glucosiminidase was reduced in concentration to that found during the 14th week of pregnancy. Table 1 lists the inactivation of the

total enzyme activity after the addition of the antisera. There was no significant inactivation after one hours incubation with the antisera. However after forty eight hours the antiserum to placental B and foetal B isoenzymes had inactivated the maternal serum enzyme by 71.9% and 75% respectively.

The total activity of N-acetyl- -glucosiminidase during the 38th week of pregnancy was reduced to the concentration found in maternal serum during the 14th week of pregnancy after the addition of antiserum to placental B and foetal B isoenzymes.

Using gel diffusion an enzyme immunoprecipitate formed when the four antisera were assayed against the purified isoenzyme extracts. The antiserum to the isoenzymes A of placenta and foetal liver did not react with pregnancy serum. The antiserum to the B isoenzymes of placenta and foetal liver formed an immunoprecipitate with maternal serum. Fig. 2 and 3 illustrates the progressive increase in the immunoprecipitate of pregnancy serum between twelve and thirty eight weeks of pregnancy. Both antisera of the foetal liver isoenzymes A and B reacted with extracts of liver, spleen, pancreas, intestine and kidney but the placental antisera did not react with the tissues tested.

The titre of all four antisera were found to be 1/32 when the concentration of the antigen was 1 mg/1 ml enzyme protein.

DISCUSSION

Two isoenzymes of N-acetyl- -glucosiminidase A and B were detected in homogenates of liver, spleen, bone, pancreas, intestine, kidney, placenta and foetal tissue. In pregnancy serum both A and a P isoenzyme were detected with the P form progressively increasing during gestation. Table 1 shows that there is no significant inactivation after the one hour incubation of serum with antiserum. However after forty eight hours antiserum to placental and foetal B isoenzyme inhibits the activity in maternal serum by 70% and 75%. These results confirm the work of Stirling who employed starch gel electrophoresis. A number of workers Robinson and Stirling[8], Dance et. al.[11], and Okada and O'Brien[9] have demonstrated two forms in human tissues. In serum from males and non-pregnant females only trace quantities of isoenzyme A was detected. Price and Dance[7] found that the heat labile form could be converted to I1 and I2 forms which were indistinguishable from the P isoenzyme found in pregnancy serum. Walker et. al.[1], found histochemically N-acetyl- -glucosiminidase in the decidual cells of the

chorion and basal plate of the placenta and speculated that the increased serum activity was derived from the placenta. Huddleston[5] did not implicate the placenta as the source of the enzyme since there were no electrophoretically similar isoenzyme to that found in pregnancy serum. Furthermore they found that elevated systemic oestrogens found during oral contraception did not produce the pregnancy isoenzyme. The source of origin of the P isoenzyme in pregnancy serum has not been established using electrophoresis, heat denaturation and by inhibition studies.

The antigenicity of N-acetyl- -glucosiminidase molecule was seen by the production of the antibody after a course of only three injections. The foetal liver A and B and the placental A and B isoenzymes reacted with their corresponding antisera. All four isoenzymes also cross-reacted with the four antisera which suggests that the four isoenzymes were antigenically similar. The isoenzyme P of pregnancy serum reacted only with antiserum of foetal liver B and placental B which would establish that the pregnancy serum P isoenzyme was either of foetal or placental origin.

Both antisera to foetal liver A and B reacted with all adult tissues and were therefore antigenically indistinct although the structures may or may not be the same. On the

other hand the placental A and B antisera did not react with other adult tissues and were therefore antigenically indistinct and have different structures.

During the passage of the isoenzyme from placenta to the maternal circulation it would be likely that a change in structure such as unfolding of the chain or a conformationa change may occur in the enzyme molecule which may produce the P isoenzyme found in pregnancy serum.

The antisera to foetal A and placental A did not react with the pregnancy enzyme of maternal serum which suggests different enzyme structure. It would be reasonable to expect the antiserum to the foetal B isoenzyme to react with the pregnancy serum enzyme since this antiserum was found to react with all adult and foetal tissue enzymes.

The concentration of the P isoenzyme in cord serum was found to be approximately one eighth of that found in maternal serum taken during the 38th week of pregnancy. It would therefore be unlikely that the pregnancy serum P isoenzyme would arise in the foetus. Since the antiserum to the placental B isoenzyme reacts with pregnancy serum it would be reasonable to speculate that the pregnancy serum enzyme is derived entirely from the placenta. Furthermore the antibodies raised against the placental B and foetal liver B

isoenzymes neutralized the major portion of the maternal serum N-acetyl- -glucosiminidase taken during the 38th week of pregnancy to an activity found during the 14th week of pregnancy. A conformational change in the placental B isoenzyme may give rise to the placental A isoenzyme although antigenically the two enzymes were similar.

The foetal and adult tissue isoenzymes A and B can be assumed to be heterogenous but the placental A and B were found to be organ specific although cross reactions were found with the foetal liver isoenzymes.

The difficulty hitherto in finding the source of origin of the pregnancy isoenzyme have been in the main due to the fact that the properties measured by electrophoresis and chromatography on DEAE cellulose would be unaltered by molecular substitutions which would not affect the size or charge but could be changed by molecular binding to other components in biological fluids.

From these data it is concluded that the pregnancy serum P isoenzyme is of placental origin and that the foetal isoenzymes are antigencially similar to that found in adult tissues. The progressive increase in the pregnancy serum during gestation can be estimated immunologically using a placental specific antiserum.

Antiserum		Pregnancy serum N-acetyl- -glucosaminidase mole/min/L		% inactivation
		Untreated	Treated	
Placental A isoenzyme	1 hr.	8.2	8.0	2.4
	2 days	7.9	7.6	3.7
Placental B isoenzyme	1 hr.	8.3	8.0	3.6
	2 days	8.2	2.3	71.9
Foetal A isoenzyme	1 hr.	8.3	8.2	1.2
	2 days	8.1	7.8	3.7
Foetal B isoenzyme	1 hr.	8.1	7.8	3.7
	2 days	8.0	2.0	75.0

Table 1. The inactivation of pregnancy serum N-acetyl- -glucosaminidase by antisera raised against placental and foetal liver isoenzymes A and B.

Fig's 1, 2 and 3 submitted for publication were similar to Fig's 46, 62 and 64 found in main thesis.

1. P. G. Walker, M. E. Woollen and D. Pugh, 'N-Acetyl- - Glucosaminidase Activity in Serum during Pregnancy,' J. Clin. Path., 13, 353-357 (1960)

2. S. Furiya and A. Fukuda, 'Estimation of Serum -acetylamino-deoxyglucosidase,' J. Biochem. Tokyo, 54, 398-402 (1963).

3. J. M. Wollen and P. G. Walker, 'The Fluorimetric Estimation of N-acetyl- - glucosaminidase and - galactosidase in Blood Plasma,' Clin. Chim. Acta, 12, 647-658 (1965)

4. D. Pugh, D. H. Seaback and P. G. Walker, 'N-Acetyl- -Glucosaminidase in Rat Tissue Preparations,' Biochem. J., 65, 16 (1957)

5. J. F. Huddleston, G. Lee and J. C. Robinson, 'Electrophoretic Characterization of Glucose Dehydroganase, -glucoromidase and N-acetyl- -glucosaminidase from Placenta and Gestational Serum,' Amer. J. Obstet. Gynec. 109, 1017-1021 (19

6. J. L. Stirling, 'Separation and Characterisation of N-Acetyl- -glucosaminidase A and P from Maternal Serum', Biochim. Biophys. Acta, 271, 154-162 (1972)

7. R. G. Price and N. Dance, 'The Demonstration of Multiple Heath Stable Forms of N-Acetyl- -glucosaminidase in Normal Human Serum,' Biochim. Biophys Acta, 271, 145-153 (1972).

8. D. Robinson and J. L. Stirling, 'N-Acetyl- -Glucosaminidase in Human Spleen,' Biochem. J., 107, 321-327 (1968)

9. S. Okada and J. S. O'Brien, 'Tay-Sachs Disease - Generalized Absence of B-D-N-Acetyl Hexosaminidase,' Science, 165, 698-700 (1969)

10. J. S. O'Brien, S. Okada, A. Chen and D. L. Fillerup, 'Tay-Sachs Disease. Detection of Heterozygotes and Homozygotes by Serum Hexosaminidase Assay,' New Eng. J. Med., 283, 15-20 (1970)

11. N. Dance, R. G. Price, B. Robinson and J. L. Stirling, ' -Galactosidase, - Glucosidase and N-Acetyl- -glucosaminidase in Human Kidney,' Clin. Chim. Acta, 24, 189-197 (1969)

PUBLICATION 5

Enzyme in press 1973

The serum activity of glucose-6-phosphatase and 5-nucleotidase during human pregnancy.

In press 'Enzyme,' 1973.

The Serum Activity of Glucose-6-Phosphatase and 5'-Nucleotidase during Human Pregnancy

D. D. Jones, G. F. Williams and B. Prochazka,
Bronglais General Hospital, Aberystwyth and
Department of Biological Sciences, University
of Aston, Gosta Green, Birmingham.

SUMMARY

An attempt has been made to show that the increase in enzyme activities in sera of pregnant women found with glucose-6-phosphate and adenosine 5-monophosphate as substrates (described as glucose-6-phosphatase and 5'-nucleotidase) was due to the increase in alkaline phosphatase. The three enzyme activities had pH optima and heat stability characteristics of alkaline phosphatase. The response to the action of inhibitors and activators was typical for alkaline phosphatase. There was an identical increase in all three enzyme activies during pregnancy. As a control similar investigations were made with liver and placental tissue extracts.

INTRODUCTION

An increase in maternal serum alkaline phosphatase (orthophosphoric monester phosphohydrolase, EC 3.1.3.1), 5-nucleotidase (5-ribonucleotide phosphohydrolase, EC 3.1.3.5), and in glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase) have been reported by Neale et. al.[1] and Elder[2]. The placenta has been presumed to be the source of these enzymes.

The changes in 5-nucleotidase and glucose-6-phosphatase during pregnancy have been controversial, Wachstein et. al.[3] found glucose-6-phosphatase during early pregnancy, whereas Curzen[4] and Carter and Weber[5] found the enzyme in the placenta during early and late pregnancy. 5-nucleotidase has been reported to exceed the normal limits during the last trimester of pregnancy Mistilis[6] but Seitanidis and Moss[7] found no such increase.

Since there is a strikingly similar increase in maternal serum 5-nucleotidase and glucose-6-phosphatase to alkaline phosphatase and also the substrates used for their determinations are related, the possibility was considered that alkaline phosphatase was responsible for all three enzyme activities.

The aim of this work was to investigate whether 5-nucleotidase and glucose-6-phosphtase activity in serum of pregnant women was due to the action of alkaline phosphtase. The parameters used in distinguishing between the activities of the enzymes in serum of pregnant women was pH optima, heat stabilities and the action of activators and inhibitors.

MATERIALS AND METHODS

Sera and tissue extracts Maternal blood was taken without anticoagulant. A serum pool consisting of 10 sera taken during the 38th week of pregnancy was used throughout. 0.5 g of liver and placenta were homogenized with 10 ml of 0.9% saline and centrifuged to remove cellular debris.

Buffer systems[8] (1) Tris-maleic acid (Tris 24.23 g + maleic acid 23.22 g + water to 1 litre). 0.2 M Sodium hydroxide. pH obtained 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5.

(2) 0.1 M carbonate-sodium bicarbonate. Anhydrous sodium carbonate 6.3 g, sodium bicarbonate 3.36 g, water to 1 litre. pH obtained 9.0, 9.5 and 10.0

Substrates Final concentrations - alkaline phosphatase 0.01 M disodium phenyl phosphate; glucose-6-phosphatase 0.01 M glucose-6-phosphate; 5-nucleotidase 0.004 M adenosine 5-mono-phosphate.

Activators and Inhibitors Final concentrations - Magnesium chloride 10 mM; manganese sulphate 1 mM; nickel chloride 10 mM

and beryllium sulphate 1.4 mM.

Heat stability The sera were heated to 65°C for 30 minutes as described by Hunter[11].

Technique for enzyme estimations All three enzymes were estimated by the method of Koide and Oda[9]. The inorganic phosphate liberated was estimated colorimetrically by the method of Fiske and Subbarrow[10]. 1 unit of enzyme activity was that amount of enzyme that release 1 g of inorganic phosphate at 37°C for one hour.

RESULTS

The results of the alkaline phosphatase, 5-nucleotidase and glucose-6-phosphtase of a pooled serum are seen in Fig. 1. No peak of enzyme activity was obtained with either enzyme between a pH of 6 and 8. Maximum hydrolysis of each substrate occurred at pH 10.0. The addition of magnesium ions activated the hydrolysis of all three substrates. At pH 10.0 the alkaline phosphatase was activated by 28%, 5-nucleotidase by 25% and glucose-6-phosphatase by 5%. Fig. 1 illustrates the results obtained. Manganese was found to activate alkaline phosphatase substrate by 17% at pH 10.0 and the 5-nucleotidase by approximately 22% throughout the pH range. The activation of glucose-6-phosphate was similar to that found for alkaline phosphatase, Fig. 2.

At a pH of 10.0 nickel ions were found to activate the three enzymes alkaline phosphatase, 5-nucleotidase and glucose-6-phosphatase by 24%, 48% and 38% respectively. In each instance the nickel inhibited the hydrolysis between

pH 6 and 9 as illustrated in Fig. 3. Beryllium sulphate was found to inhibit the three enzymes between a pH of 5 and 10 - Fig. 4.

Heating the pooled serum to 65°C for 30 minutes was found to inactivate the enzymes between pH 6 and 8. None of the above experiments showed a peak of enzyme activity between pH 6.0 and 8.0.

The activity of 5-nucleotidase, glucose-6-phosphatase and alkaline phosphatase were estimated in thirty individual samples of serum taken between the fourteenth week of pregnancy and term. The results for alkaline phosphatase were plotted against both 5-nucleotidase and glucose-6-phosphatase. In each case a straight line was obtained.

5-Nucleotidase and glucose-6-phosphatase could be demonstrated in liver extracts. Placental extract contained 5-nucleotidase with optimum activity at pH 7.0 but no glucose-6-phosphatase was detected, Fig 5.

DISCUSSION

Mistilis[6] has reported that serum 5-nucleotidase may reach or exceed the normal upper limit of activity during the third trimester of pregnancy. We have also found similar results with glucose-6-phosphatase. A number of workers[12, 13, 14, 15] have described activators and inhibitors to estimate the 'true' 5-nucleotidase and glucose-6-phosphatase in serum. During pregnancy where a progressive increase is found in

serum alkaline phosphtase the estimation of 5-nucleotidase and glucose-6-phosphatase can present difficulties due to the 'tailing' of the alkaline phosphatase activity at unoptimum pH.

The addition of magnesium and manganese both activators of 5-nucleotidase did not produce a peak of enzyme activity in the pH range 6 to 8. The addition of beryllium to inhibit the alkaline phosphatase did not isolate enzyme activity between pH 6 and 8. Elder[2] using beryllium ions found no increase in serum glucose-6-phosphatase during pregnancy which was contrary to our findings. Since beryllium ions have been found to inhibit alkaline phosphatase and the hydrolysis of glucose-6-phosphate was due to non-specific alkaline phosphatase then no increase would be expected in the serum glucose-6-phosphatase levels.

Nickel which is known to inhibit 5-nucleotidase[14] was found to inhibit the hydrolysis of all three substrates between pH 6 and 9 and to activate at a pH of 10.0. These results suggest the absence of 5-nucleotidase in pregnancy serum. It has been suggested[16] that 5-nucleotidase has a larger molecular weight than alkaline phosphatase and therefore diffusion out of the placenta into the maternal circulation may be limited.

Elder et. al.[15] found a peak of enzyme activity in serum using berrylium as inhibitor of alkaline phosphatase but the hydrolysis of the three substrates in the presence of beryllium was found by us to be similar. Although a

glucose-6-phosphatase like activity has been demonstrated histochemically in placenta by Carter and Weber[5] it would be unlikely the enzyme which is microsome bound would be liberated into the maternal circulation.

It is interesting to note that a straight lime was obtained when plotting the activity of 30 serum alkaline phosphatase against 5-nucleotidase and glucose-6-phosphatase. Although this in itself would not be sufficient evidence to implicate the non-specific alkaline phosphatase for hydrolysing glucose-6-phosphate and adenosine monophosphate it does serve as further evidence.

Alkaline phosphatase was found to be active over a wide pH range and the hydrolysis of adenosine-5-monophosphate and glucose-6-phosphate was due to non-specific alkaline phosphatase. It appears that the glucose-6-phosphatase and 5-nucleotidase reported to be found in pregnancy serum would be entirely due to the non-specific action of alkaline phosphatase.

ACKNOWLEDGEMENT

We wish to thank the Welsh Regional Hospital Board for a grant in support of this work.

Fig's 1, 2, 3, 4 and 5 submitted for publication were similar to Fig's 50, 51, 52, 53 and 56 found in main thesis.

REFERENCES

1. Neale, F. C., Chubb, J. S., Hotchkis, D. and Posen, S. (1965) J. Clin. Path., 18, 359-363.
2. Elder, M. G. (1973) J. Obst. Gyn. Brit. Comm., 80, 109-110.
3. Wachstein, M., Meagher, J. G. and Ortiz, J. (1964) Am. J Obst. Gynec., 87, 13-17.
4. Curzen, P. (1964) J. Obst. Gyn. Brit. Comm., 71, 388-340
5. Carter, J. E. and Weber, G. (1966) Am. J. Obst. Gynec., 95, 914-924.
6. Mistilis, S. P. (1968) Aust. Ann. Med., 17, 248-260.
7. Seitanidis, B. and Moss, D. W. (1969) Clin. Chim. Acta, 25, 183-184.
8. Documenta Geigy., Ed. Diem, K. and Sentner, C. p. 280 J. R. Geigy, Basle, Switzerland.
9. Koide, H. and Oda, T. (1959) Clin. Chim. Acta, 4, 554-558.
10. Fiske, C. H. and Subbarow, Y. (1925) J. Biol. Chem., 66, 375-378.
11. Hunter, R. J. (1969) J. Obst. Gyn. Brit. Coun., 76, 1057-1069.
12. Belfield, A. and Goldberg, D. M. (1969) J. Clin. Path., 22, 144-151.
13. Campbell, D. M. (1962) Biochem. J., 82, 34
14. Foz, M. (1967) Clin. Chim. Acta, 17, 13-19.
15. Elder, M. G., Wood, E. J. and Said, J. (1972) Clin. Chim Acta, 38, 211-215.
16. Moss, D. W. (1966) Nature, 209, 806-807.