

THE PHOSPHATASES AND GLYCOLYTIC ENZYMES

IN DISEASE

Thesis submitted by

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ABSTRACT.

The purpose of this work has been to correlate the level of certain enzymes in blood serum with clinical status.

The introduction is concerned with the historical background of clinical enzymology and the general conditions which cause alterations in the level of enzymes in serum. Reasons are given for studying the seven selected enzymes: acid and alkaline phosphatase, 5-nucleotidase, glucose-6-phosphatase, phosphoglucose isomerase, aldolase and iso-citric dehydrogenase.

Part I is devoted to a review of the relevant properties of each enzyme and the methods available for its determination. Details are given of the methods used in this laboratory, together with the experimental findings which led to the development of new methods for the determination of alkaline phosphatase, glucose-6-phosphatase and 5-nucleotidase. Attempts at improving the sensitivity of the existing methods for acid phosphatase are also described.

Part II gives a review of the literature related to alterations in the serum level of these selected enzymes. The results of our investigations are presented, and they have been correlated with the clinical condition of the patients. Kinetic studies have been used to investigate the origin of serum alkaline phosphatase in pathological conditions. Having established that

raised levels of 5-nucleotidase and iso-citric dehydrogenase may be taken as an indication of disease in the liver, these and other enzymes have been used to investigate the existence and site of secondaries in women with cancer of the breast.

Discussion. In this section the conditions which influence the release of enzymes and their removal from the circulating blood are discussed.

Appendix. Biochemical and case histories and detailed results of the enzyme determinations on the individual cancer patients are given in this section.

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INTRODUCTION.

This work has been directed towards establishing what useful information can be obtained from the study of serum enzymes in women with cancer of the breast. The correlation between the clinical condition of the patient and alterations in the levels of different enzymes has been investigated and attempts have been made to link the position of metastases with increases in the serum level of different enzymes. In order to establish the conditions which cause elevations in some of the less well-known serum enzymes, these have been studied in diseases other than cancer of the breast.

Enzymes are proteins with catalytic properties due to their power of specific activation (Dixon and Webb, 1958). They are produced as a result of cellular activity, but are independent of the presence of living cells for their operation. In specifying that all enzymes are proteins, the definition excludes glutathione and other coenzymes. All the enzymes so far obtained in a pure state have been found to be proteins and there is much to be said for confining the definition to protein catalysts exhibiting the property of specific activation of a substrate. This also excludes cytochrome C which is a protein catalyst but, acting as a carrier and not as an activator, can be classed with other coenzymes which work only when they themselves are activated by specific enzymes.

The early work dealing with enzymes was confined almost entirely to the process of alcoholic fermentation and it was from this work that Kühne introduced the term "Enzyme" in 1878; it is derived from a Greek word of which the English translation is "in yeast."

The beginnings of clinical enzymology may be traced to the early studies of digestive enzymes. Kirscheff (1815) found that starch could be converted to sugar and dextrin by a glutinous compound extracted from wheat. Payan and Persoz (1833) also separated an active amylase from wheat and recognized the presence of a substance having similar action in saliva. In 1846 Magendie found amylase in blood and urine and so became the first person to estimate enzyme activity in these two biological fluids. In 1908 Wohlgemuth introduced a method for estimating amylase activity in urine and found that patients with acute pancreatitis have high urinary amylase activity when compared with normal persons or patients suffering from other diseases. He was, therefore, the first person to use enzyme determinations as a diagnostic aid.

The study of the enzymes concerned with digestion and fermentation led to the discovery, at the beginning of this century, of enzymes that split phosphate esters. In 1907 Suzuki, Yoshima and Takahashi extracted an enzyme from *takadiastase* which would liberate free alcohol or sugar and inorganic phosphate from esters of phosphoric acid. Several

years later Robison (1922) found a phosphate-splitting enzyme present in yeast juice that would hydrolyse glucose monophosphate to liberate sugar and inorganic phosphate, which in the presence of calcium ions would precipitate as insoluble calcium phosphate. Robison (1923) then looked for and found a similar activity in bone, which he called phosphatase. In 1926 Martland and Robison showed that blood plasma contained a phosphatase, active in an alkaline medium, similar to that in bone. With Kay's discovery in 1929 that a marked plasma phosphatase activity was associated with osteoblastic bone diseases, the door was opened to the study of plasma enzyme levels in disease.

During the last thirty years there has been a great advancement in enzymology starting with the isolation, amongst others, of the glycolytic and Krebs cycle enzymes from muscle, liver and other tissues. When Warburg and Christian (1943) demonstrated aldolase activity in the serum of tumour-bearing rats, they made the first important step in the study of enzymes in cancer and other tissue-destructive diseases. Sibley and Lehninger (1949a) demonstrated abnormal levels of aldolase activity in the serum of cancer patients. The study of serum enzymes was thus extended to the glycolytic enzymes, where previously it had been restricted to the phosphatases and digestive enzymes; the transaminases, dehydrogenases and others followed, until at the present time more than twenty enzymes have some diagnostic usefulness.

Alterations in the enzyme levels in body fluids may reflect changes within the body in several ways. A low enzyme level in blood or urine may result from impaired function of the organ concerned in its production, thus the very low serum alkaline phosphatase encountered in hypophosphatasia is a reflection of the failure in normal bone production seen in this condition.

A raised serum enzyme level may result from one of three things: (i) overactivity in a particular organ, such as the high serum and urine amylase found in acute pancreatitis; (ii) the release of enzyme from damaged tissue as occurs in myocardial infarction; or (iii) a failure in the normal excretory mechanism, as in an obstruction of the common bile duct which blocks the normal excretory pathway of alkaline phosphatase.

With the numerous enzyme determinations now at the clinical biochemist's disposal, some discrimination has to be made as to which enzymes will give the most valuable information about the patient.

For diagnostic purposes serum enzymes can be divided into two types. Firstly, there are those which give information about disease in a particular organ, i.e. they are organ-specific. A good example of this is the tartrate-labile acid phosphatase encountered in the serum of men with metastatic carcinoma of the prostate. Secondly, there are those of wide distribution, such as the glycolytic enzymes, which give information about the

General condition of the patient, rather than about disease in a particular organ.

The main study for this thesis has been on patients with hormone-dependent cancer of the breast treated by ablation of the pituitary gland by the implantation of radioactive yttrium. It may be difficult for the clinician to assess the patient's response to treatment, and the determination of serum enzyme levels is often helpful.

By careful selection of which serum enzyme levels to investigate, information can be obtained about the presence, site, and extent of metastases and it is to these ends that the present study has been directed. Particular attention has been paid towards finding an enzyme or enzymes which will give information about secondary growths in the liver.

Some of the enzymes which have been studied have already been shown to be helpful in the management of cancer patients and efforts have been made to improve the methods for the determination of these enzymes in an attempt to extend their usefulness. Others are not so well established and in these cases their value has been assessed.

Both acid and alkaline phosphatase levels have been used clinically for thirty years and in most cases the information provided by them is clear cut, but in women with cancer of the breast the reason for an abnormal alkaline phosphatase may be

less obvious. Metastases in either the bone or the liver could be responsible for elevated levels of the enzyme in serum. The source of this serum increment has been investigated by the use of different substrates for the determination of enzyme activity and by kinetic studies.

5-Nucleotidase is a substrate-specific phosphatase, active at pH 7.5 on nucleotides with a phosphate group attached to the fifth carbon atom of the pentose. The serum level of the enzyme has been studied in conjunction with alkaline phosphatase since it has been shown to be raised only in liver disease (Dixon and Rardon, 1954; Young, 1958). A series of cancer patients has been followed to assess the value of this determination in differentiating between bone and liver metastases.

Acid phosphatase has been used in the diagnosis of cancer of the prostate for many years (Gutman, Sproul and Gutman, 1936). The specificity of this test was improved by the discovery of specific inhibitors for prostatic and red cell acid phosphatase (Abul-Fadl and King, 1949), and methods involving the use of these were soon forthcoming. Attempts have been made to improve the sensitivity of the latest method for the determination of prostatic acid phosphatase by King and Jegatheesan (1959).

Total acid phosphatase levels are useful in following

the progress of women with cancer of the breast. A few differential substrate studies have been carried out in an attempt further to fractionate this breast component.

Glucose-6-phosphatase has been shown by Swanson (1950) to be an enzyme found only in liver, and it has been reported to be raised in the serum of patients with liver cell damage (Koide and Oda, 1959). A little work has been done on this enzyme to investigate its use in the diagnosis of liver disease.

Bodanaky (1954b, 1955) showed that the glycolytic enzyme phosphoglucose isomerase was useful in the study of women with cancer of the breast and men with cancer of the prostate. Continued use has been made of this as an index of response and these results have been compared with aldolase levels determined on the same patients.

Serum iso-citric dehydrogenase has been included in the study, because of the recent claims to its specificity for liver disease (Wolfson, Spencer, Sterkel and Williams-Ashman, 1958), and results obtained with this enzyme have been compared with the phosphatases and 5-nucleotidase.

The thesis is divided into two parts. In Part I, a short survey of the relevant properties of each enzyme studied is given. The experiments described in this part are confined to work carried out to devise suitable methods of determination or to

improve existing methods. Part II describes the investigations carried out on patients with cancerous and non-cancerous disease. These new data are interpreted in the light of clinical information on the patients studied, and of the existing literature on the enzymes concerned. Conclusions are drawn as to the enzyme determination of value in these diseases.

PART I

THE PHOSPHATASES

In the years 1907 to 1913, several biochemists presented evidence for the existence of an enzyme that would liberate phosphoric acid from a great variety of phosphate esters. The enzyme was called phosphatase and in 1936 Felley and Kay proposed a scheme of classification for the enzyme which depended upon the type of substrate hydrolysed. Since then the non-specific phosphomonoesterases have received the most attention; it is now usual to class the enzyme active in a medium with a pH greater than 7 as alkaline phosphatase, while acid phosphatase denotes the enzyme optimally active at a pH below this. There are a few substrate-specific phosphomonoesterases which are optimally active at pHs characteristic for each enzyme. 5-Nucleotidase and glucose-6-phosphatase are two of these enzymes which will be discussed here, together with acid and alkaline phosphatase.

1.

Alkaline Phosphatase.

Distribution of Alkaline Phosphatase in Human Tissue.

In 1923 Robison found a phosphatase present in human bone which would split glucose-6-phosphate in an alkaline medium. Since that time, alkaline phosphatase activity has been demonstrated in a wide variety of human tissues; it has been found in all animal cells except hyaline cartilage and the tunics of vessels (MacFarlane, Patterson and Robison, 1934). Certain tissues contain more alkaline

phosphatase than others; bone, liver, kidney cortex, intestinal mucosa and lactating mammary gland are particularly rich sources. Blood serum normally contains a small quantity of alkaline phosphatase.

Purification.

The first attempt at purifying alkaline phosphatase was made by Martland and Robison in 1929. They prepared an aqueous extract of rachitic bone, allowed it to autolyse in chloroform water for several days and then precipitated the enzyme by pouring the extract into a solution of 3 parts alcohol, 2 parts ether to 1 part aqueous extract. Armstrong (1935) prepared the enzyme from an aqueous extract of dog faeces by ammonium sulphate precipitation. Schmidt and Thamhauser (1943) treated an organic extract of intestinal phosphatase with trypsin to release the enzyme into solution; it was then precipitated with ammonium sulphate. There are objections to proteolytic release of the enzyme and Morton (1954) was able to extract alkaline phosphatase from calf intestinal mucosa and cow mammary gland with butanol. He then precipitated the enzyme with acetone. Many other attempts at purification have been reported: (Mathies, 1958; Schales and Arai, 1959; Ahmed, Abul-Fadl and King, 1959). The most recent report is by Binkley (1961) who has obtained a thirty thousand fold purification of alkaline phosphatase from swine kidney.

These methods are unsuitable for the purification of alkaline phosphatase from serum, since there is a relatively small amount of enzyme in the presence of a large amount of protein. However, electrophoresis on both starch and paper has been used to advantage in the purification of the serum enzyme.

Properties of Alkaline Phosphatase.

Activation and Inhibition.

Alkaline phosphatase is activated by magnesium (Erdtmann, 1927; Jenner and Kay, 1931). 5mM is the optimum concentration for this cation; there have been reports of activation by other divalent cations, but they are less effective than magnesium (Roche, 1950; Ahmed and King, 1959).

The strongest inhibitors of alkaline phosphatase are phosphate and arsenate ions (Roche and Thosai, 1943). Bedanaky (1937) found that bile acids inhibit bone and kidney alkaline phosphatase but have no effect on the enzyme from intestinal mucosa. Other activation and inhibition studies have produced inconclusive results because of the impurity of the enzyme preparations. It is not possible to distinguish with any certainty the tissue source of serum alkaline phosphatase by the use of specific inhibitors as it is with acid phosphatase.

Rate of Hydrolysis of Phosphate Esters

The rate of hydrolysis by alkaline phosphatase depends on the nature of the substrate. Dalory and King (1943), using dog faecal alkaline phosphatase, found a relationship between pK values of the phosphate dissociation constants and the optimum pH of the enzyme reaction; the more acid the phosphate dissociation, the more alkaline is the optimum pH and the greater the amount of hydrolysis in a given time.

Thus the optimum pH for the hydrolysis of 5mM phenyl phosphate in carbonate-bicarbonate buffer is 10.1. This substrate is hydrolysed two and a half times as fast as 5mM β -glycerophosphate in veronal buffer at pH 8.6.

pH Optimum.

The pH optimum for alkaline phosphatase activity has been shown to vary with initial substrate concentration, the type of buffer, the type of substrate and the source of enzyme (Folley and Kay, 1935; Walker and King, 1950; Zittle and Della Monica, 1950; Merton, 1955; Motzok, 1959).

In addition to affecting the optimum pH, variations in the initial substrate concentration cause alterations in the shape of pH activity curves for alkaline phosphatase (Motzok and Wynne, 1950; Ross, Ely and Archer, 1951; Merton, 1957; Motzok, 1959).

Methods for Determining Alkaline Phosphatase Activity.

The nature of the physiological substrate for alkaline phosphatase is not known, so consequently the choice of artificial substrate depends upon the ease of determining the reaction products. The two most commonly used phosphoric esters are β -glycerophosphate (Bedansky, 1933) and phenyl phosphate (King and Armstrong, 1934).

The ease with which phenol can be determined colorimetrically by using either Folin Ciocalteu (1929) reagent or amino antipyrine (Kind and King, 1954), coupled with the speed at which phenol is liberated by the enzyme, make it a particularly useful substrate. The determination of inorganic phosphate is used to obtain a measure of the enzyme activity in the methods using β -glycerophosphate; this involves a high control to account for the phosphate content of the serum.

Other methods using esters which liberate easily determined substances on hydrolysis have been developed. p-Nitrophenyl phosphate has been used by Ohmori (1937) and Bessey, Lowry and Brock (1946). Bray and King (1943) used phenolphthalein phosphate, and Seligman et al. (1951) developed a method which depends on the determination of free β -naphthol from β -naphthyl phosphate by an azodye technique.

The development of spectro-fluorimeters has led to the introduction of methods based on the measurement of

fluorescent products of hydrolysis. Moss (1960a) has used α - and β -naphthyl phosphate as substrates for a method of this type.

With the present commercial availability of hexose phosphates, the possibility of their use as substrates for the determination of serum alkaline phosphatase has been investigated. Alkaline phosphatase is an example of an enzyme which will act on a number of closely-related substances and which carries out the same reaction in all cases. The rate at which hexose phosphates are hydrolysed by the enzyme has been determined to see if one was specific for serum alkaline phosphatase. Use of such a substrate would involve the measurement of a substrate-specific alkaline phosphatase activity which would be different from the non-specific activities determined by the use of unphysiological substrates.

Glucose-6-phosphatase is the only enzyme that has been shown to have specific activity on a hexose phosphate and this enzyme is optimally active at pH 6.5.

A method has been worked out using a hexose phosphate substrate and it has been compared with phenyl phosphate to see if any distinction could be made between the hyperphosphatasaemia seen in bone and liver disease

**Investigation of Hexose Phosphate Esters for use
as Substrate for the Determination of Alkaline
Phosphatase.**

The pH activity curves in carbonate-bicarbonate buffers were determined for a number of hexose phosphate substrates at 5mM concentration, and the relative rates of hydrolysis under optimum conditions were compared with the rate of hydrolysis of phenyl phosphate of the same molarity.

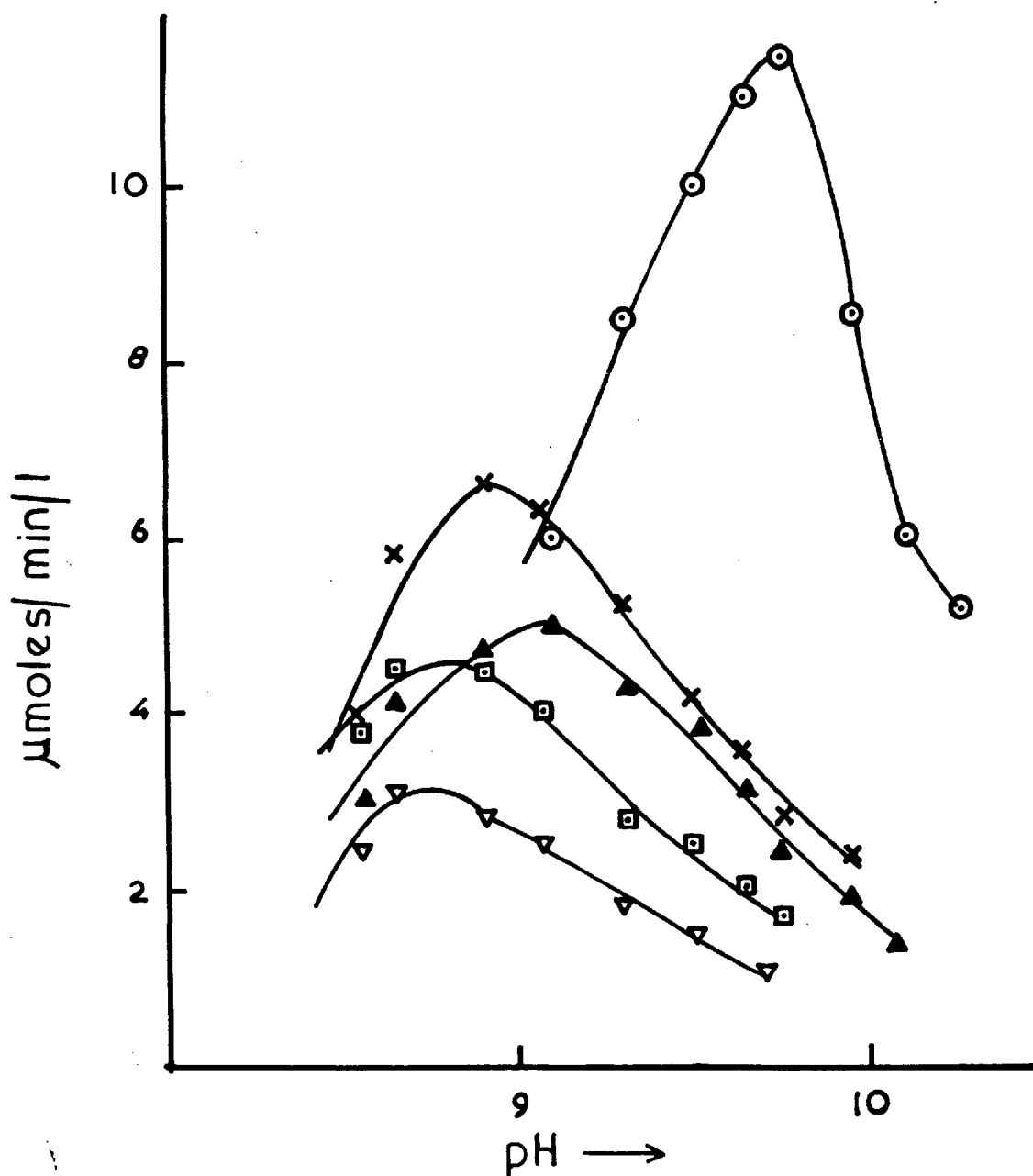
The phosphoric esters used were sodium β -glycerophosphate (β G-P), dipotassium glucose-1-phosphate (G-1-P), barium glucose-6-phosphate (G-6-P) and barium fructose-6-phosphate (F-6-P). The barium salts were dissolved in hydrochloric acid and a little water was added. Addition of sodium sulphate caused precipitation of barium as barium sulphate which was removed by centrifugation. The pH of the supernatant was adjusted to neutrality and the solution then made up to volume. 1 ml. substrate and 1 ml. buffer (0.1M carbonate-bicarbonate, King and Wootton, 1959) were incubated for 3 min. at 37°. Addition of 0.1 ml. enzyme solution (a serum of high alkaline phosphatase activity) started the reaction, which was allowed to proceed for 15 min. Addition of 10% trichloroacetic acid stopped the reaction and precipitated the protein. Inorganic phosphate was determined in the filtrate by the method of Fiske and Subbarow (1925).

The pHs of the reaction mixtures were determined at 37° with a glass electrode and these values were plotted against units which are expressed in terms of μ moles substrate utilized/min./l.

fig.1

pH Activity curves for hydrolysis
of 5 different Substrates by
alkaline Phosphatase

- phenyl Phosphate
- ▲ Glucose-1-phosphate
- x β Glycerophosphate
- Fructose-6-phosphate
- ▽ Glucose-6-phosphate



The results are shown in Fig. 1, and under these conditions the optimum pH for phenyl phosphate is 9.8, G-1-P 9.1, β -G-P 8.9, F-6-P 8.8 and G-6-P 8.7.

The rate of hydrolysis of these substrates by different enzyme solutions was determined under optimum conditions for each ester. Phenyl phosphate was hydrolysed most rapidly, and it was found that G-1-P was hydrolysed at three-fifths of this rate, and all other esters were hydrolysed at about two-fifths the rate of phenyl phosphate. These results are consistent with Delory and King's (1943) observation that the higher the optimum pH the greater the amount of hydrolysis.

Glucose-1-phosphate was chosen as the substrate and a method for its use was developed.

Determination of Serum Alkaline Phosphatase Activity using Glucose-1-phosphate as Substrate.

Non-specific alkaline phosphatase hydrolyses glucose-1-phosphate (G-1-P) and a measure of the enzyme activity may be obtained by the determination of the liberated phosphorus or glucose.

Inorganic phosphate is determined by the very sensitive method of Delsal and Manhourl (1958) which develops the molybdenum blue colour at a buffered pH of 4 with acetic acid instead of with strong sulphuric acid, as in other methods.

This method may be adapted for the determination of substrate-specific glucose-6-phosphatase (see Chapter 4).

Reagents.

Carbonate-bicarbonate buffer. (0.1 M) pH 9.1 (King and Weotten, 1959).

Glucose-1-phosphate. (10 mM) 372 mg. dipotassium salt/100 ml. kept at 4°.

Sulphosalicylic acid (10% W/V).

Acetate buffer. (2N) 2.5 g. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 46 g. crystalline $\text{CH}_3\text{COONa} \cdot \text{H}_2\text{O}$ are dissolved in 1 l. 2N-acetic acid. The pH is adjusted to 4.0 with sodium acetate crystals.

Ammonium molybdate (5% W/V).

Rhodol-sulphite reagent. (2% Rhodol (p-methylamino phenol sulphate) in 5% neutral anhydrous sodium sulphite). 2 g. Rhodol are dissolved in 80 ml. water and 10 g. hydrated sodium sulphite added (more pure than anhydrous and equal to 5 g. of it). The solution is made up to 100 ml., filtered and stored in a dark bottle at 4°.

Stock phosphate standard (1 mg.P/ml.) 2.194 g. potassium dihydrogen phosphate pure (A.R.) are dissolved in 500 ml. distilled water. The solution is preserved with a few drops of chloroform and kept at 4°.

Dilute phosphate standard (6 µg.P/ml.) 3 ml. stock solution diluted to 500 ml. with water, mixed well and kept at 4°.

Method.

Test. 1 ml. substrate and 1 ml. buffer are incubated at

37° for 3 min. 0.1 ml. serum is added and the solution incubated for 15 min. The reaction is stopped, the proteins precipitated by the addition of 0.4 ml. sulphosalicylic acid, and filtered through Whatman No.5 filter paper.

Control for non-enzymic hydrolysis and free inorganic phosphate in serum and substrate. 1 ml. substrate and 1 ml. buffer are incubated as for the test. After 15 min. 0.4 ml. sulphosalicylic acid is added followed by 0.1 ml. serum, the solution is then filtered.

Phosphate Determination.

Test. Control. 1 ml. filtrate (for test and control) and 1 ml. water are added to 3 ml. acetate buffer.

Standard. 1 ml. standard, 0.2 ml. sulphosalicylic acid and 0.8 ml. water added to 3 ml. acetate buffer.

Blank. 0.2 ml. sulphosalicylic acid and 1.8 ml. water added to 3 ml. acetate buffer.

To all tubes are added 0.5 ml. ammonium molybdate and 0.5 ml. rhodol sulphite, and mixed well after each addition. Optical densities are read after 10 min. in a Unicam spectrophotometer at 880 mμ. or in a photoelectric colorimeter with an Ilford red 608 light filter.

Calculation.

Results are given in terms of micromoles of substrate utilized per min. per litre of serum. These are the

International Enzyme Units recommended by the Sub-Commission on Clinical Enzyme Units of the Commission on Clinical Chemistry.

1 ml. filtrate $\pm$ 0.04 ml. serum

$$\frac{\text{O.D. of test} - \text{O.D. of control}}{\text{O.D. of standard} - \text{O.D. of blank}} \times \frac{6}{34} \times \frac{1}{15} \times \frac{1000}{0.04} \text{ } \mu\text{moles/min./l.}$$

$$= \frac{T - C}{S - B} \times 320 \text{ } \mu\text{moles/min./l.}$$

Results.

Using this method we determined the alkaline phosphatase activity of over one hundred specimens of blood serum and compared the results with those obtained with phenyl phosphate substrate. We found an average of 68 $\mu\text{moles/min./l.}$ for the estimations using glucose phosphate as substrate and 133 $\mu\text{moles/min./l.}$ for phenyl phosphate substrate. Thus glucose-1 phosphate is hydrolysed at 0.51 times the rate of phenyl phosphate.

We established a normal range for twenty-one specimens of 12-60 $\mu\text{moles/min./l.}$ with a mean of 33 and S.D. of 13. We were unable to demonstrate any differences in the ratios of rates of hydrolysis of the two substrates by the raised serum alkaline phosphatase occurring in different diseases. There is no obvious advantage in using glucose-1-phosphate as substrate in preference to phenyl phosphate despite its physiological nature, since we found the same type of enzyme activity with both substrates.

In arriving at the form of determination which was used, the following factors were investigated experimentally:

Phosphate Determination. Glucose-1-phosphate is acid-labile; consequently the classical method of Fiske and Subbarow for phosphate determination is unsuitable, since the addition of the strongly acid ammonium molybdate reagent causes a steady hydrolysis of the substrate during the phosphate determination (Fig. 2a).

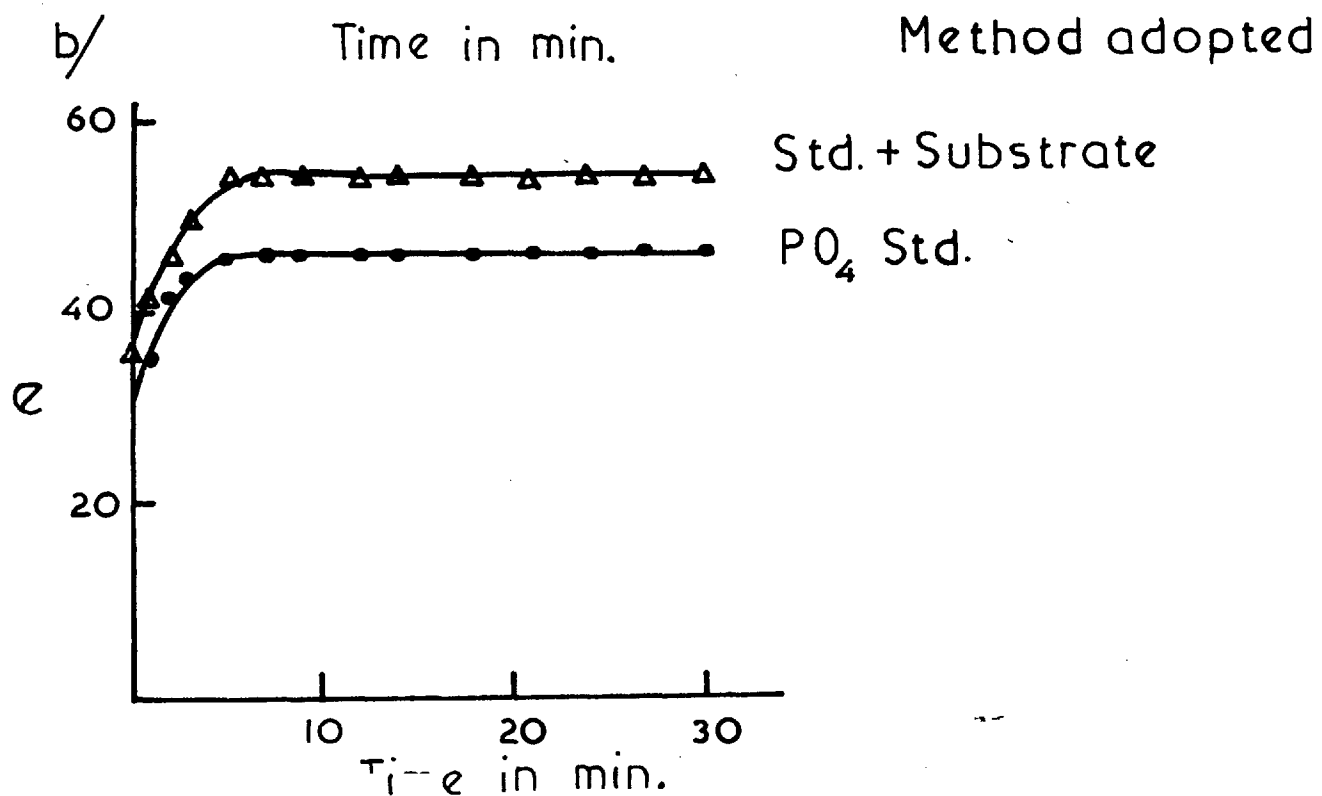
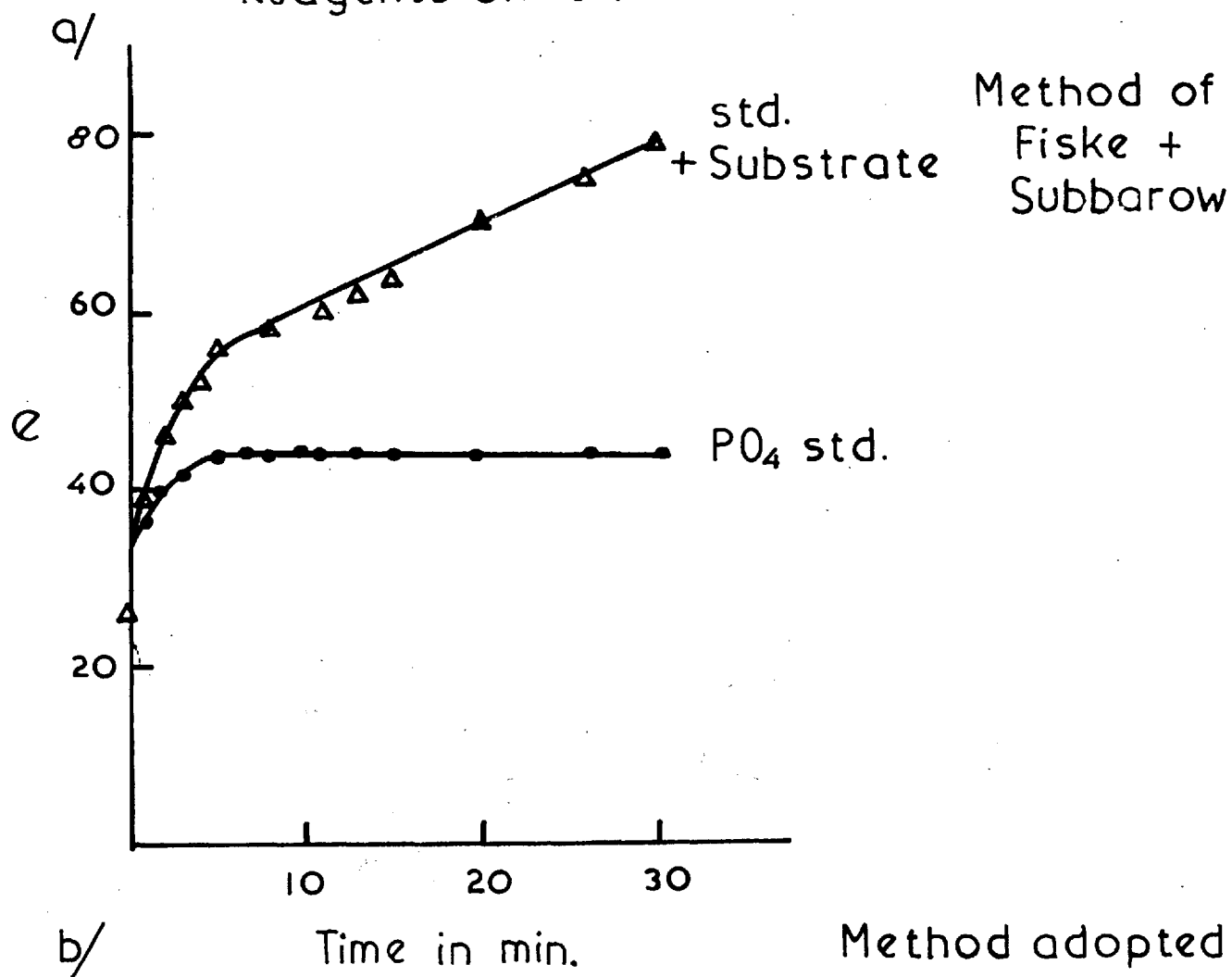
The method for the determination of phosphate in acetate buffer at pH 4.0 of Delsal and Manhourri (1958) has been used for the determination of the reaction product. The reaction mixture contains acetate buffer, a sample of filtrate after precipitation of the proteins, 5% ammonium molybdate and rhodol sulphite which acts as the reducing agent. This mixture is sufficiently acid for the formation of the phosphomolybdate complex but not strongly acid enough to cause hydrolysis of the substrate (Fig. 2b). The presence of $5 \times 10^{-3} M$ copper in the buffer hastens the colour development, which is complete in 2 min. and stable for 30 min.

The linear increase in extinction with phosphate concentration is shown in Fig. 3. All tubes were acid-washed to ensure that there was no contamination by detergent.

Protein Precipitant. The insolubility of phosphate in alkaline solution and the acid lability of G-1-P necessitates a careful choice of protein precipitant, since it must be acid enough to ensure that all the phosphate stays in solution, but it must not hydrolyse the substrate. The CCl_3COOH used in the original experiments was too strongly acid, so other reagents and methods had to be investigated.

Effect of Phosphate Colour Reagents on G-I-P

39



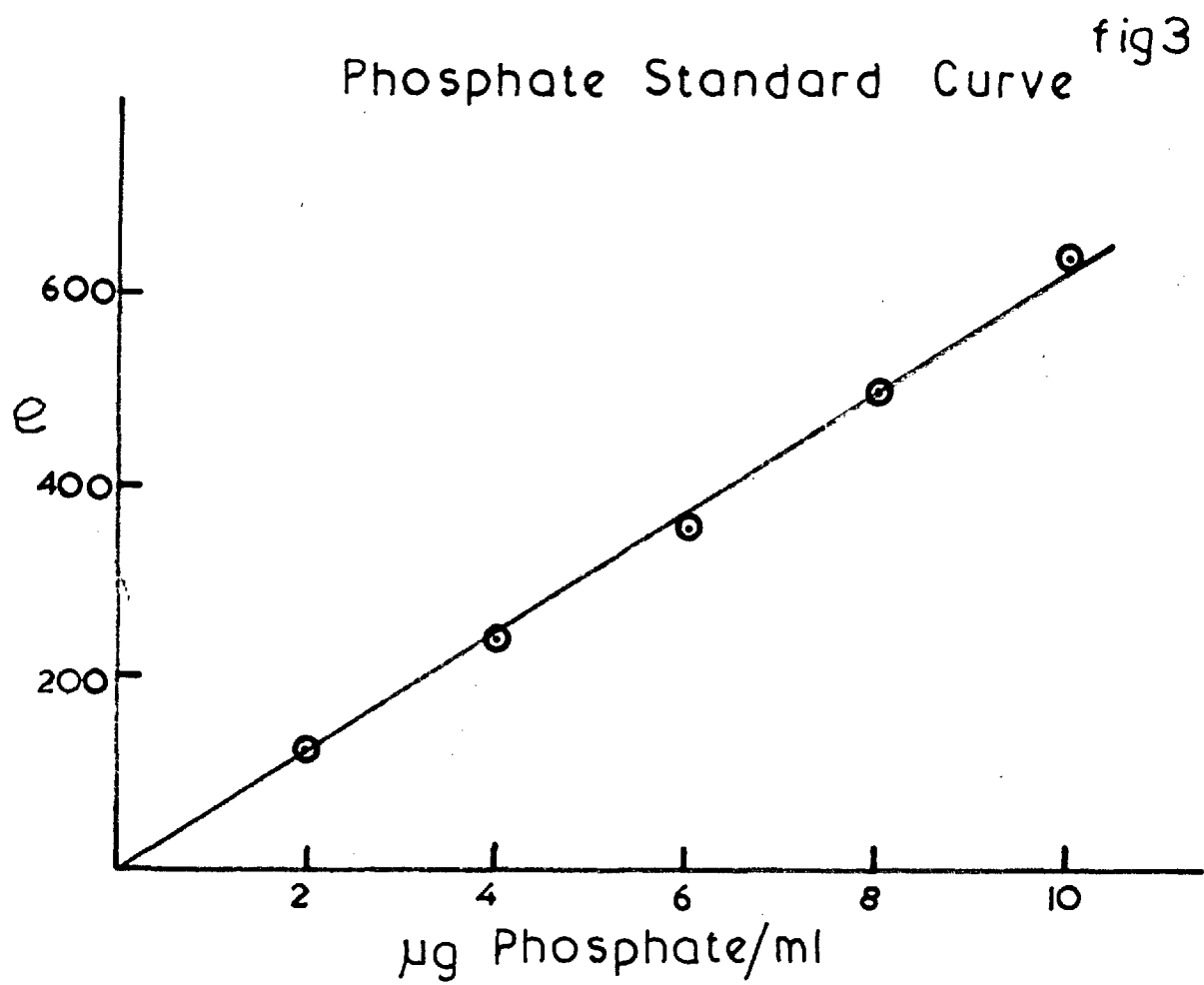
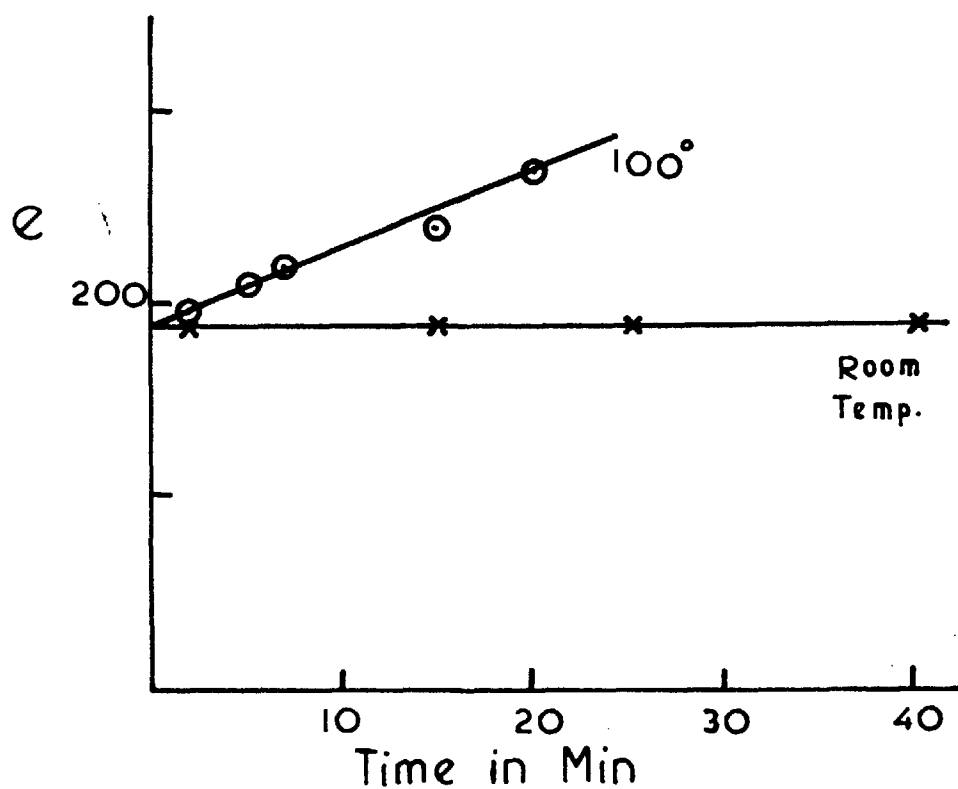


fig4

Effect of 0.05 N acetic Acid
on Glucose-1-Phosphate
at room Temperature
and 100°



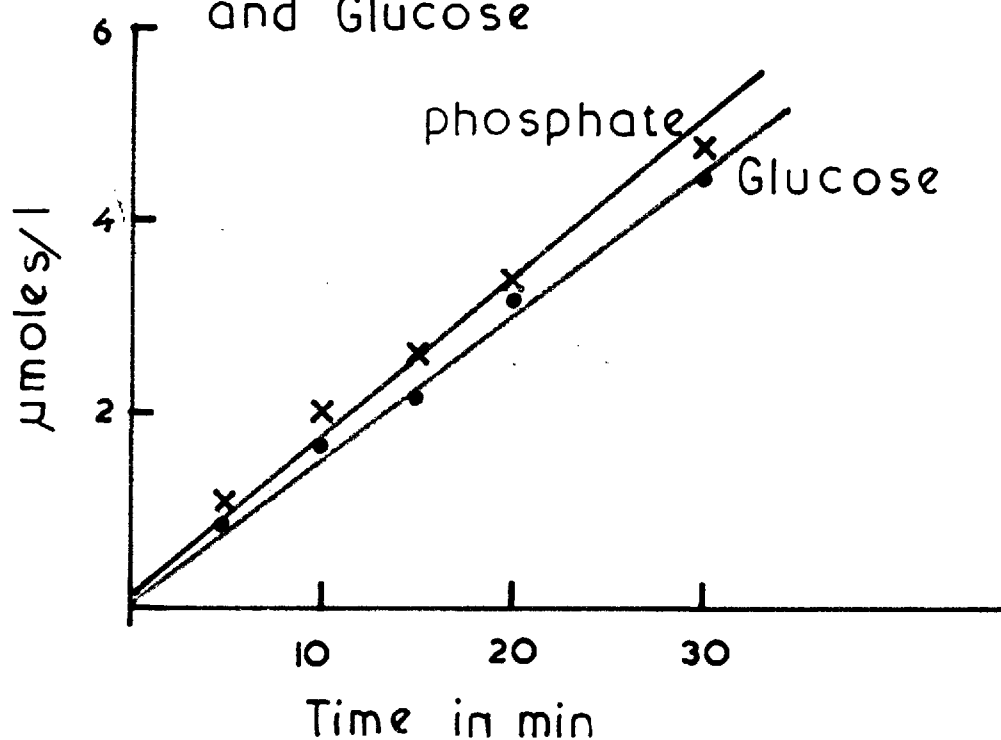
The method of Hunter (1957) for deproteinizing blood was tried. This depends upon the adjustment of pH of the solution with 0.05N acetic acid to a value suitable for the precipitation of the protein by heat, i.e. pH 5.3. Then the solution is heated for a few minutes in a boiling waterbath. The acetic acid did not affect the substrate at room temperature, but it did cause hydrolysis at 100° (Fig. 4) and so was abandoned.

Other acid protein precipitants were investigated. 10% Solutions of toluene sulphonic, perchloric and sulphosalicylic acids were allowed to stand with buffer and substrate at room temperature, 1 ml. samples were removed at intervals over a period of 60 min. and were investigated for free inorganic phosphate. Of the acids tried, only sulphosalicylic acid did not cause substrate hydrolysis at room temperature for at least an hour. To ensure that no error is introduced into the method because of the instability of substrate in an acid medium, the sulphosalicylic acid should be added to the control tube immediately after it has been added to the test and 1 ml. samples of filtrate should be transferred simultaneously to the acetate buffer as soon as filtration is complete.

Progress of Hydrolysis. This was linear over the 15 min. reaction period up to 30 min. (Fig.5); after this there was slight deviation from linearity.

Determination of Glucose. The determination of glucose as a measure of enzyme activity was investigated. The glucose oxidase

fig.5
Progress of Hydrolysis of G-I-P
by Alk.P estimation of Phosphate
and Glucose



method of Huggett and Nixon (1957) was used to determine glucose in the sulphosalicylic acid filtrate after the enzyme reaction.

Glucose oxidase will oxidise glucose to hydrogen peroxide and gluconic acid. Peroxidase will reduce the hydrogen peroxide and the oxygen thus produced is transferred to a suitable acceptor. In this method *o*-dianisidine is oxidised to a yellow-brown complex, the intensity of which is proportional to the amount of glucose initially present.

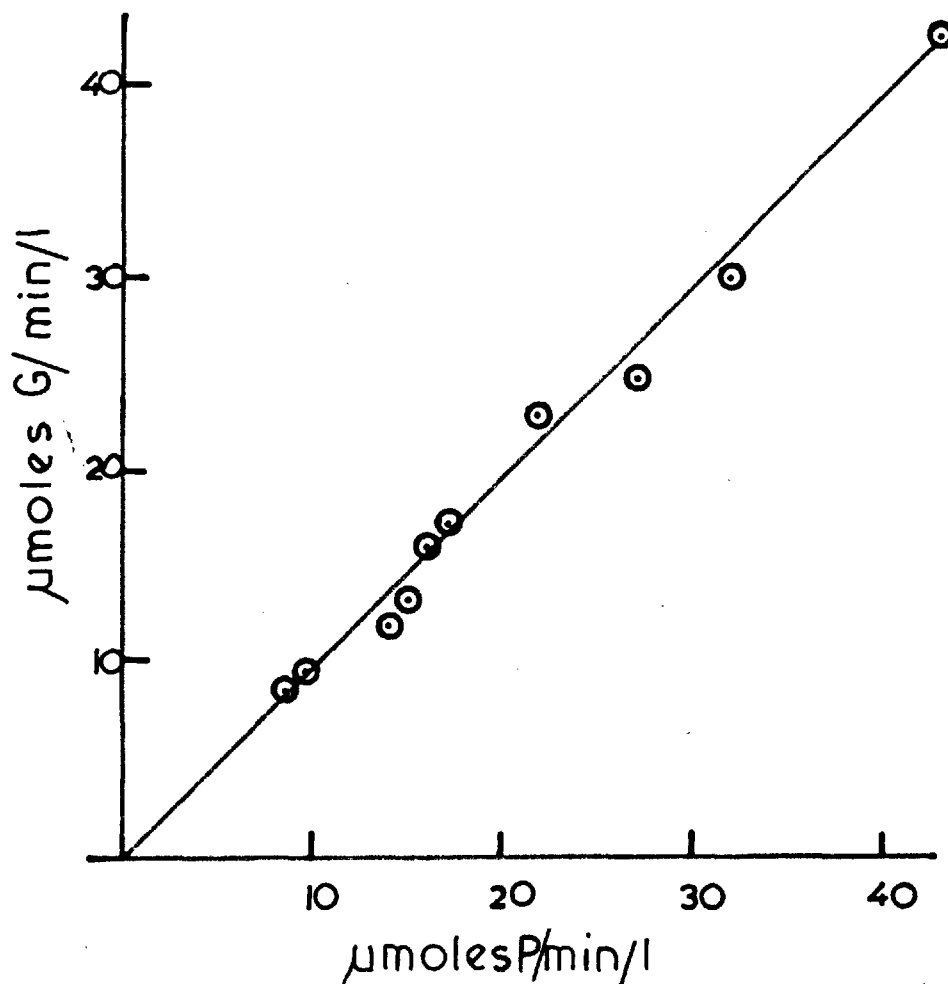
Test and Control. 1 ml. of filtrate is mixed with 5 ml. glucose oxidase reagent (125 mg. glucose oxidase, 0.5 mg. peroxidase and 0.5 ml. 1% *o*-dianisidine made up to 100 ml. in 0.5M-phosphate buffer, pH.7.0) are incubated for 30 min. at 37°. The solutions are cooled and the optical densities read in a photoelectric colorimeter with an Ilford violet 621 filter.

A glucose standard (100 µg./ml.) and a water blank are treated similarly and the results expressed in terms of µmoles glucose/min./l.

Results.

The progress of hydrolysis of G-1-P by serum alkaline phosphatase was followed over 30 min. by the determination of both phosphate and glucose and the results are shown in Fig. 5. It can be seen that both determinations were linear over the period, but the glucose results were slightly lower (5%) than the

fig6
Simultaneous determination of
phosphate and glucose for
estimation of Alk. P activity



phosphate. Since the glucose oxidase method is practically specific for glucose, these results suggest the presence of impurities in the substrate, e.g. another hexose phosphate.

Determination of glucose and phosphate was carried out on the filtrates from 10 different sera and the results are plotted in Fig. 6. It can be seen that essentially the same result is obtained whether glucose or phosphate is taken as the measure of enzyme activity.

It was decided to discontinue the estimation of glucose, since it offered no advantages over the phosphate determination. The method involves a lengthy incubation for the glucose oxidase reaction and thus considerably prolongs the total time for the method. The variable amount of glucose present in the serum may involve a deep colour in the control tube which makes the determination of small amounts of enzyme activity against this large background less accurate.

Kinetic Studies.

One of the most important factors in determining the velocity of an enzyme reaction is initial substrate concentration. It was found at the beginning of this century that the rate of reaction increases with increasing substrate concentration until a maximum is reached; after this further addition of substrate fails to affect the reaction rate.

Henri (1902) interpreted these findings as a saturation effect of the enzyme by the substrate; he proposed the formation of an unstable enzyme-substrate complex which breaks down to yield reaction products to account for this effect.

Michaelis and Menten (1913) carried out a mathematical analysis of the reaction proposed by Henri and this has been the foundation of the greater part of enzyme kinetics. They defined the Michaelis constant (K_m) which is the dissociation constant for the enzyme-substrate complex. The magnitude of the K_m gives a measure of the affinity of the enzyme for a particular substrate, thus a low K_m indicates that the enzyme binds the substrate strongly, whereas higher constants are evidence of low affinities of the enzyme for the substrate.

This constant can be determined graphically by several methods of plotting reaction velocities determined at different low substrate concentrations. One of the most convenient is that described by Lineweaver and Burk in 1934; we have used this method of determining the Michaelis constants for alkaline phosphatase with phenyl phosphate, glucose-1-phosphate and β -naphthyl phosphate.

Experimental.

The Michaelis constants for homogenates containing alkaline phosphatase activity from human bone and liver were determined

with phenyl phosphate and glucose-1-phosphate substrates. pH activity curves were plotted for each substrate concentration (5mM, 2mM, 1mM, 0.75mM and 0.5mM) and the maximum velocity at the optimum pH was used in the Lineweaver-Burk (1934) reciprocal plot. Metzok (1959) has shown that the pH activity curves for alkaline phosphatase are not similar in shape for all concentrations of substrate. He has also shown that Michaelis constants (K_m) for phosphatases determined at constant pH give unreliable results; the values depending on the activity of the enzyme solution. This error is removed by determining the activity at the optimum pH for each concentration of substrate and under these conditions the K_m is independent of enzyme activity.

Comparison between Phenyl Phosphate and
Glucose-1-phosphate Substrates.

Fig. 7 shows pH curves for alkaline phosphatase activity in the liver homogenate with glucose-1-phosphate substrate in veronal-bicarbonate buffers (King and Delory, 1940), and Fig. 8a shows the plot of the reciprocal of each substrate concentration against the reciprocal of the optimum velocity at that concentration. The second line of the graph shows points similarly determined for the bone enzyme. Fig. 8b shows reciprocal plots for the two alkaline phosphatases with phenyl phosphate as substrate. The buffers used for these determinations were carbonate-bicarbonate (King and Wootton, 1959).

fig. 7
pH Curves for liver
alkaline phosphatase
G-I-P Substrate
Veronal Buffers

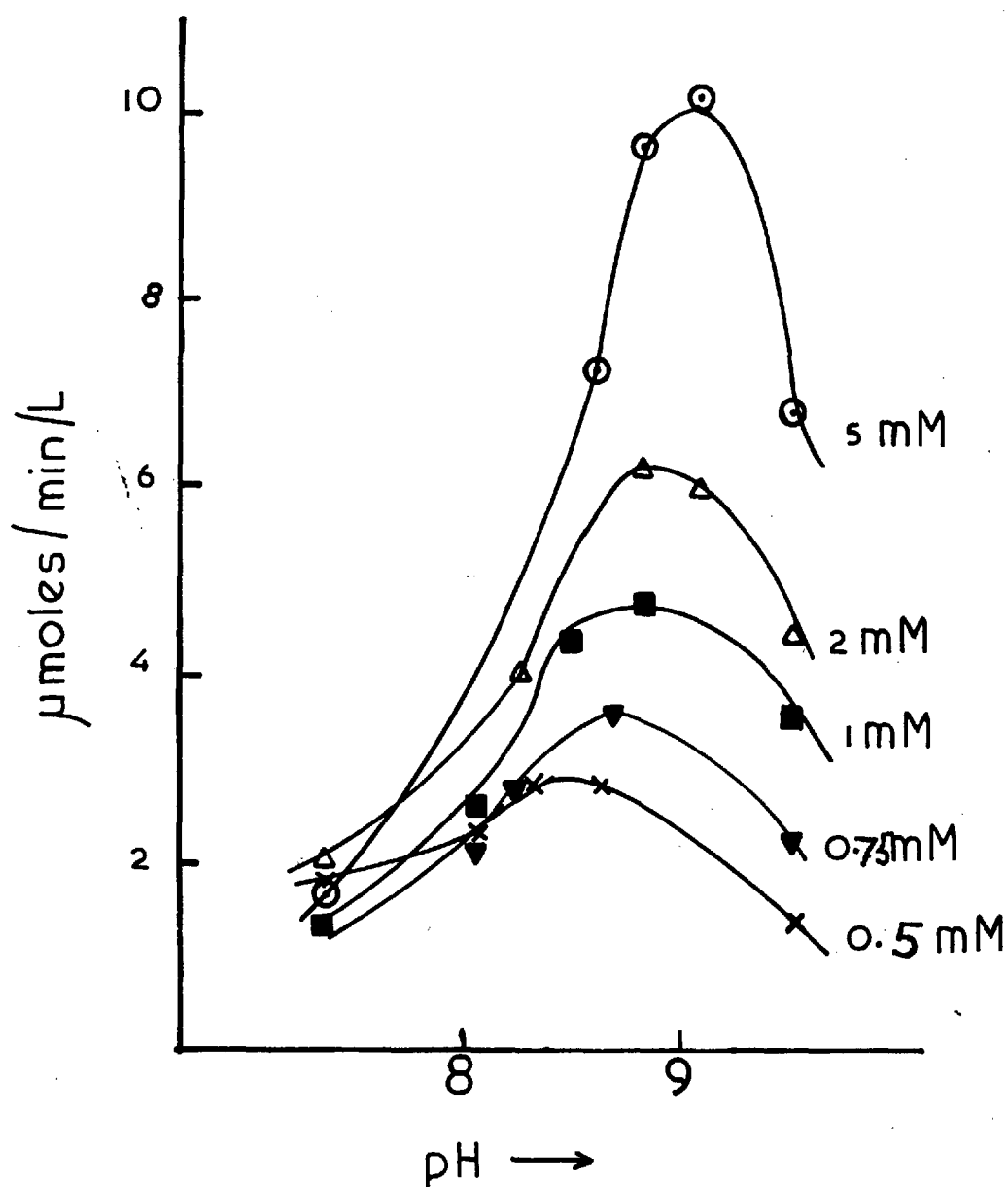
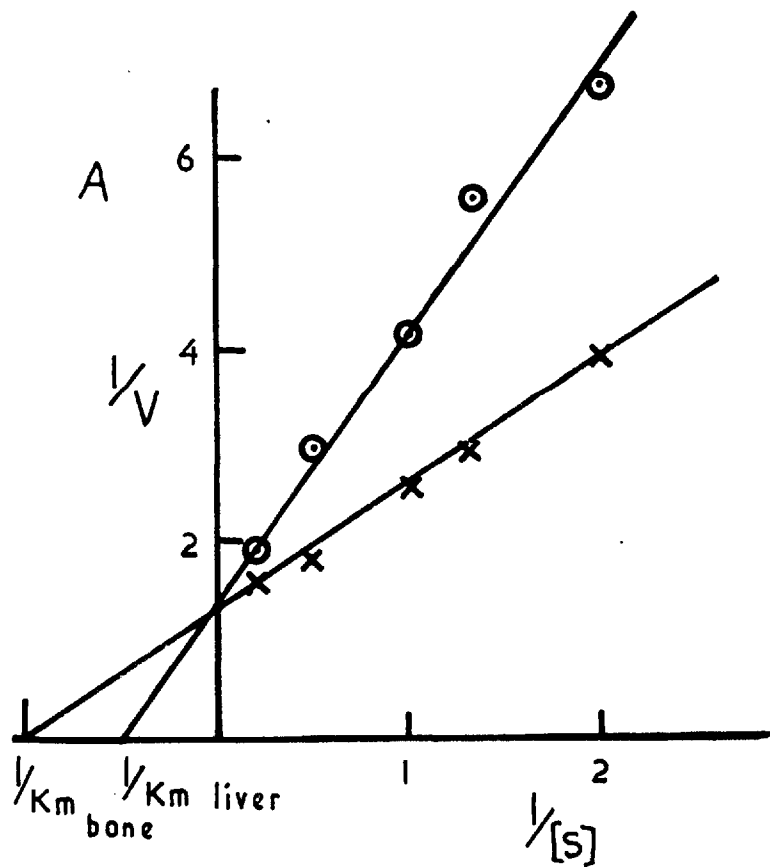


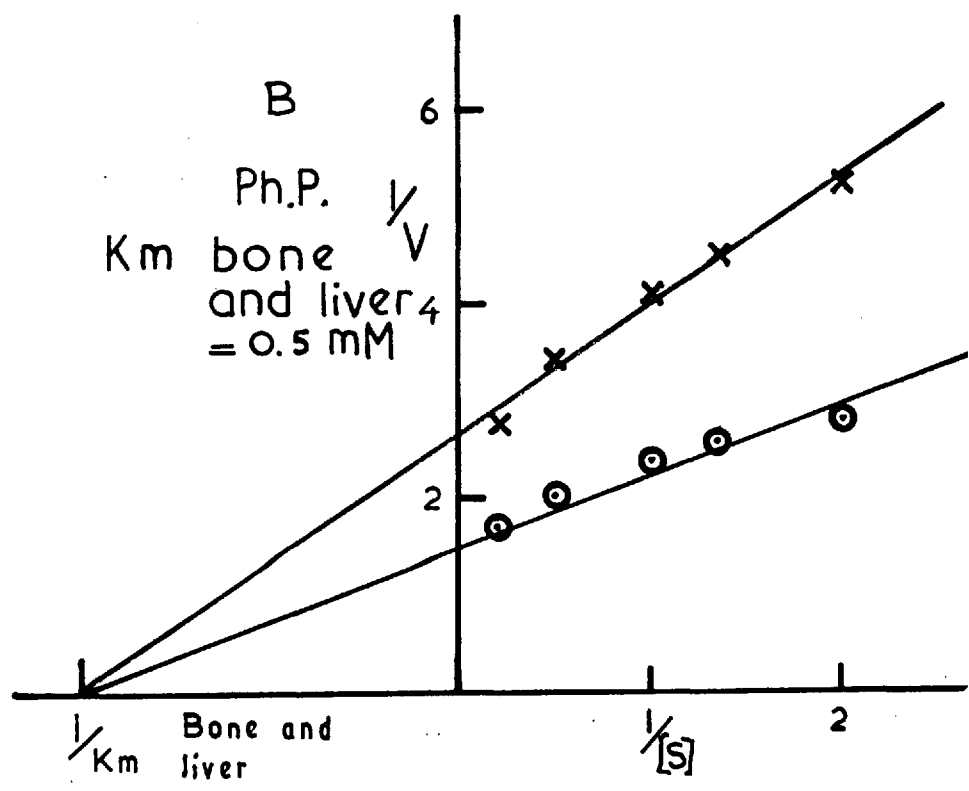
fig.8

Reciprocal plots for
bone(x) and liver(o)
Alk.P. with G-I-P
and Ph.P.



G-I-P

K_m bone=1mM
liver=2mM



B

Ph.P.
 K_m bone
and liver
= 0.5 mM

It can be seen from the graphs that under these conditions the bone and liver gave the same apparent Michaelis constant of 0.5mM with phenyl phosphate, and different constants, bone 1mM, liver 2mM, with glucose-1-phosphate. It was thought that kinetic studies using glucose-1-phosphate might be used to characterize the serum phosphatase seen in the hyperphosphatasemia.

Four sera were investigated in this manner. The pH optimum having been previously determined for each substrate concentration, 0.1 ml. serum was incubated with 1 ml. substrate of appropriate concentration and 1 ml. buffer of appropriate pH. At the end of 15 min. the reaction was stopped, and the amount of phosphate liberated determined. The reciprocals of these velocities were plotted as before, and the Michaelis constants determined graphically (Fig. 9). The sera investigated were:

- (1) D.G. 120 K.A. Units (obstructive jaundice);
- (2) S.B. 60 K.A. Units (carcinoma of prostate with bone secondaries);
- (3) M.H. 45 K.A. Units (carcinoma of head of pancreas);
- (4) B.C. 36 K.A. Units (hyperparathyroidism).

Apparent Michaelis constants of 1mM, i.e. the same as those previously found for bone enzyme, were found in each case.

The clinical details on these patients indicated that in

fig.9
 Reciprocal plots for
 Alk.P. Activity in 4 patholo-
 gical sera

G-I-R substrate

○ D.G.
 × S.B.
 □ M.H.
 ▲ B.C.

Km 1 mM

$\frac{1}{V}$

$\frac{1}{K_m}$

$\frac{1}{[S]}$

2

1

1.0

2.0

cases (2) and (4) the serum alkaline phosphatase did come from bone and the results are therefore what might be expected from this type of patient. But in cases (1) and (3) there was considerable liver involvement and it is likely that there was at least some liver alkaline phosphatase present in the serum which this method failed to demonstrate. For this reason further studies on sera along these lines were abandoned.

β -Naphthyl Phosphate Substrate.

The Michaelis constants for crude tissue extracts of human bone, kidney, liver and intestine were determined, using β -naphthyl phosphate as substrate (Moss, 1960a); the fluorescence intensity of the liberated β -naphthol was measured in a spectrofluorimeter (Moss, 1960b) and the Michaelis constants were determined as in the previous experiments with phenyl phosphate and glucose-1-phosphate. The tissue extracts were then added to normal serum and partially purified by starch gel electrophoresis using the method of Smithies (1955). The enzyme was eluted from the gel by macerating it with buffer, and Michaelis constants determined on these enzyme solutions.

It was found that electrophoresis changed the K_m values for the kidney and liver phosphatases while bone and intestine were relatively unaltered. After the tissue extracts had been purified in this way, small but reproducible differences in K_m values were

found for the individual tissue phosphatases (see Table 1) and these have been used for comparison with K_m values of phosphatases recovered from starch gel after electrophoresis of pathological sera (Moss, Campbell, Anagnostou-Kakaras and King, 1961 a and b).

These results are extremely encouraging, since electrophoresis separates the enzyme protein from the other modifying factors present in serum, and so in one simple operation a relatively pure enzyme solution is obtained. The volume of enzyme solution recovered from the gel is only 1 - 1.5 ml. and it is, therefore, necessary to have a very sensitive technique to determine the enzyme activity; spectrofluorimetry provides this.

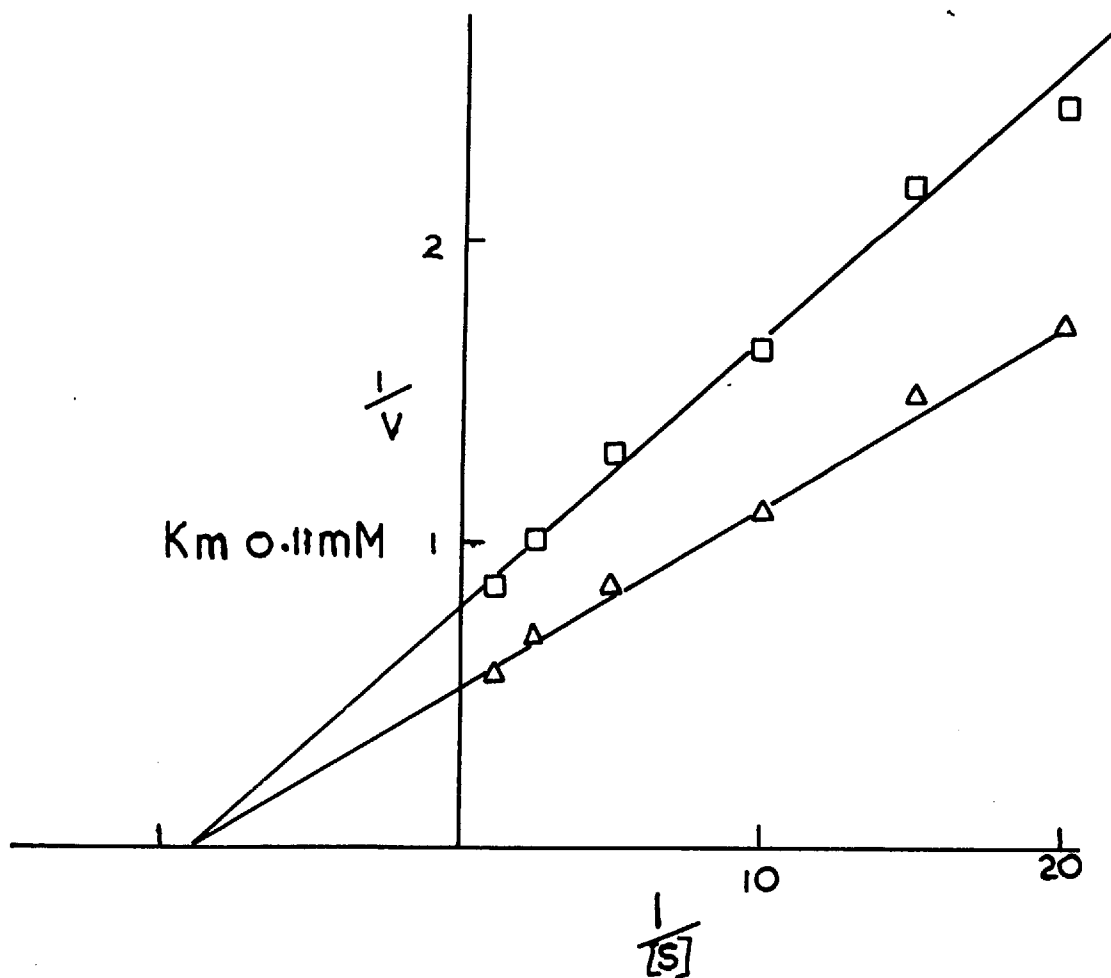
TABLE 1. Mean K_m values (mM 6-naphthyl phosphate) for alkaline phosphatases partially purified from human tissues by starch gel electrophoresis.
(Mg^{2+} concentration 5 mM).

<u>Tissue of origin</u>	<u>K_m (mM).</u>
Bone	0.110
Liver	0.067
Kidney	0.103
Intestinal mucosa	0.090

It was thought more accurate to determine the points required to define the peak of each pH curve rather than make a single estimation at a pH appropriate for each substrate concentration. Carbonate-bicarbonate buffers were used with substrate concentrations ranging from 5 mM to 0.25 mM β -naphthyl phosphate. The reaction mixture was made 5 mM with respect to magnesium.

Fig. 10 shows the Lineweaver-Burk plots for points determined in this way on enzyme solutions recovered from starch gel after electrophoresis of serum from a patient with Paget's disease, and a tissue homogenate containing human bone alkaline phosphatase. It can be seen that the K_m for both enzyme solutions is 0.11 mM. This is in full agreement with the assumption that in Paget's disease the raised serum alkaline phosphatase is a result of hyperactivity of the osteoblasts. Results will be given in Part II for other pathological sera treated in a similar manner.

fig 10
Reciprocal plots for bone(Δ)
and serum Alk. P. with β -NP



2.

Acid Phosphatase.Distribution of Acid Phosphatase in Human Tissues.

Martland, Hansman and Robison (1924) found acid phosphatase activity in erythrocytes and plasma. The following year, Demuth (1925) found the enzyme in human urine and this was confirmed in 1935 by Kutscher and Wolbergs who traced the source of this enzyme activity to the prostate gland. Other workers have demonstrated acid phosphatase activity in many human tissues and Gomori (1941), using histochemical techniques, demonstrated its wide distribution. Walker et al. (1954) think that the enzyme is present in all cells at some stage of their life.

Properties of Acid Phosphatase.

Activation and Inhibition. Tissue acid phosphatases can be separated by differential inhibition by various substances. Abul Fadl and King (1949) carried out an extensive study on the inhibition of acid phosphatase from erythrocytes, prostatic tissue and other sources. They found complete inhibition of the prostatic enzyme by $\text{L}(+)\text{tartrate}$ and fluoride, while red cell acid phosphatase is quite unaffected by the former and only slightly inhibited by the latter. Formaldehyde (0.5%) completely inhibits red cell acid phosphatase but has no effect on the prostatic enzyme; it causes 30 - 40% inhibition of the

enzyme from liver, kidney, spleen and normal serum. It was found that ethyl alcohol, tauroglycolic acid, arsenate and oxalate inhibit the enzyme for both red cells and the prostate to a lesser extent. There was no evidence of any activation of acid phosphatase by divalent cations but copper completely inhibits the red cell enzyme while not affecting prostatic acid phosphatase (Abul Fadl and King, 1949).

Methods for the Determination of Acid Phosphatase Activity in Serum.

The methods available for the determination of acid phosphatase are the same in principle as those for alkaline phosphatase, and they employ the same substrates. An acid buffer of pH 5 replaces the alkaline buffer and the incubation times are usually longer, since there is comparatively less acid phosphatase activity present in normal serum.

Inhibitors have been used to identify the source of serum acid phosphatase. The first to do this was Herbert (1946), who used 40% ethyl alcohol to inhibit prostatic acid phosphatase. Delory, Sweetser and White (1954) used formaldehyde to inhibit the red cell, spleen, kidney and liver contribution to total serum acid phosphatase. Fishman and Lerner (1955) were the first to introduce a method taking advantage of the specific inhibition of prostatic acid phosphatase by L(+)-tartrate. King and Jegatheesan (1959)

have also published a method using this inhibitor to determine the prostatic component.

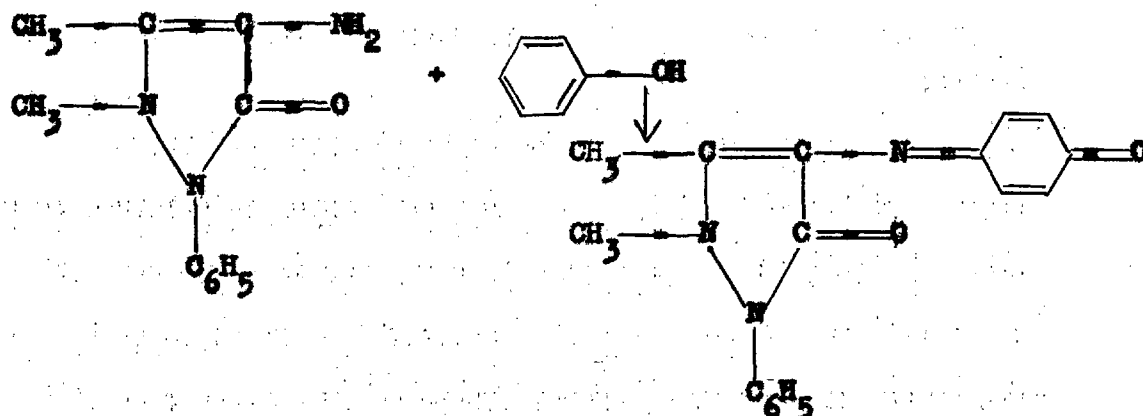
Babson and Reed (1959) advocated the use of α -naphthyl phosphate as a specific substrate for prostatic acid phosphatase, since they showed that it is not hydrolysed by the red cell enzyme. They have assumed that the contribution to the total serum acid phosphatase from other sources may be neglected; however, it has been shown by Campbell and Moss (1961) that there is an enzyme present in the serum of women with cancer of the breast with bone secondaries which is not inhibited by L(+)-tartrate but will hydrolyse α -naphthyl phosphate, i.e. in this case the substrate is being hydrolysed by a non-prostatic, non-erythrocytic acid phosphatase.

Method, as used in this laboratory.

(Kind and King, 1954; King and Jethessee, 1959).

Principle.

Serum is incubated with phenyl phosphate in an acid medium and the amount of phenol liberated in a fixed time is taken as a measure of the enzyme activity. Phenol is determined colorimetrically; Emerson (1943) found that it condenses with amino antipyrine (AAP) to form a compound which is oxidised to a coloured quinone in the presence of an alkaline oxidising reagent.



Incubation of a second tube containing 1 drop M-tartrate in addition to the other reagents estimates the non-prostatic acid phosphatase activity. Subtraction of the optical density of this from the first tube will give the prostatic fraction.

Procedure.

Serum (0.1 ml.) is added to 1 ml. phenylphosphate (10M) and 1 ml. citrate buffer (0.1 M, pH 4.9) in a water bath at 37°. After 1 hr. addition of 1 ml. NaOH (0.5N) stops the reaction. 1 ml. NaHCO₃ (0.5N) adjusts the pH to 10.2 which is required for colour development. 1 ml. AAP (0.6%) and 1 ml. K₃Fe(CN)₆ (2.4%) are then added, with shaking, and the coloured solutions read immediately in a photoelectric colorimeter with an Ilford green 624 filter. Results are given in King-Armstrong units, i.e. mg. phenol per 60 min., per 100 ml. serum.

Handling of Specimens - Heat Stability and Haemolysis.

Prostatic acid phosphatase loses its activity very rapidly when heated to 37° in slightly alkaline solution. The

acid phosphatases from other sources are similarly, though not quite so rapidly, inactivated; platelet acid phosphatase appears to be the most stable in this respect. This instability of the enzyme at physiological temperature and pH could account for the known rapid disappearance of the enzyme from the circulation.

Care has to be taken in the handling of specimens for determination of serum enzyme activity. King and Jegatheesan (1959) found that sera should be placed in the refrigerator at 0° immediately after separation from the clot. This should be done as soon as possible after taking the blood, since there is leakage of the enzyme from the red cells, even in the absence of any visible haemolysis. Because of the high acid phosphatase content of erythrocytes, it is impossible to use haemolysed specimens for determining the total serum enzyme activity.

Attempt at Improving Present Method (By the extraction of the phenol-amine antipyrine colour)

The narrow limits of the normal range for tartrate-labile acid phosphatase in the method of King and Jegatheesan (0 - 0.7 K.A. units per 100 ml.) and the consequent small difference in colour between the tubes determining total and non-prostatic enzyme activity, prompted us to try to improve accuracy by increasing this colour difference. We did this by increasing the amount of the reaction mixture five times; we then extracted the coloured quinone into a small volume of a suitable solvent.

Selection of Solvent

A series of standards was prepared containing 10 µg. phenol per ml., as in the original method; a small volume of organic solvent was added and the solutions well shaken. The results are shown in Table 2.

TABLE 2. Selection of Solvent.

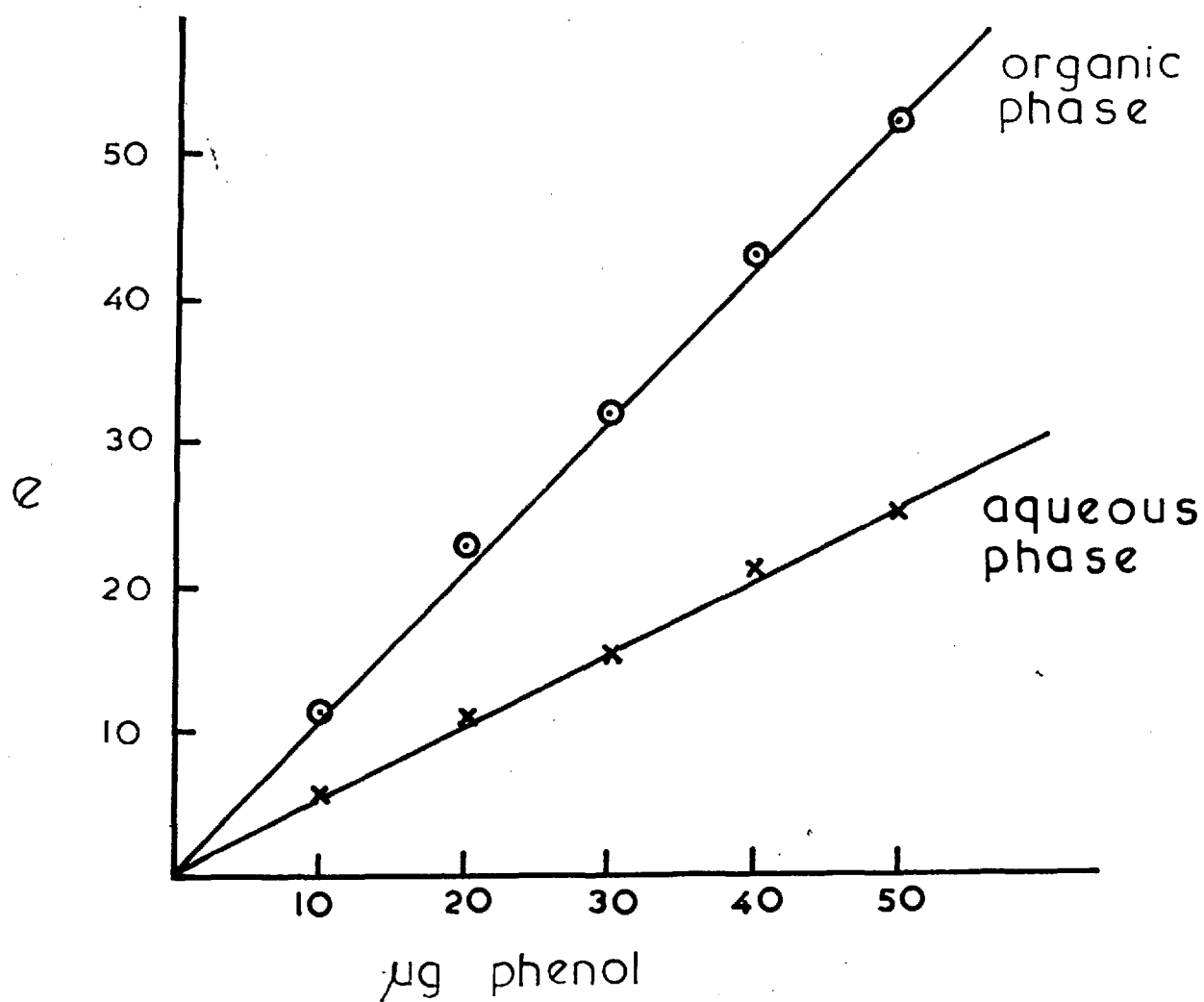
<u>Solvent</u>	<u>Power of extraction</u>
Ether	No good
Chloroform	Poor, organic layer becomes cloudy and sinks to bottom.
Carbon tetrachloride	Equal distribution of colour between the two layers
Toluene	Quite good - a few bubbles were present
Ethyl acetate	Quite good
Amyl alcohol	Good
Butyl alcohol	Good

Butanol was chosen for use since it is equally as good a solvent for the quinone as amyl alcohol and has a more pleasant smell.

Investigation of Standards

A series of phenol standards was prepared using five

fig.11
Graph to show linearity
of standard colour before
and after Butanol
extraction

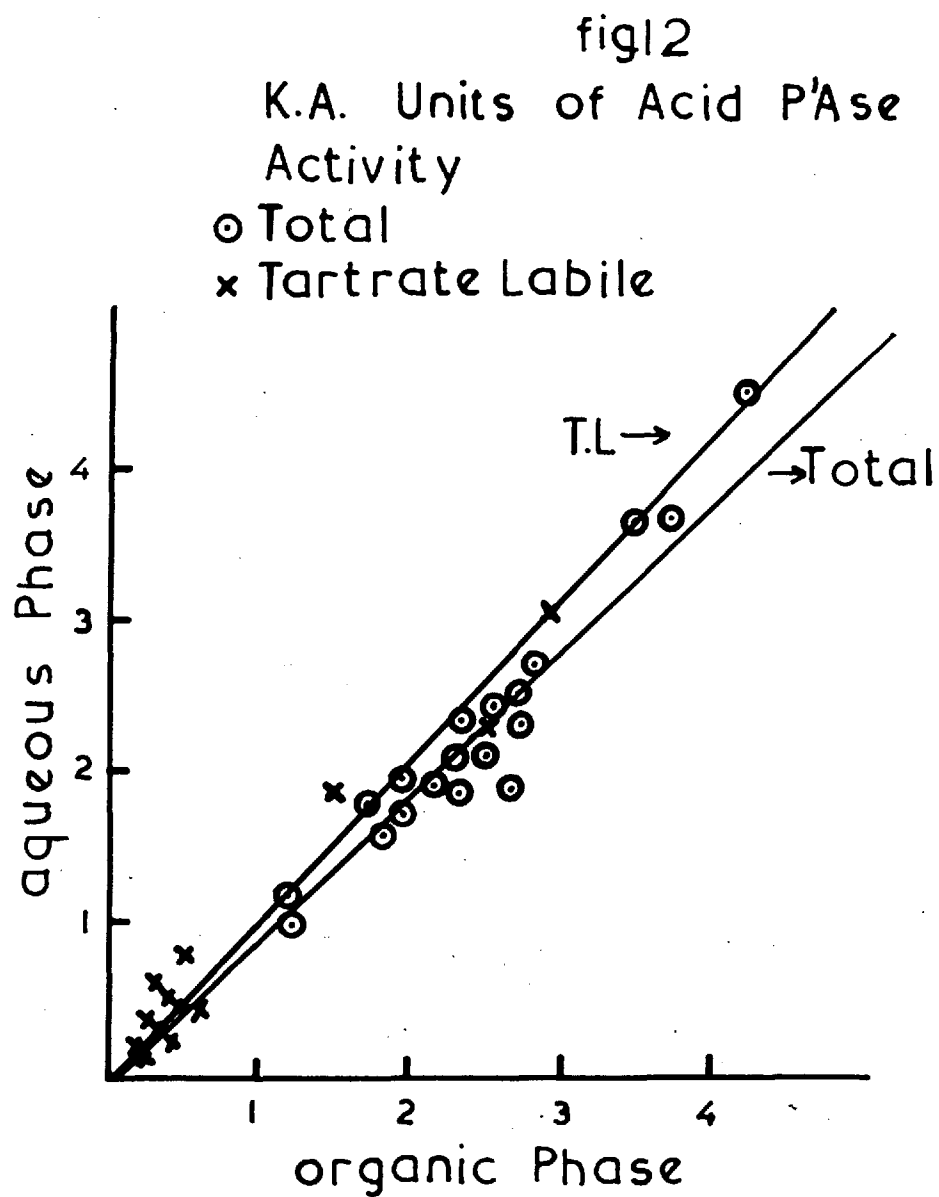


times the volumes of reagents with concentrations of phenol ranging from 10 - 50 μ g. The optical densities of the tubes were read after the addition of the colour reagents. 10 ml. of butanol were then added, the solutions well shaken and the optical densities of the organic layers were then measured. The results are shown in Fig. 11; it can be seen that there is a linear increase in colour in both the aqueous and the organic phases. There is, however, only 100% increase in colour, despite a reduction in volume by one third.

Use of the Method with Serum

It was found that serum caused an emulsion to form between the butanol and the aqueous phase, and it was, therefore, necessary to centrifuge the solution to facilitate the separation of the two layers.

The reaction was carried out in 50 ml. centrifuge cups. 5 ml. substrate and 5 ml. buffer were incubated for a few min. at 37°. Addition of 0.5 ml. serum started the reaction which was allowed to proceed for 1 hr. After this time 5 ml. NaOH (0.5M) stopped the reaction; then 5 ml. NaHCO₃ (0.5M) were added, followed by 5 ml. AAP (0.6%) and 5 ml. K₃FeON₆ (2.4%). The solutions were carefully shaken between each addition. For the determination of the prostatic fraction, 5 drops M-tartrate were included with the buffer and substrate. A control was run for each specimen in which the serum was added after the addition



of the NaOH. a 50 μ g. standard was run with each batch.

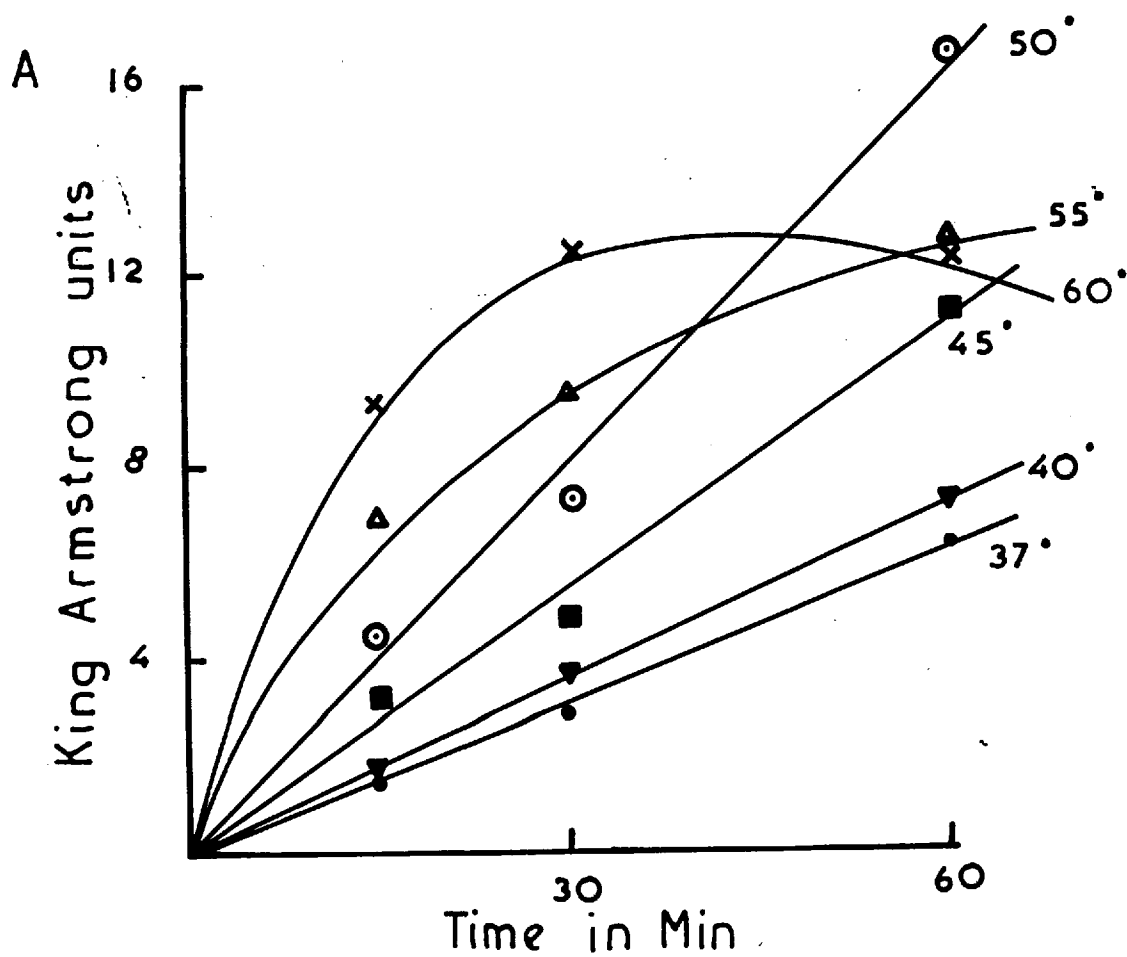
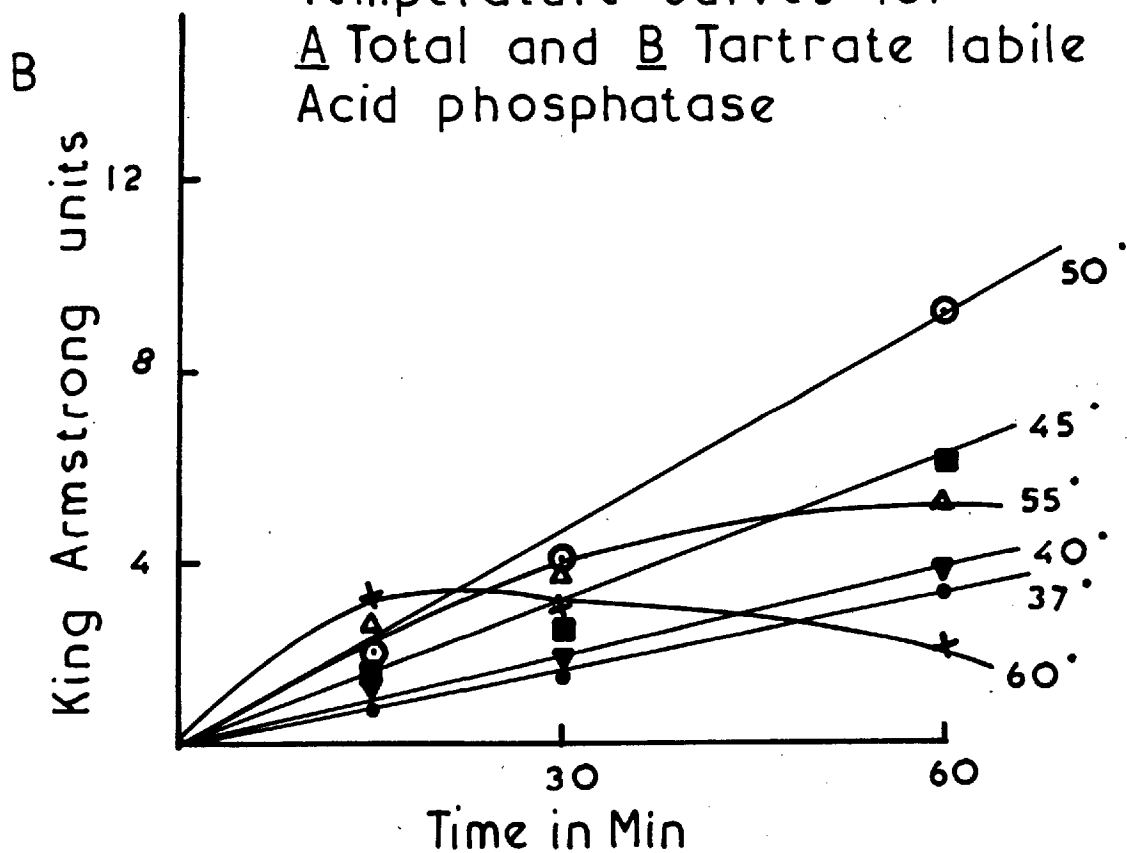
The colours of the solutions were read with an Ilford 624 green filter in a photoelectric colorimeter after the addition of potassium ferricyanide. 10 ml. butanol were then added, the tubes well shaken and centrifuged. The butanol layer was removed with a Pasteur pipette and the optical density measured. The results were calculated in K.A. units for both sets of readings and then compared (Fig. 12).

Results.

The average values for 24 determinations by each method were calculated. There was an average of 2.26 K.A. units of total activity calculated from the colour of the aqueous solutions, and 2.46 K.A. units from the organic extracts. The aqueous colours gave an average of 0.66 K.A. units of tartrate-labile acid phosphatase, while the organic extracts gave a value of 0.61.

It can be seen, therefore, that for total activity the butanol colour gives values a little higher than the aqueous colour, while for the tartrate-inhibited fraction the butanol values are slightly lower than the aqueous ones. This discrepancy in the results, together with the difficulty of handling 40 ml. of solution, led us to the conclusion that the procedure was unsuitable for routine purposes.

Temperature Curves for **fig.13**
A Total and B Tartrate labile
 Acid phosphatase



Effect of Temperature on the Rate of Reaction.

The rate of hydrolysis of phenyl phosphate by acid phosphatase was studied at different temperatures, and compared with the rate at 37° . The temperature of the water bath was raised to 40° and then in 5° increments up to 60° . Total and tartrate-labile acid phosphatase activities were determined by the method of King and Jegatheesan (1959) after 15, 30 and 60 min. incubation at each temperature.

Fig. 13 shows that up to 50° there is a linear relationship between length of incubation and amount of hydrolysis. Above this temperature there is deviation from linearity, presumably due to denaturation of the enzyme by heat. It can be seen that above 50° the tartrate-labile enzyme is more sensitive to heat than the total acid phosphatase.

Methods Using α -Naphthyl Phosphate as Substrate.

Colorimetric Method.

Following Eabsen and Read's (1959) claim that α -naphthyl phosphate could be used as a specific substrate for prostatic acid phosphatase, investigations were made into this by adapting the colorimetric method for determination of phenol for the estimation of α -naphthol. The principle of the colour reaction is exactly the same, but the colour produced is slightly less stable than the quinone formed between phenol and amine antipyrine.

Using this method, Babson and Read's findings that red cell acid phosphatase does not hydrolyse α -naphthyl phosphate were confirmed. A 1 in 10 dilution of erythrocytes had 575 K.A. units of activity with phenyl phosphate, but it had no effect on the α -naphthyl ester. Using an extract of prostatic acid phosphatase the total and tartrate-inhibited enzyme activities were then investigated with both substrates. We found that the enzyme would hydrolyse each substrate in the absence of tartrate, and that inclusion of tartrate in both reaction mixtures inhibits this activity.

We then compared the hydrolysis of the two substrates by serum acid phosphatase, determining both the total and prostatic fractions. We found that the results obtained with α -naphthyl phosphate paralleled those with phenyl phosphate; a small portion of the total enzyme activity in normal serum was inhibited by L(+)-tartrate; the remainder hydrolysed both substrates equally well. In some cases of cancer of the breast there was a considerable acid phosphatase activity that was not inhibited by tartrate, and is clearly non-prostatic in origin. While α -naphthyl phosphate is useful because of its inability to be hydrolysed by red cell acid phosphatase, it is not entirely specific for the prostatic enzyme.

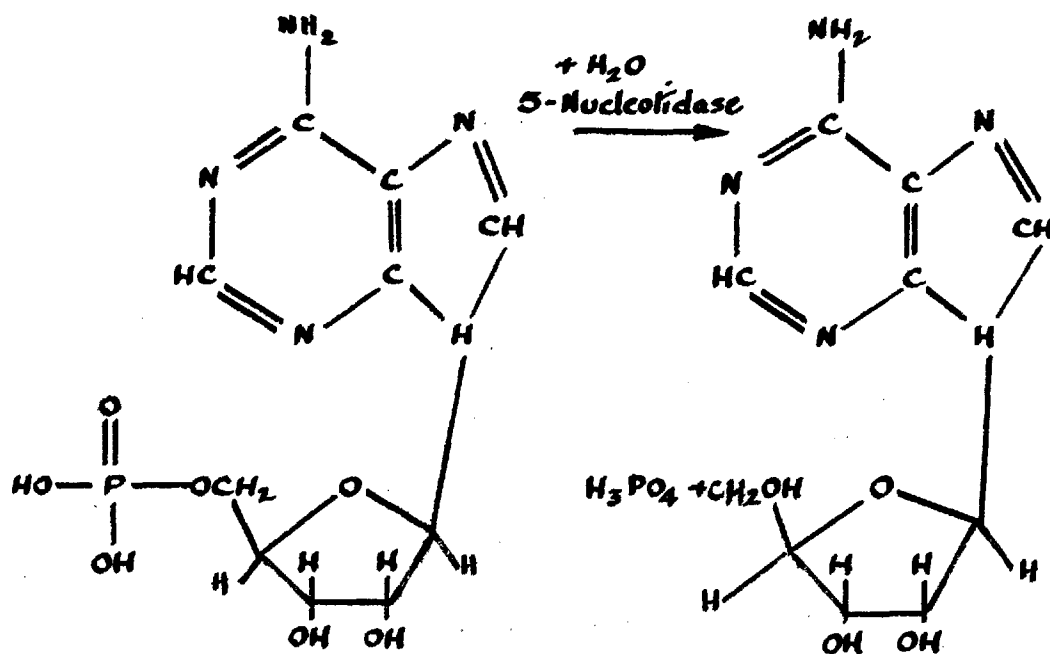
Spectrofluorimetric Method.

Advantage was taken of the fact that α -naphthyl phosphate and α -naphthol are fluorescent substances and a method was developed which depends on measuring the fluorescence intensity of the liberated α -naphthol (Campbell and Moss, 1961). This is more sensitive than the colorimetric method, and it was, therefore, possible to reduce the incubation time from 1 hr. to 15 min. The results with this method were essentially the same as those obtained with the colorimetric determination of the free α -naphthol. It was found that phenyl phosphate is hydrolysed 1.3 times as fast as α -naphthyl phosphate.

3.

5-NucleotidaseIntroduction.

5-Nucleotidase catalyses the dephosphorylation of nucleotides which have the phosphate attached to the fifth carbon atom in the ribose radical. Although the enzyme has become known as 5-nucleotidase, this terminology is not strictly correct, since from the name it might be expected that the phosphate group is attached to the nitrogenous base and not to the glycosidic part of the molecule. It should be called 5'-nucleotidase, but to conform with the literature the usual name 5-nucleotidase will be used here.



Adenosine - 5'- Phosphate

Adenosine

Occurrence and Substrate Specificity

In 1934 Reis described an enzyme present in the brain of rat and rabbit and in the retina of the frog, rat, rabbit and calf that would liberate inorganic phosphate from muscle adenylic acid and inosinic acid many times more rapidly than it would from yeast adenylic acid or glycerophosphate. Because of the attachment of the phosphate group to the carbon in the fifth position on the pentose in adenylic and inosinic acids, he called this enzyme 5-nucleotidase. Reis (1937) provided further confirmation of the specificity of this enzyme for 5-nucleotides by extending the list of phosphate esters that are only slowly hydrolysed by it. He attributed this slight hydrolysis to the presence of a small amount of non-specific phosphatase present in the extracts of calf brain and horse nerve that he was using as the source of enzyme.

Gulland and Jackson (1938) found that various snake venoms were rich sources of 5-nucleotidase uncontaminated by other phosphatases. They extended to 15 the number of esters of monophosphoric acid that were unattacked by the enzyme and thus gave a further demonstration of its specificity. With the discovery by Hatcher and Welbergs (1935) of non-specific acid phosphomonoesterase activity in human semen, Reis (1938) and Marm (1945) looked for and found 5-nucleotidase activity in the semen of bull, bear and ram. In 1949 Gomori confirmed the substrate specificity of 5-nucleotidase by histochemical techniques using

slices of tissue prepared from various organs of different animals. He found different patterns of distribution of phosphatase activity when the substrate was adenosine-5-phosphate from those with any other phosphate ester as substrate.

Heppel and Hilmeo (1951) undertook the purification of 5-nucleotidase from bull semen, and were able to remove the slight activity towards yeast adenylic acid that had contaminated mammalian preparations of the enzyme in earlier work. They extended the number of 5-nucleotides investigated and found that the enzyme would also liberate phosphate from nicotinamide-ribose-5'-phosphate (nicotinamide mononucleotide), uridine-5'-phosphate and cytidine-5'-phosphate, thus further demonstrating its high specificity for esters containing the ribose-5-phosphate group. They found that the pH optimum was pH 8.5 for this 5-nucleotidase. Reis (1950) demonstrated the wide distribution of the enzyme in human tissues. He found the highest activity in the wall of the aorta, and here there was little contamination by non-specific phosphatase. He also found high concentrations of the enzyme in the thyroid, testicle, brain and pancreas. The pH optimum for the 5-nucleotidase from these tissues was between 7.5 and 8.

Kaye (1960) using dialysed cobra venom found optimum 5-nucleotidase activity between pH 6.8 and 7.0 in contrast to the earlier work on other snake venoms, whose pH optima were between 8.5 and 8.9. He was able to demonstrate enhancement of activity

after dialysis, and concluded that this was due to the removal of Zn ions which have been shown to be inhibitory.

Activation and Inhibition

Heppel and Hilme (1951) found that Mg^{2+} activated a 5-nucleotidase preparation from bull semen, while Ca^{2+} and Mn^{2+} had no effect on the enzyme from this source. Mg^{2+} activation could be prevented by the presence of Ca^{2+} , but this Ca ion effect could again be overcome by the addition of excess Mg^{2+} . Zeller (1950) has shown that the 5-nucleotidase in snake venom exhibits activity in the absence of divalent ions but the dephosphorylation rate is enhanced by the addition of Mg^{2+} , Ca^{2+} , Mn^{2+} or Cu^{2+} . He noted that Ni^{2+} and Zn^{2+} were powerful inhibitors. Kaye (1955) studied the effect of zinc on the 5-nucleotidase of snake venom.

Reis (1950) studied the 5-nucleotidase activity of various human tissues. He demonstrated activation of the enzyme by Mg^{2+} from these sources but did not state the concentration used in the experiments. In 1958 Ahmed and Reis investigated the activation and inhibition by metal ions of 5-nucleotidase from human aorta and human placenta. The results from the two tissues were essentially the same. The final concentration of all ions studied was mM , and there was strong activation by Mn^{2+} and only slight activation by Mg^{2+} at this concentration. Young (1958) has found activation by Mg^{2+} at $40mM$ concentration of the enzyme found in serum, so it is likely that the variation in Reis'

results is due to difference in concentration of Mg^{2+} . Ahmed and Reis (1958) found no inhibition by excess phosphate ions, contrary to their effect on non-specific alkaline phosphatase.

Methods Used to Study 5-Nucleotidase Activity.

When investigating the 5-nucleotidase activity in blood and other human tissues, the non-specific alkaline phosphatase activity at pH 7.5 has to be estimated. Dixon and Purdon (1954) took the hydrolysis of β -glycerophosphate at pH 7.5 as giving a measure of the alkaline phosphatase activity at this pH in the 5-nucleotidase-containing samples. They subtracted this value from the total activity obtained with adenosine-5-phosphate substrate. Similarly Ahmed and Reis (1958) used phenyl phosphate as the substrate to estimate the alkaline phosphatase. Both these methods give erroneous results, since β -glycerophosphate is hydrolysed by non-specific alkaline phosphatase more slowly than adenosine-5-phosphate and phenyl phosphate more quickly, so in the first case too low values are subtracted giving too high values for the 5-nucleotidase activity, the reverse being true in the second case.

Young (1958) overcame the problem by pre-incubating the serum with 0.0015M ethylenediamine tetraacetic acid. This inactivates the alkaline phosphatase but has no effect on the 5-nucleotidase. Ahmed and Reis (1958) suggested making use of

Ni^{2+} inhibition to suppress the 5-nucleotidase activity and thus obtain a measure of the hydrolysis of adenosine-5-phosphate by alkaline phosphatase. We have developed a method along these lines.

Colorimetric Determination of 5-Nucleotidase Activity

Principle

The method involves two enzyme determinations at pH 7.5. Firstly the sum of the alkaline phosphatase and 5-nucleotidase activities on adenosine-5-phosphate is determined, and then by inhibiting the 5-nucleotidase with nickel the alkaline phosphatase is determined separately with the same substrate. Subtraction of one from the other gives the 5-nucleotidase activity. To conform with Young's method (1958), the reaction mixtures are made 40mM with respect to magnesium which activates both enzymes. The liberated phosphate is determined colorimetrically by the method of Delsal and Manhourl (1958) to give a measure of the enzyme activity.

Reagents.

For enzyme determination

Veronal buffer (0.04 M, pH 7.5)

Adenosine-5-phosphate (10 mM): dissolve 34.7 mg. in 18 ml. 0.1 M NaOH and make up to 100 ml. with water.

These solutions should be kept at 4°.

Nickel chloride (0.1 M)

Magnesium chloride (0.8 M)

Trichloroacetic Acid (10% W./V.)

For phosphate determination

Acetate buffer (2 N pH 4.0): In 1 l. of 2N-acetic acid are dissolved 2.5 g. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 46 g. pure crystalline $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$. The pH is adjusted to 4.0 by the addition of $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ crystals.

Ammonium molybdate (5%): 5 g. Ammonium molybdate are dissolved in 100 ml. water and filtered.

Rhodol sulphite (2% Rhodol (p-methyl aminophenol sulphate) in 5% neutral anhydrous Na_2SO_3): 2 g. Rhodol are dissolved in about 80 ml. water and 10 g. hydrated Na_2SO_3 added (more pure than the anhydrous and equal to 5 g. of it). The solution is made up to 100 ml., filtered and stored in a dark bottle at 4°.

Stock phosphate standard (1 mg. phosphorus/ml.): 2.194 g. potassium dihydrogen phosphate pure (A.R.) are dissolved in 500 ml. water and preserved with a few drops of chloroform and kept in the cold.

Working phosphate standard (10 µg. phosphorus/ml.): 1 ml. of stock standard is diluted to 100 ml. with water. The solution is mixed well and preserved with chloroform.

Method.

For each determination a test and a control tube are necessary.

Test. 1.5 ml. veronal buffer, 0.1 ml. $MgCl_2$ and 0.2 ml. serum.

Control. 1.3 ml. veronal buffer, 0.1 ml. $MgCl_2$, 0.2 ml. $NiCl_2$ and 0.2 ml. serum.

The solutions are mixed and both tubes are placed in a water bath at 37° for a few minutes, and then 0.2 ml. adenosine-5-phosphate is added to each. The reaction is allowed to proceed for 30 min., and then 2 ml. of CCl_3COOH added and mixed well. The tubes are centrifuged and 2 ml. of the supernatant used for the phosphate determination.

Colour Reaction. 2 ml. of supernatant are added to 3 ml. acetate buffer, and 0.5 ml. ammonium molybdate added, followed by 0.5 ml. rhodol sulphite. The tubes are shaken and left for 10 min. The extinction at 880 m μ is measured in a Unicam SP 500 spectrophotometer.

Standard. To 1 ml. 10 $\mu g./ml.$ standard, 1 ml. 10% CCl_3COOH and 3 ml. of the acetate buffer are added the colour reagents as for the test and control solutions.

Blank. 1 ml. water, 1 ml. 10% CCl_3COOH , 3 ml. acetate buffer and the colour reagents.

Calculation.

Results are expressed in terms of micro-moles of substrate utilized per min. per litre of serum (International Units per litre).

The 2 ml. of supernatant represents 0.1 ml. serum and therefore

$$\begin{aligned} \text{International Units per l.} &= \frac{\text{Reading of Test-Control}}{\text{Reading of Standard-Blank}} \times 10 \times \frac{1000}{0.1} \times \frac{1}{30} \times \frac{1}{31} \\ &= \frac{T - C}{S} \times 108 \text{ } \mu\text{moles/min./l.} \end{aligned}$$

Results.

We established a normal range of 2-15 with a mean of 7.7 and S.D. of 3.9 for 20 healthy laboratory workers. The precision of the method is 1.6 $\mu\text{moles/min./l.}$ calculated from duplicate determinations of 16 sera by means of the formula $\sqrt{\frac{\sum d^2}{2n}}$ where d is the difference between duplicates and n the number of pairs.

This method was used to investigate the 5-nucleotidase activity in the serum of patients with various diseases. The results will be discussed in Part II.

Studies on 5-Nucleotidase Procedure

Source of Enzyme.

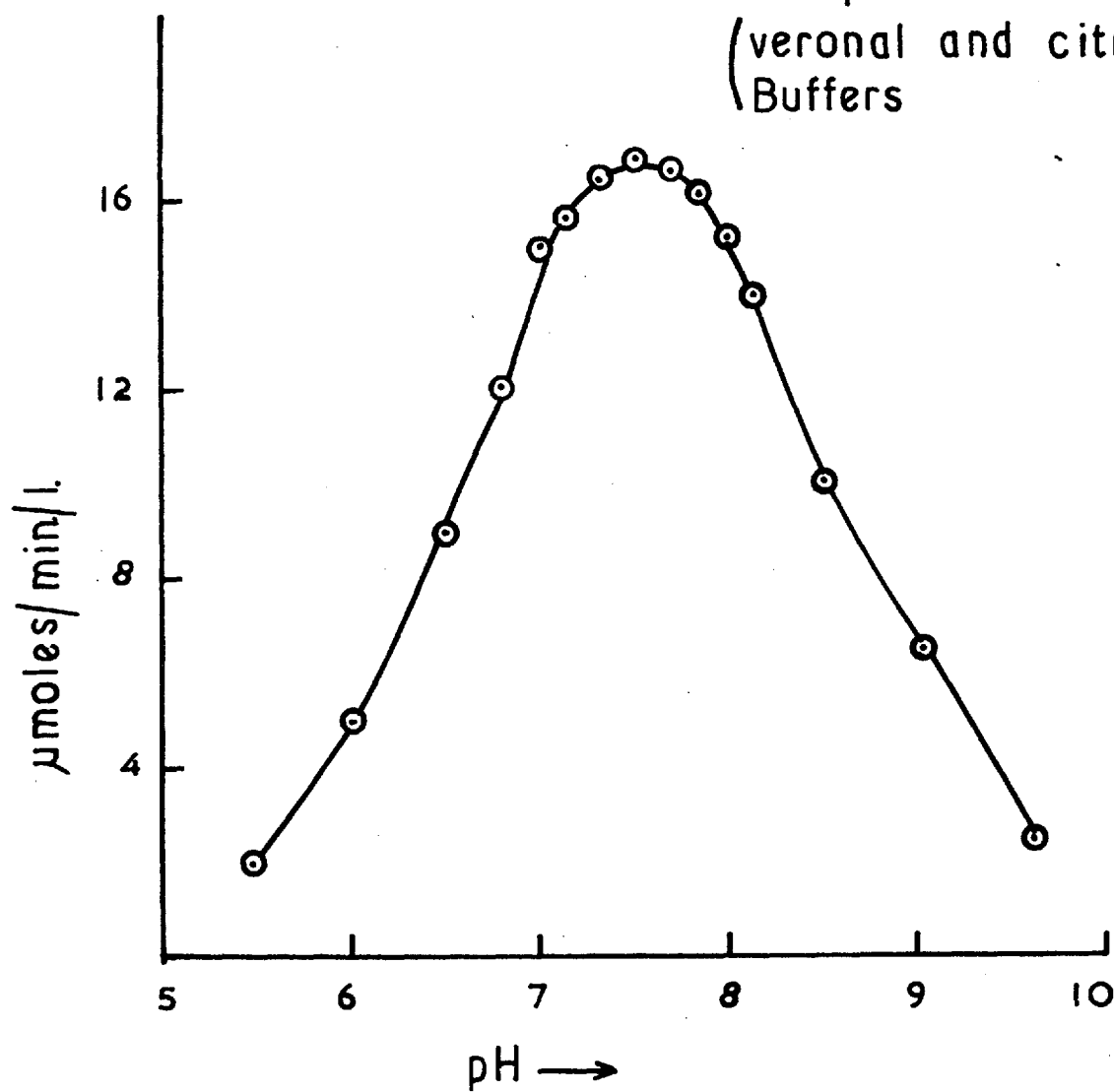
Partially purified aqueous extracts of human aorta were used as the source of 5-nucleotidase in inhibition and kinetic studies. The aorta was taken at autopsy, and after removal of the intima and adventitia was ground up in 10 vol. water with sand in a pestle and mortar, and then left for 3 days at room temperature with a few drops of chloroform. The solution was then centrifuged and the supernatant used as tissue extract. This enzyme solution was used in the study of the properties of 5-nucleotidase since it is free from any alkaline phosphatase activity, shown by its inability to hydrolyse phenyl phosphate, and thus avoids any complications which may arise from its presence.

For estimation of serum activity fresh serum was used. If it was not possible to carry out the test on the same day, the specimens were kept at 4° to preserve the activity.

Effect of pH on 5-Nucleotidase Activity.

Determination of pH optimum in veronal and citrate buffers carried out for the action of partially purified aorta 5-nucleotidase on A-5-P gave a peak at pH 7.5, with a 1 mM final substrate concentration (Fig. 14). This pH was, therefore, adopted in the method for determination of the enzyme activity in serum, since it was established that 5-nucleotidase from all human tissues had the same pH optimum (Reis, 1950).

fig 14
Variation of Aorta
5-Nucleotidase activity
with pH
(veronal and citrate)
Buffers



Increase in Enzyme Concentration

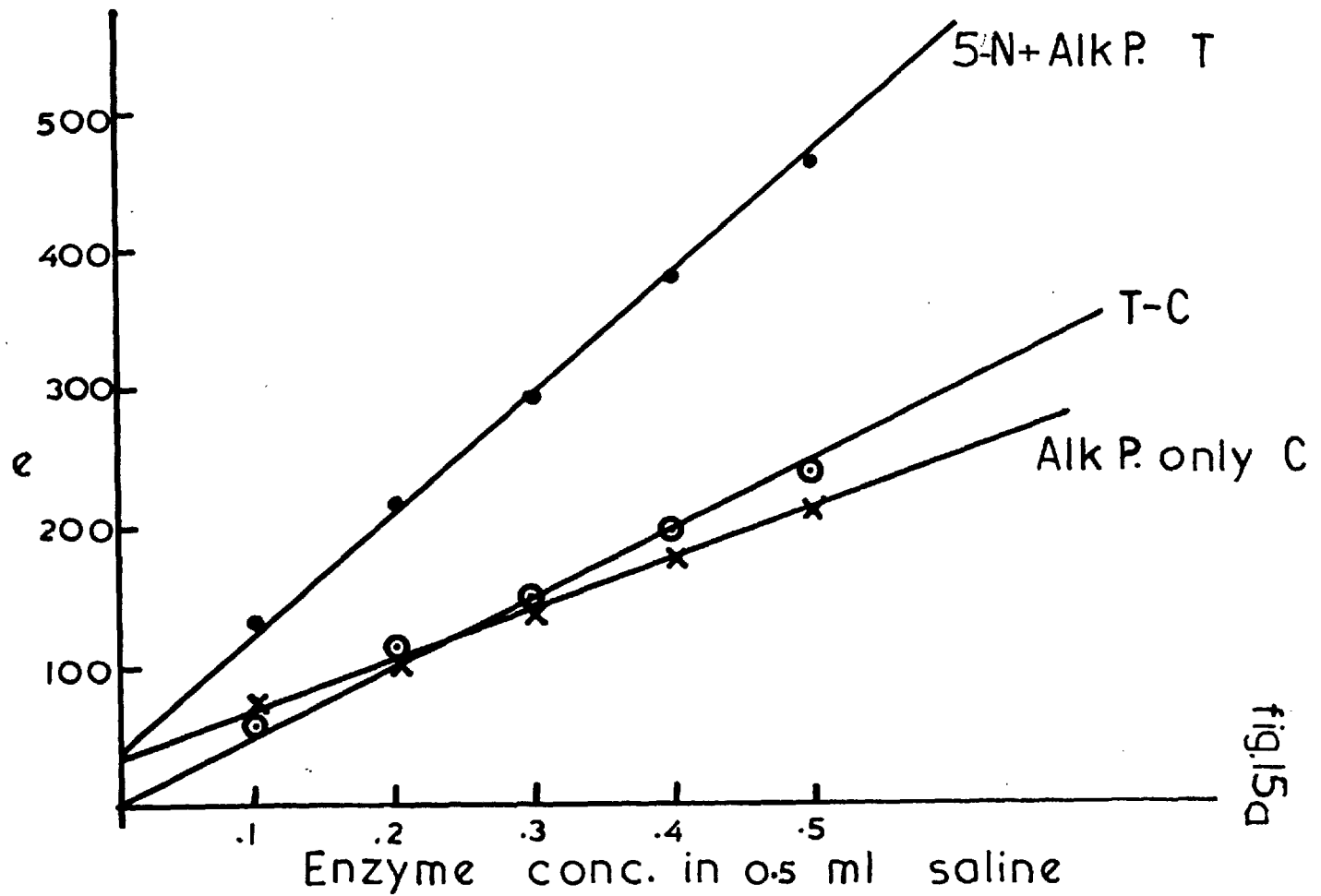
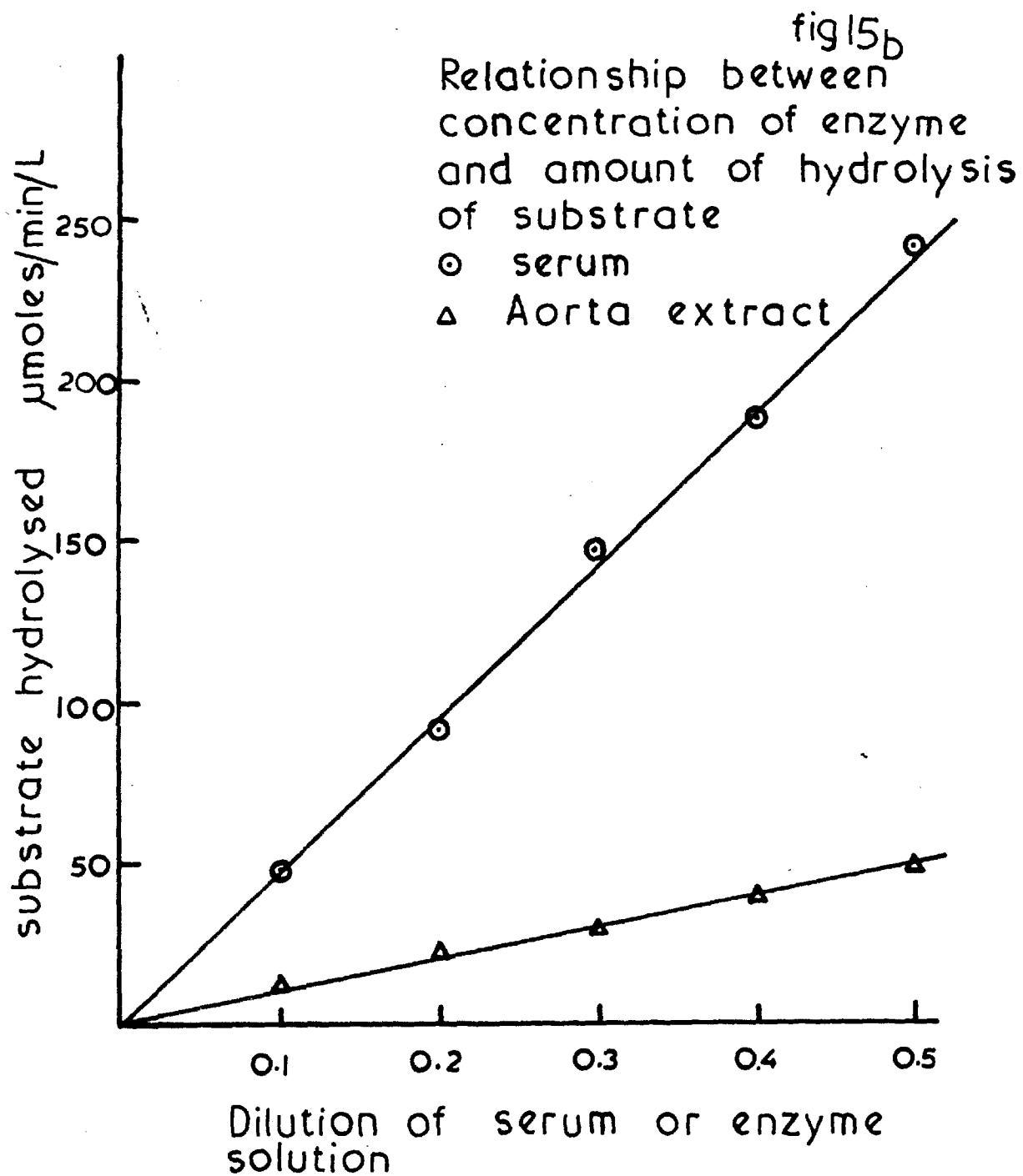
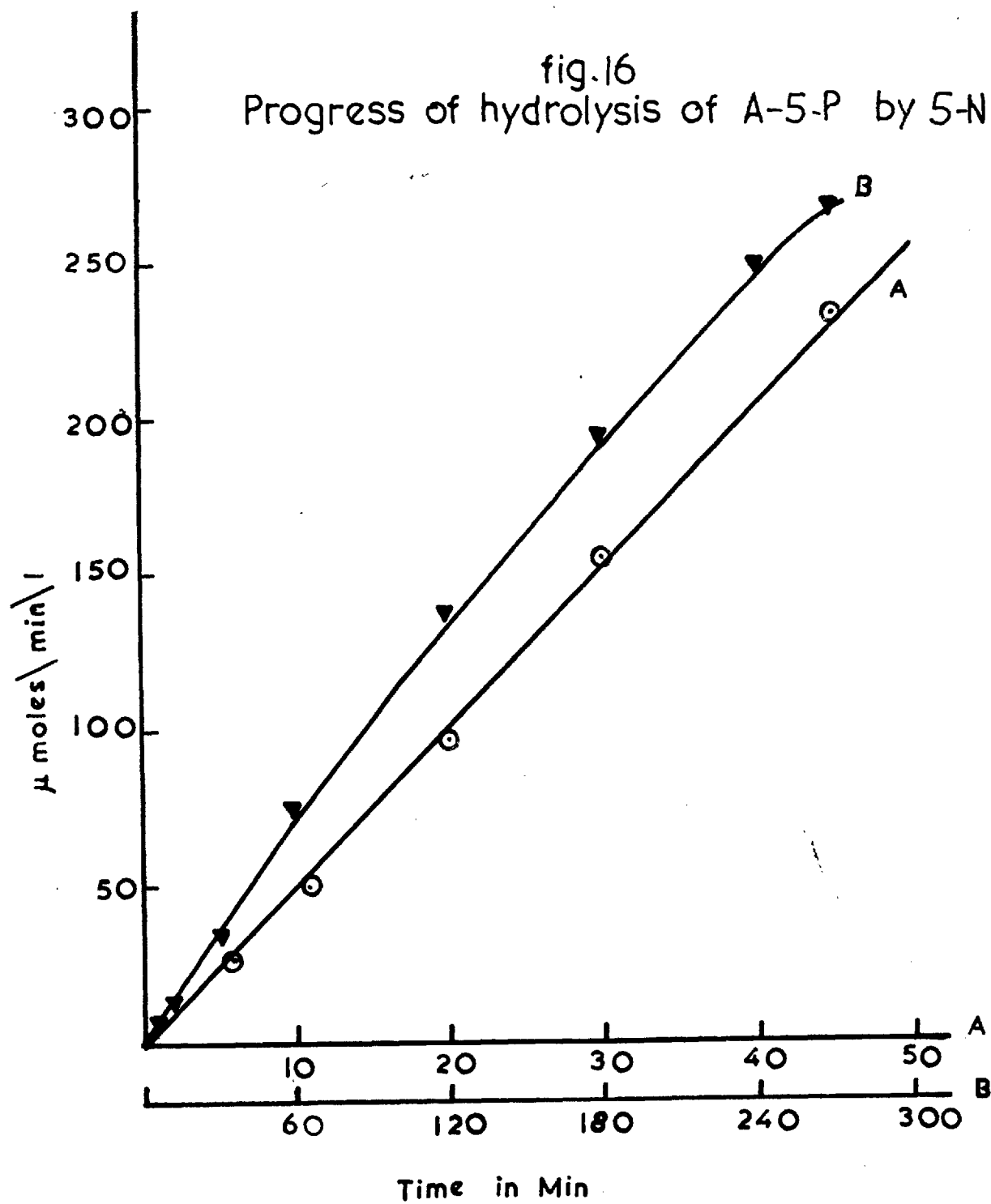


fig.15a





Effect of Enzyme Concentration

The relation between enzyme concentration (varied by taking serial dilutions of aorta extract) and amount of hydrolysis was linear over a range 1 - 3 times the normal serum activity (Fig. 15 a and b).

This relationship was also linear when dilutions of a serum of high activity (240 μ moles/min./l.) was used (16 X normal). For activities higher than this, a shorter incubation time should be employed.

Progress of Hydrolysis.

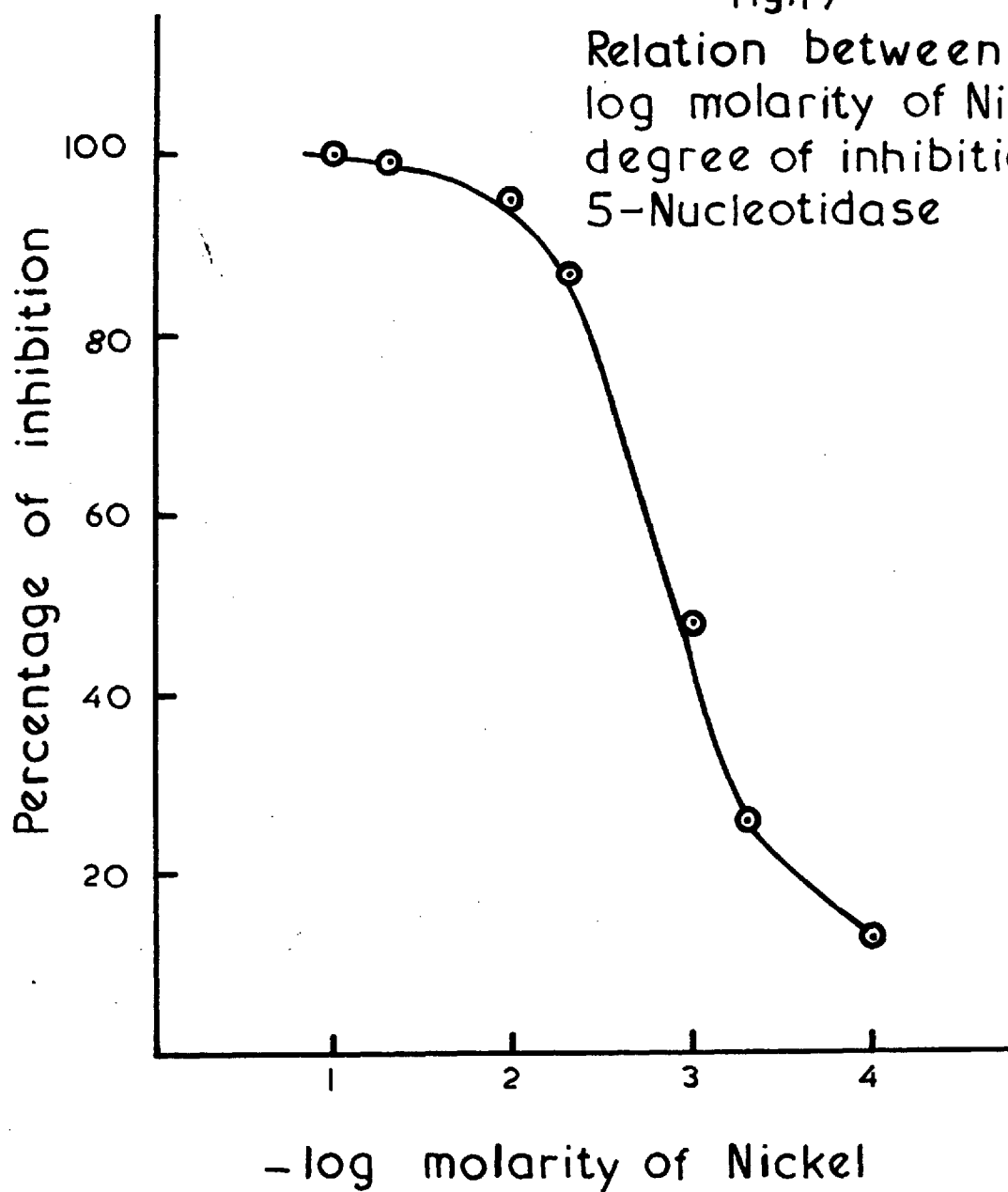
This was linear over the 30 min. incubation period and up to 120 min. There was slight deviation from linearity when the incubation was prolonged up to $4\frac{1}{2}$ hr. (Fig. 16).

Inhibition and Kinetic Studies.

As previously mentioned, the hydrolysis of A-5-P at pH 7.5 by aorta 5-nucleotidase is inhibited by Ni^{2+} . The percentage inhibition was determined by incubating aorta extract with A-5-P and concentrations of nickel chloride ranging from 100 mM to 0.1 mM ; the activities thus obtained were compared with the activity in the absence of nickel. It was found that inhibition becomes 95% complete when the concentration of nickel exceeds 10 mM with a 1 mM substrate concentration. These results are shown in Fig. 17 in which the negative log molarity of nickel is plotted against percentage inhibition.

fig.17

Relation between negative
log molarity of Nickel and
degree of inhibition of
5-Nucleotidase



Lineweaver - Burk Reciprocal
plot to determine K_m of Aorta
5-Nucleotidase

$K_m = 0.1 \text{ mM}$

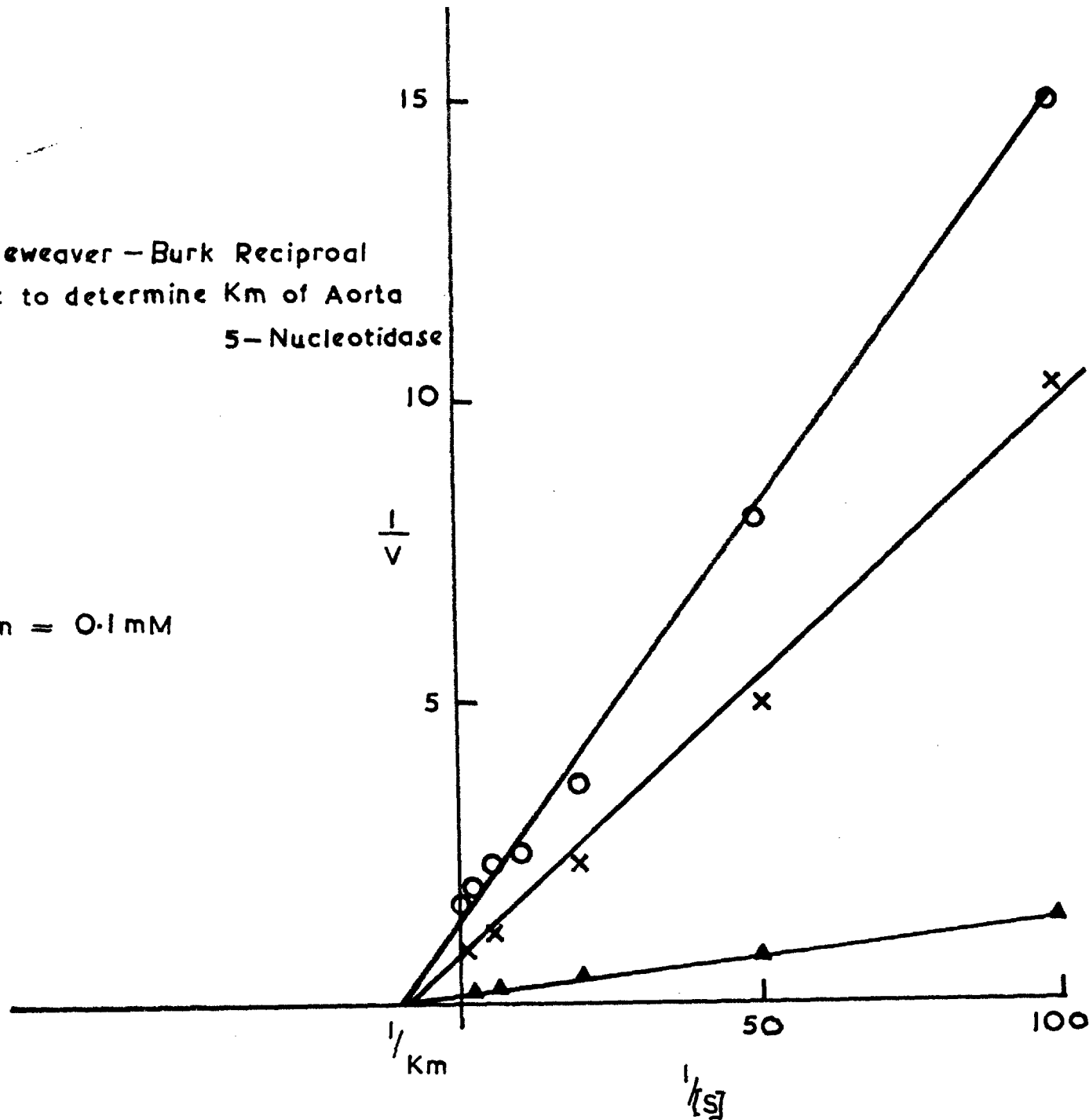
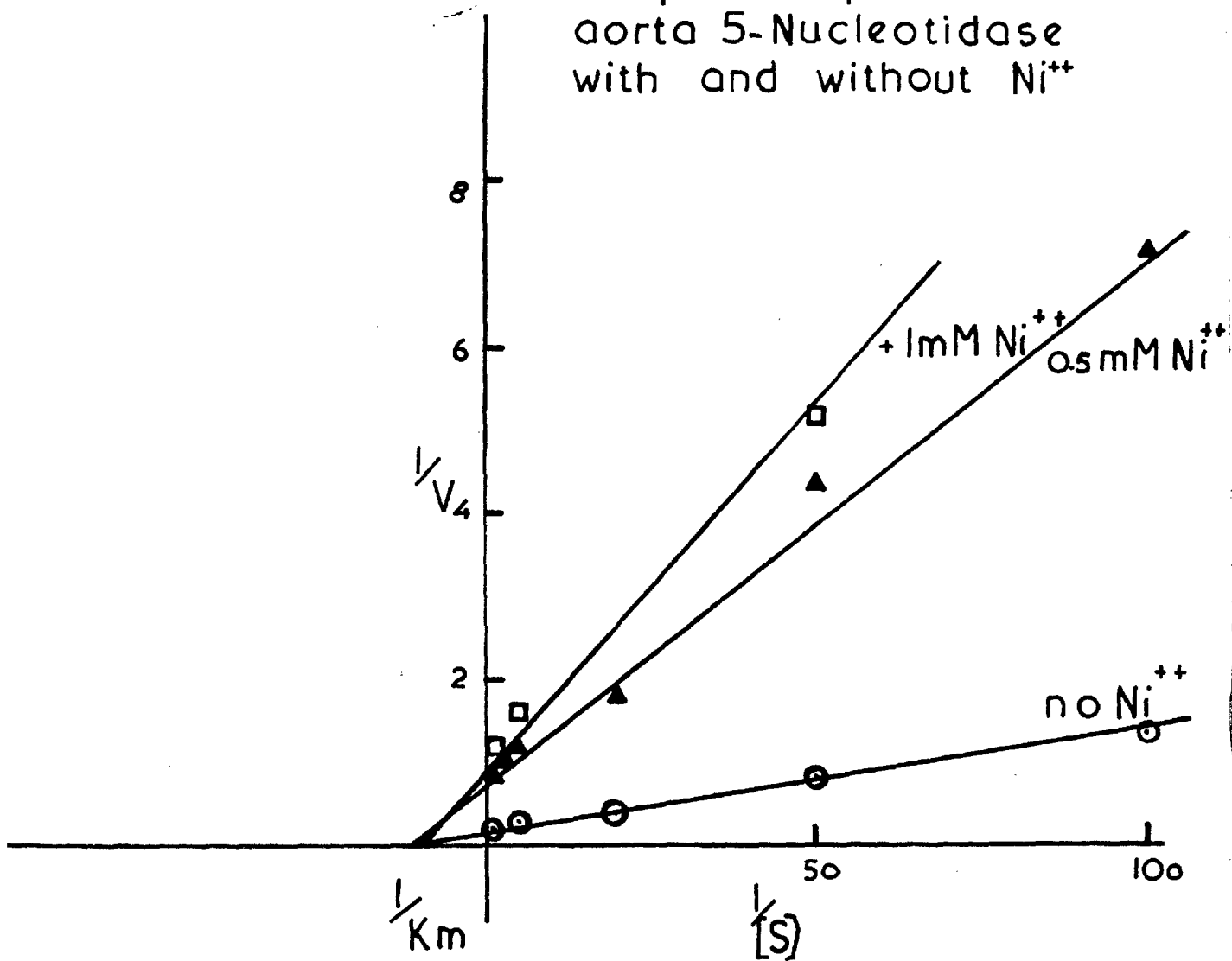


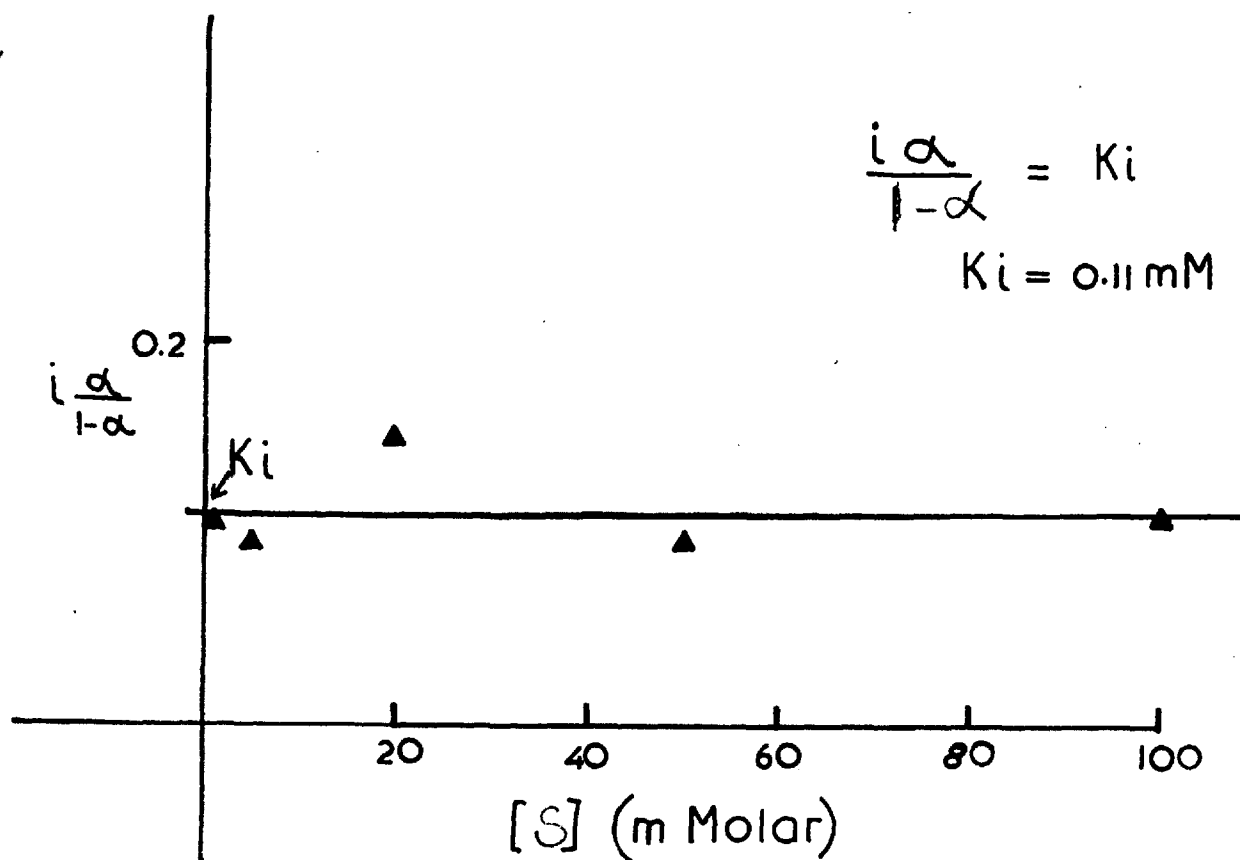
fig.18

fig 19
 Reciprocal plots for
 aorta 5-Nucleotidase
 with and without Ni^{++}



Graphical Determination
of inhibitor constant for
Nickel inhibition of 5-N

fig20



The Michaelis constant for aorta 5-nucleotidase was determined by measuring the velocities with a series of substrate concentrations varying from 1 - 0.01 mM and the results were plotted by the method of Lineweaver and Burk (1934); a constant of 0.1 mM was obtained with 3 enzyme preparations of different activities. The results are shown in Fig. 18.

The type of inhibition by Ni^{2+} was similarly determined by plotting $1/v$ against $1/S$ with and without nickel. It was found that V_{max} was altered by the presence of nickel whereas K_m was not, a result consistent with non-competitive inhibition (Fig. 19).

The inhibitor constant was determined by plotting the results by the method of Hunter and Downs (Dixon and Webb, 1958) (Fig. 20) where $1 \frac{\alpha}{1 - \alpha}$ is plotted against substrate concentration S . i = concentration of inhibitor, $\alpha = \frac{v_1}{v}$ where v_1 is velocity in presence of inhibitor and v the velocity in its absence, with the same substrate concentration. It can be seen that $1 \frac{\alpha}{1 - \alpha}$ is independent of substrate concentration, another fact that is consistent with non-competitive inhibition.

Effect of Alkaline Phosphatase on Determination of 5-nucleotidase.

The effect of variation in alkaline phosphatase activity on the determination of 5-nucleotidase was investigated to ensure that at high alkaline phosphatase concentration there was no

competition for the substrate between this enzyme and 5-nucleotidase, thus giving an apparently low value for 5-nucleotidase.

A series of enzyme solutions were prepared containing a constant volume of aqueous aorta extract and increasing concentrations of partially purified bone alkaline phosphatase which was free from any 5-nucleotidase activity. The total and alkaline phosphatase activities were determined for each solution. The results are shown in Fig. 21.

The alkaline phosphatase activity varied between 14 and 54 $\mu\text{moles/min./l.}$, and it can be seen from the graph that this had no significant effect on the 5-nucleotidase activity, it being 54 $\mu\text{moles/min./l.}$ with the maximum and minimum amount of alkaline phosphatase present.

Investigation of Precipitate Formed Occasionally in Control Tubes.

In some cases a precipitate appears when the serum is added to the buffer and nickel chloride in the control tube. This precipitate was investigated to ensure that this was not an additional cause for the reduction in activity in the control tube as well as the nickel inhibition of the enzyme. Buffer, nickel chloride and a serum previously shown to cause this precipitation effect were mixed in the proportions

Effect of Alkaline Phosphatase
on Determination of
5-Nucleotidase

○—○ Alk P+5'-N activity
x x Alk P activity
▲▲ 5'-N activity

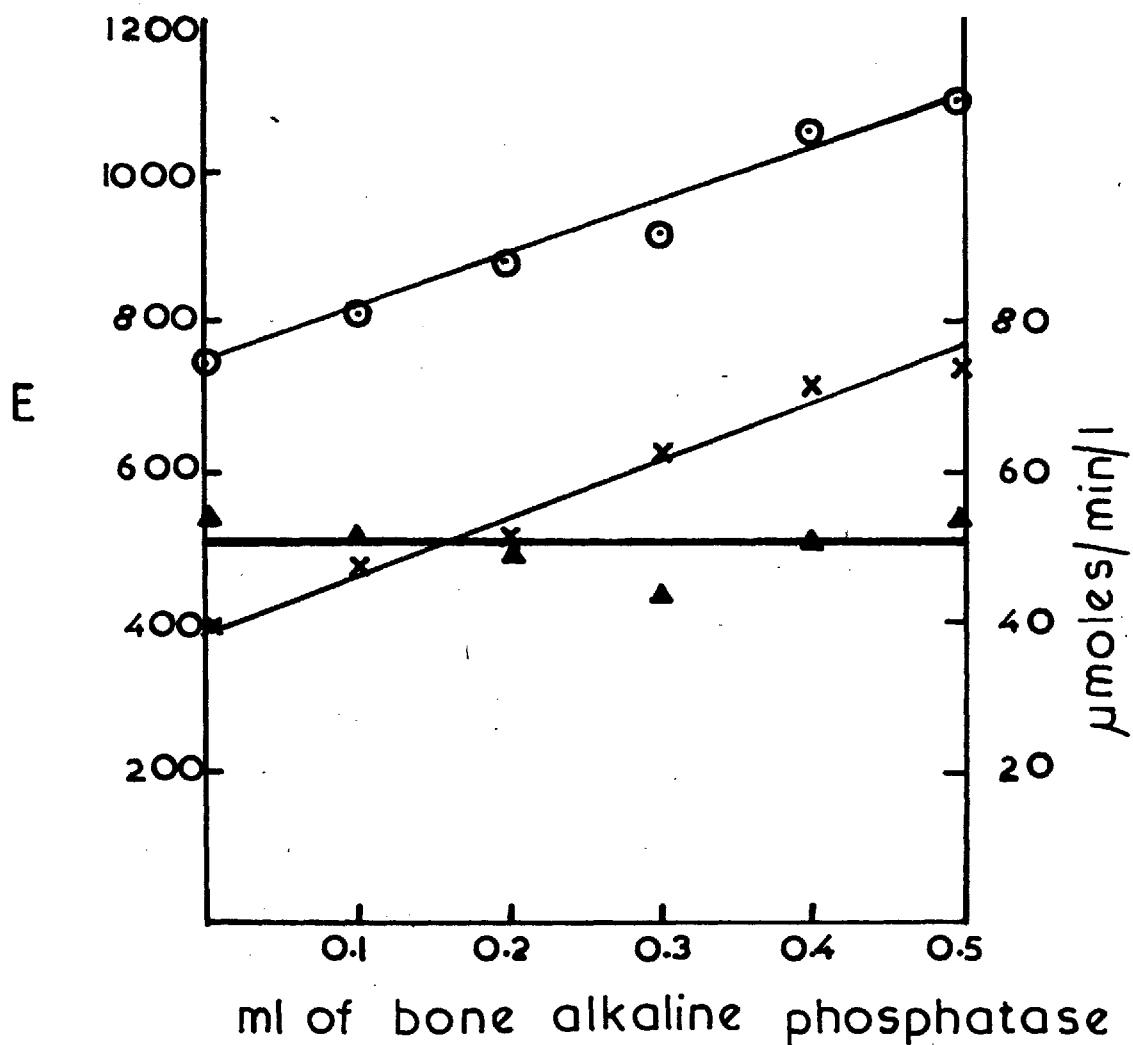


fig. 21

used in the method for estimation of serum 5-nucleotidase to give a total volume of 9 ml. The precipitate which formed was removed by centrifugation and was then suspended in buffer. Both this and the supernatant were dialysed against running tap water overnight to remove nickel ions.

The original serum sample, the supernatant and the precipitate were then investigated for total 5-nucleotidase and alkaline phosphatase activity as in the method described. The inhibition caused by the addition of nickel to the supernatant had been removed during dialysis, and this solution exhibited 67% of the total activity shown by the original serum sample. The addition of nickel again caused inhibition of the 5-nucleotidase in the control tube. There was no activity in the precipitate after dialysis.

4.

Glucose-6-Phosphatase

Glucose-6-phosphatase is a substrate-specific phosphatase present in large amounts in liver. Swanson (1950) was the first to prepare the enzyme in a purified form; several workers, Cori, Cori and Schmidt (1939), Fantl and Rose (1945), Broh-Kahn and Mirsky (1948) and de Duve *et al.* (1949) had previously provided indirect evidence for its existence while investigating the nature of the liver phosphatase which hydrolyses glucose phosphate, but they all used crude liver extracts with high activities of other enzymes. Swanson's preparation had high activity towards glucose-6-phosphate with minimal activity towards other phosphate esters.

Langdon and Weakley (1957) showed that glucose-6-phosphatase is a tightly bound microsomal enzyme. Dixon and Webb (1958) suggest its use during the preparation of sub-cellular fractions as an index of contamination of other fractions by microsomes.

Properties of the Enzyme.

Swanson (1950) found glucose-6-phosphatase to be extremely labile to heat and observed that most of the activity was lost after a short time at room temperature. She, therefore, carried out all her experiments at 30° to avoid

inactivation of the enzyme during the reaction period. Swanson found the optimum pH to be 6.5, in agreement with Fantl and Rome (1945). This is the pH at which acid and alkaline phosphatase activity is at a minimum and provides further evidence for the specific nature of glucose-6-phosphatase. Langdon and Weekley (1957) have been unable to demonstrate any metal cofactor requirements.

Serum Enzyme.

Koide and Oda (1959) reported the presence of glucose-6-phosphatase in the serum of patients with liver disease although the enzyme can scarcely be detected in normal serum (Bruna, 1954). Since the enzyme is present only in liver, they suggested adopting its use in the investigation of patients with liver damage. They demonstrated a rise in serum glucose-6-phosphatase activity in dogs with experimental liver damage, produced by the injection of carbon tetrachloride, which was accompanied by a decrease in the enzyme activity in the liver. They found that the serum level reached a peak of 5 - 20 times normal six hours after the injection and returned slowly to the normal level within seven days. The serum transaminase levels were also studied in these dogs and it was found that peak activity was reached three days after the injection. In view of this, Koide and Oda (1959) suggest

that glucose-6-phosphatase is a more sensitive index of liver damage than serum transaminases.

Methods.

These results encouraged us to investigate the use of this as a liver-specific enzyme. We adapted our glucose-1-alkaline phosphatase method (see Chapter 2) for use with glucose-6-phosphate and citrate buffer at pH 6.5, and we compared these results with those obtained by the method of Koide and Oda. They used a 20 mM concentration of substrate, 0.2 ml. serum in a 1 ml. reaction mixture and they incubated the test solution for 1 hr. at 37°.

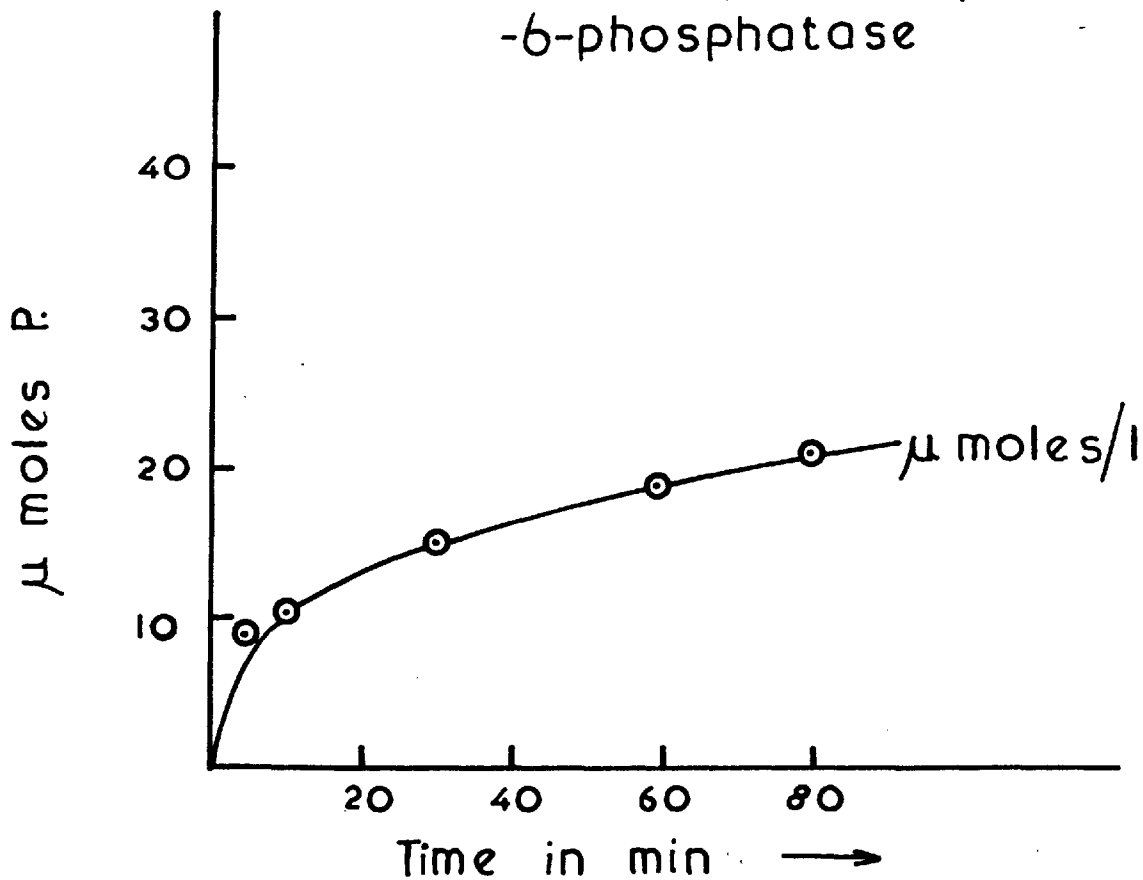
Addition of trichloroacetic acid stopped the reaction and inorganic phosphate analysis was carried out on a 1 ml. sample of filtrate by the method of Fiske and Subbarow (1925).

The results by both methods were most disappointing. Swanson (1950) reported the extreme sensitivity of the enzyme to heat and, despite determinations being carried out immediately after the blood had been taken, we were unable to get satisfactory results because of inactivation of the enzyme during the incubation period (Fig. 22).

This difficulty could be overcome by reducing the length of the reaction period and the temperature at which it is carried out. But the serum needs special handling; separation and estimation as soon as possible after it has been

fig22

Graph to show progress
of hydrolysis of Glucose
-6-Phosphate by Glucose
-6-phosphatase



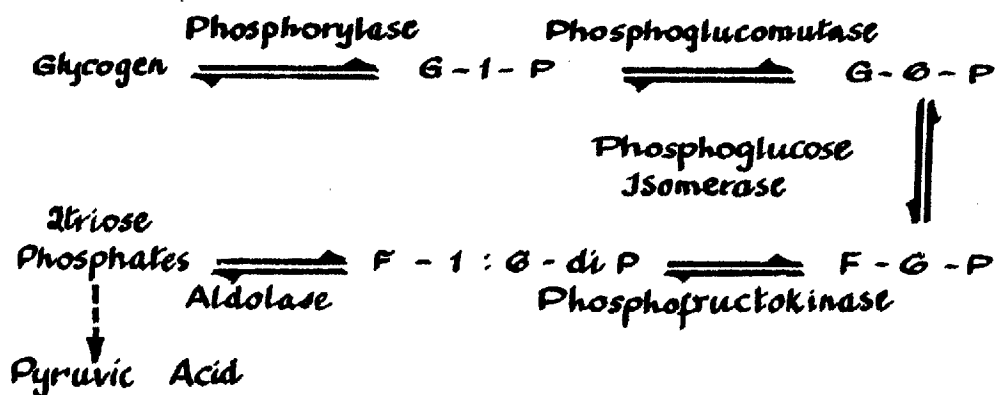
taken. This is unsuitable for use in a routine laboratory, and is difficult even for research purposes, so further investigation of glucose-6-phosphatase was not undertaken.

5.

THE GLYCOLYTIC ENZYMES.

Glycolysis is the term applied to the anaerobic breakdown of carbohydrate (glucose or glycogen) with the production of pyruvate and the liberation of energy. It originally referred to glycogen breakdown in muscle, but it now has a wider implication. The process of glycolysis is elaborate and takes place in many stages, each one controlled by a glycolytic enzyme.

The sequence of reactions is as follows:



Warburg and Christian (1943) prompted the study of glycolytic enzymes in disease with their work on aldolase levels in the serum of tumour-bearing rats. Sibley and Lehninger (1949a) extended this work to human subjects and since then serum phosphoglucose isomerase and phosphoglucose mutase levels in disease have also been studied by many workers. We have studied the level of the first two enzymes in the serum of patients with cancer and other diseases.

Phosphoglucose Isomerase.

Introduction.

Phosphoglucose isomerase, the enzyme which brings about the interconversion of glucose-6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P) was first demonstrated by Lohmann in 1953.

He prepared extracts from various animal tissues and found that the reaction did not reach completion but attained an equilibrium mixture of approximately 70% G-6-P and 30% F-6-P.

The enzyme was initially called phosphohexose isomerase, but Slein (1949, 1950) demonstrated the presence of an enzyme in non-purified preparations of Lohmann's

isomerase that caused the interconversion of F-6-P and mannose-6-phosphate (M-6-P). To avoid confusion, Slein (1954) suggested that the two enzymes should be called more descriptively phosphoglucose isomerase and phosphomannose isomerase.

Phosphoglucose isomerase has been found in pea meal (Tanko, 1936; Somers and Cosby, 1945). In 1951 Cori and co-workers showed that the enzyme is present in most animal tissues but it received little attention until Bodansky (1953a) demonstrated considerable phosphoglucose isomerase activity in the sera of several species, including man.

The equilibrium point of the reaction depends upon the experimental conditions and source of enzyme (Lohmann, 1933). Values between 30 and 40% F-6-P have been reported; Bodansky (1953a) found equilibrium at 40% F-6-P for the serum enzyme.

Properties of the Enzyme.

Phosphoglucose isomerase has not yet been isolated in a pure state. Slein (1954), using ammonium sulphate fractionation, and Ramasarma and Giri (1956), using acetone precipitation, have obtained a six-fold purification. The enzyme has been shown to be relatively stable at room

temperature, but is rapidly inactivated at temperatures above 45° (Ramadas and Giri, 1956). They have shown also that the enzyme prepared from green gram is more stable at an alkaline than an acid pH. The optimum pH is 7.8 for G-6-P substrate and 9.0 when F-6-P is the substrate.

Method for Determination of Serum Phosphoglucose Isomerase.
(Bodansky, 1954a).

Principle.

When serum is added to glucose-6-phosphate (G-6-P) at pH 7.5, the phosphoglucose isomerase present in the serum converts the G-6-P to fructose-6-phosphate (F-6-P). The F-6-P thus formed is determined by Ree's method (1954) for fructose, based on the Seliwanoff reaction for ketoses.

Method.

Enzyme Reaction. Serum is diluted 1 in 5; 0.4 ml. serum is added to 0.4 ml. saline in the reaction tube; this is placed in a water bath at 37° for a few minutes and 2 ml. of buffer-substrate mixture (veronal buffer 0.0715 M, pH 7.5 and G-6-P 0.0025 M) previously warmed to 37° , is added. The reaction is terminated after 30 min. by the addition of 2.5 ml. trichloroacetic acid (10% W/V). The solution is filtered and a 2 ml. sample is taken for fructose determination. A control tube for each set of determinations is prepared by adding the trichloroacetic acid to the diluted serum before the buffered-substrate.

Colour Reaction.

2 ml. resorcinol (0.1% W/V) and 6 ml. HCl (10 N) are added to 2 ml. of filtrate, the tubes are well shaken and placed in a water bath at $80^{\circ} \pm 1^{\circ}$ for exactly 15 min. The tubes are then cooled and the optical densities of the cherry-red solutions are read in a photoelectric colorimeter with an Ilford 623 green filter.

A F-6-P standard (50 μ g. fructose/ml.) and a colour reagent blank are similarly treated with resorcinol and HCl.

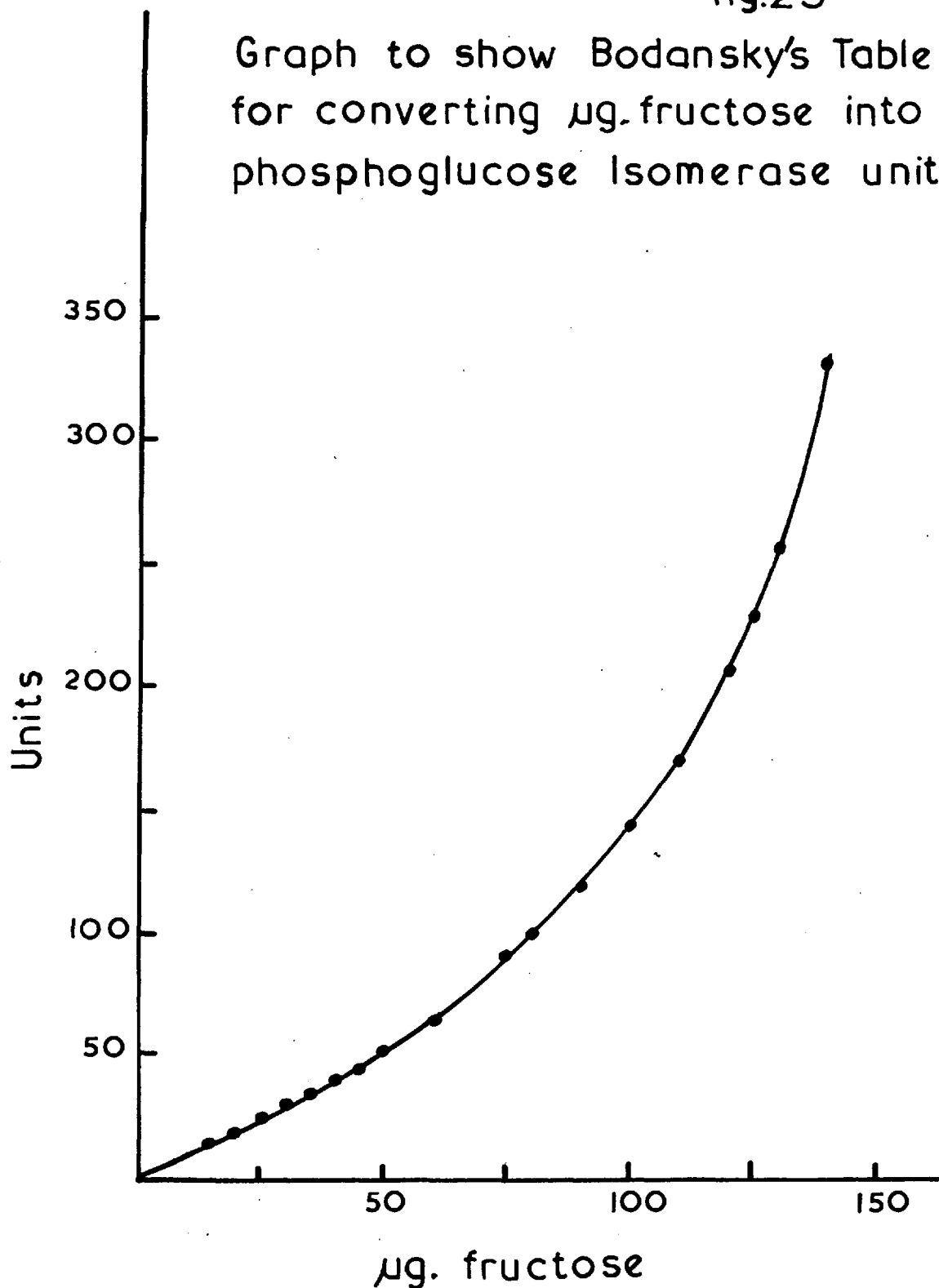
Since the reaction reaches equilibrium at 40% F-6-P under these conditions, with this molarity of substrate a maximum of 144 μ g. fructose as F-6-P can be formed per ml. of reaction mixture. Specimens with low enzyme activities will form less than 144 μ g. of fructose during the reaction and in these cases the equilibrium point will not be reached. If 144 μ g. of fructose is formed during the 30 min. incubation period, the test must be repeated with a greater dilution of serum.

Units.

Bedansky (1953b) has shown how enzyme activity may be expressed simply in terms of the amount of substrate changed at any stage of a reaction. He has applied this treatment to the expression of phosphoglucose isomerase activity (1954a) and defines the unit as the reciprocal of that concentration of

fig.23

Graph to show Bodansky's Table
for converting $\mu\text{g. fructose}$ into
phosphoglucose Isomerase units



substrate expressed as millilitres per millilitre of reaction mixture that will cause the formation of 25 μ g. of fructose as F-6-P in 30 min. per ml. of reaction mixture from 0.002 M-G-6-P at pH 7.4 at 37°.

There is a linear relationship between rate of reaction and enzyme activity up to 12% conversion of the substrate. After this there is increasing deviation from linearity, and Bodansky has constructed a table relating μ g. of fructose to units. This is shown graphically in Fig. 23.

We have recalculated Bodansky's units for phosphoglucose isomerase in terms of International Units per litre - μ moles/min./l. His units are expressed in terms of μ g. fructose per 0.04 ml. of serum per 30 min. Therefore

$$1 \text{ Bodansky unit} = 1 \times \frac{1}{180} \times \frac{1}{30} \times \frac{1000}{0.04} \mu\text{moles/min./l.}$$

$$= 4.63 \text{ International Units/l.}$$

We read off the μ g. fructose from his table for conversion to Bodansky units and then multiply by 4.63 for conversion to International Units. Bodansky's normal range of 8 - 40 units is equivalent to 37 - 180 International Units per litre.

We have established a normal range for 30 healthy laboratory workers of 12 - 29 Bodansky units, with a mean of 20 and standard deviation (S.D.) of 5.5. This is equivalent to a range of 55 - 130 International Units per litre.

In our hands the precision of the method was 5 μ moles/min./l., calculated from duplicate determinations on ten sera by means of the formula $\sqrt{\frac{\sum d^2}{2n}}$ where d is the difference between duplicates and n the number of pairs.

Bodansky found that serum retained its activity for 8 hr. at room temperature and for at least 2 weeks in the ice box. To ensure that there was no loss of activity while the specimen was waiting for estimation, the blood was separated as soon as possible and the serum placed in the deep freeze at -15° . We have found that the enzyme retains its activity for at least 5 months under these conditions.

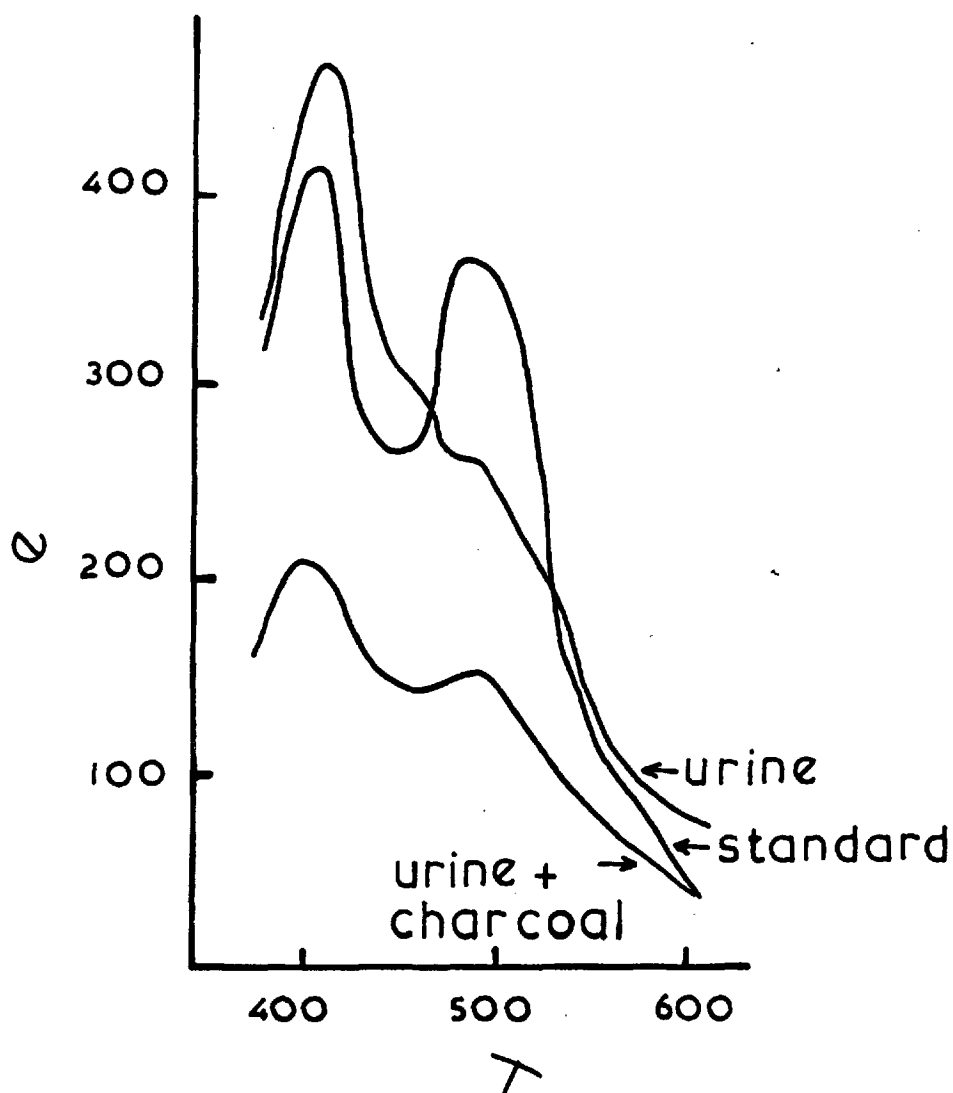
The results on the patients will be given in Part II.

Phosphoglucose Isomerase in Urine.

During a survey of the serum enzyme level in other diseases, it was found that there were large variations in the day to day level of phosphoglucose isomerase in patients in acute renal failure. The serum level of the enzyme fell during the diuretic phase, and so a study of the enzyme in urine was undertaken to find out if this fall during recovery indicated a cessation in the release of the enzyme or clearance of it by the kidneys. Bodansky's (1954a) method for determination of the enzyme in serum was adapted for use with urine.

fig.24

Absorption Curve showing
the effect of treating urine
with charcoal



Adaptation of Method for Use with Urine.

0.5 ml. urine is substituted for the diluted plasma and the method then carried out just as for serum. Results are expressed in terms of micromoles of substrate transformed per minute per 24 hr volume of urine.

A normal range of 0 - 15 μ moles/min./total vol. was established for 20 healthy laboratory workers and patients with non-cancerous diseases, all with normal serum phosphoglucose isomerase levels.

Investigation of Colour in Control.

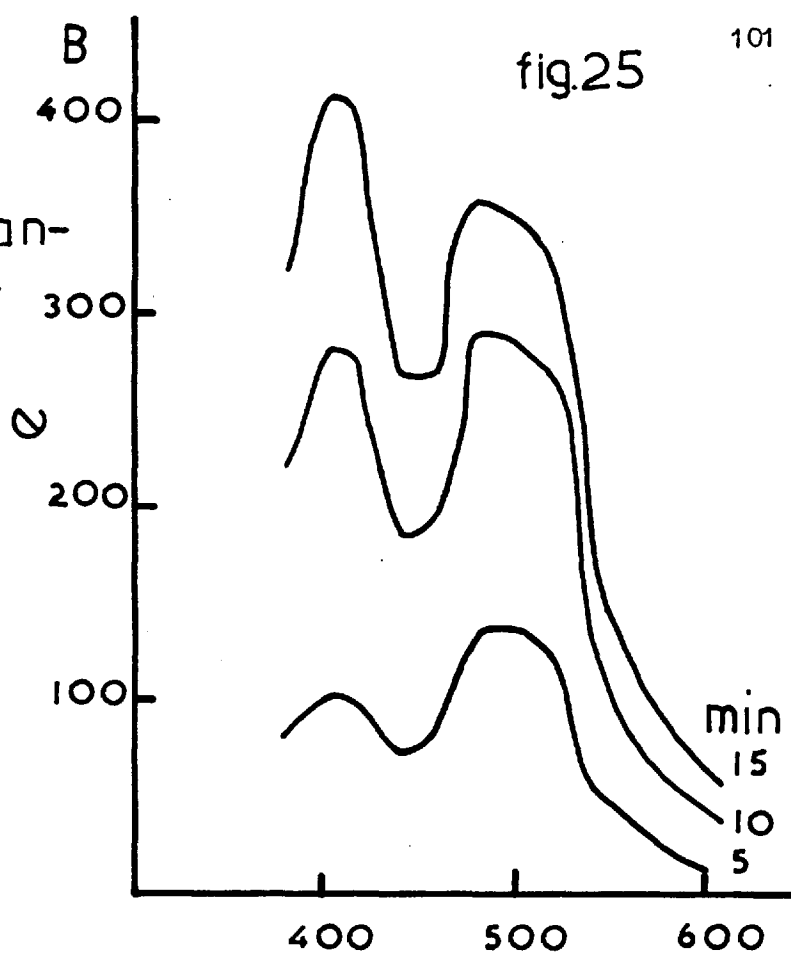
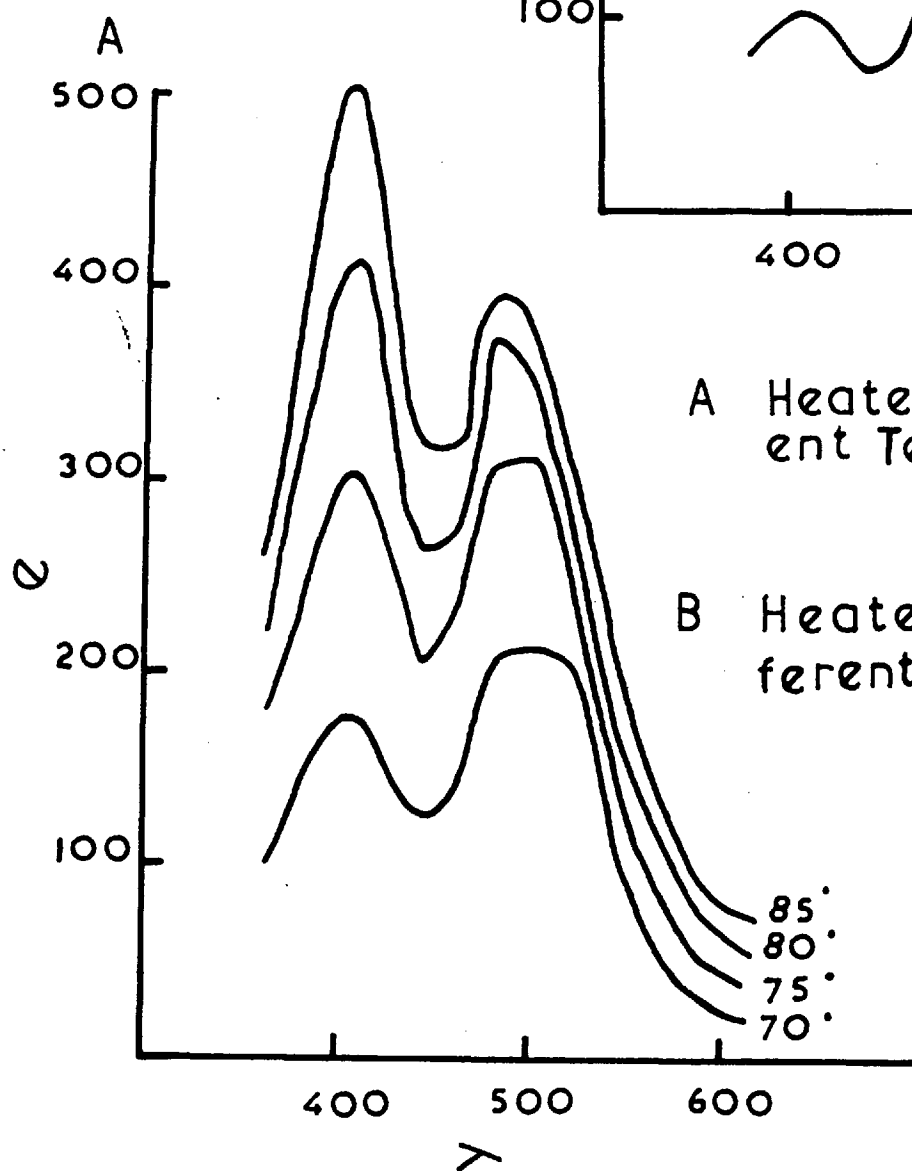
It was found that there were variable amounts of substances in the urine which reacted with the colour reagents, thus giving erroneously high results for enzyme activity, so it was necessary to include a control tube for each specimen.

Attempts were made to remove these substances by treating the urine with acid-washed charcoal.

Absorption curves were plotted for the colour in the urine control before and after charcoal treatment and these were compared with a fructose standard curve (Fig. 24). It can be seen that the standard has two absorption peaks, one at 410 m μ . and the other at 490 m μ . The urine before treatment with charcoal has a much higher peak at 410 m μ . than at 490 m μ . when compared with the standard. Shaking with acid-washed charcoal followed by filtration removes some of the impurities

fig.25

Fructose stan-
dard Absorp-
tion Curves



A Heated at differ-
ent Temperatures
for 15 min

B Heated for dif-
ferent periods of
time at 80°

and the shape of the curve is much more like the standard, the peak at 490 m μ . becoming more apparent. However, this treatment did not completely remove these impurities and, as they are less important at 490 m μ ., the wave length at which the optical densities are read, than at 410, this procedure was not adopted routinely.

Standard Curves.

Absorption curves of the standard colour under different heating conditions were also plotted. It was found that the temperature and the duration of the heating were both important factors in determining the shape of the absorption curve (Fig. 25 a and b). Consequently the heating conditions were rigidly controlled.

Results.

It was found that there was little phosphoglucose isomerase activity in urine, unless the patient had proteinuria, in which case some isomerase passed into the urine along with other proteins. This is in agreement with the results of Kemp and Laurson (1960) who studied the level of several enzymes in the urine of humans and rats. They found that even in the presence of high enzyme levels in the serum there was little demonstrable activity in the urine.

There was no relationship between the level of phospho-

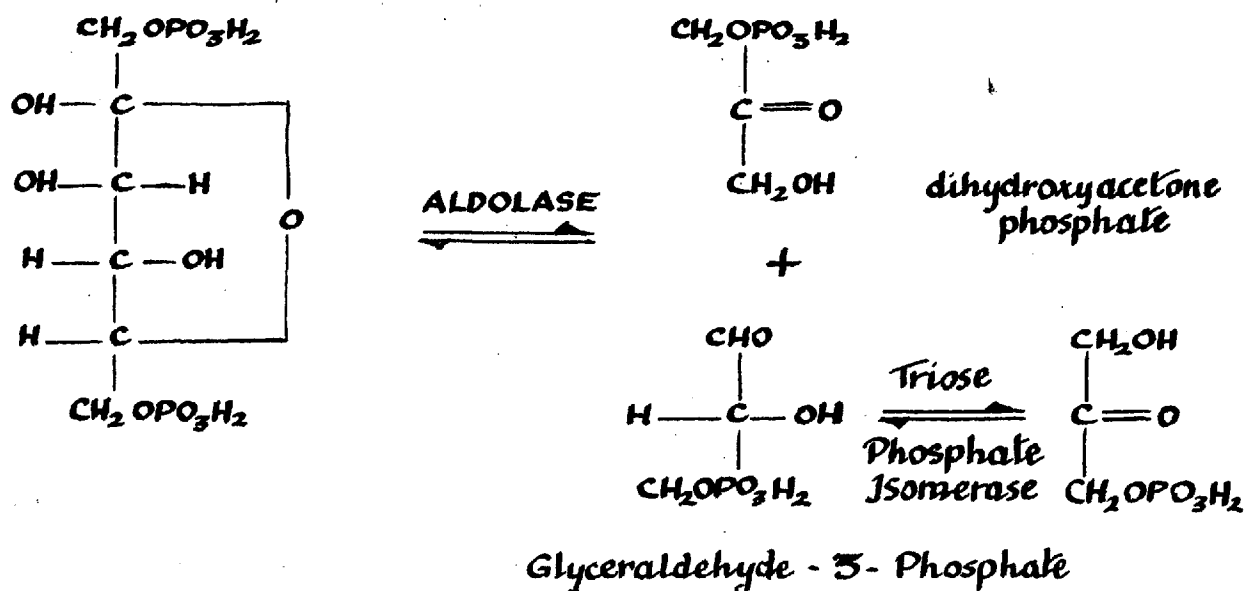
glucose isomerase in the serum and the level in the urine of patients in the diuretic phase of renal failure. Fluctuations in the serum level of the enzyme were observed in early diuretic phase while the urinary output of the enzyme was constant if the patient had albuminuria. This appearance of enzyme in the urine continued after the serum level had reached normal limits until the amount of protein in the urine became negligible.

In the light of these results we did not continue these investigations of urine.

6.

Aldolase

Meyerhof and Lohmann (1934) discovered an enzyme in rabbit voluntary muscle and in yeast which catalysed the reversible cleavage of 1 molecule fructose-1:6-diphosphate into 2 molecules triosephosphate. The stages of the reaction were elucidated during the next few years by Meyerhof and Küssling (1935), Meyerhof, Lohmann and Schuster (1936) and Fischer and Baer (1937). They found that the reaction is catalysed by two enzymes, aldolase and triosephosphate isomerase.



Occurrence.

Like the other enzymes of fermentation and glycolysis, aldolase is present in practically all cells, higher concentrations being found in those with a high rate of carbohydrate metabolism. It is present in a soluble form in the cytoplasm, as are the other enzymes of glycolysis. The enzyme has also been found in yeast by Meyerhof and Lohmann (1934) and in 1948 Stumf reported it in green plants.

Properties.

Warburg and Christian (1943) showed that muscle aldolase was not activated by metals, but they found that the enzyme from yeast requires bivalent metal ions for its function, indicating the existence of two types of aldolase.

Substrate Specificity.

Muscle aldolase is a highly specific enzyme and requires the presence of fructose-1,6-diphosphate (FDP) for the reaction to proceed to the right. For the reaction to occur in the opposite direction, dihydroxyacetone must be present. Hers (1957) found an aldolase in liver which acts on fructose-1-phosphate (F-1-P); in view of this, Dreyfus, Schapira and Schapira (1958) suggested using differential substrate studies to trace the source of the enzyme.

which occurs in serum. Only the FDP aldolase has been studied in this research.

pH Optimum.

Sibley and Lehninger (1949a) found the optimum pH for aldolase activity to be between pH 8.5 and 9; this result agrees with that of Herbert et al. (1940).

Methods of Estimation.

Meyerhof and Lehmann (1934) estimated aldolase activity by measuring the alkali-labile phosphate groups of the triose reaction products. This method is reliable under carefully controlled conditions, but is inconvenient owing to the necessity of performing many blank determinations of inorganic and alkali-labile phosphate.

Warburg and Christian (1943) used a spectrophotometric method based on the reduction of diphosphopyridine nucleotide (DPN) by triose phosphate dehydrogenase in the presence of phosphoglyceraldehyde and arsenate. Sibley and Lehninger (1949a) have found that this method is unreliable when used for the assay of crude tissue extracts, owing to the presence of other enzymes capable of reducing DPN, so they presented a new method. This depends on the measurement of the colour produced by the hydrazones formed by the action of 2:4-dinitrophenylhydrazine on those phosphates produced

during the incubation of the enzyme solution with FDP. In 1948 Dounce and Beyer published a method based on the colorimetric determination of acetaldehyde formed from the triose phosphates. Jegannathan et al. (1956) estimated aldolase activity in the mould Aspergillus niger by measuring the light absorption of the triose hydrazones at 240 mμ.

The method of Sibley and Lehninger (1949a) can be applied to enzymes from various sources and is much less subject to errors than the other methods. It is the one commonly used for serum and has been used by us in our investigations.

Determination of Aldolase Activity in Serum.
(Sibley and Lehninger, 1949a).

Principle.

Serum is incubated with buffered substrate and hydrazine which fixes the aldehyde and ketone groups as soon as they are formed, thus eliminating the action of triose phosphate isomerase. The reaction is stopped with trichloroacetic acid, and a sample of supernatant is treated with alkali followed by 2:4-dinitrophenylhydrazine. Further addition of alkali produces the characteristic colour of 2:4-dinitrophenylhydrazones of the triose, which has a maximum absorption at 540 mμ. The intensity of this colour is directly proportional to enzyme concentration.

Method.

Enzyme Reaction. 1 ml. Tris buffer (0.1 M, pH 8.6) is mixed with 0.2 ml. serum and 0.25 ml. hydrazine (0.56 M, pH 8.6), 0.8 ml. water is added and the tube placed in a water bath at 37° for a few minutes. Addition of 0.25 ml. FDP (0.05 M) starts the enzyme reaction, which is allowed to proceed for 30 min. 2 ml. 10% Trichloroacetic acid stops the reaction after this time. The solution is centrifuged and 1 ml. of supernatant is taken for colour development. A control tube is run for each specimen, this is treated in the same way as the test, except that addition of FDP is delayed until the reaction has been stopped with trichloroacetic acid.

Colour Reaction. The test and control are now treated similarly. 1 ml. NaOH (0.75 N) is added to 1 ml. filtrate and the mixture is allowed to stand at room temperature for 10 min. 1 ml. 2:4-Dinitrophenylhydrazine (0.1%) is added to each tube and they are then placed in a water bath at 37° for 10 min. After this time 7 ml. NaOH (0.75 N) is added and 10 min. later the intensity of the purple colour is read on a photoelectric colorimeter with a 540 m μ . filter (Ilford 625 yellow-green filter).

Preparation of Standard Curve. Since the chromogen measured is of unknown structure and pure samples of triose

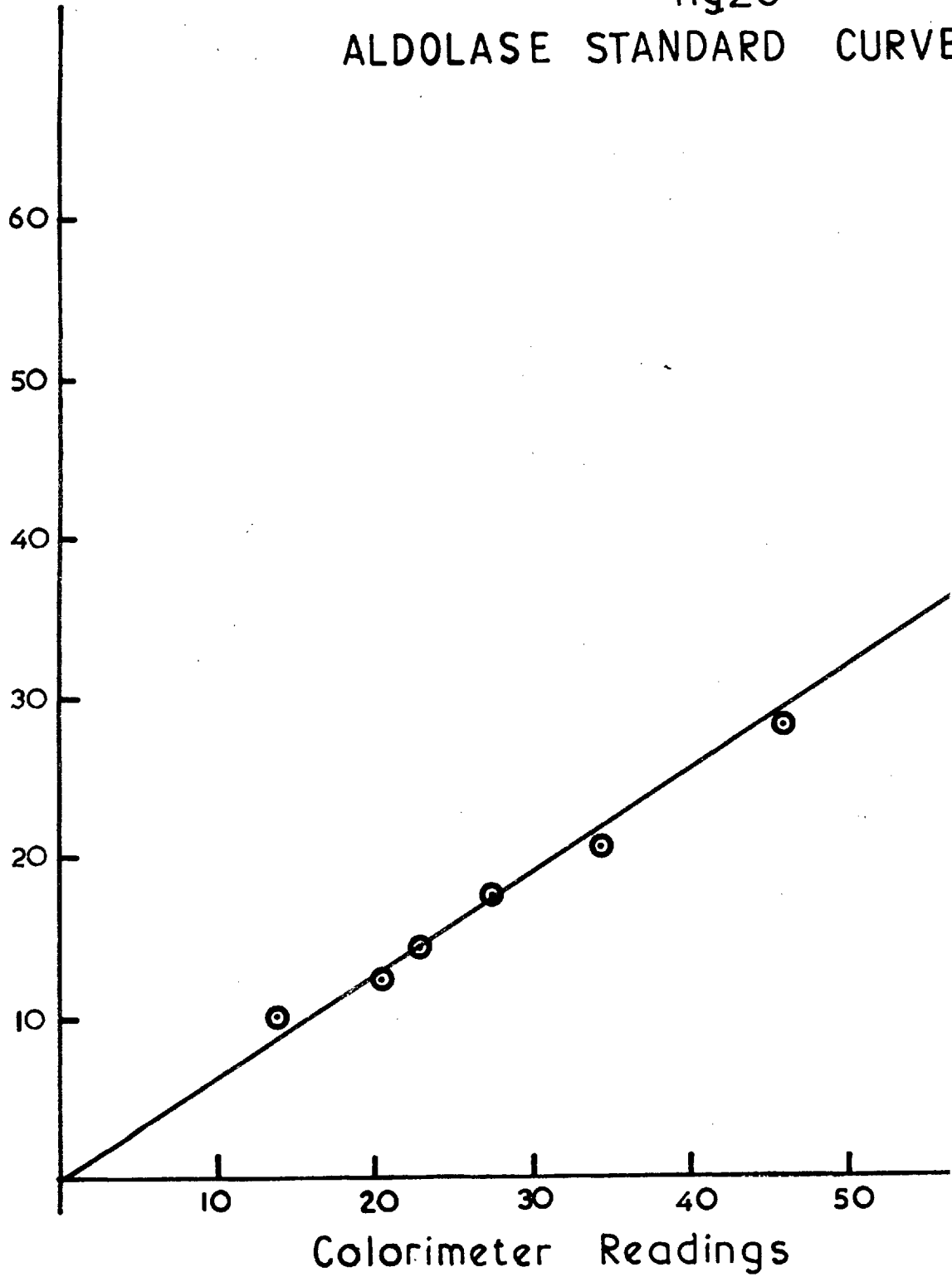
phosphates are not readily available for use as primary standards, a standard curve is necessary to relate per cent transmission to the amount of FDP split. This is prepared by submitting serial dilutions of some source of aldolase to the analysis just described, the colour reaction being carried out on 0.5 ml. of supernatant. The alkali-labile phosphate (ALP) in the supernatant is determined by measuring inorganic phosphate in two 1 ml. samples, one of which is allowed to remain for 20 min. at room temperature with 1 $\frac{N}{4}$ NaOH which liberates the phosphate from triose phosphate. The solution is then neutralized with H_2SO_4 , and the phosphate in each tube is determined.

The ALP of the control is subtracted from the ALP of the test, thus giving the ALP corresponding to the triose phosphate produced by each enzyme concentration. From these results a standard curve is constructed relating ALP to units (Fig. 26).

Units. Sibley and Lehninger (1949a) proposed that 1 unit of aldolase activity should be 1 μl . FDP split in 1 hr. by 1 ml. serum under the conditions of the test. To convert colorimeter readings on 1 ml. samples of trichloroacetic acid filtrate to units of activity, micrograms ALP liberated per sample are transformed into μl . FDP split per hr. per ml. of serum.

Aldolase Units: μ l. FDP split per ml. serum per hour

fig26
ALDOLASE STANDARD CURVE



For this calculation $22.4 \text{ ml. FDP} = 1 \text{ } \mu\text{mole FDP}$
 $= 2 \text{ } \mu\text{moles triose P} = 2 \text{ } \mu\text{moles ALP} = 62 \text{ } \mu\text{g. P}$

The colorimeter readings of the triose chromogen per 1 ml. sample are then plotted against $\mu\text{l. FDP}$, yielding a standard curve (Fig. 26).

Effect of Haemolysis and Keeping Properties of Serum Aldolase.

Red cells contain one hundred and fifty times as much aldolase as is found in serum, and therefore haemolysed sera are unsatisfactory specimens on which to estimate serum aldolase activity (Sibley and Lehninger, 1949a). If it is impossible to perform the assay on fresh specimens, they should be kept at -15° in order to preserve the activity. The enzyme is stable under these conditions, but loses activity if left for any length of time at room temperature.

Normal Range.

A range of 2 - 5 units was established for a series of 20 healthy laboratory workers. This is somewhat lower than the range of 3.5 - 8 units given by the original authors.

Sibley and Lehninger's aldolase units, $\mu\text{l.FDP/ml./hr.}$ can be recalculated in terms of International Units per litre, $\mu\text{moles/min./l.}$ by use of the appropriate factor. In this case 1 aldolase unit equals 0.74 International Units per litre.

Therefore, our range of 2 - 5 Sibley and Lehninger Units is equivalent to 1.5 to 3.8 International Units.

We have studied the serum aldolase levels in a number of patients with cancer of the breast, and the results of these investigations will be given in Part II.

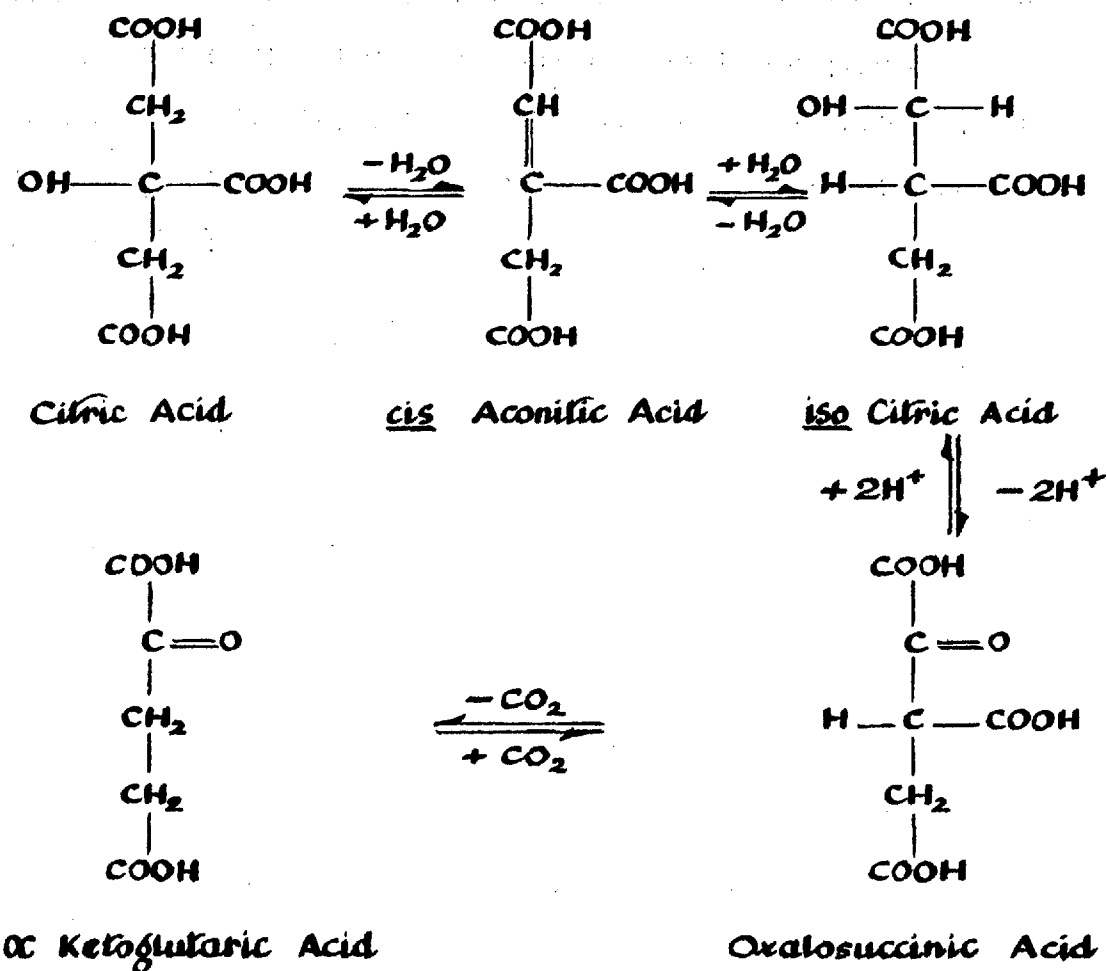
CITRIC ACID CYCLE ENZYMES

In the presence of an adequate supply of oxygen, pyruvate is oxidized to carbon dioxide and water. This process takes place by the citric acid cycle of Krebs which operates in the mitochondria. Iso-citric dehydrogenase is one of the enzymes controlling this cycle, and this and malic dehydrogenase have received the most attention from workers studying serum enzyme levels in disease in this group. We have made a few determinations of the first of these two enzymes, to supplement the information gained from the phosphatases and glycolytic enzymes.

7.

Iso-citric Dehydrogenase

Thimberg (1910) discovered an enzyme which oxidised citric acid; he called it citric acid dehydrogenase and by 1929 had found it in most animal tissues, moulds and bacteria. Martius and Knoop (1937) showed that the reaction involves several stages.



It was assumed that oxalosuccinic acid was produced by enzymic dehydrogenation and this spontaneously lost carbon dioxide to form α -ketoglutaric acid.

Adler et al. (1939) showed that the enzyme system required triphosphopyridine nucleotide (TPN) and Mn^{2+} for its activity. In 1945 Ochoa and Weiss-Taberi, working with acetone powder of pig heart, showed that the second stage of the reaction was also enzymic and reversible. It was not clear if the same enzyme was responsible for both reactions until Moyle and Dixon (1955, 1956) showed that a single protein possessed both iso-citric dehydrogenase and oxalosuccinic decarboxylase activity; they called this protein the iso-citrate enzyme. Siebert, Carsiotis and Plant (1957) were unable to isolate free oxalosuccinate during the dehydrogenation of the iso-citric acid and in view of this suggest that it only exists in an enzyme-bound form during the reaction and as such is not a true intermediate of the Krebs cycle.

Distribution of Iso-citric Dehydrogenase.

Iso-citric dehydrogenase is found in all animal tissues and requires TPN for its action. In 1932 Anderson found an iso-citric dehydrogenase in plant tissue that required diphosphopyridine nucleotide (DPN) as coenzyme. Further evidence has been presented for the existence of a

DPN dependent enzyme (Kornberg and Pricer, 1954; Flaut and Sung, 1954). The two iso-citric dehydrogenases are situated in different subcellular areas; the DPN specific enzyme is found in the mitochondria and the TPN specific is a soluble enzyme found in the cytoplasm (Dixon and Webb, 1958). Small amounts of this latter enzyme are present in normal serum (Wolfson and Williams-Ashman, 1957).

Methods for the Determination of Iso-citric Dehydrogenase Activity.

Wolfson and Williams-Ashman (1957) published a method based on the increase in optical density at 340 m μ , as TPN is reduced by the hydrogen produced during the enzyme reaction. Bowers (1959) simplified this method by eliminating the serum blank, and reducing the sample size to 0.2 ml., thus cutting the volume of serum necessary for a determination by one-fifth. He studied the factors which influence iso-citric dehydrogenase activity and made certain adjustments to improve accuracy. We have used this method in our study of the enzyme.

Bell and Baron (1960) introduced a method based on the colorimetric determination of the phenylhydrazone of α -ketoglutaric acid formed during the enzyme reaction. This method is similar in principle to that used for the transaminases (Reitman and Frankel, 1957). The authors

consider it more suitable for routine analysis than the spectrophotometric methods, since it can be applied to batches of specimens and does not require an ultraviolet photometer.

Method used in this Laboratory.
(Bowers, 1959)

Principle.

The overall enzyme reaction is



The substrate and coenzyme are mixed with the serum in a suitable buffer in a cuvette and the production of TPNH_2 is followed spectrophotometrically at 340 mμ.

Method.

Serum (0.2 ml.) is added to 2.2 ml. tris buffer (0.1 M) pH 7.5, containing MnCl_2 (0.01 M) and 0.5 ml. TPN (0.001 M)). The addition of 0.1 ml. iso-citrate (0.1 M) starts the reaction and the solution is immediately transferred to a cuvette of 3 ml. capacity. The increase in optical density is recorded at 340 mμ. in a spectrophotometer, readings being taken at 1 - 3 min. intervals for at least 10 min. The temperature at which the reaction occurs is recorded for conversion to activity at 25° in the calculation.

Calculation.

Bowers units are in terms of millimicromoles of TPNH_2

formed per ml. of serum per hour at 25°. We have expressed our results in International Units per litre, i.e. micromoles TPNH₂ formed per minute per litre at 25°. To convert Bowers units to International Units it is necessary to divide by 60.

The micromolar extinction of TPNH₂ at 340 mμ. is 6.22 for a 1 cm. light path. If V = total volume of reaction mixture; S, the volume of serum and Tf the temperature factor for conversion to 25°, then

$$\begin{aligned} \text{International Units per l.} &= \frac{\Delta \text{ O.D./min.}}{6.22} \times \frac{V}{S} \times \frac{1}{Tf} \text{ } \mu\text{moles/min./l.} \\ &= \frac{\Delta \text{ O.D.}}{6.22} \times \frac{5}{0.2} \times \frac{1}{Tf} \text{ } \mu\text{moles/min./l.} \\ &= \frac{\Delta \text{ O.D.} \times 2.41}{Tf} \text{ } \mu\text{moles/min./l.} \end{aligned}$$

Effect of Temperature on Reaction Rate.

Estimations of sera of various enzyme concentrations were carried out at temperatures ranging from 18 - 28° and a temperature coefficient (Q₁₀) of 2.3 was established over this range, for conversion to activity at 25° in the calculation.

Normal Range.

Our normal range of 0.5 - 3.6 μ moles/min./l., with a mean of 2.0 and S.D. of 0.86 for 20 healthy laboratory workers is a little higher than Bowers's range of 40 - 160 units which when recalculated represents 0.7 - 2.7 International Units.

The precision of the method is 0.2 μ moles/min./l., calculated from duplicate determinations on 10 sera by means of the formula $\sqrt{\frac{\sum d^2}{2n}}$ where d is the difference between duplicates and n the number of pairs.

IGD is unstable at room temperature; Welfsen and Williams-Ashman (1957) found that it would retain its activity for only five hours under these conditions, but they found that it was stable for many days at 4°. Bedansky et al. (1960) report that the enzyme loses its activity after twenty-one days at -15°. Our findings do not support this. We have found the same activity in specimens after five months at -15°. Our routine procedure for handling the specimens was, therefore, separation as soon as possible and then storage at -15° until the determination was carried out.

We have investigated the serum IGD level in patients with various diseases and the results will be discussed in Part II of this thesis.

Thermal Stability of Heart and Liver Iso-citric Dehydrogenase.

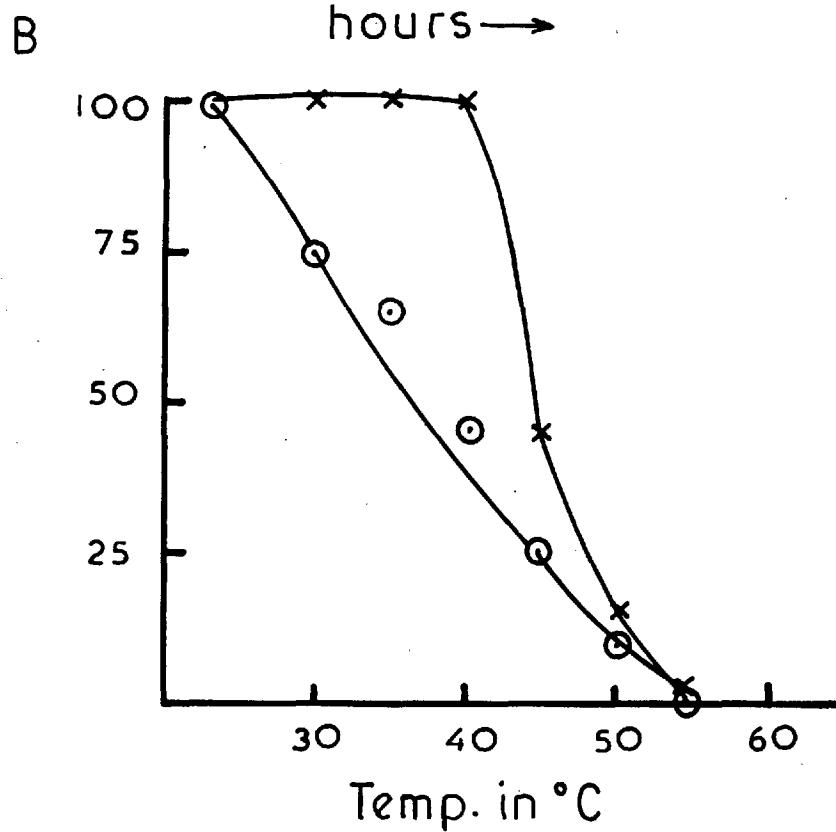
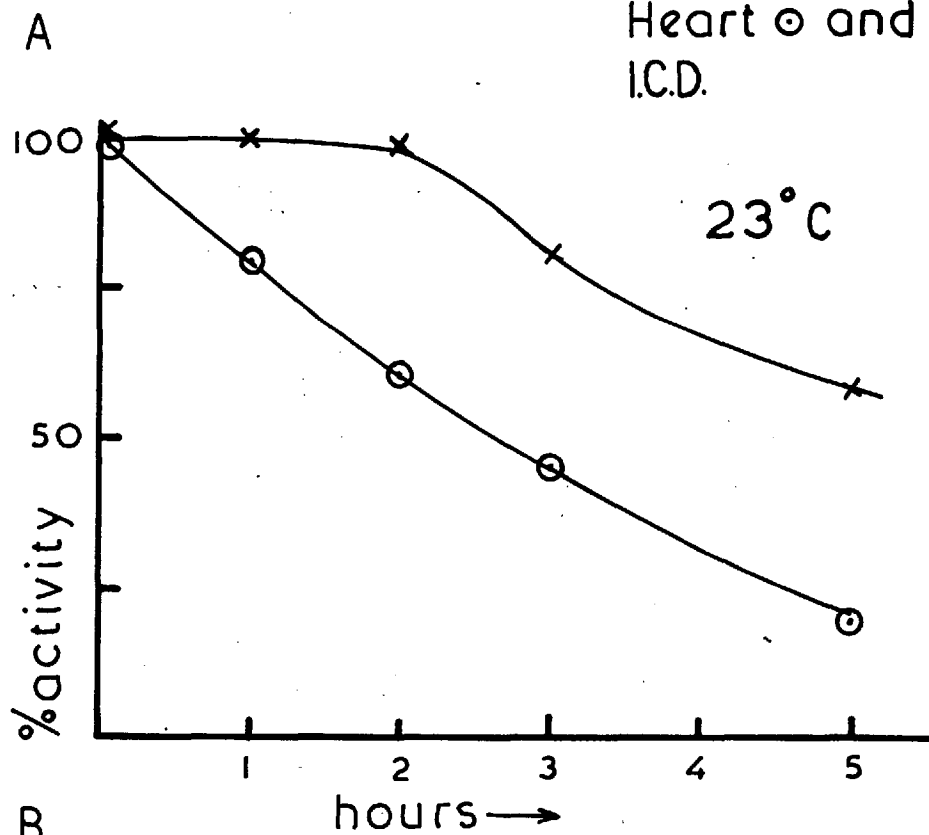
The rise in the serum level of iso-citric dehydrogenase (ICD) has been well established in certain types of liver disease, but there is apparently no such rise following myocardial infarction. It has been reported that myocardium is rich in ICD activity (White, 1958; Wolfson et al., 1958). Therefore, it might be expected that the serum level of this enzyme would rise along with lactic dehydrogenase, the transaminases and aldolase following a myocardial infarction, if it is assumed that increase in serum enzyme activity is related to the release of enzymes from damaged tissue.

Strandjord, Thomas and White (1959) found that ICD is released into the circulation from injured heart muscle after experimental myocardial infarctions in dogs. They have shown that it is released shortly after the infarction and that the serum enzyme level is only briefly raised. They have observed that there is also a rapid removal of the enzyme from the circulation of the dog after injection of ICD prepared from pig heart. It was found that the rate of clearance was unaltered by hepatectomy or nephrectomy.

We undertook a study of the stability of ICD of human heart and liver. We prepared saline extracts from post-mortem specimens of the two tissues and investigated their

fig.27

Heat stability of
Heart $\circ$ and Liver $\times$
I.C.D.



relative activities after exposure to room temperature (23°) and after 20 min. in a water bath at various temperatures. The results are shown in Fig. 27.

A. shows that the heart extract steadily lost its activity when left at 23° and after 5 hr. only 20% of the original remained. The liver extract was stable for 2 hr; it did begin to deteriorate after this, but 58% of the original activity remained at the end of 5 hr.

B. shows the effect of exposing the two extracts to different temperatures for 20 min. The heart extract steadily deteriorated with the rise in temperature, and it was completely inactivated after 20 min. at 55° . The liver retained its activity up to 40° , after which it became denatured until it was completely inactive after 20 min. at 55° .

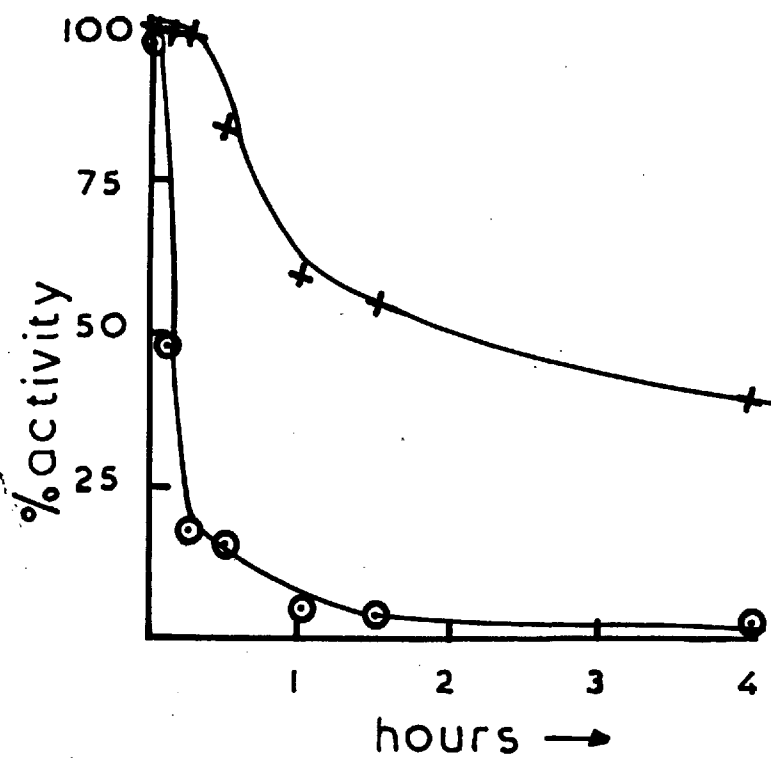
We then studied the effect of prolonged incubation at 37° , in an attempt to inactivate the heart extract and retain the activity of the liver, with a view to fractionating the serum enzyme. We prepared acetone powders by the method of Siebert, Dubuc, Warner and Flaut (1957), and made saline solutions of these powders and incubated them at 37° . 0.2 ml. samples were withdrawn at intervals and the ICD activity was estimated.

The results are shown in Fig. 28. It can be seen that the heart extract was rapidly inactivated after exposure to the temperature and less than 5% of the original activity was retained after 1 hr. incubation. The liver was stable for 15 min. and then slowly began to deteriorate, but 35% of the original activity still remained after 4 hr.

The experiments suggest that heart and liver ICD are isoenzymes; they have the same type of activity but differ in their protein structure, since they differ in their stability on exposure to heat. The extreme heat lability of heart ICD could explain the absence of this enzyme in the serum after myocardial infarction.

Wroblewski and Gregory (1961) have shown that lactic dehydrogenase (LDH) can be separated into five different protein fractions by starch gel electrophoresis. They have shown that the fraction which is mainly present in liver is easily destroyed by heat, so that the lack of any significant rise of LDH in liver cell damage could also be explained on the grounds of heat lability of this enzyme from the liver.

fig.28
Stability of Heart $\odot$
and Liver $\times$ ICD at 37°C



PART II

INTRODUCTION

Using the methods described in Part I, the enzyme levels have been estimated in the sera of patients with a variety of diseases, in order to establish the clinical conditions under which increases in the serum enzyme activity occur. Particular attention has been paid to the specificity of each enzyme for disease in certain organs, in order to decide on the range of tests which will provide maximum information about the patient with the minimum number of investigations. Having established the enzyme patterns for disease in certain organs, particularly liver and bone, this information has been used as a basis for investigating the sites of secondaries in breast cancer patients.

8.

Serum Alkaline Phosphatase in Disease.

Robison's (1923) discovery of high alkaline phosphatase activity in bone prompted the investigation of the effect of skeletal disease on the level of the enzyme in serum. Kay (1929) and Roberts (1930) found a marked increase in the serum alkaline phosphatase level in patients with some types of bone disease. It became obvious after work by Franseen and Maclean (1935) and Gutman, Tyson and Gutman (1936) that the increases occur only in osteoblastic diseases such as Paget's disease, rickets in children and osteomalacia in adults, hyperparathyroidism and osteoplastic metastases.

Roberts (1930, 1933), investigating the serum alkaline phosphatase activity in other diseases, found increased levels in patients with jaundice due to obstruction of the extra-hepatic biliary tract. He found normal and only slightly elevated values in all other types of jaundice, and he proposed the use of the determination of serum alkaline phosphatase to distinguish obstructive from other types of jaundice. His results were reproduced by Armstrong, King and Harris (1934) and by Bodansky and Jaffe (1933) who obstructed the common bile duct in the dog and observed a rise in the serum alkaline phosphatase, which subsequently returned to normal after removal of the obstruction. Bodansky and Jaffe (1933) found the distinction between the serum alkaline phosphatase in obstructive and other types of jaundice

less clear-cut than Roberts had shown. Others supported these findings, but it was concluded from these early studies that the determination of serum alkaline phosphatase affords a sensitive criterion of the integrity of the intra- and extra-hepatic biliary excretory channels.

In obstructive jaundice the serum alkaline phosphatase levels are usually over 50 King-Armstrong (K.A.) units, and in hepatocellular jaundice the serum levels are usually less than this. This arbitrary dividing line is useful in practice, but anomalies may occur. Raised serum alkaline phosphatase levels are seen in hepatic metastases, even without jaundice or bone involvement, but a normal level does not exclude liver secondaries.

There are two theories concerning the cause of the elevation of serum alkaline phosphatase activity in diseases of the liver and biliary tract. The retention theory proposed by Armstrong et al. (1934) and supported by Herbert (1935) and Gutman et al. (1940) proposes that the serum enzyme originates in bone and is actively secreted into the bile; any increase in the serum level is attributable to intra- or extra-hepatic obstructive retention of the enzyme. The evidence for this theory is based on the known occurrence of alkaline phosphatase in bone, the elevation of the enzyme level in osteoblastic bone disease, and its presence in the bile. This theory correlates so well with clinical experience that it has gained wide support.

The second, or hepatogenic, theory proposed by Bodansky and Jaffe (1933) and Bodansky (1937) supposed that the enzyme was produced largely by the liver and that it leaked into the bloodstream from injured liver parenchymal cells. Most observers have failed to find any proportionality between the extent of hepatocellular necrosis and the degree of rise in serum alkaline phosphatase activity (Gutman, 1959). This school of thought now supports the ideas of Winkelman and Schiffmann (1939), who suggest that the serum increment depends on two factors: the extent to which the functional state of liver parenchyma is preserved and the degree of obstruction of the biliary tract. Clinical and experimental observations also support this theory, but until the routes and mechanisms of alkaline phosphatase transfer through the liver are clarified, it is impossible to say which is correct.

The kinetic properties of tissue alkaline phosphatases have been studied in an attempt to identify the source of the serum increment seen in hyperphosphatasaemia.

The use of Michaelis Constants in the Investigation of
the Source of Serum Alkaline Phosphatase.

The preparation of partially purified specimens of alkaline phosphatase by starch gel electrophoresis described in Part I was applied to ten pathological sera. The Michaelis constants of these enzyme solutions towards β -naphthyl phosphate were determined, and the results compared with the constants for individual tissues (see Table 1, page 45), in an attempt to characterize the serum increment. Results of these investigations and other data are shown in Table 3.

For the purpose of this discussion, we have, in general, ignored any contribution from kidney alkaline phosphatase, since Schlamowitz and Bodansky (1959), using immunochemical techniques, have shown that its effect is negligible.

**TABLE 3. Michaelis Constants and Clinical Details of
8 Patients with Hyperphosphatasemia.**

<u>Patient.</u>	<u>Serum Alkaline Phosphatase level.</u> (K.A. units)	<u>Km.</u>	<u>Diagnosis.</u>	<u>Other data.</u>
<u>Group A</u>				
B.C.	53	0.11	Paget's disease	
M.G.	57	0.11	Ca. of breast with bone secondaries	ICD and 5-N normal. No liver second- aries at post-mortem.
A.G.	96	0.094	Ca. of pancreas with obstruction of common bile duct	ICD normal 5-N raised turbidity tests normal.
<u>Group B</u>				
B.I.	200 310	0.067 0.061	Metastatic Ca. of pancreas	Jaundice ICD raised 5-N " GP-T "
E.C.	97	0.071	Biliary cirrhosis	
C.B.	120	0.074	Metastatic Ca. of stomach	
<u>Group C.</u>				
H.W.	14	0.080	Uraemia. Congestive cardiac failure	ICD raised 5-N normal GP-T raised.
B.H.	160	0.081	Lymphosarcoma	Jaundice ICD slightly raised 5-N raised
A.C.	56	0.083	Metastatic Ca. of pancreas	Jaundice ICD and 5-N raised. Turbidity tests normal.

ICD = iso-citric dehydrogenase

ICD = iso-citric dehydrogenase
 5-N = 5-nucleotidase
 GP-T = glutamic pyruvic transaminase
 Ca. = carcinoma

For pathological sera a value of 0.11 mM. would be expected from specimens in which the phosphatase is mainly of bone origin, with lower values for those with contributions from other sources. Values below 0.09 mM. , the K_m for intestinal phosphatase, indicate the presence of liver phosphatase whose K_m is 0.067 mM. We have divided the results into three groups: A, those with K_m values resembling bone; b, those which resemble the liver enzyme; and C, those lying between these two extremes.

In Group A, the K_m of 0.11 for the serum enzyme of the first patient, B.C., is in agreement with the diagnosis of Paget's disease, and the assumption that in this condition the serum alkaline phosphatase is of bone origin. The second patient, M.G., died from metastatic carcinoma of the breast, and at post-mortem was shown to have extensive bone secondaries while her liver was perfectly normal. A K_m of 0.11 mM. for the partially purified serum enzyme might, therefore, be expected from this patient. The third patient, A.C., was a case of carcinoma of the head of the pancreas. At the time of determination, normal iso-citric dehydrogenase (ICD) and turbidity tests demonstrated an undamaged liver, while the raised 5-nucleotidase (5-N) indicated that there was obstruction of the bile duct (see section on 5-N and ICD). The K_m of 0.094 suggested that the raised serum phosphatase had resulted from a failure to excrete the normal bone enzyme, with perhaps some contribution from intestinal phosphatase which would account for the constant being a little below the bone value.

The second group of patients all have Km.'s. which indicate liver as the source of serum enzyme. The jaundice, elevated glutamic pyruvic transaminase and ICD levels suggest liver cell damage, which resulted in the release of alkaline phosphatase into the bloodstream.

In the third group of patients, it appears from the Km. values that the serum enzyme is of mixed origin, there being some contribution from the liver, as might be expected from the raised ICD levels indicating liver cell damage. The first patient, H.W., in this group was suffering from congestive cardiac failure with secondary liver damage, as indicated by the raised transaminase and ICD. The normal 5-N and lack of jaundice show that there was no obstruction of the bile duct, so the normal excretory pathway was open and the total serum increment only just outside the normal range. Since the Km. value is 0.080 mM. , midway between the constants for liver and bone, the liver component must be relatively small. B.H. was a case of lymphosarcoma with bone and liver secondaries, the contributions from both sources resulted in a constant midway between the two. The last case in this group is a second investigation of A.C. This specimen was taken a month after the first one, during which time the patient had had an operation. The bone enzyme had been released, as shown by the

drop from 96 to 56 K.A. units for the total serum alkaline phosphatase.

The now raised IGD and abnormal turbidity tests indicate a spread of the disease to the liver; this is reflected in the drop in Km. from 0.094 mM. to 0.083 mM., caused presumably by a hepatic contribution.

These ten examples demonstrate the possibilities of applying sensitive techniques to the investigation of the source of raised serum enzyme levels. The necessity for carrying out some form of purification of the enzyme is shown by the success of this method, as compared with the failure of the method using serum and glucose-1-phosphate, as described in Part I.

9.

Serum Acid Phosphatase in Disease.

There is a small amount of acid phosphatase present in normal circulating blood, and in diseases involving certain organs there is a characteristic rise in serum level of this enzyme. The best known condition is cancer of the prostate. Gutman, Sproul and Gutman (1936) were the first people to demonstrate the clinical importance of acid phosphatase, when they discovered that bones which were the site of metastases for prostatic carcinoma had acid phosphatase activity up to one hundred times that found in normal bone. In 1938, the Gutmans reported increased acid phosphatase activity in the serum of men with metastases in bone from prostatic cancer. The determination of this enzyme activity in serum was promptly adopted as a aid in diagnosing cancer of the prostate and in following the patient's progress during the course of therapy. Woodard and Dean (1947) assessed the information provided by this test, and it was clear that, when treatment was successful, an initial high level fell to near normal within three or four days after surgical castration or within two to three weeks after the institution of oestrogen therapy. During a remission the low levels were maintained and sometimes, but not always, rose again with relapse. Patients whose serum acid phosphatase level did not decrease failed to respond to treatment.

As well as the contribution to normal serum acid phosphatase from the prostate, it is likely that erythrocytes, spleen and liver are sources of some of the serum enzymes. (Woodard, 1959). Zucker and Borrelli (1958, 1959) have shown that blood platelets liberate acid phosphatase during clotting and this must also contribute to the total activity estimated in serum.

There is a rise in serum acid phosphatase in osteoclastic bone diseases, such as Paget's disease and hyperparathyroidism (Sullivan, Gutman and Gutman, 1942; and Woodard, 1956). Elevated acid phosphatase levels are frequently associated with bone metastases, and King and Jegatheesan (1959) found elevated acid phosphatase levels in women with cancer of the breast with bone and liver secondaries. Farber (1952) reported increased acid phosphatase activity in the spleen of a small girl with Gaucher's disease, while Crocker and Landing (1960) have found that it is a characteristic of the abnormal cells found in Gaucher's disease. They found that patients with the disease show a mild to moderate increase in serum acid phosphatase.

We have used the method of King and Jegatheesan (1959) for the determination of prostatic acid phosphatase and have continued to find it a sensitive test for the diagnosis of cancer of the prostate.

10.

Serum 5-Nucleotidase in Disease.

Following Reis's (1950) observations on the wide distribution of 5-nucleotidase throughout human tissues, Dixon and Purdon (1954) investigated the activity of the enzyme in serum and compared it with alkaline phosphatase activity in a number of diseases. They were able to show that a high alkaline phosphatase level in liver disease was usually accompanied by a 5-nucleotidase value increased as much as one hundred-fold, whereas that from bone disease was not. They, therefore, assumed that bone was not a likely source of serum 5-nucleotidase.

Young (1956, 1957, 1958) confirmed Dixon and Purdon's findings that 5-nucleotidase was not raised in bone disease, and he extended the study of the enzyme in various types of liver disease. He found that in obstructive jaundice the levels of alkaline phosphatase and 5-nucleotidase paralleled each other, but after relief of the obstruction the 5-nucleotidase fell more rapidly than the level of the alkaline phosphatase. He also found that the overall distribution of values in patients with hepatobiliary disease was fairly similar for both enzymes. In cases of hepatocellular jaundice there was not a large increase in serum 5-nucleotidase; most patients showed elevations of less than three times the upper limit of normal. Infiltrative lesions of the liver were found to cause a rise in serum 5-nucleotidase

while similar lesions in bone did not affect the serum level; contrary to the results obtained with alkaline phosphatase.

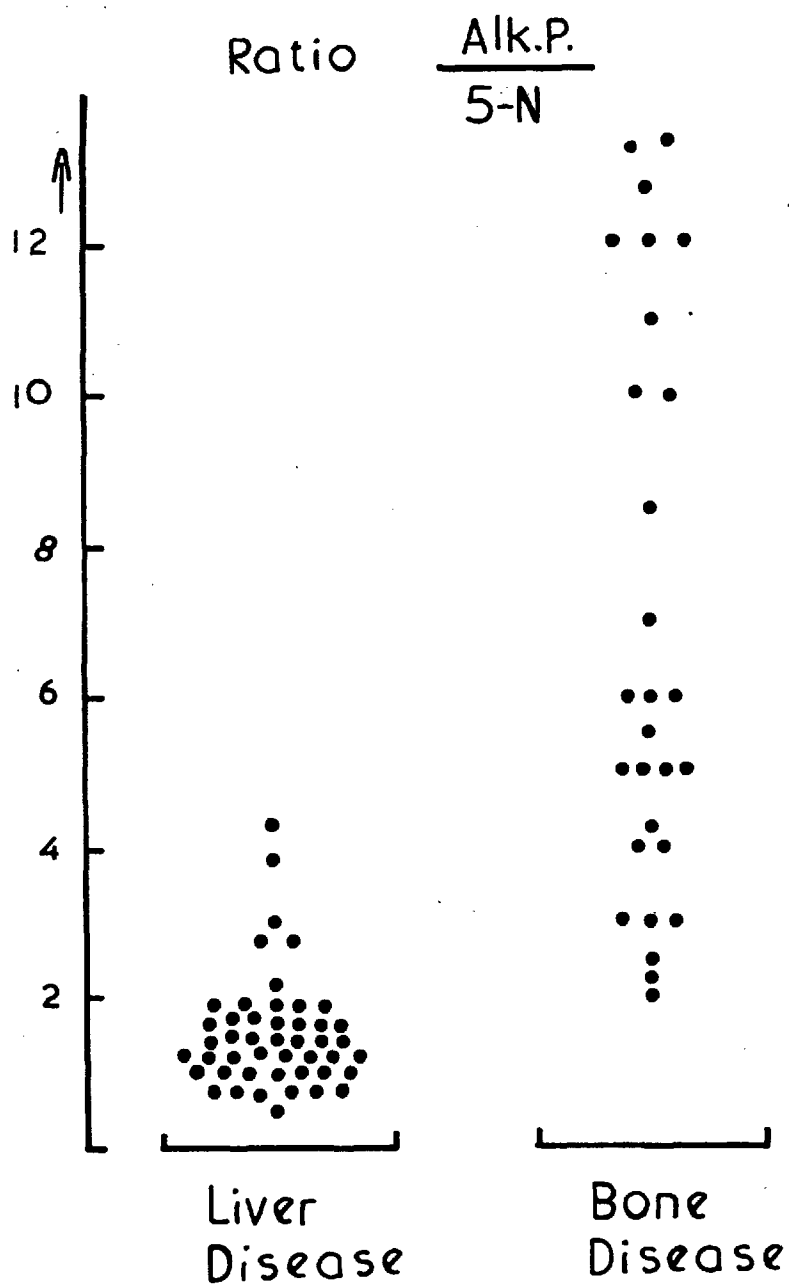
From his results, Young concluded that the liver is the most likely source of the increase in serum 5-nucleotidase levels found in hepatobiliary disease, and considered the estimation of this enzyme to be at least as sensitive as alkaline phosphatase in detecting biliary tract obstruction.

Serum 5-Nucleotidase in Non-Breast Cancer Patients.

The level of 5-nucleotidase was studied in the sera of seventy-five patients, all of whom had a raised serum alkaline phosphatase. Taking the results of these determinations, together with other biochemical and clinical findings, it has been found that the serum 5-nucleotidase level is raised only in liver disease, whereas alkaline phosphatase may be raised in bone and liver disease. These results are in agreement with the findings of Dixon and Purdom (1954) and Young (1958).

Fig. 29 shows the ratio of alkaline phosphatase in K.A. units to 5-nucleotidase in $\mu\text{moles/min./l.}$ in these patients. It can be seen that in liver disease the majority of the ratios fall between 0.5 and 2, with only a few values outside these limits. These exceptions may be explained by the fact that the level of both enzymes has fallen with treatment from initial high values, but the level of 5-nucleotidase falls more rapidly than alkaline phosphatase.

fig.29



An example of this is a patient who was suffering from obstructive jaundice, with initial values of 96 and 83 for alkaline phosphatase and 5-nucleotidase respectively, giving a ratio of 1. Several days after relief of the obstruction, these serum enzymes were again determined and the alkaline phosphatase level was 58 while the 5-nucleotidase had fallen to 22 units, thus giving a ratio of 3 : 1. But, as can be seen from the figure, the occasions when the ratio is above 2 in cases of liver disease are rare. However, the reverse is true for bone disease, and ratios ranging from 2 to 26 have been found during this study.

We have not found any cases where raised serum alkaline phosphatase levels resulting from bone disease are accompanied by abnormal 5-nucleotidase values, although there have been one or two instances where the 5-nucleotidase has been normal in the presence of a raised alkaline phosphatase and biochemical evidence of liver disease. M.A. was a case of drug-induced jaundice with liver damage, with raised serum transaminase levels and iso-citric dehydrogenase values three times the upper limit of normal. The alkaline phosphatase was just outside the normal range (15 units) but the 5-nucleotidase varied between 3 and 6 units throughout the ten days' serial study, giving ratios ranging from 3.8 to 5.

Of the cases of liver disease studied, 6% failed to show raised 5-nucleotidase levels. These included a case of jaundice with mitral incompetence, a case of hypertension with icteric sclerae,

a woman with jaundice who was thirty-two weeks pregnant, and a case of recovering hepatitis.

Fig. 30 shows serial studies on the 5-nucleotidase and alkaline phosphatase levels in three patients.

A was a case of obstructive jaundice caused by carcinoma of the head of pancreas. It can be seen that the pre-operative levels of the two enzymes ran closely together, but that after the operation the 5-nucleotidase level fell more rapidly than the alkaline phosphatase. B was suffering from obstruction of the bowel following an appendectomy. This patient again showed the two enzymes paralleling each other, although the rise in 5-nucleotidase was not as great as the increase in serum alkaline phosphatase. C is a case of osteoporosis and demonstrates the normal serum 5-nucleotidase levels in the presence of a raised alkaline phosphatase originating in bone.

Serial enzyme studies

units μ moles/
/min/L
fig.30
▼ Alk.P
• 5-N

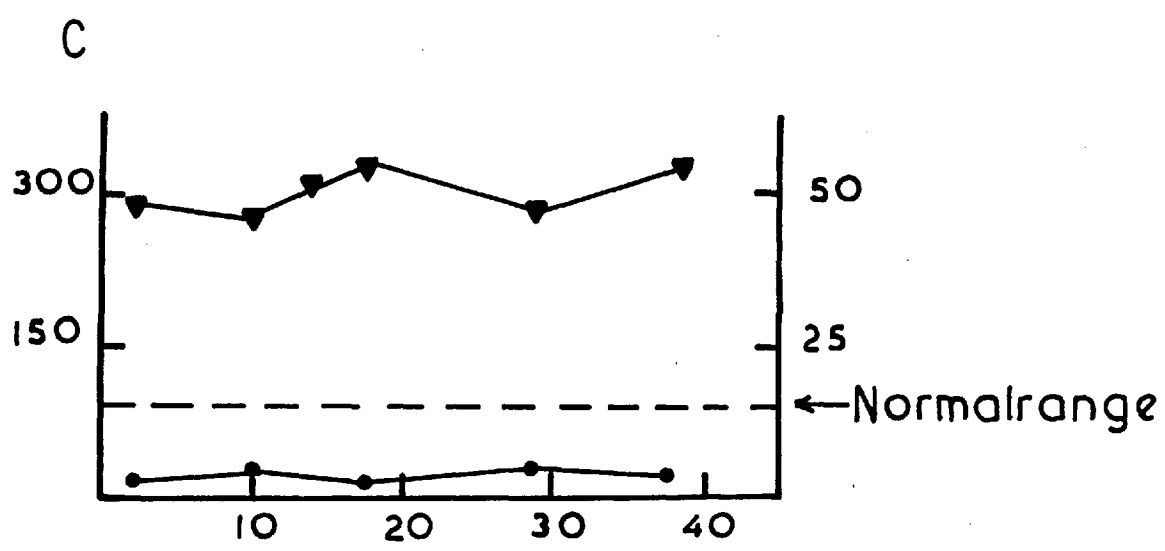
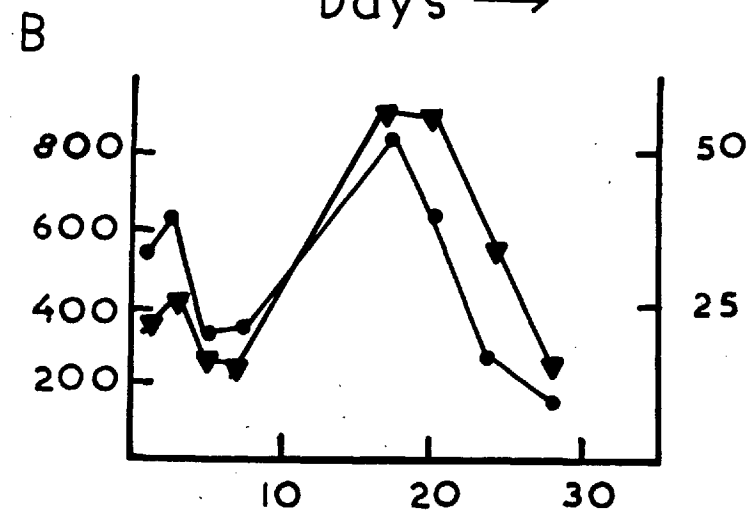
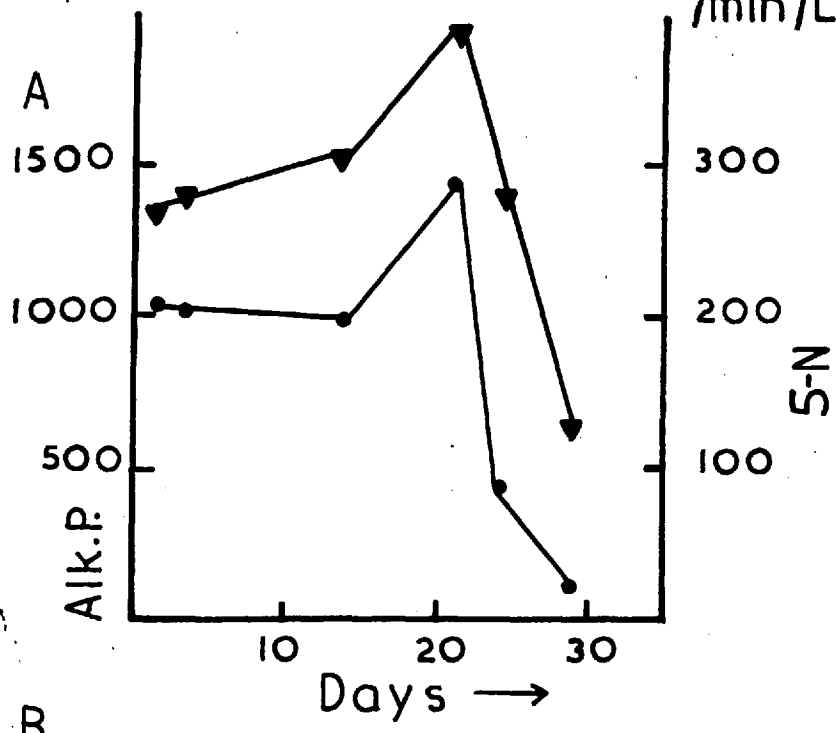


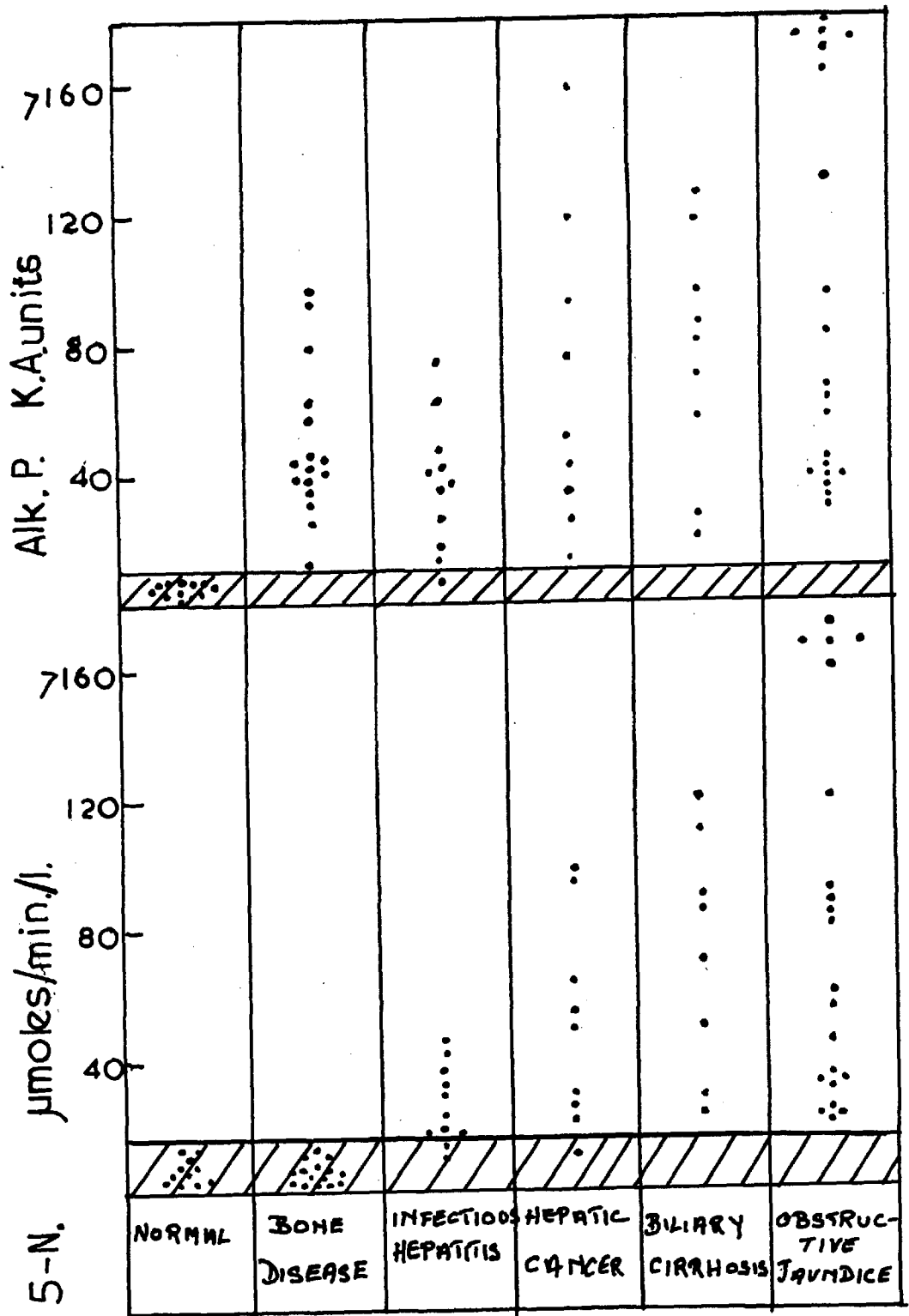
TABLE 4. Range of Values for 5-Nucleotidase and Alkaline Phosphatase in Various Disease States.

<u>Disease State.</u>	<u>5-Nucleotidase</u>			<u>Alkaline Phosphatase</u>		
	<u>Range.</u> (μ moles/min./l.)	<u>Ave.</u>	<u>X Normal.</u>	<u>Range.</u> (K.A.Units)	<u>Ave.</u>	<u>X Normal.</u>
Normal	2 - 15	8	1	2 - 13	9	1
Bone disease	2 - 11	7	1	3 - 95	47	4
Infectious hepatitis	10 - 45	25	3	12 - 76	33	3
Primary and metastatic hepatic carcinoma	10 - 90	49	6	14 - 160	70	8
Biliary cirrhosis	3 - 120	65	8	19 - 128	78	8.5
Extra hepatic biliary tract obstruction	30 - 250	96	12	30 - 310	104	12

The range of values found for 5-nucleotidase in different disease states is shown in Table 4. It can be seen that the values for various types of liver disease are similar to those for alkaline phosphatase also shown in the table, slightly higher levels being found for the latter enzyme, while in bone disease the phosphatase is usually much greater (Fig. 31).

fig 31

5-N. & Alk.P. in different disease states

normal range 

The range of values up to only three times the upper limit of normal for 5-nucleotidase found in infectious hepatitis, as opposed to figures sixteen times outside the normal range which may occur in obstructive jaundice, suggests that the serum enzyme is not wholly hepatic in origin, an assumption made by Dixon and Purdom (1954), Kowlessar et al. (1958) and Young (1958). We have found grossly elevated values when the excretory function of the liver is impaired either by intra- or extra-hepatic obstruction. These findings are similar to the observations of others for alkaline phosphatase (Gutman, 1959) and contrast with the levels of the transaminases and iso-citric dehydrogenase which are grossly elevated in hepatitis and are normal or only slightly raised in obstructive jaundice. We have found that when extra-hepatic obstruction is removed by operation the level of both 5-nucleotidase and alkaline phosphatase return to normal. These observations suggest to us that the substrate-specific 5-nucleotidase shares the same excretory mechanisms as alkaline phosphatase, and any impairment to this function results in abnormal levels of the enzyme occurring in serum. Thus a rise in serum 5-nucleotidase is a good index of liver dysfunction, and is more sensitive than alkaline phosphatase, since it is not raised in bone disease.

Based on these assumptions, we have extended this study to patients with cancer of the breast to evaluate the use of 5-nucleotidase as an index of hepatic metastases.

11. Iso-Citric Dehydrogenase in Disease.

The discovery by Wolfson and Williams-Ashman (1957) of iso-citric dehydrogenase (ICD) activity in serum and erythrocytes of humans and rats prompted the study of this enzyme in disease. Wolfson, Spencer, Sterkel and Williams-Ashman (1958) studied the serum ICD activity in two hundred and fifty patients. They found that the enzyme levels seldom fell outside the normal range, unless hepatic disease was present. Increases of up to forty times normal were seen in patients with hepatitis, and in patients with hepatitis complicating infectious mononucleosis. In portal cirrhosis the serum ICD was usually within the normal range and was never elevated more than three times the normal mean, even in hepatic coma. They never found ICD values outside the normal range in extra-hepatic obstruction of the common bile duct. In about 50% of the patients studied with liver metastases from malignant disease the serum ICD activities were greater than the upper limit of normal. A wide variety of malignant tumours did not cause elevations of serum ICD, but if it is raised they consider that involvement of the liver is indicated.

These workers observed normal values in subjects with a wide variety of infectious, pulmonary, gastrointestinal, cardiovascular, urological and neoplastic diseases. The specificity of this enzyme for diseases involving the liver has been further demonstrated by White (1958), Baron and Bell (1959)

and Kerppola, Nildkita and Pitkanen (1959).

We have investigated iso-citric dehydrogenase levels in liver disease and have included it in the study of some of our cancer patients to see if it adds anything to the information provided by phosphoglucose isomerase, 5-nucleotidase and alkaline phosphatase about the integrity of the liver.

Iso-citric Dehydrogenase in Non-Breast Cancer Patients.

Serum iso-citric dehydrogenase (ICD) levels were studied in fifty patients, the majority of whom were suffering from liver disease. We confirmed the findings of Wolfson et al. (1958) that the level of the enzyme did not fall outside the normal range, except in the presence of liver disease, and then only when there was liver cell damage, such as occurs in hepatitis. The range of ICD levels in various disease states is shown in Table 5 and Fig. 32.

TABLE 5. Range of ICD Values in Liver Disease.

<u>Disease.</u>	<u>Range.</u> (units μ moles/min./l.)	<u>No. of cases.</u>
Normal range	0.5 - 3.5	20
Obstructive jaundice	1.5 - 3.5	8
Biliary cirrhosis	1.5 - 12	7
Hepatic carcinoma	2 - 13	8
Infectious hepatitis	1 - 60	10
Toxic hepatitis	4 - 10	5

Not one of our seven patients with extra-hepatic obstructive jaundice, uncomplicated by malignancy, had ICD levels outside the normal range, a finding which agrees with Wolfson et al. (1958) and Kerpola, Nikkita and Pitkanen (1959).

All but one of our seven cases of primary or metastatic hepatic carcinoma had abnormal ICD levels. This is higher than the 50% reported by Wolfson et al. (1958), but the number of cases studied is small. We have concluded that a raised serum ICD in a patient with cancer indicates liver involvement, but a normal level does not completely exclude this.

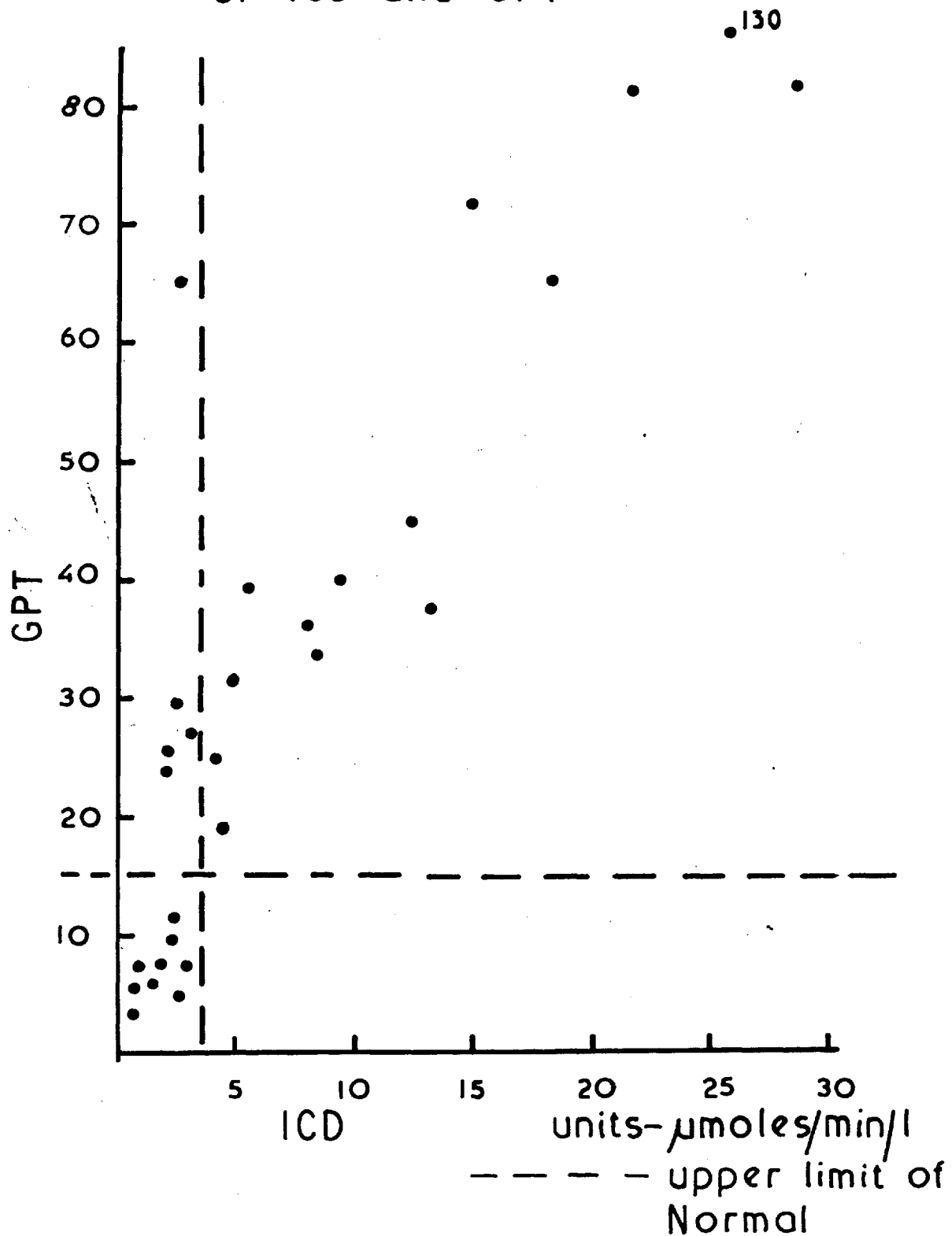
Values from within the normal range to three times the upper limit of normal have been found in seven cases of

cirrhosis. The degree of elevation depends on the acuteness of the disease, normal levels being found in slowly-progressing cirrhosis and higher values occurring in acute phases. High levels of the enzyme are found in hepatitis, but they soon fall after the peak of icterus and reach the normal range some time before the transaminases. From these results we have concluded that a raised serum ICD is indicative of severe liver cell damage. Normal levels occur in cirrhosis, hepatitis and cancer depending on the phase of the disease, so it is not possible to use ICD to distinguish one from another.

In general, we have found that ICD adds little to the information provided by the transaminases, particularly glutamic pyruvic transaminase (GP-T). The results of simultaneous determination of these two enzymes in patients with liver disease are plotted against each other in Fig. 33. It can be seen that, except for a small number of cases, the two enzyme levels are either both normal or both raised. The exceptions are patients in the recovery phase in which the ICD has fallen to within the normal range before the GP-T.

Stranford, Thomas and White (1959) studied the rate of clearance of enzymes from the circulation after injection of purified samples; they found that ICD disappears more quickly than the transaminases. So, although all three enzymes are raised in an acute phase of liver disease, it might be expected

fig 33
Simultaneous Determination
of ICD and GPT



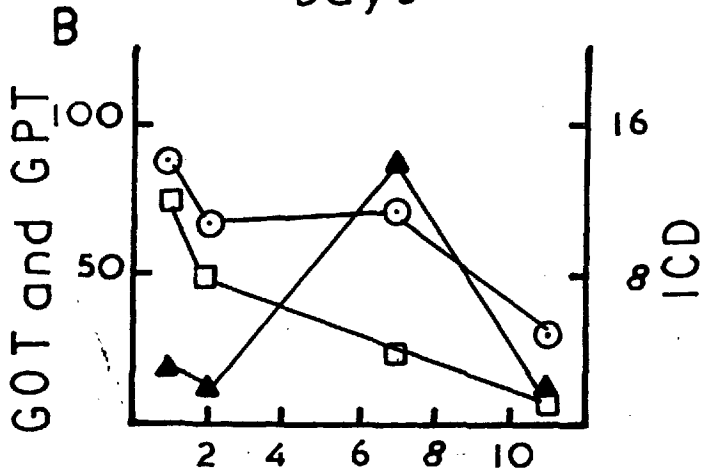
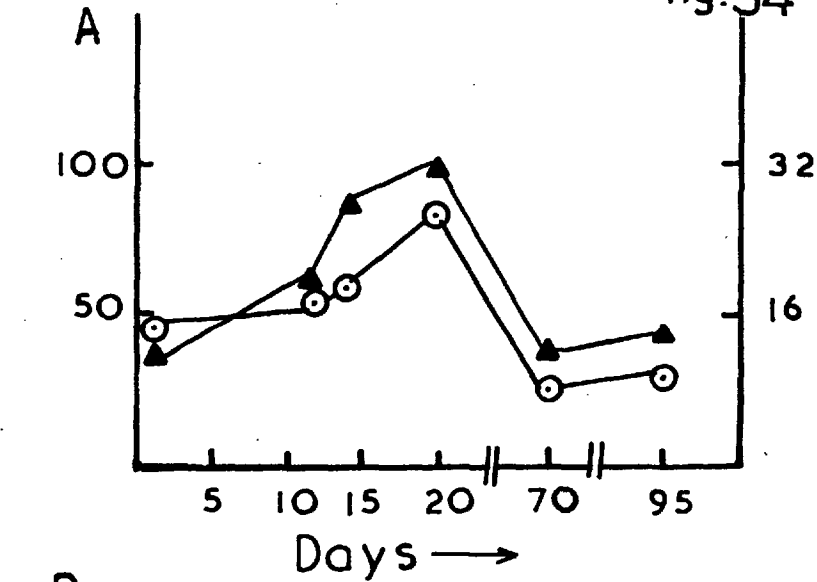
that the level of ICD would fall rapidly with improvement in the patient's condition and reach the normal range before recovery is complete. In no case of acute liver disease did we encounter a raised ICD with normal GP-T.

Serial Studies of ICD.

During serial studies on patients with acute liver disease, fluctuations in ICD levels are very similar to alterations in GP-T. This is well illustrated in Fig. 34A. The patient was a baby who was born with intra-hepatic obstructive jaundice with liver cell damage; over the ninety-five day study the levels of the two enzymes closely paralleled each other. On the twentieth day of study a laparotomy was performed; there was no marked atresia and the baby improved after the operation. This is reflected by the fall in ICD and GP-T levels.

Norberg (1961) in a study of serum enzymes in icteric conditions suggests the use of ICD as a supplementary test to alkaline phosphatase and the transaminases for the diagnosis of liver disease. He has found that it gives added information in special cases of liver damage, and we agree with this. We have found it useful in cases of secondary liver damage, particularly when it follows a myocardial infarction; a raised ICD will confirm liver damage and help to explain the

fig. 34



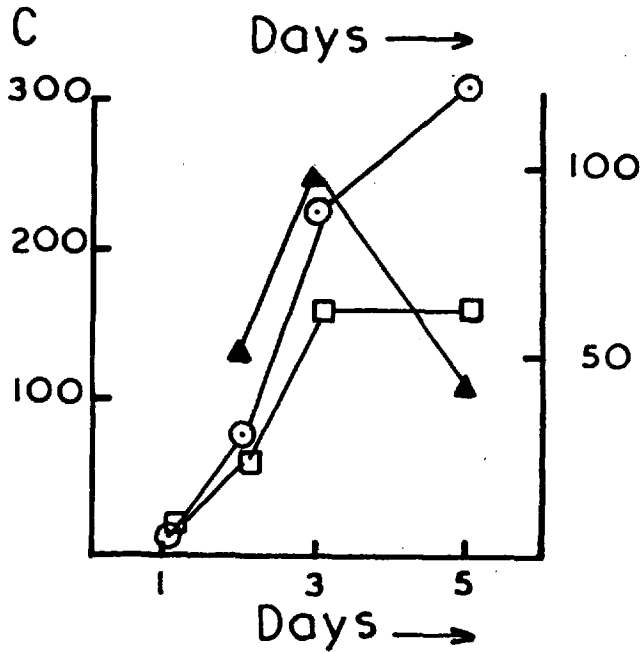
Serial enzyme
Studies

▲ ICD

○ GPT

□ GOT

units $\mu\text{moles/min/L}$



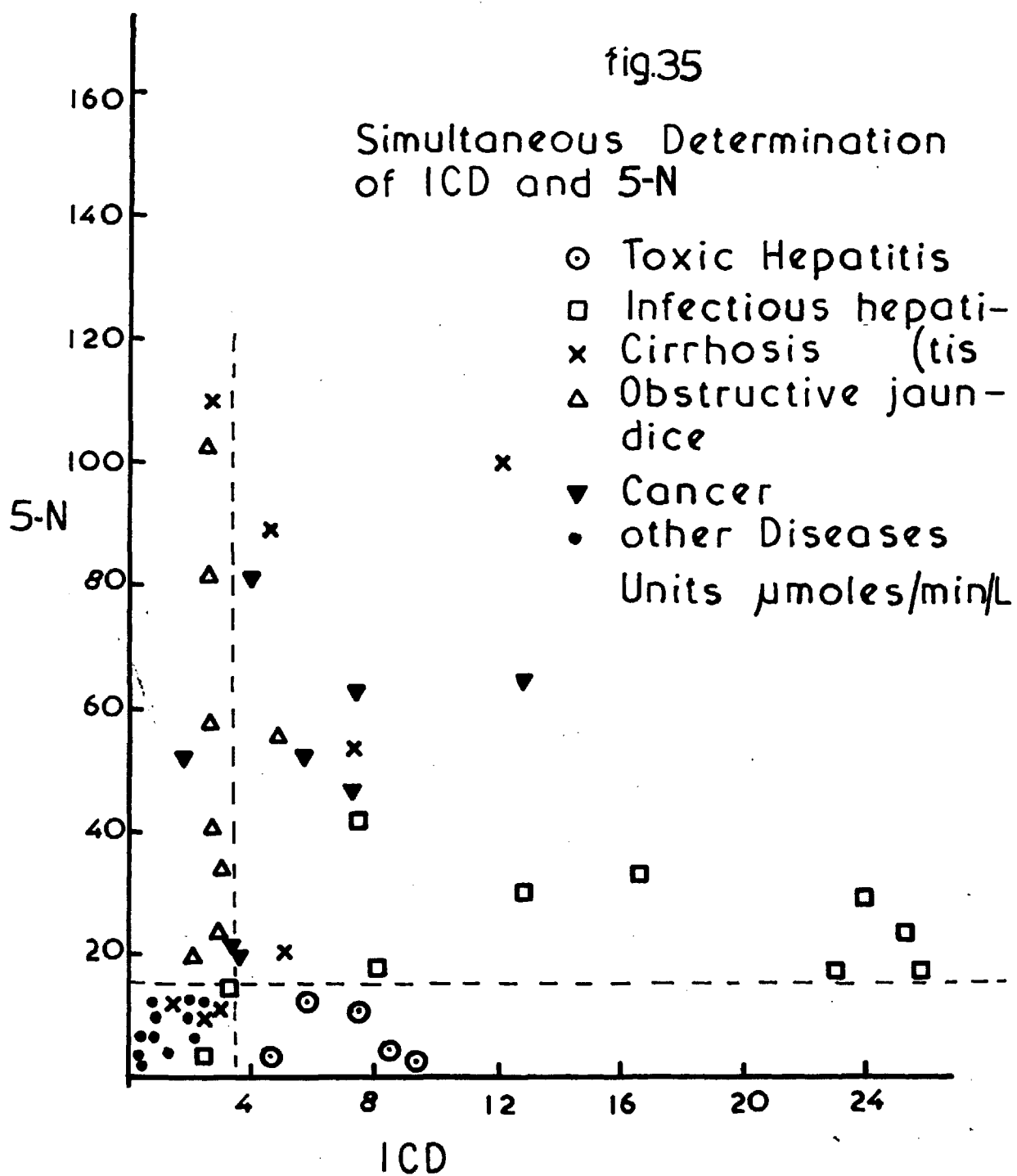
rise in GP-T which also occurs.

Serial studies on two such special cases are shown in Fig. 34. B was a case of carbon monoxide poisoning which caused muscle necrosis followed by damage to the liver. Initially the transaminases were raised and the ICD within the normal range. The level of glutamic oxaloacetic transaminase (GO-T) fell steadily during the eleven days of study, but there was a secondary rise in GP-T on the fourth day, accompanied by a sharp rise in ICD, indicating liver damage. By the eleventh day, both GO-T and ICD levels were normal, while the GP-T was still slightly raised.

C was a patient who had suffered a small cardiac infarction several days before the first enzyme determinations were made. At post-mortem the liver was found to be fatty with congestive changes, which would account for the grossly elevated enzyme levels shown in the figure.

Comparison of ICD and 5-Nucleotidase in Non-Breast Cancer Patients.

Serum 5-nucleotidase levels were estimated in the fifty patients studied; the levels of the two enzymes are plotted against each other in Fig. 35. It can be seen from the figure that in obstructive jaundice and hepatitis the two enzymes complement each other; large elevations in 5-nucleotidase occurring in the first case in conjunction with normal ICD levels, and in the second case high ICD activities are accompanied by slightly raised 5-nucleotidase levels.



There were five cases of liver damage with raised transaminases and ICD, but with normal 5-nucleotidase. All but one of the patients with carcinoma of the liver had abnormal levels of both enzymes, indicating liver cell damage and either a leakage of 5-nucleotidase from the cells or impaired excretory function.

In cirrhosis normal levels of both enzymes occurred in the inactive phase of the disease, and all except one patient in active phase had raised values for both enzymes. The exception had a raised 5-nucleotidase and normal ICD.

A variety of diseases had normal levels for both enzymes; they included multiple myeloma, polyarteritis, bone metastases, myxoma, nephritis, osteoclastoma and malabsorption syndrome. These results indicate that the estimation of ICD in conjunction with 5-nucleotidase can be used to advantage in the diagnosis of several types of liver disease, particularly hepatitis and obstructive jaundice.

12.

Serum Enzyme Studies in Patients with
Cancer of the Breast.

Introduction.

Some breast cancers depend upon a supply of sex hormones for their continued growth; in these cases removal of the source of these endocrine secretions may result in clinical improvement. The patient is usually treated initially by oophorectomy or adrenalectomy, and clinical improvement following this therapy demonstrates the hormone-dependant nature of the cancer. In these cases, if the patient's condition should later deteriorate, ablation of the pituitary may be performed with some confidence that the treatment will be successful.

It is sometimes difficult to assess the effect of endocrine ablation on the progress of breast cancer, and any addition to the usual parameters is valuable. We have followed the serum levels of several enzymes during treatment by ablation of the pituitary by the implantation of seeds of radio-active yttrium by the method of Fraser et al. (1959), in order to investigate their value as an additional index of clinical status and as an indication of the presence and position of metastases. The serum levels of six enzymes have been studied in these patients in the hope that several enzymes in parallel would provide a clearer picture than isolated investigations of individual enzymes.

Phosphoglucose isomerase has been used as an indication of the presence of metastases. Bedansky (1954b) showed that there is considerable association between rises in the serum level of this enzyme and the growth of metastatic tumours in bone, liver and spleen. Other workers have shown that it is raised in myocardial infarction (Bing, Castellanos and Siegal, 1957), leukaemia (Israels and Delory, 1956), hepatitis (Bruns and Jacob, 1954; Bing *et al.*, 1957; Ward, Krugman, Giles, Jacob and Bedansky, 1958), and various myopathies (Schapira *et al.*, 1955). These observations indicate that destruction of any phosphoglucose isomerase-rich tissue will result in a rise in the serum enzyme activity. It is not known whether the increase in serum glycolytic enzymes, which occurs in cancer, comes from the host tissue or the tumour itself, since both are rich in phosphoglucose isomerase, however, we have chosen this non-specific enzyme to provide an indication of the presence of metastases.

Alkaline phosphatase is known to be in high concentration in bone and liver, hence a raised serum level in cancer of the breast indicates a spread of the disease to either one or other of these organs, or to both of them. In these cases there is a variety of factors which could cause a rise in serum alkaline phosphatase. Metastases in bone may stimulate osteoblastic activity, in an attempt to repair the damage done by the invading cancer, or the cancer itself may be of an osteoblastic type. Alternatively,

osteolytic secondaries may break down normal bone and release alkaline phosphatase into the blood plasma.

Liver metastases could cause an increase in serum alkaline phosphatase, either from the release of enzyme from damaged liver cells or by effecting a failure in the normal excretory mechanism for the enzyme. This latter may result from intra- or extra-hepatic obstruction, or from a functional failure of the diseased cells.

It can be seen, therefore, that determination of alkaline phosphatase by itself does not provide information of great specificity. We think that its value can be greatly increased by simultaneous determinations of total acid phosphatase and 5-nucleotidase.

Total acid phosphatase has been shown to be raised in osteoclastic bone disease (Woodard, 1959), and Burstone (1959) has demonstrated histochemically a high acid phosphatase activity associated with osteoclasts. We have, therefore, used this enzyme in conjunction with alkaline phosphatase as an index of bone metastases, and have investigated the correlation between osteolytic secondaries and elevations in the serum level of acid phosphatase. We have assumed that a raised acid and/or alkaline phosphatase with normal 5-nucleotidase is indicative of bone metastases. The liver is also a rich source of acid phosphatase, and a contribution to the total serum activity from this organ

cannot be completely ignored, although there are few reports of raised acid phosphatase in liver disease.

5-Nucleotidase has been taken together with alkaline phosphatase as an indication of secondaries in the liver. The reports by Dixon and Fardom (1954) and Young (1958) assume that the observed elevations of this enzyme in liver disease are caused by release of 5-nucleotidase from liver cells. Reis (1950) has shown that the enzyme is widely distributed throughout the human body; and while, undoubtedly, some 5-nucleotidase is released from the liver cells, it seems to us more likely that the elevations are caused by a failure to excrete the enzyme. We have found high concentration of the enzyme in fluid from a biliary fistula and our observations of moderate increases in serum 5-nucleotidase in hepatitis and of gross elevations of the enzyme in obstructive jaundice, a pattern similar to that shown by alkaline phosphatase in these different liver diseases, suggest to us that the two enzymes share the same excretory pathway. In support of this, Wachstein (1959) has shown histochemically that the concentrations of both 5-nucleotidase and alkaline phosphatase are high in the bile ducts and bile canaliculi, and low in liver parenchymal cells. It seems likely, therefore, that the increase in 5-nucleotidase in liver disease reflects either a mechanical or a functional failure in the excretory mechanism in the liver, and can be used as an indication of hepatic metastases.

We have taken a normal 5-nucleotidase with increased levels of acid and/or alkaline phosphatase as an indication of metastases in bone, and a raised alkaline phosphatase with a raised 5-nucleotidase as a sign of liver involvement. If the levels of all three enzymes are above their upper limits of normal, we think it is likely that both bone and liver secondaries are present.

We have investigated the level of iso-citric dehydrogenase in these patients, to assess its use as an indication of liver secondaries, following the claims by Wolfson et al. (1958) of its specificity as an indication of liver disease.

Serum aldolase levels were investigated in the early part of this study, but its determination was discontinued when it became apparent that the results added nothing to the information provided by phosphoglucose isomerase.

By the determination of these selected enzymes we hope to be able to distinguish bone, liver and soft tissue metastases to cancer of the breast, in the presence of each other.

During this study there has been close co-operation with Dr. G.F. Joplin, Registrar to Professor Russell Fraser, and the clinician in charge of the patients. He has made an

independent assessment of clinical progress by the measurement of visible masses, radiology, studies of calcium metabolism and haematology. His clinical findings have been compared with our assessment based on our assumptions about the significance of the serum enzyme determinations.

Clinical Assessment of the Patients.

Eighteen patients will be discussed here. The group is comprised of all those on whom we made a detailed pre-treatment study and have sufficient clinical data to assess the response. We have excluded some of the patients at the beginning and end of the two-year study period, because of insufficient data to present a complete picture.

The patients have been divided clinically into six groups on a basis of objective cancer response to treatment. These are shown in Table 6.

**TABLE 6. Clinical Classification of Response
to Treatment.**

	<u>Group.</u>	<u>Cancer response.</u>
Responders	A	Clear improvement. No new lesions.
	B	Clear improvement in some lesions, but new ones appearing. Overall picture - remission.
Mixed Responders	C	Cancer arrest
	D	Improvement in some lesions, deterioration in others. Overall picture - no change.
Non-responders	E	Improvement in some lesions, appearance of new ones. Overall picture - deterioration.
	F	All lesions advanced.

Table 7 shows the groups into which the eighteen patients have been classified, with the position of metastases as assessed clinically. Seven patients were classed as responders, six in group A and one in group B; six were classed as mixed responders in group D, and five patients failed to respond and so were classed as group F.

Each patient is discussed individually in the appendix. For the calculation of pre-treatment average enzyme levels, the values found in the week immediately following the pituitary implant have been included with those determined before the operation. The condition of the patients has been assessed from a clinical point of view into periods of remission and relapse or failure to respond, and average enzyme levels for each clinical state calculated.

In Figs. 37 and 38 the number of determinations which have been used to calculate the average enzyme levels in each clinical state are shown in brackets.

TABLE 7. Classification of Patients on Clinical Assessment.

<u>Number</u>	<u>Patient</u>	<u>Group</u>	<u>Position of Secondary Lesions</u>
1	F.B.	A	lytic + blastic + other
2	M.E.	A	lytic + blastic + other
3	I.H.	A	lytic + other
4	F.P.	A	lytic + other + liver
5	M.R.	A	lytic + other
6	C.W.	A	lytic + blastic + liver
7	E.T.	B	lytic
8	I.G.	D	lytic + other
9	R.G.	D	lytic + other
10	L.L.	D	blastic + liver
11	A.S.	D	liver + other
12	I.S.	D	blastic + other
13	D.S.	D	lytic + other
14	D.C.	F	liver + other
15	L.G.	F	other
16	M.G.	F	lytic + blastic + other
17	T.M.	F	other
18	E.W.	F	lytic + other

Serum Enzyme Levels as an Index of Clinical Status.

The average levels of phosphoglucose isomerase, 5-nucleotidase and acid and alkaline phosphatase for these eighteen patients in three different clinical states: pre-treatment, remission and relapse or failure to respond are shown in Fig. 36. The average levels for phosphoglucose isomerase and acid phosphatase on the individual patients are shown in Figs. 37 and 38.

It can be seen from Fig. 36 that the levels of phosphoglucose isomerase fall with clinical improvement and rise to above the pre-treatment levels with relapse or failure to respond. 5-Nucleotidase in these patients failed to show any change between pre-treatment and response levels, but it did rise above the upper limit of normal with clinical deterioration. Acid and alkaline phosphatase failed to show any significant alterations during any of the three clinical phases.

For the individual patients (Fig. 37), six of the seven responders had initial phosphoglucose isomerase levels above the upper limit of normal, and in five of these there was a significant fall with clinical improvement. Two patients were followed into relapse, and they both showed increases above the pre-treatment level of this enzyme. The

Average serum enzyme levels

■ pre treatment units $\mu\text{moles/min./l.}$
 ▨ remission
 □ relapse

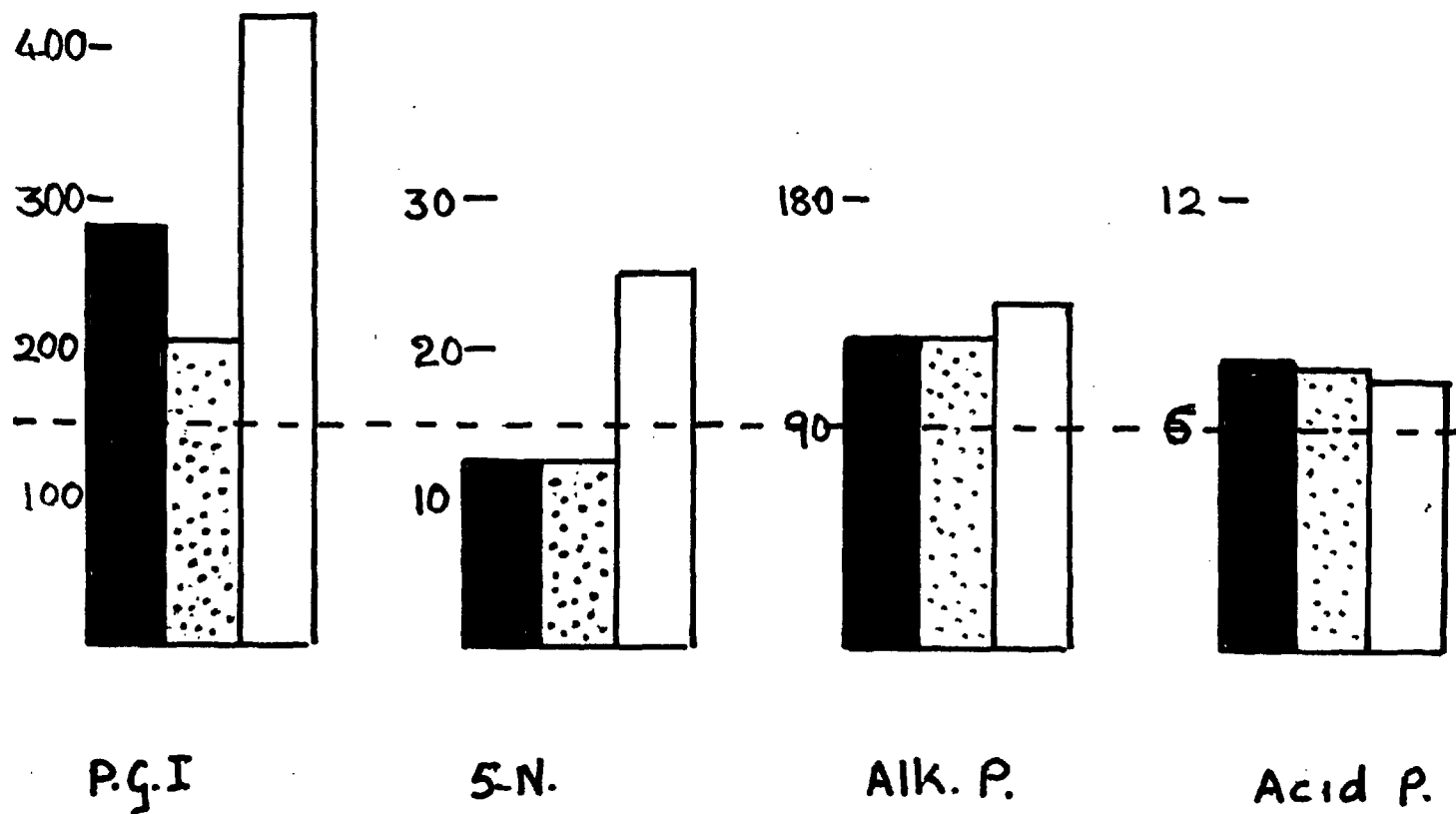


fig. 36

effect of pituitary ablation on breast cancer correlated with

SERUM P.G. I.

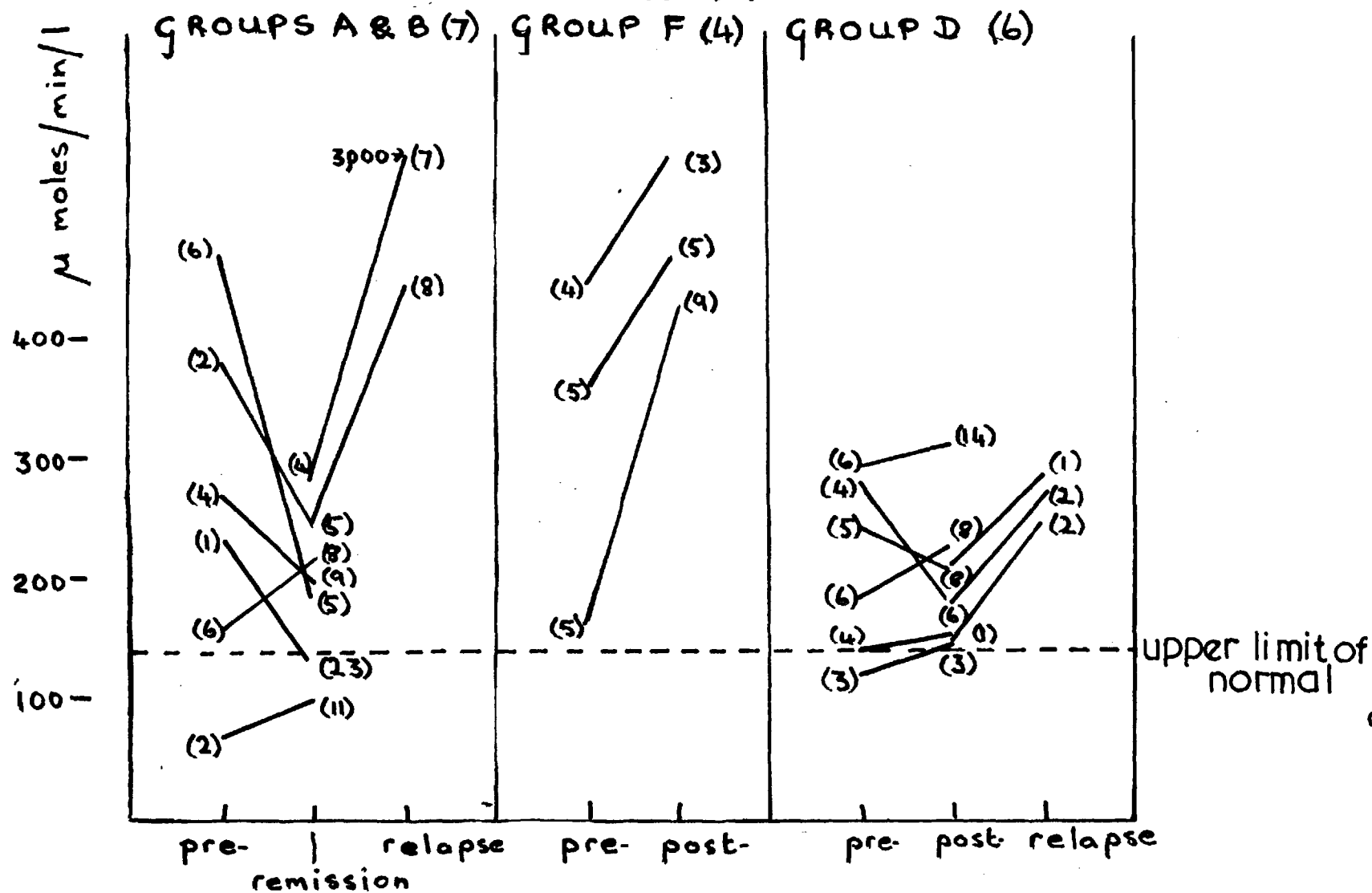


fig. 37

effect of pituitary ablation on
breast cancer correlated with serum
total acid phosphatase
groups A & B (7) group F (4) group D (6)

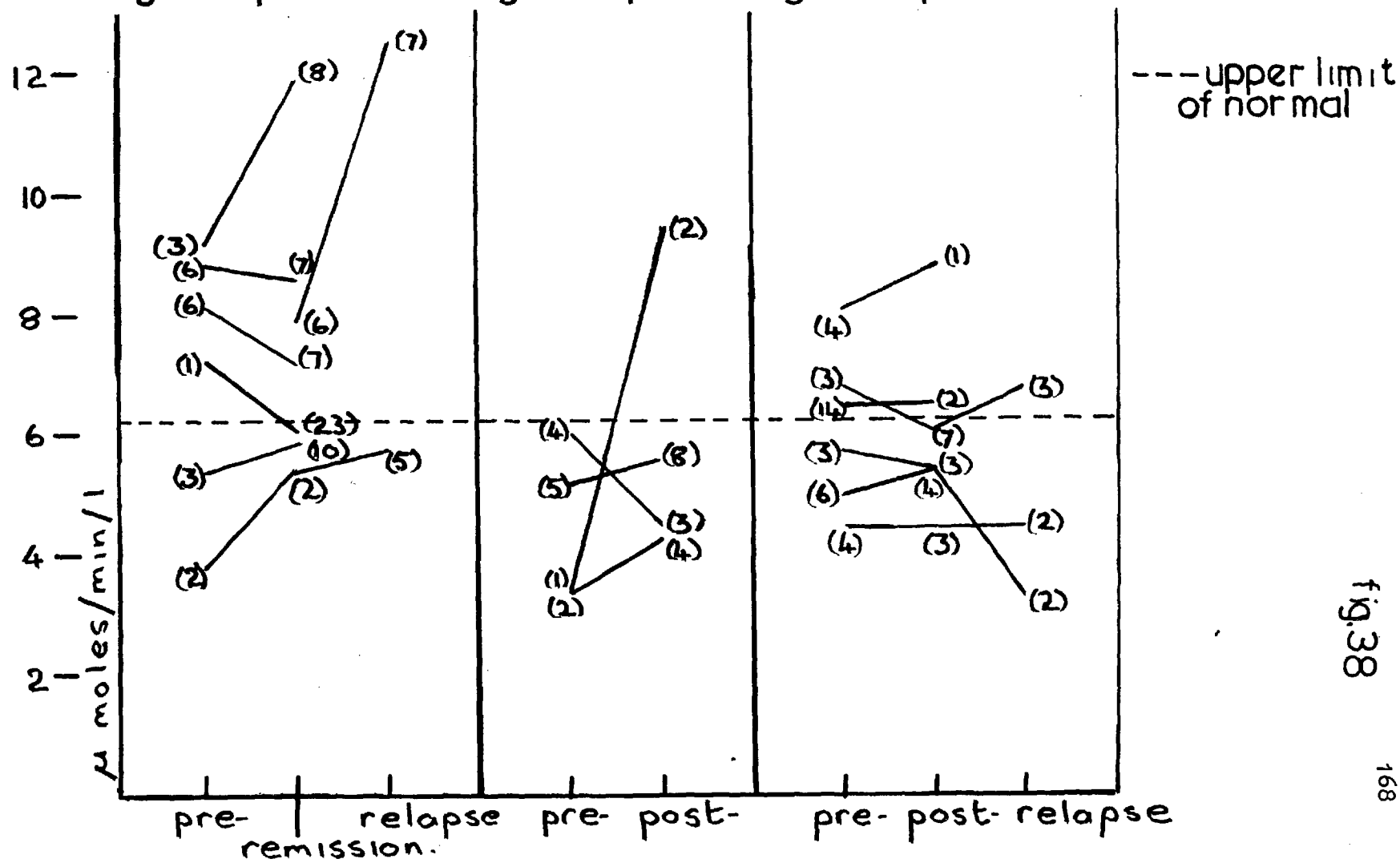


fig. 38

three non-responders all showed a rise with advancement of the disease, and there was little significant change in the level of the enzyme in the serum of the six mixed responders.

Table 7 shows that all the eighteen patients investigated had metastatic cancer and of these M.E. and R.G. were the only patients on whom we were unable to demonstrate the presence of metastases by raised levels of phosphoglucose isomerase. The alterations in acid phosphatase (Fig. 38) are less significant. It appears that, of the four enzymes studied, phosphoglucose isomerase gives the best indication of the clinical condition of the patient.

Serum Acid and Alkaline Phosphatase in Breast Cancer.

We have found that the combined study of serum levels of acid and alkaline phosphatase does provide a good indication of the presence and extent of bone secondaries in general, although we have not found it possible to use the levels of serum phosphatases to distinguish osteolytic from osteoblastic secondaries. The levels of both enzymes have been determined in the eighteen patients under consideration. Only four of them were free from secondary deposits in the bone and three of these, A.S., T.M. and L.G., had normal levels of both phosphatases. The fourth, D.C., had hepatic metastases which caused an increase in alkaline phosphatase.

Initially the acid phosphatase in this patient was normal, but the serum activity increased just before death as liver involvement increased. This organ could have been the source of this serum increment, since there is quite a high concentration of acid phosphatase in liver cells.

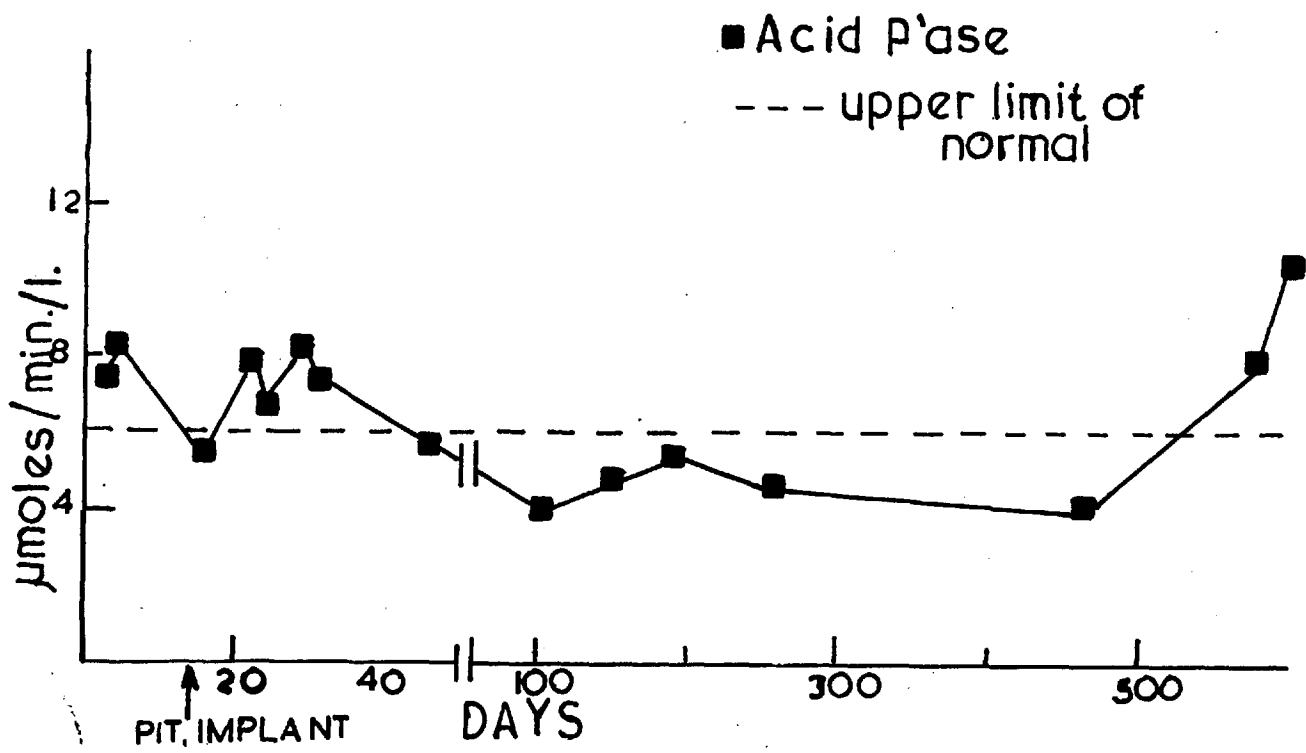
The other fourteen patients all had bone secondaries of some sort, and all except one (I.G.) had increased levels of either acid and/or alkaline phosphatase at some time during the investigations. Eight patients had osteolytic metastases and five of these (I.H., M.R., E.T., D.S. and F.P.) had abnormal levels of both enzymes. Of the remaining three, R.G. had increased levels of acid phosphatase only, while E.W. had an abnormal amount of alkaline phosphatase in her serum, with normal levels of acid phosphatase. I.G. had normal levels of both enzymes in the presence of osteolytic lesions.

Two patients had osteoblastic secondaries. L.L. had a raised alkaline phosphatase only, while I.S. had increased levels of both enzymes. Four patients had both osteolytic and osteoblastic metastases, and three of these (F.B., C.W. and M.G.) had raised serum activity of both phosphatases, while M.E. had normal alkaline phosphatase and raised acid phosphatase levels.

Alterations in serum enzyme levels may indicate a successful response or a deterioration in the condition of the

fig.39

Serial enzyme studies



patient, but we have found that acid phosphatase does not provide as reliable an index of progress as does phosphoglucose isomerase. An example of serial studies of serum total acid phosphatase is shown in Fig. 39. In this patient, the treatment was successful and her response is indicated by a fall from slightly raised levels of acid phosphatase to values within the normal range during her period of remission. Her subsequent relapse was obvious biochemically from a rise in all serum enzyme levels, including acid phosphatase.

Serum 5-Nucleotidase and Iso-citric Dehydrogenase in Breast Cancer.

Our experience with 5-nucleotidase and alkaline phosphatase in patients with bone and liver disease was repeated in the majority of breast cancer patients with bone and liver secondaries. Serum iso-citric dehydrogenase (ICD) levels were used as additional biochemical evidence to support our findings.

5-Nucleotidase was determined in twelve out of the eighteen patients and in ten of these the serum ICD was also studied. Out of the twelve patients, seven had clinical evidence of liver secondaries. In four patients, F.P., M.R., C.W. and D.C., the clinical evidence was supported by biochemical findings of abnormal levels of 5-nucleotidase. The level of ICD was determined in the first three patients and this also was raised. These patients had all been in Grade A remission, and

the rise in the serum level of these two enzymes occurred as their clinical condition began to deteriorate and the disease spread into the liver.

Of the remaining three patients with liver secondaries, when assessed from a clinical view point, one of them, L.L., had normal 5-nucleotidase but elevated ICD levels. In I.S. the disease only spread to the liver at the beginning of the terminal phase. The patient died at another hospital and so we were not able to follow this deterioration biochemically. The level of ICD had become slightly abnormal on the last two occasions of our investigations, indicating that the disease was affecting the liver, but at this stage the 5-nucleotidase had not become abnormal.

The investigation of serum 5-nucleotidase levels was only introduced half-way through the study of patient A.S. The first determination showed a raised level of the enzyme, but this fell during remission and failed to rise as she went into relapse. ICD was determined on two occasions and normal levels were found both times.

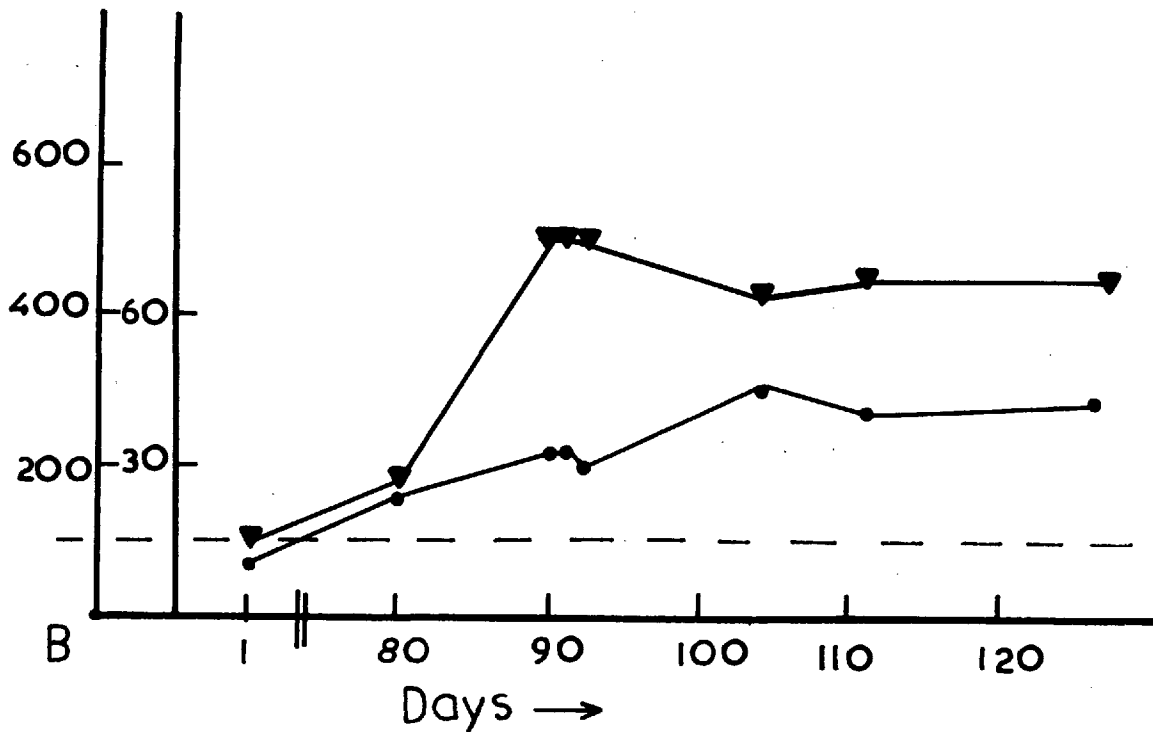
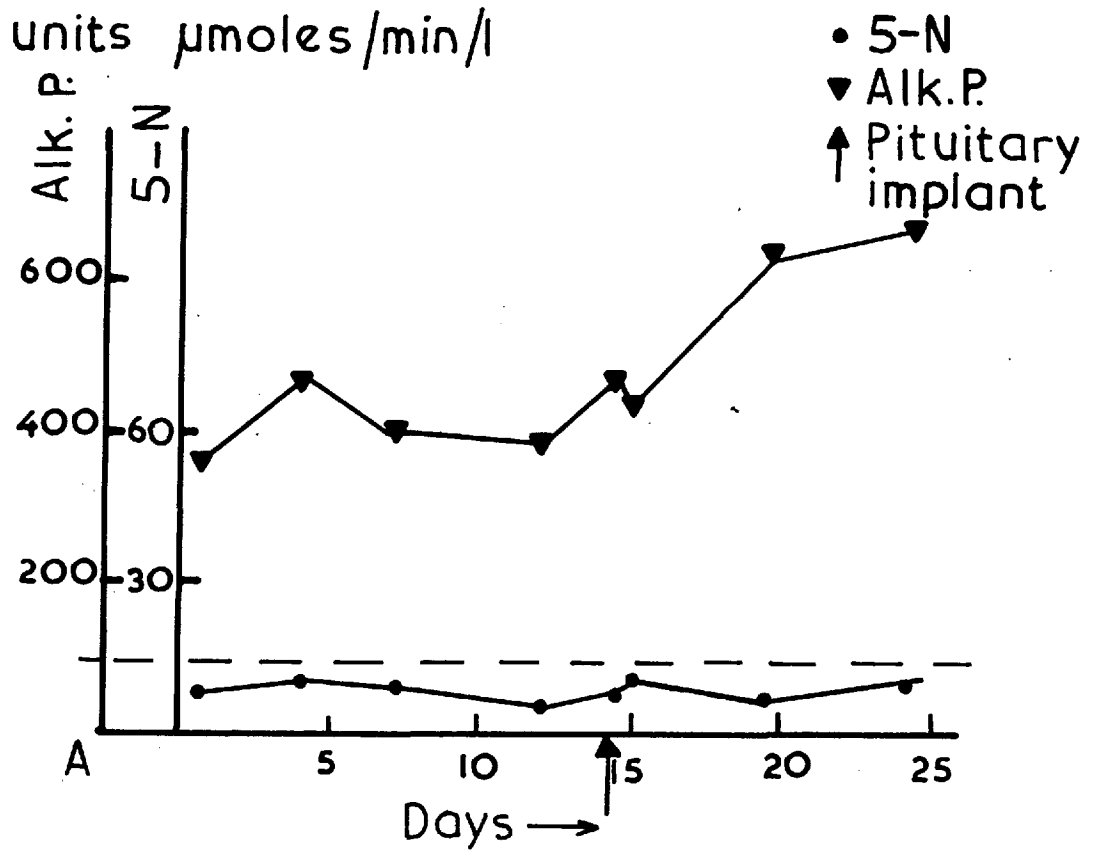
Of the five patients with no clinical evidence of liver involvement, two (R.G. and M.G.) had bone secondaries indicated by raised levels of acid and/or alkaline phosphatase, with levels of 5-nucleotidase which remained within the normal range.

The three remaining patients of this group of twelve all had raised levels of 5-nucleotidase and ICD for some part of the time during which biochemical investigations were carried out, indicating liver damage, but this was not apparent from clinical observations. One patient, D.S., died suddenly and no post-mortem was carried out. The other two, I.H. and E.T., are still in remission, so at this stage it is not possible to say whether the clinical or biochemical assessment is correct. However, since both enzymes are raised, it is likely that these increases resulted from damage of some sort to the liver, but whether it is malignant or not remains to be seen.

Fig. 40 shows serial studies of levels of alkaline phosphatase and 5-nucleotidase in two patients. A was patient No.16, M.G., on whom the pituitary implantation treatment was unsuccessful. She died nearly a month after admission and at post-mortem was found to have secondary growths in her bones, adrenal glands and myocardium. Her liver was perfectly clear, as was indicated biochemically by her persistently normal 5-nucleotidase.

B was patient No.6, C.W., who was in remission when the first investigations shown here were carried out. Three months later her condition had begun to deteriorate

fig.40
Serial enzyme studies



clinically and this was reflected by a rise in serum alkaline phosphatase and 5-nucleotidase. The levels of the two enzymes remained elevated until her death six weeks later. The abnormal 5-nucleotidase indicated hepatic metastases which were shown to be present at post-mortem.

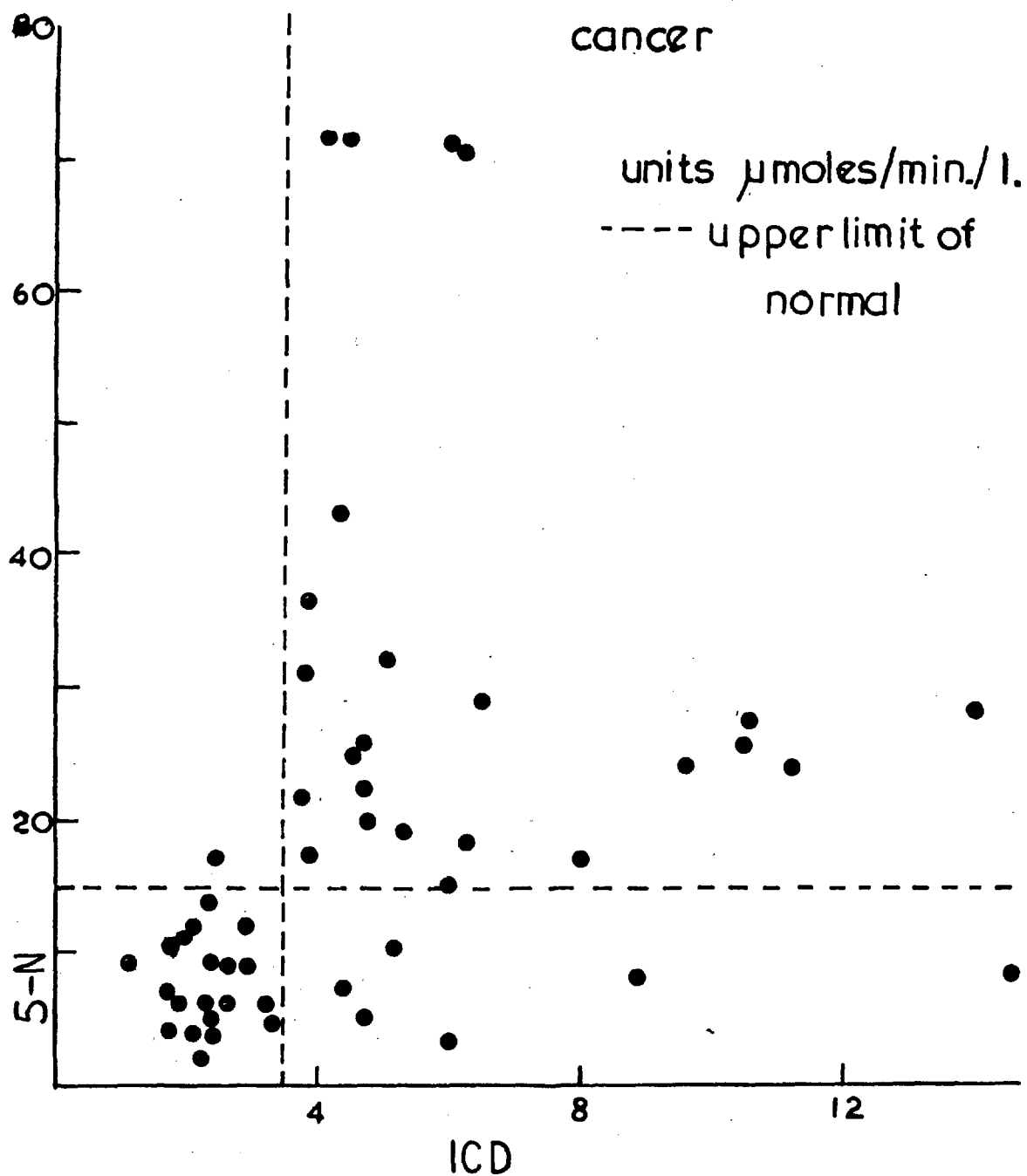
In the two examples under discussion here, Case A had normal levels of ICD throughout her illness, and in Case B the raised 5-nucleotidase was accompanied by raised ICD, providing further evidence of liver involvement in this patient.

The spectrophotometric determination of ICD in the serum of cancer patients may not be possible because of the precipitation of cryoglobulins while the specimens are kept at -15° awaiting investigation. Use of such a specimen causes turbidity of the reaction mixture and, therefore, prevents the measurement of the increase in optical density as the coenzyme is reduced.

However, we made some determinations on fresh specimens and the pattern of elevations of this serum enzyme is similar to that shown by 5-nucleotidase.

Fig. 41 shows the simultaneous determination of 5-nucleotidase and ICD on breast cancer patients. This diagram shows that, in general, the two enzymes are either both normal or both raised. However, there are exceptions:

fig.4
5-N and ICD in breast
cancer



four determinations are shown on patient L.L. who had clinical evidence of liver secondaries. This was indicated biochemically by raised ICD, but the normal 5-nucleotidase did not substantiate this. There are two determinations on I.S. when the disease had started to infiltrate into the liver. This was indicated by an increase in ICD, but the patient then moved to another hospital, so that we were unable to follow the complete deterioration and did not observe a rise in 5-nucleotidase. The raised 5-nucleotidase with normal ICD was a determination on patient No.7, E.T., whose clinical condition improved with treatment. The improvement was reflected by a fall from moderately elevated values of the two enzymes to values within the normal range. The levels of ICD fell more rapidly than did the level of 5-nucleotidase, thus giving on one occasion abnormal values of one enzyme but not the other.

Apart from the two patients with clinical evidence of liver secondaries which were indicated biochemically by iso-citric dehydrogenase but not by 5-nucleotidase, the two enzymes appear to provide the same information. In view of this, we suggest the routine use of 5-nucleotidase as an index of liver metastases in these patients, since it does not depend on the optical clarity of the serum for a successful determination as does ICD. In cases of disagreement between clinical and biochemical findings, or to provide further confirmation, ICD may be a useful additional test.

Serum Aldolase

Aldolase was the first glycolytic enzyme to be studied by Warburg and Christian in their classic experiments in 1943 on tumour-bearing rats. They found that the aldolase content of malignant tumour is very low when compared with muscle on the same species, but they found that the serum content of the enzyme in rats with large tumours was greatly increased.

Sibley and Lehninger (1949a) confirmed Warburg's observations on increased serum aldolase levels in rats and also observed increases of the serum enzyme in patients with cancer and other diseases. Sibley and Fleisher (1954) and Bruns and Fuls (1954) reported greatly increased activity in the serum of patients with acute hepatitis but normal values in portal cirrhosis and obstructive jaundice. Schapira, Demos, Schapira and Dreyfus (1957) found that patients with progressive muscular dystrophy had increased serum aldolase activity, and White (1956) has found increases in the enzyme level in patients with myocardial infarction and dermatomyositis. Baker and Gevan (1953) reported increases in serum aldolase in most patients with advanced prostatic carcinoma. It is clear that increased serum aldolase levels indicate the existence of tissue damage, but owing to its widespread distribution, its use in the diagnosis of cancer is limited.

Serum Aldolase in Breast Cancer.

Using the method of Sibley and Lehninger (1949a), described in Part I, the aldolase activity was determined in one hundred and twenty sera from fifteen cases of cancer of the breast, to assess its use as an index of response to treatment by pituitary ablation. The results were compared with levels of phosphoglucose isomerase determined on the same specimens, and the findings on each enzyme were related to clinical observations.

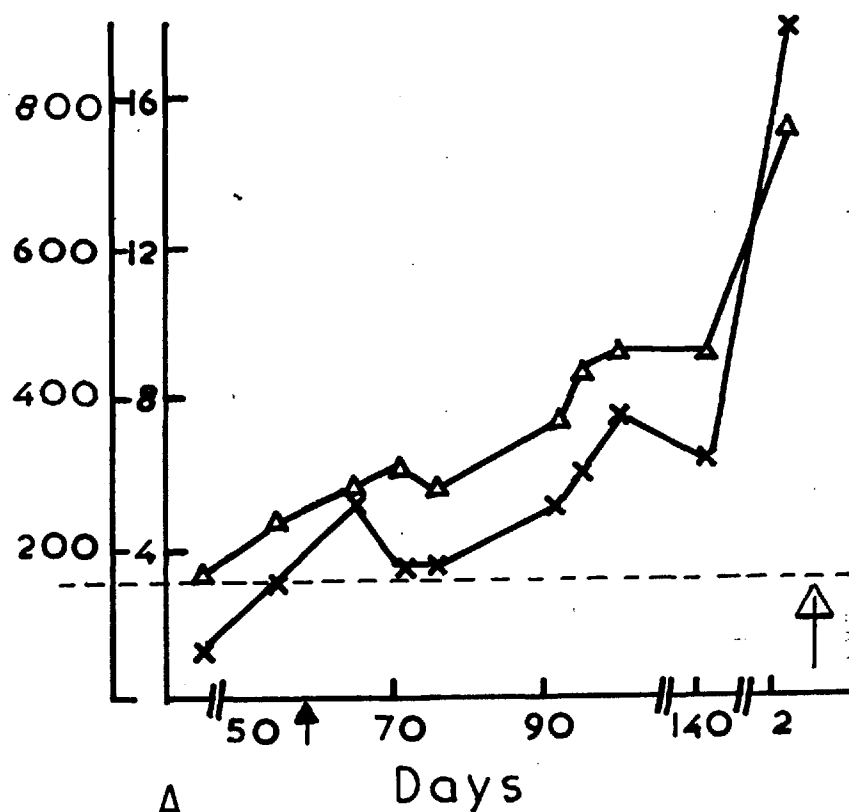
Significant elevations were found in only 18% of the aldolase determinations, which agrees well with Sibley and Lehninger (1949b), who found raised values in 20% of the one hundred and four cases which they studied. However, 66% of the phosphoglucose isomerase determinations were above the upper limit of normal, and these figures showed a better correlation with the patients' condition than did the serum aldolase.

It was observed by Sibley and Lehninger (1949b) that surgical removal of tumours from rats caused a fall in serum aldolase. We were unable to demonstrate a similar fall in response to pituitary ablation in the fifteen patients studied. The mean pre- and post-ablation levels were determined for both aldolase and phosphoglucose isomerase, and only a slight drop in aldolase activity from 3.1 to 2.5 $\mu\text{moles/min./l.}$ was found. Neither value is outside the

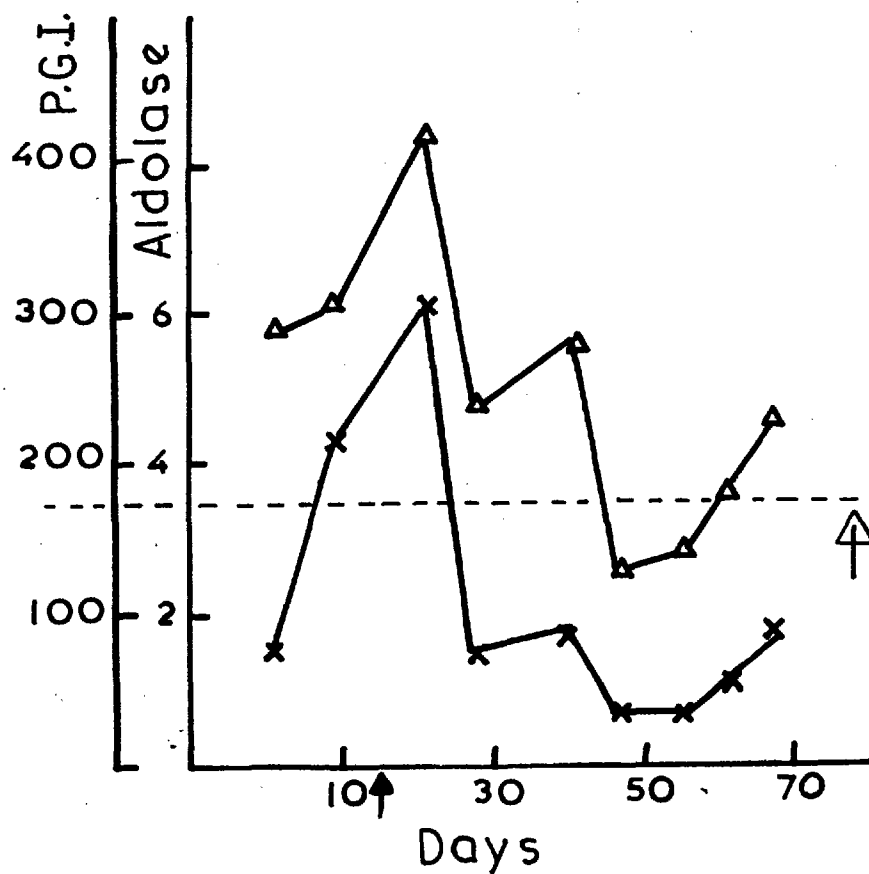
normal range and the figures cannot be considered to be significant. The mean phosphoglucose isomerase values gave a much better indication of the successful response in these patients, falling from 270 to 180 $\mu\text{moles/min./l.}$ after the operation.

During serial studies on the levels of serum aldolase and phosphoglucose isomerase on several patients, it was observed that fluctuations in the level of one enzyme were reflected by the other, but again it was found that aldolase did not give as good an indication of the patient's clinical status as did phosphoglucose isomerase. On no occasion did we find a raised serum aldolase level with a normal phosphoglucose isomerase. Fig. 42 shows serial studies on three of these cases. In A, the early abnormal levels of the two enzymes fell following treatment by pituitary ablation, but the clinical response was not as dramatic as might be expected from the fall in the serum aldolase levels. Unlike phosphoglucose isomerase, the aldolase activity failed to rise above the upper limit of normal when the condition of the patient deteriorated. B is an example of fluctuations in the levels of both enzymes, in which aldolase stayed within the normal range while phosphoglucose isomerase levels were outside it. The successful response to treatment is reflected by both enzymes.

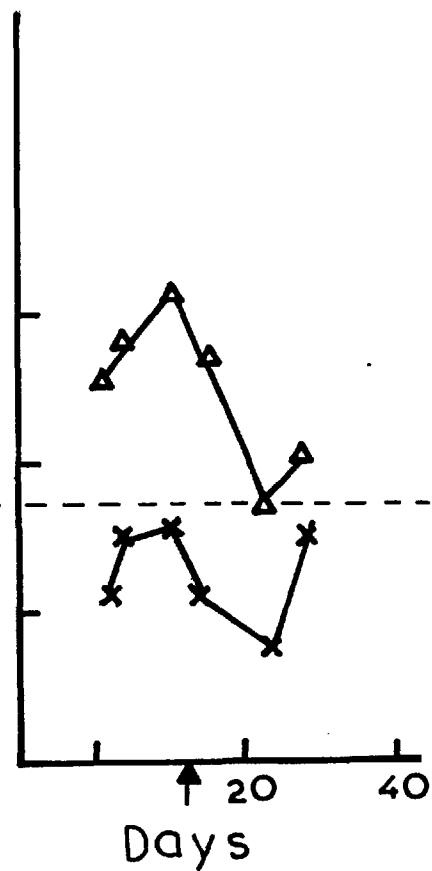
C



A



B



The levels of the two enzymes closely parallel each other in Case C, and, apart from one or two early determinations, abnormal activities of both enzymes occurred in the serum. The continued increase in activity reflected the decline in the patient's condition.

Since both enzymes are widely distributed throughout the body, any increased glycolytic activity by tumour tissue or breakdown of normal tissue by invading cancer cells, which results in the release of glycolytic enzymes into the serum, will cause a rise in the level of both aldolase and phosphoglucose isomerase.

We have concluded that it is unnecessary to perform estimations of both enzymes and, as aldolase is not as sensitive an index of the patient's condition as phosphoglucose isomerase, we have discontinued its estimation.

Conclusion.

From these results we conclude that the serial determination of the levels of acid and alkaline phosphatase, 5-nucleotidase and phosphoglucose isomerase in serum can be used to advantage in the biochemical investigation of the sites of metastases in women with cancer of the breast. By

the combined use of these four enzymes, it is possible to indicate the presence of lesions in bone, liver or soft tissue. If these biochemical data are interpreted in the light of clinical findings, they make a useful additional parameter in the assessment of the response to treatment by the implantation into the pituitary of seeds of yttrium 90.

DISCUSSION

The Origin and Fate of Enzymes in Serum

The small amounts of enzymes normally present in the circulating blood are provided by the physiological breakdown of cells, and as this is a continuous process there must, at the same time, be a continuous inactivation and/or excretion process. It follows, therefore, that a rise in the level of serum enzymes will result from either increased release of enzyme or decreased removal. It is the balance between these two processes which determines the serum level of activity of each enzyme: but little is really known about the mechanism of the release of enzymes from tissue or how they are removed from the circulation. There are various theories about the release of enzymes in cancer, with experimental evidence to support each one. It is probable that the serum enzymes are released from several sources.

In 1943 Warburg and Christian reported that five of the enzymes involved in glycolysis were present in the serum of rats and they found that the levels of two of these - aldolase and triose isomerase - were consistently raised in the serum of rats bearing large tumours. They concluded that the tumour itself was not the source of excess enzyme, but that the enzymes might be released from muscle as the tumour acted as a

biological parasite. With improved methods for measuring aldolase activity, Sibley and Lehninger (1949 a and b) found large amounts of aldolase in other normal tissues, such as liver and kidney, and they suggested that these might also be sources of serum aldolase. It soon became obvious that high concentrations of glycolytic enzymes were well distributed throughout the tissues of the body, and break-down of any of these tissues would result in a release of enzymes into the blood stream.

Bođanaky (1954a) has shown that the organs which are the chief sites of metastases are rich in phosphoglucose isomerase, and destruction of these tissues by the invading tumour could result in the release of the enzyme into the circulation. Sibley (1958) has found that the aldolase content of blood from a vein leaving a tumour was significantly higher than that of arterial blood drawn at the same time. It is, therefore, possible for both the tumour and the host tissue to be the source of the increased glycolytic enzymes in serum.

An increase of up to 17 times the normal level of phosphoglucose isomerase was demonstrated by Bođanaky (1958) in the serum of animals given 6-methylpurine. This was associated with a decrease in the liver content of the enzyme, but there was no histological evidence of definite cell necrosis in the liver or skeletal muscle. These findings

could be explained either by alteration in the permeability of the cell wall or a change in the binding of the enzyme within the cell and should be considered when investigating the origin of serum enzyme increments.

Other workers suggest that the increase in the level of enzymes in serum is a reflection of alterations in normal tissue in sites removed from the tumour or its host.

White (1956) postulated that the increase in serum enzymes reflects the break-down of muscle tissue to supplement the patient's protein intake, which is usually insufficient to support tumour growth. He has found that serum enzyme changes seen in cancer are very similar to those found in muscle-wasting diseases. Most of his patients had creatinuria; on feeding a high protein diet in most cases there was a fall in the serum enzyme levels and a reduction in the amount of creatine in the urine. Notable exceptions were the patients with liver metastases. This fall in serum enzyme levels was not observed in patients with muscular dystrophy when fed on a similar diet.

Hauss and Lippelman (1958) believe that enzyme changes in serum represent a complete non-specific reaction on the part of the organism towards a variety of excitations. They cite the fall in serum level of tributyrinase and cholinesterase, which accompanies the rise in glutamic

oxaloacetic transaminase, lactic dehydrogenase and aldolase following myocardial infarction and other acute diseases, as evidence of a non-specific reaction to stress. They think that, while release of enzyme from damaged tissue could explain the rise in the serum level of some, it does not account for the fall in the level of others.

The now well-known and characteristic appearance in the serum of glutamic oxaloacetic transaminase, which occurs after a myocardial infarction, and the high concentration of this enzyme in heart tissue, is a good example of tissue necrosis releasing enzyme into the circulation. The rise in lactic dehydrogenase, which also occurs, can be similarly explained, but the absence of any increase in iso-citric dehydrogenase (ICD) activity appears not to fit into this pattern, since heart tissue is also rich in this enzyme. However, Stranjord, Thomas and White (1959) showed that there was an increase in serum ICD activity immediately after experimental myocardial infarction in dogs, but that the serum level rapidly returned to normal. A similar rapid disappearance of the enzyme in the human subject would explain the absence of this enzyme several hours after a myocardial infarction. In our experiments on the heat stability of ICD from human heart and liver tissue we have demonstrated that the enzyme from heart tissue is rapidly inactivated at 37° , and this could account for its rapid disappearance from the circulation.

These experiments demonstrate the importance of the rate of inactivation of the enzyme on the serum level. Several people have injected purified enzymes into the circulation and have noted the rapid rate of clearance. But where do they go? Stranjord et al. (1959) found that neither hepatectomy nor nephrectomy affected the rate of clearance of ICD or lactic dehydrogenase injected into rats.

The experiments by Armstrong et al. (1934), in which they tied off the common bile duct in dogs and so caused a rise in the level of alkaline phosphatase in serum, demonstrated the excretory pathway for this enzyme, but it has been impossible to ascribe a similar route for others. In later experiments, Frankl and Merritt (1959) found some transaminase and lactic dehydrogenase activity in the bile of dogs, but were unable to demonstrate any relationship between the level of enzyme in bile and the serum level. Carbon tetrachloride poisoning produced a rise to ten times the upper limit of normal in the serum of dogs but the enzyme level in the bile was unaltered. Injection of purified enzyme to a concentration of fifteen times the normal level also failed to produce an increase in the bile enzyme level, despite the fact that these enzymes are rapidly cleared after injection.

In our studies we have chosen enzymes which have been shown to be concerned in several of these different mechanisms

of release and removal which result in changes in the level of activity in the serum.

Our work on the kinetic properties of alkaline phosphatase demonstrates that both increased release of enzyme and decreased removal can result in hyperphosphatasaemia. The bone-like characteristic of the enzyme in the serum of patients with osteoblastic bone disease, and the appearance of an alkaline phosphatase with a Michaelis constant similar to that of the enzyme purified from liver in hepatic disease, again shows that damage or overactivity of an organ will result in an outpouring of enzyme into the serum.

However, the kinetic studies on patients with obstruction of the common bile duct show that in these instances the increase in serum alkaline phosphatase is caused by a failure in the excretion of enzyme with bone characteristics.

We have demonstrated the increases in serum 5-nucleotidase in liver disease and if, as we think, this enzyme does share the same excretory pathway as alkaline phosphatase, then the parallel estimation of the two phosphatases will indicate whether the increase in alkaline phosphatase is the result of increased release of enzyme into the serum from bone or decreased removal.

Phosphoglucose isomerase has been used by us as an indication of the presence of metastases. It provides an index

as does serum aldolase, of the release of glycolytic enzymes from either the tumour itself, or the host tissue, or from normal muscle. Despite the fact that both the origin and the fate of this enzyme are uncertain, we have demonstrated its use clinically and consider that it is a useful addition in the study of patients with cancer.

Until the facts about the release of enzymes into serum and their removal have been elucidated, their physiological significance cannot be fully realized. Meanwhile, however, we have shown that the measurement of the level of enzyme activity in serum can be an advantageous addition to the clinical study of disease.

APPENDIX.

Each patient is considered individually in this appendix and the results of the serum enzyme determinations are given in terms of μ moles of substrate utilised/min./l.

<u>Enzyme</u>	<u>Abbreviation</u>	<u>Normal range</u> (μ moles/min./l.)
Alkaline phosphatase	Alk. P.	20 - 90
Acid phosphatase	Acid P.	1.8 - 6.2
Phosphoglucose isomerase	PGI	16 - 140
Aldolase	Aldol	1.5 - 3.8
5-Nucleotidase	5-N	2 - 15
<u>Iso</u> -citric dehydrogenase	ICD	0.5 - 3.5

No. 1, F.B., aged 50.

<u>Date</u>	<u>Alk.</u>	<u>Acid.</u>	<u>FGI</u>	<u>Aldol.</u>	
17.2.60	175	9.4	330		} Pre treatment
3.3.60	340		200		
7.3.60	270	8.3	175	4.1	
10.3.60	355	13.2	320	1.5	
11.3.60		IMPLANT		1.1	
19.3.60	255	10.6	200		} Grade A remission
21.3.60	225	10.3	145	0.7	
24.3.60	290	15.8	310	0.7	
29.3.60	300	12.0	250	0.7	
9.5.60	185	11.3	270	0.7	
20.5.60		IMPLANT			
30.5.60	380	13.5	205	1.1	}
8.6.60	300	14.0	190	1.1	
9.7.60	225	9.8	115		

No. 1, F.B.

The patient was first seen in February, 1960. She had bone and soft tissue secondaries to cancer of the breast. These lesions were apparent biochemically from raised serum acid and alkaline phosphatase and phosphoglucose isomerase. A pituitary operation was carried out a month later and her condition began to improve. Grade A type of remission was maintained with clear bone recalcification when compared with pre-implant X-ray pictures, and the breast mass became smaller. A second pituitary implant was carried out in May, as it was found that ablation had not been complete the first time. Her condition continued to improve until her death in August, which was sudden and due to causes other than cancer. The serum level of phosphoglucose isomerase reflected the clinical improvement and it had reached the normal range by August. The phosphatases, however, did not show the expected fall, and both remained above their upper limits of normal. This patient was followed during the early part of this study, and we did not determine the levels of serum 5-nucleotidase or iso-citric dehydrogenase. Serum aldolase remained within normal limits the entire time, and did not provide any useful information about the clinical condition.

No. 2, M.E., aged 50.

<u>Date.</u>	<u>Alk.</u>	<u>Acid</u>	<u>PGI.</u>	<u>5-N.</u>	<u>ACD.</u>	
27.12.60	42	4.0				} Pre treatment
22. 2.61	56	5.8	70	6	1.9	
23. 2.61	49	6.5	70	10		
3. 3.61	IMPLANT					
24. 3.61	56	6.0	115	6	2.3	} Clear remission Grade A
6. 3.61	63		92	9	2.4	
8. 3.61	71	5.4	100	6		
17. 3.61	84	4.8	87	5		
24. 4.61	56	7.6	106	6		
24. 6.61	71	4.1	160			
6. 8.61	63	4.0	135	5		
11. 9.61	56	5.6	160	6		
12. 9.61	63	5.4	41	4		
13. 9.61	63	6.5	69	8		
19. 9.61	56	5.0	152	8		

No. 2. M.E.

This patient was first seen in December, 1960, at which time she was found to have osteoblastic and osteolytic and soft tissue secondaries. These lesions were not apparent biochemically, as both acid and alkaline phosphatases were normal. She was seen again in February, when there had been a rise in acid phosphatase, but all the other enzymes remained within their normal ranges. Seeds of yttrium 90 were implanted into the pituitary at the beginning of March and her condition improved. The serum level of acid phosphatase fell again to within normal limits, and has remained there except for two isolated occasions. The normal levels of other enzymes were maintained throughout her Grade A remission.

No. 3. I.H., aged 40.

<u>Date.</u>	<u>Alk.</u>	<u>Acid.</u>	<u>PGI.</u>	<u>5-N.</u>	<u>IOD.</u>	
28.4.61	275	11.2	160	4	2.4	Pre treatment
29.4.61	305	11.2	155	4	1.9	
2.5.61	200	8.3	130	8		
5.5.61	IMPLANT					
6.5.61	275	7.7	200	18		Grade A remission
8.5.61	270	7.4	160	18		
10.5.61	250	7.4	140	13		
17.5.61	210		250	43	4.3	
24.5.61	320	7.4	295	34		
26.5.61	435	7.7	200	36	3.9	
30.5.61	520	7.4	170	25	4.6	
19.9.61	250	9.3	170	11		
21.9.61	250	10.1	250	14		
23.9.61	270	10.1	250	14		
26.9.61	210	6.5	190	8		

No. J I.H.

Admitted at the end of April, 1961, this patient came to Hammersmith Hospital with osteolytic and soft tissue secondaries to cancer of the breast. At that time, the presence of bone lesions was indicated biochemically by raised acid and alkaline phosphatase, and the slightly raised level of phosphoglucose isomerase was in keeping with the presence of soft tissue metastases. A week after admission, seeds of yttrium 90 were implanted in the pituitary and the patient responded well to this treatment. She went into Grade A remission, but this was not reflected by dramatic changes in serum enzyme levels. Immediately post-implant the serum levels of 5-nucleotidase and iso-citric dehydrogenase rose, suggesting injury to the liver. This was accompanied by a slight rise in phosphoglucose isomerase and alkaline phosphatase. By September the levels of 5-nucleotidase had returned to normal and at the moment there is no clinical evidence of hepatic metastases. As the rise in alkaline phosphatase accompanied the increase in 5-nucleotidase, this could be a reflection of a failure in the excretory mechanism of the liver. There was a subsequent fall in the level of alkaline phosphatase to values similar to those obtained before the pituitary operation by September. The levels of acid phosphatase and phosphoglucose isomerase remained elevated.

No. 4, F.P., aged 56.

<u>Date.</u>	<u>Alk.</u>	<u>Acid.</u>	<u>FGI.</u>	<u>5-N.</u>	<u>ICD.</u>	
16. 8.60	49	3.4	385			} Pre treatment
17. 8.60	49	4.0	365			
19. 8.60		IMPLANT				
31. 8.60	77		370			} Grade A remission
1. 9.60	84		315			
29. 9.60	100	6.1	305			
31.12.60	56		92			
23. 2.61		4.7	135		3.0	} Relapse
20. 3.61	84	6.0	230	5		
21. 3.61	100	6.5	300	9		
11. 4.61			410	19	5.3	
12. 4.61	100	6.0	440	28	5.0	
14. 4.61		IMPLANT				
17. 4.61	100	5.1	515	29	6.5	} Grade B remission
19. 4.61	107	4.9	750	24	11.2	
24. 4.61	121	6.2	620	24	9.6	
29. 4.61	113	9.8	1050	20	4.7	
1. 5.61	128	7.1	500	17	8.0	
5. 5.61	135	6.0	440	18		
9. 5.61	135	6.8	620	22		
4. 7.61	480					} Relapse
17. 7.61		DIED				

No. 4. F.P.

The patient was admitted in August, 1960, with osteolytic and intra-cranial secondaries to cancer of the breast. The first implantation of seeds of yttrium 90 into the pituitary was carried out that month, and as her clinical condition improved, the raised phosphoglucose isomerase level fell to within normal limits.

Initially, serum acid and alkaline phosphatase levels were normal, and, therefore, did not indicate the presence of osteolytic secondaries which were apparent from X-ray and clinical observations. Six weeks after the operation, both phosphatase activities were slightly increased, but these fell to within the normal range and remained there for about five months.

In March, phosphoglucose isomerase and acid and alkaline phosphatases were again raised, indicating a return of the disease to the bones and soft tissues. Three weeks later, 5-nucleotidase and iso-citric dehydrogenase levels, which had previously been normal, also became elevated, indicating a spread of the disease to the liver. A second pituitary operation was carried out in April, but this was unsuccessful and the patient died three months later. There was post-mortem confirmation of bone, liver and soft tissue metastases.

No. 5, M.R., aged 48.

<u>Date.</u>	<u>Alk.</u>	<u>Acid.</u>	<u>FGI.</u>	<u>Aldol.</u>	<u>5-N.</u>	<u>IOD.</u>	
13.11.59							Taken as pre-treatment level
30.11.59	156	7.2	230	2.2			
2.12.59	120	8.1	156	1.3			
15.12.59							
	<u>IMPLANT</u>						
15.12.59	212	5.4	120	1.1			Post ⁹⁰ Y and oophorectomy Grade A remission
17.12.59	205	7.9	165	2.2			
21.12.59	310	6.6					
23.12.59	230	8.1	192	5.5			
28.12.59	285	7.4	120	2.2			
7. 1.60	185	5.7	175				
1. 2.60	100	6.5	100	2.2			
27. 2.60	80	4.0	130				
23. 3.60		5.9	175	1.8			
30. 3.60	56	4.8	110	2.2			
28. 4.60	64	5.6	138	1.5			
8. 6.60		4.5	190				
15. 9.60		4.0	180				
20. 4.61	100	4.8	106		7		
28. 4.61	80	6.5	160		8		
16. 8.61	128	5.4	110		16		
17. 8.61	170	7.8	295		21		
18. 8.61							
	<u>IMPLANT</u>						
18. 8.61	142	10.5	195		11		Relapse
22. 8.61	150	4.5	175		2		
1. 9.61	156	4.0	125		10		
15. 9.61	156	8.1	250		8		
9.11.61	255	8.6					
9.12.61	370	10.0	240		9		
13.12.61	325	7.6	305		27		
19.12.61	350	9.0	212		28		
27.12.61	335	8.7	415		18	6.5	

No. 5. M.R.

This patient was initially treated by eophorectomy in November, 1959. The indications of the success of this type of therapy prompted the implantation of yttrium 90 into the pituitary a month later. On admission, she had osteolytic and soft tissue secondaries and Grade A remission followed the two operations. The improvement in her clinical condition was reflected by a return to the normal range from initially elevated levels of acid and alkaline phosphatase and phosphoglucose isomerase. Serum aldolase remained normal throughout the study period. The patient's condition began to deteriorate in April 1961. This was indicated biochemically by a rise in acid and alkaline phosphatase. By August, phosphoglucose isomerase and 5-nucleotidase had risen above their upper limits of normal. A second pituitary implant was carried out that month and this produced a transitory clinical improvement, reflected by a decrease in the levels of all the enzymes except alkaline phosphatase. This second remission was only temporary and, by December, there had been an increase in the levels of all the enzymes. The rise in 5-nucleotidase indicated the infiltration of the disease into the liver and this was substantiated by abnormal levels of iso-citric dehydrogenase and jaundice.

No. 6. C.W., aged 60.

<u>Date.</u>	<u>Alk.</u>	<u>Acid.</u>	<u>FGT.</u>	<u>5-H.</u>	<u>IOD.</u>	
4. 2.60		7.4	195			Grade A response
28. 4.60	85	5.9	275			
25. 6.60		6.6	138			
3.11.60		9.3	405			
12. 1.60	100	8.5	325	10		
5. 4.61	185	10.0	350	25		Terminal relapse
15. 5.61	235	14.2	660	72	4.4	
16. 5.61	235	13.7	820	71	6.0	
17. 5.61	215	11.2	970	71	6.0	
29. 5.61	330	12.8	1200	61		
6. 6.61	285	14.8	415	64		
21. 6.61	300	11.2	875	64		
10. 7.61	290	11.0	665	43		

No. 6. C.W.

We began the biochemical investigation of this patient in February, 1960, at which time she was in Grade A remission following a pituitary implant in December, 1957. Her clinical condition remained good until May, 1961, when it began to deteriorate. Biochemically she had fluctuating elevated levels of phosphoglucose isomerase and acid phosphatase, with normal alkaline phosphatase during the remission period. The relapse was indicated by a rise in alkaline phosphatase and 5-nucleotidase in April, at which time she had a B.S.P. retention of 25%, suggesting a spread of the disease to the liver. There was a further rise in the level of all the enzymes as the disease progressed and serum iso-citric dehydrogenase also became elevated, providing further evidence of the presence of secondary growths in the liver. Death followed two months later and there was post-mortem confirmation of osteolytic, osteoblastic and hepatic metastases.

No. 7. E.T., aged 67.

<u>Date.</u>	<u>Alk.</u>	<u>Acid.</u>	<u>IGI.</u>	<u>5-N.</u>	<u>ICD.</u>	
5. 4.61	114	9.1	300	16		Pre treatment
13. 4.61	128	6.8		26	10.5	
14. 4.61	114	6.8	440	26	4.7	
21. 4.61	IMPLANT					
21. 4.61	114	5.4	460	22	2.2	Grade B remission
22. 4.61	121	10.1	430	17	2.2	
24. 4.61	121	10.1	275	14	1.7	
29. 4.61	121	8.6	210	10	1.9	
1. 5.61	156	6.8	235	11		
4. 5.61	156	8.1	165	14	2.2	
8. 5.61	170	7.2	165	7		
10. 5.61	170	9.5	138	15		
29. 7.61		5.8	185			
23. 9.61	84	6.2	165	7		
28. 9.61	63	5.6	92	6		
9.12.61	71	4.5	74	10		

No. 7. E.T.

This patient was first seen in April, 1961, at which time she had extensive osteolytic secondaries to cancer of the breast. Her poor clinical condition was reflected by raised levels of serum acid and alkaline phosphatase, phosphoglucose isomerase, 5-nucleotidase and iso-citric dehydrogenase. These latter two enzymes indicated secondary growths in the liver which were not apparent from clinical observations. Seeds of yttrium 90 were implanted in her pituitary two weeks after admission and this resulted in a general improvement in her condition. The patient has remained in Grade B remission since the operation and the serum levels of all the enzymes have returned to normal. At this stage, the presence of metastases in the liver cannot be confirmed, but the biochemical indications for them are strong, since both 5-nucleotidase and iso-citric dehydrogenase were raised.

No. 8, I.G., aged 50.

<u>Date</u>	<u>Alk.</u>	<u>Acid.</u>	<u>FGI.</u>	<u>Alcal.</u>	
30.5.60	49	4.3	260	2.3	} Pre treatment
1.6.60	42	4.7	275	3.0	
3.6.60	IMPLANT				
9.6.60	42	5.2	315	3.0	} Grade D response
10.6.60	49	5.8	270	2.3	
20.6.60			165	1.5	
25.6.60	71	3.4	195	1.5	
14.7.60			195		
15.7.60	49	4.2	195		
2.8.60	78	4.0	190		
15.8.60	IMPLANT				
15.9.60	49	4.5	180		} Relapse
17.9.60	71	4.5	370		

No. 8, I.C.

This patient was first seen at the end of May, 1960. She was admitted with osteolytic and soft tissue metastases and a pituitary operation was carried out at the beginning of June. The only enzyme level to be raised was phosphoglucose isomerase, the normal acid and alkaline phosphatase failed to demonstrate the presence of osseous metastases which were obvious from X-ray examination. However, the raised phosphoglucose isomerase did indicate that the primary cancer had metastasised. The level of this enzyme fell as her clinical condition improved slightly and she went into Grade D remission. Serum aldolase levels remained normal throughout the investigations. The necessity of a second pituitary operation was indicated by a decline in the patient's clinical condition, and this was performed in August, 1960. The patient failed to respond and she died a month later. The last determination of serum enzymes showed an increase in phosphoglucose isomerase, but the phosphatases still remained within their normal ranges.

No. 9, R.G., aged 56.

<u>Date.</u>	<u>Alk.</u>	<u>Acid.</u>	<u>PGI.</u>	<u>5-N.</u>	
22.2.61	100	8.5	96	12	} Pre treatment
2.3.61	78	8.5	156	8	
3.3.61	78	8.0	100	8	
10.3.61	DIETARY				
10.3.61		9.4	124	9	} Grade D response
17.3.61	85	7.0	96	8	
27.3.61	106				
29.3.61	85	10.2	195	12	

No. 9, R.G.

This patient was first seen in February, 1961, and it was apparent from X-ray and clinical examination that she had osteolytic and soft tissue secondaries. Seeds of yttrium 90 were implanted in her pituitary in mid-March and there was some improvement in her clinical condition, although some new lesions did appear; thus she exhibited a Grade D type of response. Initially, the only serum enzyme to have consistently raised levels was acid phosphatase. Alkaline phosphatase levels were near the upper limit of normal and occasionally just above it. The level of phosphoglucose isomerase was slightly raised on one occasion before the operation, while 5-nucleotidase remained within its normal range, the latter enzyme providing biochemical confirmation of the clinical assumption of absence of liver secondaries. The patient died at the end of March from non-cancer causes.

No. 10, L.L., aged 57.

<u>Date.</u>	<u>Alk.</u>	<u>Acid.</u>	<u>PGI.</u>	<u>5-N.</u>	<u>IGD.</u>	
27. 4.61	107	5.2	210	15		
9. 5.61	135	5.0	124	5		
12. 5.61	IMPLANT					
12. 5.61	170	4.8	96	12		Pre treatment
13. 5.61	190	5.0	170	4		
15. 5.61	184	5.4	320	3	6.0	
17. 5.61	175	4.7	200	8	8.9	
19. 5.61	184	4.8	480	10	5.1	
23. 5.61		5.0	650	9	21.0	
3. 6.61	230	5.4	150	12		
26. 7.61	71	6.6	190	3		
11. 8.61	85		175	17		
14. 8.61	78	4.7	96	3		
9. 9.61	120	4.5	65	13		Grade D remission
14.10.61	100	6.5	55	6		

No. 10, L.L.

This patient was first seen at Hammersmith Hospital at the end of April, 1961, at which time she had osteoblastic secondaries and a large liver suggesting that the disease had spread to this organ. A pituitary operation was performed in mid-May. After this her clinical condition improved and she went into Grade D remission. On admission she had raised levels of serum alkaline phosphatase and phosphoglucose isomerase, acid phosphatase levels within normal limits, and 5-nucleotidase levels near the upper limit of normal. Fluctuations in the level of all the enzymes were observed during remission, but there was a general downward trend in the phosphoglucose isomerase and alkaline phosphatase. 5-Nucleotidase failed to confirm the presence of hepatic metastases. However, serum iso-citric dehydrogenase was raised and this did provide a biochemical indication of liver involvement. The patient is still in remission, so confirmation of these findings is impossible at the moment.

No. 11, A.B., aged 64.

<u>Date.</u>	<u>Alk.</u>	<u>Acid.</u>	<u>WBC.</u>	<u>5-N.</u>	<u>ICD.</u>	
28.10.60	49	3.1				}
31.10.60			305			
1.11.60		IMPLANT				
1.11.60	71		255			} Pre treatment
5.11.60	56	6.1	200			
8.11.60	56	6.1	175			
15.11.60			245			
16.11.60			230			
14. 1.61			185			} Grade D remission
23. 1.61	56		185			
22. 2.61	64	5.7	138	18		
2. 3.61	64	5.7	185	11	2.0	
3. 3.61	71	4.5	315	11		
6. 5.61	78	3.9	175	7	1.7	} Terminal relapse
10. 5.61		2.4	280	7		
early August		DIED				

No. 11, A.S.

This patient was admitted to Hammersmith Hospital at the end of October, 1960, with evidence of liver and soft tissue metastases to cancer of the breast. Seeds of yttrium 90 were implanted in the pituitary at the beginning of November, and a Grade D type of remission ensued. Initially, the only enzyme to be elevated was phosphoglucose isomerase, while the phosphatases remained normal throughout the study. The determination of 5-nucleotidase had not been introduced as a routine investigation at the time of the operation. The clinical improvement in her condition was reflected by a fall in the level of phosphoglucose isomerase; this rose again as her condition began to deteriorate in May. 5-Nucleotidase was first determined at the end of February, and this was just above normal, but the level fell to within the normal range and remained there even when the patient went into terminal relapse. A few determinations of the level of iso-citric dehydrogenase were made, but this enzyme failed to indicate liver involvement. The patient died early in August, 1961.

No. 12, I.S., aged 51.

<u>Date.</u>	<u>Alk.</u>	<u>Acid.</u>	<u>PGI.</u>	<u>5-N.</u>	<u>ICD.</u>	
2.12.60	128	6.8				} Pre treatment
5.12.60	135	7.2	180			
6.12.60	100	6.3	115			
8.12.60			135			
9.12.60		IMPLANT				
21.12.60	100		175			} Grade D remission
28. 1.61	220	6.0	110			
27. 2.61		6.3	160	2	2.2	
7. 3.61	178	7.1	125	5	2.4	} Relapse
8. 3.61	192	7.4	160	5	4.7	
22. 4.61	165	6.1	460	7	4.3	

No. 12, I.S.

This patient was first seen in December 1960, when she was admitted to Hammermith Hospital with osteoblastic and soft tissue metastases. The presence of bone lesions was indicated by abnormal levels of acid and alkaline phosphatases, and an initial raised phosphoglucose isomerase provided biochemical evidence of soft tissue metastases. A pituitary operation was performed, and she went into Grade D type of remission for three months. During this time there was a fall in acid phosphatase and a rise in alkaline phosphatase. There were no significant alterations in the level of phosphoglucose isomerase during remission, but the patient relapsed in March and by April the level of this enzyme was more than three times the upper limit of normal. Determinations of 5-nucleotidase and iso-citric dehydrogenase activity were started in February, and at this time they were both normal. There was a slight rise in the level of iso-citric dehydrogenase in March, but this was not at this stage accompanied by an increase in 5-nucleotidase.

The patient died in August at another hospital, and there was post-mortem confirmation of osteoblastic and soft tissue metastases. The disease had spread to the liver during the terminal phase, as had been indicated by serum levels of iso-citric dehydrogenase but not by the levels of 5-nucleotidase.

No. 13, D.S., aged 47.

<u>Date</u>	<u>Alk.</u>	<u>Acid.</u>	<u>FGI.</u>	<u>5-N.</u>	<u>ICD.</u>	
1.3.61	71	5.8	170	5		Pre treatment
8.3.61	71	6.1	200	14		
14.3.61	92	6.1	325	11		
17.3.61	IMPLANT					
18.3.61	107	6.8	420	30		Grade D remission
20.3.61	85	5.9	325	11		
22.3.61	71	5.4	270	12		
27.3.61	71	5.8	255	8		
29.3.61	78	11.2	270	5		
4.4.61	71	6.5	500	10		
7.4.61	78	6.3	310	6		
21.4.61	92	8.2	265	15	6.0	
24.4.61	85	7.2	285	17	3.8	
26.4.61	IMPLANT					
28.4.61	85	7.4	305	31	3.8	
29.4.61	100	6.7	570	30		
1.5.61	85	6.8	275	17		
4.5.61	78	5.9	260	14		
5.5.61	13	5.2	260	18		
8.5.61		4.3	280	13		
10.5.61	115	5.8	260	15		
16.5.61	115	6.3	380	15	3.1	
22.6.61	DIED					

No. 13. D.S.

This patient was first seen in March, 1961, at which time she had osteolytic and soft tissue metastases. Seeds of yttrium 90 were implanted in her pituitary in the middle of the same month, and this brought about some clinical improvement. At first, serum acid and alkaline phosphatase were normal and on only one occasion before the operation were they both raised. Phosphoglucose isomerase was above the upper limit of normal before the operation, and there was no significant drop in the serum level of this enzyme as the patient went into Grade D remission. 5-Nucleotidase levels fluctuated near the upper limit of normal before the pituitary operation. Her clinical condition indicated that a second implant might bring about a more lasting remission, so this was done at the end of April. At this time, phosphoglucose isomerase, acid phosphatase, 5-nucleotidase and iso-citric dehydrogenase were all raised, the latter two enzymes suggesting a spread of the disease to the liver, although the level of alkaline phosphatase was only near the upper limit of normal. There was a fall in the acid phosphatase levels after the second implant, and a rise in alkaline phosphatase, suggesting reparative osteoblastic activity rather than further liver involvement, since there was a slight fall in levels of 5-nucleotidase.

Her progress was not maintained and the patient died in June. There was no post-mortem, so it was impossible to confirm the presence of liver secondaries which were indicated by 5-nucleotidase and iso-citric dehydrogenase.

No. 14, D.C., aged 48.

<u>Date.</u>	<u>Alk.</u>	<u>Acid.</u>	<u>PGI.</u>	<u>5-N.</u>	
24.10.60	590				} Pre treatment
1.11.60	640	3.4	415	98	
3.11.60	550		395		
18.11.60	IMPLANT				
30.11.60	470		390	39	} Steady deterioration
5.12.60	540	11.5	275	58	
6.12.60	610		350	63	
22.12.60	1000	7.9	220		} Grade F response

No. 14, D.C.

This patient was first seen in November, 1960. At that time she has soft tissue and liver secondaries to primary cancer of the breast. On admission, the serum levels of alkaline phosphatase, 5-nucleotidase and phosphoglucose isomerase were elevated, while acid phosphatase was normal. This is the enzyme pattern which we would expect from a patient with this distribution of metastases. At the time of the operation, the patient was very breathless due to a chest infection, which may also have contributed to serum level of phosphoglucose isomerase. Yttrium 90 seeds were implanted in her pituitary in the middle of the month, but her condition did not improve and her response was graded as type F. Two weeks after the implant there was a slight fall in level of alkaline phosphatase and 5-nucleotidase, but these rose again as she continued to deteriorate clinically. There was a downward trend in levels of phosphoglucose isomerase, but this did not reach the normal range. Serum acid phosphatase rose above the upper limit of normal, a fact which is rather surprising, in view of the absence of osseous metastases. However, since the other phosphatases indicate that the liver involvement is quite considerable, this organ may also be the source of acid phosphatase. The patient died a few weeks after the last determination, and there was post-mortem confirmation of the clinical and biochemical findings of hepatic and soft tissue metastases.

No. 15, L.G., aged 58.

<u>Date</u>	<u>Alk.</u>	<u>Acid.</u>	<u>PGI</u>	<u>Aldol.</u>	
6.2.60			380	1.5	} Pre treatment
12.2.60			395		
16.2.60	63	3.8	340		
18.2.60	49	3.1	340	1.0	
26.2.60	IMPLANT				
2.3.60	49		370	0.4	} Grade F response
8.3.60	63	2.9	625	1.1	
11.3.60	56	4.1	520	3.4	
14.3.60	56		405	2.3	
19.3.60	49	4.8	360	2.3	
24.3.60	42	5.0	510	1.5	

No. 15. L.G.

This patient was admitted at the beginning of February, 1960, with soft tissue metastases to cancer of the breast, and an operation was performed on her pituitary gland at the end of the month. The treatment was unsuccessful, and the patient showed a Grade F type of response and died at the end of March. Throughout the study period, the only enzyme to be elevated was phosphoglucose isomerase. Although there were no metastases in the liver, this organ could have made a contribution to the serum level of this enzyme, since at post-mortem C.V.C. were found due to pulmonary obstruction. The normal levels of acid and alkaline phosphatase in this patient are in keeping with the absence of any bone metastases.

No. 16. M.G. aged 56.

<u>Date.</u>	<u>Alk.</u>	<u>Acid.</u>	<u>FGI.</u>	<u>5-N.</u>	<u>ICD.</u>	
3.2.61	415	12.6	195	9		Pre treatment
4.2.61	425					
7.2.61	470	22.0	185	12	2.9	
10.2.61	405	20.0	170	9	2.9	
15.2.61	390	22.0	147	4	3.5	
17.2.61	IMPLANT					
17.2.61	470	19.5	142	6	2.4	Grade F response
18.2.61	458	24.0	156	9	2.4	
22.2.61		17.6	132	5		
27.2.61	660	9.5	152	9	1.1	

No. 16. M.C.

This patient was first seen at the beginning of February, 1961. On admission she was found to have osteolytic, osteoblastic and soft tissue metastases. An unsuccessful implantation of yttrium 90 seeds was performed in the middle of the month. Her clinical condition continued to deteriorate, and she showed a Grade F type of response until she died at the beginning of March.

Initially, acid and alkaline phosphatase and phosphoglucose isomerase were all raised, and 5-nucleotidase and iso-citric dehydrogenase were normal. This pattern of enzyme levels is consistent with the presence of bone and soft tissue metastases and absence of any liver involvement. At post-mortem, the patient was found to have secondary growths in the adrenal glands and myocardium and bone, but her liver was quite normal, as was expected.

No. 17, T.M., aged 53.

<u>Date</u>	<u>Alk.</u>	<u>Acid.</u>	<u>FGI.</u>	<u>Alcal.</u>	
6.4.60	64	7.2	550	3.8	} Pre treatment
20.4.60	49	5.2	385	1.9	
21.4.60	49	6.1	460	2.3	
26.4.60	IMPLANT				
26.4.60	56	4.8	370	4.2	} Grade F response
3.5.60		5.2	570		
16.5.60	107	4.1	595	1.9	
23.5.60	135	3.9	530	2.3	

No. 17. T.M.

This patient was first seen in April, 1960, and was found to have soft tissue secondaries. A pituitary implant was done at the end of that month, at which time the only raised enzyme levels were phosphoglucose isomerase and aldolase. The operation did not benefit the patient, and her response was classed as Grade F. There was no significant alteration in level of phosphoglucose isomerase, although serum aldolase levels did return to the normal range. Acid phosphatase remained normal throughout the period, as would be expected from the absence of any bone deposits. Levels of alkaline phosphatase became abnormal at the end of the study period.

Although increases in the serum level of phosphoglucose isomerase might be expected from this patient, the values found were grossly elevated. Chronic pleural effusions of the lung were present, and this may have contributed to the total enzyme in serum. The patient died a few weeks after the last biochemical investigation.

No. 18, E.W., aged 58.

<u>Date</u>	<u>Alk.</u>	<u>Acid.</u>	<u>PGI.</u>	<u>Aldol.</u>	
9.12.59	64	3.1	166	1.3	Pre treatment
1. 2.60	78	5.6	240		
3. 2.60	93	6.1	240	3.4	
6. 2.60	IMPLANT				
9. 2.60	49	5.2	255	5.3	Grade F
12. 2.60	64	5.7	310	3.0	
18. 2.60	107	6.0	255	3.0	
23. 2.60	114	4.7	320		
4. 3.60	IMPLANT				
4. 3.60	200	3.4	370	5.3	response
11. 3.60	165	4.7	435	6.1	
12. 3.60	150				
16. 3.60	190	4.7	430		
19. 3.60	230	5.8	460	8.0	
30. 4.60	230	5.6	460	6.5	
9. 7.60	730	8.8	750	19	
11. 7.60	DIED				

No. 18, E.W.

This woman was first seen at the end of December, 1959, and it was apparent from X-ray and clinical examination that she had osteolytic and soft tissue lesions secondary to cancer of the breast. She was admitted to Hammersmith Hospital in February, 1960, and a pituitary implant was done. At the time of the operation, the only enzyme to be abnormally raised in the serum was phosphoglucose isomerase. The treatment failed to bring about any clinical improvement, and serum aldolase and alkaline phosphatase also became elevated. A second pituitary implant was made in March, but this was also without effect. Grade F response with a steady deterioration continued. This was reflected biochemically by a steady rise in serum enzyme levels and even acid phosphatase, which had previously been normal, was elevated two days before her death in July.

SUMMARY

Part I

1. Alkaline Phosphatase.

The distribution of alkaline phosphatase in human tissues, its purification, properties and methods for its determination are reviewed. Glucose-1-phosphate was investigated as a substrate for determination of its activity and a method involving its use is described. Kinetic studies were used to investigate the source of the alkaline phosphatase of blood serum, partially purified by starch gel electrophoresis and using β -naphthyl phosphate as substrate.

2. Acid Phosphatase.

The distribution of acid phosphatase, its properties and methods for determination of activity are reviewed. Details of the method used in these investigations are given, together with attempts at improving its sensitivity by extracting the final colour into a small volume of a suitable solvent. Colorimetric and spectrofluorimetric methods using α -naphthyl phosphate are also described.

3. 5-Nucleotidase.

The distribution of 5-nucleotidase, its properties and methods used for determination of activity are described. A new method for the determination of the enzyme in serum has been developed. Kinetic and inhibition studies have been carried out.

4. Glucose-6-Phosphatase.

The discovery and properties of glucose-6-phosphatase are reviewed. Experiments to investigate the presence of the enzyme in serum are described.

5. Phosphoglucose Isomerase.

The discovery and properties of phosphoglucose isomerase are described. Details of the method used for the determination of activity in serum and urine are given.

6. Aldolase.

The discovery, occurrence and properties of aldolase are described. Details of the method used for determination of activity in serum are given.

7. Iso-citric dehydrogenase.

A review of the discovery, distribution and methods of determining iso-citric dehydrogenase activity is given; followed by details of the method used in these investigations. Thermal stability studies on the enzyme from human heart and liver were carried out.

Part II.

8. Serum Alkaline Phosphatase in Disease.

Conditions which cause elevations in the serum level of alkaline phosphatase in bone and liver disease are described. The

results of kinetic studies with β -naphthyl phosphate as substrate to investigate the source of the serum increment seen in hyperphosphatasemia are given.

9. Serum Acid Phosphatase in Disease.

The clinical conditions which cause increases in the level of serum acid phosphatase are described.

10. 5-Nucleotidase in disease.

The conditions which cause elevations in the serum level of this enzyme are reviewed. This is followed by the results of our investigations.

11. Serum Iso-citric Dehydrogenase in Disease.

A review of the literature concerned with the elevation of the level of this enzyme in hepatocellular necrosis is given, followed by the correlation of our results with clinical status. The levels of iso-citric dehydrogenase and 5-nucleotidase in non-breast cancer patients were compared.

12. Serum Enzymes in Patients with Breast Cancer.

The levels of acid and alkaline phosphatase, 5-nucleotidase, iso-citric dehydrogenase, phosphoglucose isomerase and aldolase have been investigated in the serum of eighteen women with cancer of the breast treated by endocrine ablation. The condition of the patients has been assessed on clinical grounds and this has been compared with the biochemical findings. The detailed results on these individual patients are given in the appendix.

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