RAT-LIVER PHOSPHATASES.

A

T H E S I S

presented by

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Contents

GENERAL INTRODUCTION.

The earliest reference to phosphatase activity in plant or animal tissues was the report by Suzuki, Yoshimura and Takaishi (1907) that rice and wheat bran extracts decomposed phytin with the liberation of inorganic phosphate. Phytin was also shown to be split by extracts of calf-liver and blood, but not by muscle or kidney by McCollum and Hart (1908).

The first reference to the splitting of a phosphate monoester was that of Levene and Medigreceanu (1911) who showed that extracts of pancreas, liver, kidney, heart-muscle and intestinal mucosa hydrolysed nucleotides with the production of inorganic phosphate, kidney and intestinal mucosa appearing to be the most active sources of this enzymic activity.

Glycerophosphate, a substance which has come to be widely used in estimations of phosphatase activity, was first shown by Grosser and Husler (1912) to be hydrolysed by animal tissues, their results indicating that kidney, intestine and lung had considerable glycerphosphatase activity, liver much less and spleen only a trace of activity. Blood, skeletal and heart muscle were reported to have no activity. Plimmer (1913) confirmed these results for the phosphatase activity of extracts of intestinal mucosa,

kidney and lung but found liver to have only slight activity. He also found that phytic acid was attacked only very slowly, if at all, by extracts of liver and intestine and that the Harden and Young ester was attacked by intestinal extracts. Plimmer also demonstrated the hydrolysis of sodium ethyl phosphate, caseinogen and nucleic acid preparations with the liberation of inorganic phosphate by intestinal extracts. Neither sodium diethyl phosphate nor hydroxymethyl-phosphinic acid ($HO.CH_2PO_3H_2$), where the phosphate group was attached to a carbon atom, was attacked.

Although this early work was carried out with crude tissue preparations and sometimes impure substrates, with only crude pH control, the results indicated the existence in mammalian tissues of enzyme systems capable of hydrolysing many organic phosphate esters.

The splitting of guanylic acid, inosinic acid and pyrimidine nucleotides by extracts of kidney and intestinal mucosa was demonstrated by Levene and Medigreceanu (1911). Neuberg and his co-workers studied the action of kidney extracts on a number of phosphorus compounds. Sucrose phosphate (Neuberg and Behrens, 1926), diphenyl phosphate, diphenyl pyrophosate (Neuberg and Wagner, 1926), methyl-, methylpropylcarbinyl-, cetyl-, and cholesteryl-phosphate and di-o-cresyl- and di-m-cresyl-pyrophosphate (Neuberg and

Jacobsen, 1928) were all shown to be attacked. Kidney extracts were shown by Akaswa (1929) to hydrolyse acetonyl-, propyl-, isopropyl-, phenyl-, benzyl-, cresyl-, diethyl-, diglyceryl- and dicresyl-phosphate.

Jacobsen and Tapadinus (1931) however found no hydrolysis of menthyl- or dimenthyl phosphate by sheep- and rabbit-liver and kidney although bornyl phosphate was split.

It was realised at this time that enzymes existed which split monoesters of alcohols and phenols of the type $(R\text{-}O)O{:}P(OH)_2$, the nature of the R group influencing specificity. These enzymes can hydrolyse the phosphate radical from either a primary or secondary alcoholic or phenolic hydroxyl group. Phosphodiesterases are known which split esters of the type $(R\text{-}O)_2O{:}P(OH)$ and have been classified as distinct from the phosphomonoesterases in mammalian tissues by Roche and Latreille (1937). Triesters of orthophosphoric acid, although very labile in aqueous solution, appear to be immune from enzymatic attack (Roche, 1950). Also found in living tissues are the phosphoamidases and acylphosphatases which hydrolyse phosphate in combination with a primary amino group or acyl phosphatases respectively. Both of these enzymes can be differentiated from phospho-monoesterases. The present work is concerned only with the first class of enzymes - the phosphomonoesterases - and, in particular, those which are found in rat-liver.

METHODS OF DETERMINING PHOSPHATASE ACTIVITY.

In 1930 Kay introduced a method for the determination of phosphatase activity based on the liberation of inorganic phosphate from sodium /β-glycerophosphate by enzyme preparations. This method was improved by Bodansky (1933) who proposed a simple and rapid method for serum phosphatase. Although this technique was designed for alkaline phosphatase activity, it can be applied with equal accuracy to estimations of phosphatases optimally active at more acid pHs. The method can also be employed using many other phosphate esters as substrates.

The chief disadvantage of the method lies in the fact that its accuracy depends on the sensitivity of the technique used to estimate inorganic phosphate. In crude enzyme preparations, the amount of inorganic phosphate initially present in the tissue may be considerable, giving rise to high blank values. More recently methods have been introduced, however, where the substrate employed is an ester of phosphoric acid with a chromogenic alcohol molecule. The liberation of the latter can be followed by highly sensitive and specific colour reactions.

King and Armstrong (1934) devised a method using phenyl phosphate, the liberated phenol being estimated by the Folin-Ciocalteu method. A similar method utilising

tyrosine phosphate was devised by Binkley, Shank and Hoagland (1944).

The use of phenolphthalein phosphate as a substrate in phosphatase estimations was introduced by Huggins and Talalay (1945). In this method the colourless ester is converted to free phenolphthalein which is pink, the colour being intensified, stabilised and the enzyme inhibited by the addition of an alkaline buffer containing an inhibitor. The use of this substrate also enables a direct and continuous study of alkaline phosphatase kinetics to be made. With this substrate it was found, however, that although the amount of phosphate liberated was proportional to the enzyme concentration, the relation between phenolphthalein liberated and the enzyme concentration was not strictly linear.

In 1946 Bessey, Lowry and Brock developed a method using p-nitrophenyl phosphate, a substrate which had previously been used by Ohmori (1937) and Fujita (1939). p-nitrophenyl phosphate itself is colourless but on hydrolysis the yellow salt of p-nitrophenol is obtained. This substance had an absorption maximum at 400 mμ. A micromethod for estimating the phosphatase activity in 5 μl. of serum was described. Unfortunately p-nitrophenol has been shown to inhibit certain alkaline phosphatases (Green and Meyerhof, 1952). Hudson, Brendler and Scott (1947) introduced some modifications in the technique and adapted the method for

assay at acid pHs.

A method employing /β -naphthyl phosphate as substrate was introduced by Seligman, Chauncey, Nachlas, Mannheimer and Ravin (1951) for serum acid and alkaline phosphatases. The /β -naphthol liberated during the reaction is coupled with tetrazotised diorthoanisidine to give an insoluble purple dye which is dissolved in ethyl acetate for colorimetric estimation.

HISTOCHEMICAL METHODS OF DEMONSTRATING PHOSPHATASE ACTIVITY.

Gomori (1939) and Takamatsu (1939) independently devised a method employing /β -glycerophosphate as substrate. In this method the fresh tissue is fixed in cold acetone, treated with absolute alcohol and benzene and embedded in paraffin wax at 56°. Sections are incubated on microscope slides at pH 9.4 in a buffer containing substrate and Mg^{++} and Ca^{++} ions, Ca^{++} ions precipitating the liberated phosphate. The slide is immersed in cobalt nitrate solution and the calcium phosphate is converted to cobalt phosphate. This latter compound is in turn converted by ammonium sulphide to black cobalt sulphide which is located microscopically. Gomori (1943) introduced a variation whereby the calcium phosphate deposit was coloured with acridine red.

This method has been severely criticised by many workers. Danielli (1946) found that cell nuclei adsorbed calcium phosphate from solution and also that nuclei which stained strongly with this method were not stained by other histochemical methods using azo dyes. The inaccuracy of the method was further demonstrated by Martin and Jacoby (1949), who demonstrated that there was a diffusion of enzyme and/or calcium phosphate from regions of high activity. Their work was confirmed by Novikoff (1951), who showed that liver cells and Drosophila salivary gland cells, where alkaline phosphatase had been inactivated, showed a negative Gomori reaction, but if either soluble alkaline phosphatase or hydrogen peroxide were included in the incubation medium, a preferential staining of the nuclei of liver cells and chromosome bands of salivary glands was observed.

In 1951 Gomori himself criticised his method and concluded that positive staining of nuclei could not be taken to mean that they possessed alkaline phosphatase activity. The method cannot be entirely discarded, however, for in cases such as the brush border of the kidney proximal tubules and the striated border of the intestine a strongly positive reaction is obtained rapidly, which cannot be explained by adsorption phenomena (Novikoff, 1952).

This method has been adapted for other substrates and

for investigations on acid phosphatase. In the latter case, Pb^{++} ions are substituted for Ca^{++} ions.

Other techniques consist in incubating the fixed section of tissue with α - or β -naphthyl phosphate, a diazonium compound incorporated in the medium couples with the liberated naphthol to form an insoluble dye. This is assumed to precipitate with little diffusion and minimise misleading adsorption effects. Several diazonium compounds have been used, for example, Mannheimer and Seligman (1948) employed diazotised α -naphthylamine, while Gomori (1951) used diazotised 4-benzoylamino-2,5-dimethoxyanisole. Novikoff (1952) criticised this technique on the grounds that, due to the instability of the diazonium compounds, the incubation has to be carried out at temperatures below 20°, which are well below the temperature of optimal activity; even at these low temperatures there is still a rapid deterioration of the diazonium compound. In addition, diazonium compounds have been reported as inhibiting certain alkaline phosphatases (Gomori, 1951; Loveless and Danielli, 1949).

Loveless and Danielli (1949) have modified this method by using a phosphorylated azo dye which precipitates on dephosphorylation.

Acid and Alkaline Phosphatases.

When the influence of pH on the phosphatase activity of tissue extracts was first studied, pH-activity curves were obtained showing two or three peaks of maximum activity. Davies (1934) found that liver and spleen showed two such maxima and suggested that these tissues contained two separate and distinct phosphatases; one of which was identical with the enzyme found in bone and the other, which was optimally active around pH 5-6, showed properties which differentiated it from the enzymes of bone and red cell. The work of Bamaan and Riedel (1934), Belfanti, Contardi and Ercoli (1935) and Munemara (1933) also indicated the multiple nature of the phosphatases. Bamaan and Diederichs (1934 & 1935) put forward the isodynamic theory of phosphatases in which it was proposed that enzymes exist with the same substrate specificity but differing in their pH optima, activators and inhibitors. It soon became obvious that phosphatases could be divided into two groups depending on the pH of their optimum activity. Those optimally active at pHs 8-10 were termed "alkaline" phosphatases, while those optimally active at pHs below 7.0 were called "acid" phosphatases. Certain tissues such as bone, intestinal mucosa and renal cortex were found to have a high alkaline phosphatase content whereas others, such as liver and prostate, were shown to have high acid phosphatase activity. As more

purified preparations became available the separate identity of the two types of enzyme was proved beyond doubt. A closer examination of acid phosphatase activity, largely the work of Folley and Kay (1936) and Roche and Courtois (1943), indicated that three distinct types of this enzyme were recognisable in animal and plant tissues. The following table is an attempt by Roche (1950) to summarise the present state of knowledge of the phosphatases (Table I).

It should be mentioned here that there is evidence for the existence of two alkaline phosphatases, a matter which will be discussed in a later section.

THE PURIFICATION AND PROPERTIES OF ALKALINE PHOSPHATASES.

A. Purification Studies.

Earlier workers in this field encountered difficulties in the extraction of alkaline phosphatase from tissues (Bamaan and Salzer, 1937) and this fact may be partly explained by the finding of Gold and Gould (1951) that collagen fibres strongly bind alkaline phosphatase and also by the finding that the enzyme is associated with the microsomal fraction in guinea-pig and rabbit intestine (Hers, Berthet, Berthet and De Duve, 1951). Three methods have been introduced to overcome this difficulty.

TABLE I

A SUMMARY OF THE PROPERTIES OF PHOSPHATASES

(after Roche (1950)).

Type	Optimal pH	Chief Sources	Chief characteristics in cell or tissue extracts
I	8.6-9.4	Bone, kidney, intestinal mucosa, mammary gland	Activation by Mg^{++}; inhibition by -SH; more active on β- than α-glyceroPO_4; optimal stability at pH 7.5-8.5
II	5.0-5.5	Liver, seeds fungi and prostate	No action of Mg^{++}; inhibition by F^-; more active on β- than on α-glycerophosphates; optimal stability at pH 4.5-5.5
III	3.4-4.2	Liver, top yeasts	Inhibition by Mg^{++}; more active on β- than on α-glyceroPO_4; optimal stability at pH 4.5-5.5
IV	5.0-6.0	Red blood cells and bottom yeasts	Activation by Mg^{++}; more active on α- than on β-glyceroPO_4; optimal stability at pH 6.5-7.5

Albers and Albers (1935) used an autolysis of the minced tissue for 48 hours in 50% ethanol containing 5% of a mixture of equal parts of ethyl acetate and toluene to release the enzyme. Abul-Fadl and King, Roche and Nguyen-van-Thoai (1949) autolysed the tissue, in this case ox-kidney, for 2-3 days with an equal amount of 25% aqueous acetone containing 10% of a mixture of equal parts of toluene and ethyl acetate.

Trypsin was found by Ehrenward (1933) to destroy contaminating proteins without affecting alkaline phosphatase and a preliminary tryptic digest was used with success by Schmidt and Thannhauser (1945) in the purification of intestinal phosphatase and also by Abul-Fadl and King (1949a) in the purification of faecal alkaline phosphatase. The value of this method was critically studied by this latter group of workers (Abul-Fadl and King, 1949b).

The third method, introduced by Morton (1950) for the purification of intestinal alkaline phosphatase, involved the treatment of a mucosal extract with 2/5 of its volume of n-butanol for five minutes at 32°; the aqueous layer, after centrifugation, contained 50% of the original phosphatase activity.

Further purification of the enzyme has been effected by alcohol or acetone precipitation from aqueous solutions or from solutions saturated with magnesium acetate (Caputto and Marsal, 1941). Roche, Nguyen-van-Thoai and Roger (1947) found that most of the alkaline phosphatase activity of an autolysed extract of intestinal mucosa was precipitated at -5° by 55% acetone. On redissolving the precipitate in water and fractionally precipitating with acetone, the fraction between 35 and 40% acetone contained most of the activity and was relatively free from protein impurities. Ethanol fractionation was introduced by Albers and Albers (1935) for the purification of kidney alkaline phosphatase and Caputto and Marsal (1941) utilised this procedure for the purification of mammary gland phosphatase. Cohn, Surgenor and Hunter (1951) studied the solubility of various protein constituents in liver, in solutions of varying ethanol concentrations and ionic strength and found that a fraction rich in acid and alkaline phosphatase was precipitated between 0.032 and 0.066 mole fraction ethanol at -5°.

Ammonium sulphate fractionation has also been employed by some workers. Perlmann and Ferry (1942) claimed a separation of kidney acid and alkaline phosphatase with ammonium sulphate solutions at pH 6.3. No such separation was achieved by Sarles (1947), however, when he applied this

technique to dog-and ox-liver and kidney. In their purification of intestinal phosphatase Schmidt and Thannhauser (1943) also utilised an ammonium sulphate precipitation.

Adsorbents such as aluminium hydroxide gel, which was used by Schmidt and Thannhauser (1943) and others for the adsorption of protein impurities, and calcium phosphate gel, which has been used to adsorb the enzyme (Morton, 1953) have proved useful in purification studies.

Preparations of the phosphatase of intestinal mucosa are the purest which so far have been obtained. Nguyen-van-Thoai, Roche and Roger (1947) obtained a preparation from this source with a Q_p (number of cu. mm. of H_3PO_4 liberated per mg. of tissue per hour under standard conditions of temperature, pH, etc.) of 210,000.

In 1947 Sarles reported a partial purification of the alkaline phosphatase of liver. The autolysate was precipitated at 60% acetone, the precipitate redissolved in water or 0.5 M NaCl solution and the solution fractionated with acetone when it was found that the fraction precipitating between 42 and 50% had most of the enzyme optimally active at pH 9.2. The product was dissolved in 25% ethanol, precipitated at 0° with an equal volume of acetone and partially dissolved in a saturated solution of magnesium acetate.

Finally, the enzyme was precipitated from this solution with ethanol. By this procedure an acetone fraction of activity 199 µg.P/mg.N/min. was purified to give 1000 µg.P/mg.N/min. at pH 9.2 and 11.2 µg.P/mg.N/min. at pH 5.1.

B. Properties of Alkaline Phosphatase.

When considering the general properties of alkaline phosphatase the following question has to be borne in mind: Are the alkaline phosphatase activities of different organs due to the same enzyme?

Belfanti, Contardi and Ercoli (1935) put forward the view that two different types of alkaline phosphatase existed in animal tissue, the enzymes in kidney and liver appearing to be similar in their behaviour towards oxalates and fluorides but differing from those of bone and serum. Bile salts were shown by Bodansky (1937) to inhibit the alkaline phosphatase activity of bone and kidney, while that of intestinal mucosa was not affected. This worker (Bodansky, 1948) also showed that the basic amino acids, L-histidine and L-lysine, exerted a greater inhibitory effect on the enzyme from bone and kidney than on that from the intestinal mucosa. Glutamic acid, on the other hand, was shown to be about three times more inhibitory to the mucosal phosphatase than to the phosphatases of other tissues. Zittle and Dellamonica (1950),

working with purified bovine milk and intestinal alkaline phosphatases, showed differences in the degree of inhibition by tetraborate, phosphate, pyrophosphate, arsenate and ethanolamine.

Cloetens (1939a) in an investigation of this problem examined the effect of Mg^{++} and CN^{-} ions on the alkaline phosphatases of rat tissues. From his results he concluded that there were two types of alkaline phosphatase:

a) Type I, which was inactive in the absence of Mg^{++} ions and which was not influenced by KCN,

b) Type II, which was influenced in a variable manner by Mg^{++} and strongly inhibited by KCN.

He believed that these two enzymes exist in different proportions in different organs, type I being especially abundant in liver. He claimed (Cloetens, 1939b) the isolation of a preparation of this enzyme free from type II from hog-liver. His method of fractionation was based on the greater lability of the type II enzyme in more acid media and on the greater lability of acid phosphatase in alkaline media. Although his work received little acceptance at first, it has recently received some confirmation from the results of Rosenthal, Fahl and Vars (1952) on a study of the response of alkaline phosphatase of rat-liver to dietary protein depletion. Those workers found that after two weeks of protein starvation, the

alkaline phosphatase content of rat-liver increased by 28%, while 43% of the hepatic protein was lost. This enzyme increase was accompanied by an increased sensitivity to cyanide, diminished stimulation by Mg^{++}, and a higher optimum pH.

Roche and Sarles (1948) determined the K_m values for the alkaline phosphatases of dog-liver, dog-intestinal mucosa, bone (human foetus at term and sheep), and ox- and dog-kidney, using partially purified preparations. The following values for the affinity of each enzyme for sodium β-glycerophosphate at pH 9.2 were obtained:

Source	Affinity (I/K_m)
Dog-liver	500
Intestinal mucosa	80
Bone	830
Ox- kidney) Dog-kidney)	360

It has been claimed that an estimation of the affinity constant of plasma alkaline phosphatase in cases of hyperphosphatasaemia can indicate the source of the excess alkaline phosphatase.

Abul-Fadl and King (1949c), in work with purified preparations, reported a difference in the behaviour of alkaline phosphatases of faeces, intestine and liver on the

one hand, and kidney on the other during electrodialysis. The kidney enzyme appeared to be more stable and to be more strongly activated by Mg^{++} ions, whereas the other enzymes were sensitive to anions and inactivated by the anode liquid.

At present, therefore, no clear classification of alkaline phosphatases is possible and the necessity of obtaining still more highly purified preparations and of studying the properties of these is apparent.

Alkaline phosphatases, in general, are characterised by high pH optima which lie between pH 9.2 and 9.6 for the purified enzymes. Since pH values in living cell structures have been estimated at 6.9 for the cytoplasm and 7.6 for the nucleus (Chambers, 1929; Chambers, 1950; Dubin and Yen, 1950) the significance of this high pH optima has been an enigma. At pHs below 8.0 the activity of alkaline phosphatase was found to be negligible and at high pHs the enzyme was shown to be inactivated (Roche, 1950). However, two lines of investigation have indicated possible explanations for this paradox.

King and his co-workers have studied the pH optima for the hydrolysis of a series of substrates (King and Delory, 1939; Delory and King, 1943; Walker and King, 1950) with a preparation of faecal alkaline phosphatase prepared according

to Armstrong (1935). Decreasing values of optimal pH were associated with an increasing pK of the substrates and increased values of K_m and the following theory was proposed to explain their findings. The enzyme has basic properties which enable it to combine with the substrate and the affinity of the enzyme for the substrate increases with decreasing pK of the substrate. The greater this affinity, i.e., the smaller the Michaelis constant, the greater is the degree of protection given to the enzyme against the inactivation of hydroxyl ions and, in consequence, the higher the optimum pH.

The second group of experiments of interest in this connection were those carried out by Ross, Ely and Archer (1951). They showed that the optimal pH of a preparation of intestinal alkaline phosphatase varied with the concentration of substrate for a number of different substrates. The lower the substrate concentration, the lower the optimum pH and at concentrations of substrate such as would normally be found in the cell the authors claimed that the pH fell to between pH 7.5 - 8.0.

Certain divalent cations have been shown to be important activators of alkaline phosphatase. Erdtmann (1928) found that Mg^{++} ions activated the enzyme in kidney and Mn^{++} ions have also been shown to activate, although in

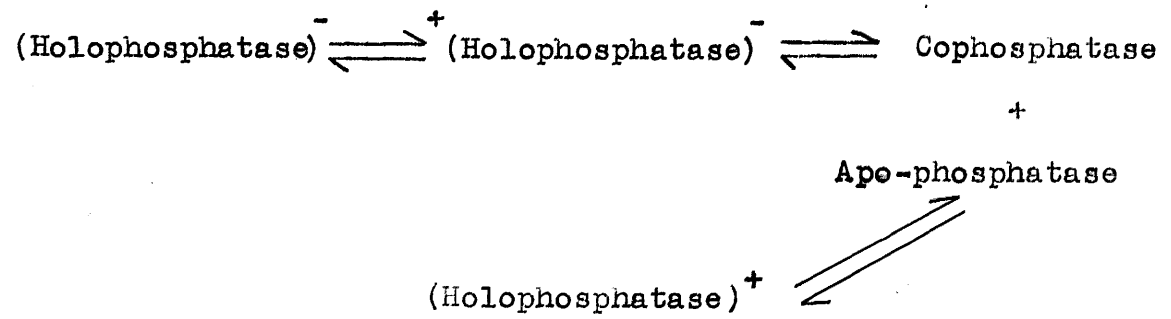
this case the effect is more irregular (Albers, 1940). Zn^{++} ions also enhance the activity of this enzyme and since with some tissues the activation was very feeble it was suggested that this ion was specific for certain phosphatases. Roche, Bouchilloux and Roger (1948) analysed various phosphatase preparations during purification for their content of magnesium and zinc. All preparations contained both metals but no parallel was observed between activity and content of either metal. The authors concluded that their preparations were not pure enough to permit any conclusions as to the essential role of either metal. Co^{++}, Ni^{++}, Fe^{++}, and Ca^{++} ions have also been shown to activate alkaline phosphatase under certain conditions (Bamaan and Heumüller, 1940; Roche and Nguyen-van-Thoai, 1943; Roche, Nguyen-van-Thoai and Durand, 1943). As purification proceeded, an increased sensitivity of the enzyme towards divalent cations and a greater regularity in the activation produced was observed by Bamaan, Riedel and Diederichs (1934). Roche and Bouchilloux (1948) found that the type of substrate employed was significant when examining divalent cationic activation and they found that purified intestinal phosphatase was much more strongly activated by Mg^{++} or Mn^{++} ions when hydrolysing the glycerophosphates than when hydrolysing phosphoryl-choline or phosphorylcolamine. Although divalent cations

may be interchanged as activating agents each ion exhibits its optimal effectiveness at a particular concentration (Nguyen-van-Thoai, Roche and Roger, 1947).

The role of Mg^{++} ions in the action of alkaline phosphatase is still far from clear, various theories having been proposed. Erdtmann (1928), Holmberg (1935) and Cloetens (1939) suggested that the metal ion protected the enzyme from the phosphate liberated in the course of the reaction. The view that Mg^{++} was concerned in the formation of an active enzyme - Mg complex was put forward by Jeener and Kay (1931), whereas Bauer (1937) postulated that activation of the substrate, S, occurred by combination with Mg^{++}, and the compound - SMg^{++} formed an easily dissociable compound $^{+}E\ Mg\ S^{-}$ with the enzyme, E. Roche and Nguyen-van-Thoai (1942) did find that intestinal phosphatase at pH 9.2 showed an increase in its affinity for the substrate when the medium is enriched with Mg^{++} ions. Finally there is some evidence to suggest that the metal ion is involved with a coenzyme of alkaline phosphatase, a matter which is worthy of further study.

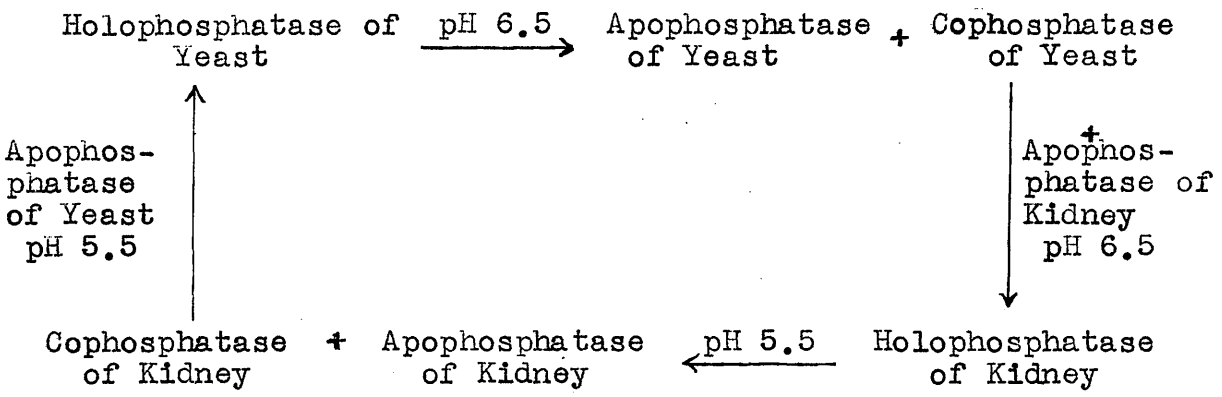
Erdtmann (1928) studied the effect of dialysis on kidney alkalaine phosphatase and found that the dialysate activated the dialysed enzyme in the presence of Mg^{++} ions more strongly than did an ash prepared from the dialysate.

The work of H. Albers (Albers, 1936; Albers, Beyer, Bohnenkamp and Müller, 1938) showed that kidney alkaline phosphatase was irreversibly denatured above pH 9.0 and reversibly denatured at pHs from 6.0 - 3.0. If the enzyme were dialysed against buffer solutions at pH 3.0 - 6.0 the recombination of diffusible and non-diffusible fractions restored the activity. Albers (1936) put forward the following hypothesis in an attempt to explain the pH-stability and dialysis effects. He suggested that the enzyme consisted of a protein apo-enzyme and a dialysable cophosphatase. At pHs above 9.0 the enzyme formed an inactive cationic form and at pHs between 6.0 and 3.0 the enzyme dissociated into the cophosphatase component and an apo-phosphatase protein:-



Albers, Beyer, Bohnenkamp and Meuller (1938) made the interesting observation that it was possible to reconstitute a kidney alkaline phosphatase preparation which had been inactivated by dialysis at pH 5.5 by adding to its inactive protein the inactive dialysate obtained from dialys-

ing type III acid phosphatase from top yeasts at pH 6.5. The inactive protein of the type III enzyme could in turn be reactivated by inactive dialysate from the kidney enzyme. Their theory of "Co-phosphatase transport" is summarised in the following diagram:



A similar cycle has also been demonstrated in the case of alkaline phosphatase and the type II acid phosphatase of liver (Ruffo, 1944; Roche, Nguyen-van-Thoai and Michel-Lila, 1944).

The nature of this cophosphatase is at present unknown. von Euler and his co-workers have shown that the active factor in the dialysate at pHs 3.0 - 6.0 can be replaced by a boiled enzyme solution or by products of dialysis which had been adsorbed on alumina (Ek, von Euler and Hahn, 1948; von Euler, and Fono, 1947). In all cases the reactivation took place slowly and only if pH had been readjusted to between pH 7 - 8.

In 1951 Tamayo and Municio claimed the isolation from dialysis liquid of a substance which had a coenzymic effect on kidney phosphatase. The analytical data and spectral analysis suggested that the active substance was a derivative of adenosine.

Apart from this observation there is no positive evidence at present to suggest that cophosphatase is a specific compound. On the other hand, however, Hove, Elvehjem and Hart (1940) showed that certain amino acids increased the activating effect of divalent cations to a significant degree. This led Abul-Fadl and King (1949c) to study the effect of electrodialysis on the activity of kidney alkaline phosphatase and it was found that a substance with strong reactivating properties migrated towards the cathode. The anode liquid had no effect on the electrodialysed enzyme and Mg^{++} ions alone produced only a partial reactivation. The nature of the reactivating substance was not determined but protein appeared absent and from analysis the presence of a peptide or amino acid was suggested. Some amino acids (excluding cysteine and cystine) were found to reactivate electrodialysed kidney and faecal alkaline phosphatase and this activating effect was considerably increased by Mg^{++} ions.

Roche, Nguyen-van-Thoai and Roger (1947) also studied the effect of amino acids and divalent metal ions on alkaline

phosphatase and found that the divalent cations only exert their full effect in the presence of amino acids. Purified dog intestinal phosphatase with an initial Q_p of 23,000 was totally inhibited by 15 - 20 days' dialysis at pH 5.8 and at 37°. On incubation for two hours at pH 8.8 a partial reactivation was observed (Q_p = 3,000), but if the incubation were carried out in the presence of Mg^{++} or Mn^{++} ions a more significant reactivation was observed (Q_p = 11,000 in presence of Mg^{++}). When the inactive enzyme was incubated in the presence of alanine alone a Q_p of 40,000 was found, but in the case of incubation with alanine in the presence of Mg^{++} or Mn^{++} a further marked activation was observed (Q_p = 170,000 - 180,000).

Roche and Nguyen-van-Thoai (1950) drew attention to the facts that amino acid must be incubated with the dialysed enzyme since its addition to the medium after the metal and before the substrate is without effect, that the addition of the metal after the amino acid has been incubated with the protein still produces activation and that the reactivation was less efficient if fluorides, phosphates or pyrophosphates were present. It has been found, however, that after reactivation with alanine and Mg^{++} ions, fluoride and pyrophosphate are not inhibitors and phosphate only inhibits to the extent it did in the original enzyme. Roche compares

the necessity for a preliminary incubation with the amino acid with the fact that a time factor is involved in the reactivation of apophosphatase with a cophosphatase as above. It would appear therefore, that if the cophosphatase is not a metal-amino acid complex, such a complex is a good substitute for the cophosphatase. It must be borne in mind that Mg^{++} ions have little effect on acid phosphatases and, in some cases, may even act as inhibitors, while the cophosphatase of the alkaline enzyme can activate the apo-enzyme of types II and III phosphatase.

Many metal forming complexes have been shown to inhibit alkaline phosphatase, for example, phosphate and arsenate as shown by Roche and Nguyen-van-Thoai (1943), oxalate (Belfanti, Contardi and Ercoli, 1935a, b & c) and fluorides (Massart and Dufait, 1942). Inhibition by sodium diethyldithiocarbamate, phenanthroline, L-$\alpha\alpha'$-dipyridyl and tungstates by Cloetens (1941a & b) and Roche and Durrand (1943), while cyanides, cysteine and glutathione were found to be inhibitory, although at low concentrations a slight activation has been observed. Eldbacher and Kütscher (1932) proposed that in this last case activation was due to blockage of heavy metal inhibitors, while inhibition was due to a combination with the constituent metal of the enzyme. Although amino acids at low concentrations cause increased

activation of alkaline phosphatase in the presence of divalent cations, they cause inhibition at higher concentrations (Bakwin and Bodansky, 1933; Bodansky, 1936a, 1936b, 1937, 1946 and 1948).

Gould (1944) showed that formaldehyde, ketene and phenyl isocyanate, substances which combine with $-NH_2$ groups, caused inhibition of alkaline phosphatase, which suggested a primary amino group may be involved in the enzymic reaction.

Roche, Danzas and Nguyen-van-Thoai (1944) found that iodides and monoiodoacetic acid were without effect on the enzyme and Sizer (1942) showed that various oxidising and reducing substances over a large pH range caused no significant change in activity, which suggests that -SH groups play no significant part in phosphatase activity.

Schmidt and Thannhauser (1943) when using purified intestinal phosphatase found that the alcohol moieties formed by phosphatase action had no effect on enzyme activity, whereas the phosphate ion always caused a marked inhibition which was partly competitive in nature. These facts suggest that phosphatases may combine with the substrate ions through the phosphate group.

Cohn (1949) studied the point of attack of the enzyme, intestinal alkaline phosphatase, on glucose-1-phosphate using a reaction mixture containing $H_2{}^{18}O$ and from the ^{18}O content

of the phosphoric acid formed she found a cleavage of the O - P bond on enzymic action which differs from the acid catalysed reaction where splitting was between the C and O atoms:-

alkaline phosphatase hydrolysis

acid hydrolysis

In view of the considerable speculation concerning the action of alkaline phosphatase mention should be made of the experiments of Bamaan (1939) and Bamaan and Meisenheimer (1938), who showed that colloidal hydroxides of many metals split glycerophosphates in a weakly alkaline medium with an optimal pH around 9.0. The hydroxides of many rare earths were the most active; those of manganese, iron and alkaline earths the least. In 1948 Bamaan and Nowotny showed that lanthanum β-glycerophosphate in aqueous suspension hydrolysed spontaneously at pH 9.5 at 37°, conditions where the glycerophosphates of the alkali earth metals are stable.

THE ACID PHOSPHATASES.

The purification and characterisation of the non-specific phosphatases optimally active at acid pHs is less complete than that of the alkaline phosphatases.

Three different acid phosphatases have been recognised

as occurring in animal tissues.

Phosphomonoesterase type II which is characterised by having a pH optimum between 5.0 and 6.0 is found in higher plants, fungi, mammalian spleen and liver and in human prostate. Most of the information concerning this enzyme has been obtained from studies on the prostate enzyme. Electrodialysis, adsorption and precipitation by neutral salts have been employed in the purification of this enzyme by Kutscher (1935) and Kutscher and Pany (1938). Bamaan and Diederichs (1934, 1935) showed that this enzyme is not influenced by Mg^{++} and other divalent cations, sulphydryl compounds and alkali cyanides. Fluoride ions, even at low concentrations (Roche, Nguyen-van-Thoai and Danzas, 1944), and molybdate ions (Courtois and Bossard, 1944) were found to be inhibitory.

The type III phosphatase which is optimally active at pH 3.4 - 4.2 appears to be present in spleen and liver (Nguyen-van-Thoai, 1941; Bamaan and Salzer, 1937; Abul-Fadl and King, 1949d), but this enzyme is very labile and no purification of it from mammalian sources has as yet been achieved.

There are claims for the existence of a type IV acid phosphatase which is optimally active at pHs 5.5 - 6.2. This enzyme differs from the other acid phosphatases in being

strongly activated by Mg^{++} and Mn^{++} ions and being more active towards α-glycerophosphate than towards β-glycerophosphate. It appears to be present in mammalian tissues in red blood cells (Abul-Fadl and King, 1949d).

It has already been mentioned that both type II and type III phosphatases can be separated into apophosphatase and cophosphatase components and the cophosphatase of alkaline phosphatase can apparently be replaced by that of either type II or type III enzyme and vice versa.

THE SYNTHETIC AND PHOSPHOTRANSFERASE ACTIVITIES OF PHOSPHOMONOESTERASES.

Plant extracts incubated with inorganic phosphate and alcohol were found to cause a disappearance of inorganic phosphate (Bodnar, 1925). A similar phenomenon was observed in animal tissues by Martland and Robison (1925). These workers showed that glycerol, glycol and mannitol in fairly high concentrations caused a decrease in the inorganic phosphate content of the medium when incubated in the presence of bone phosphatase. Kay (1926) also found an uptake of inorganic phosphate by methanol, ethylene glycol and glycerol which was catalysed by kidney or intestinal phosphatase and this worker isolated the barium salts of the monophosphoric esters of glycerol and glycol from the incubation mixture (Kay, 1928a & b).

An interesting property of the phosphomonoesterases which has recently been discovered is their ability to catalyse the transfer of phosphate groups from compounds having a high free energy of hydrolysis to alcohols such as glycerol, glucose, fructose, etc., where the phosphate compound has a lower free energy of hydrolysis.

Appleyard (1948) studied the effect of various alcohols on the hydrolysis of phenolphthalein phosphate by prostate extracts at pH 5.2 and found that methyl, ethyl, n-propyl and iso-propyl alcohols and ethylene glycol caused an increase in the liberation of free phenolphthalein. Free inorganic phosphate liberation showed, however, no change in the presence of methyl, ethyl and n-propyl alcohols, an increase in the presence of iso-propyl alcohol and a decrease in the presence of ethylene glycol. The phosphate arising from hydrolysis of phenolphthalein phosphate could not be accounted for entirely as inorganic phosphate and the formation of a stable phosphate ester was suggested.

In 1948 Axelrod showed the formation of methanol phosphate from p-nitrophenyl phosphate in the presence of navel orange juice phosphatase. When ^{32}P-labelled p-nitrophenyl phosphate was used the methanol phosphate formed was radioactive, but when unlabelled p-nitrophenyl phosphate was used and the reaction carried out in the presence of

labelled inorganic phosphate the methanol phosphate was unlabelled. This indicated a direct transfer of phosphate catalysed by phosphatase without passing through the stage of free inorganic phosphate.

Meyerhof and Green (1949) demonstrated transphosphorylation by intestinal alkaline phosphatase in the absence of nucleotides from ^{32}P labelled phosphocreatine to glycerol. Similar results were found when the glycerol was replaced by fructose. Compounds such as phosphopyruvate and glucose-1-phosphate which have a relatively high free energy of hydrolysis could also participate as phosphate donors in this direct phosphate transfer. Green and Meyerhof (1950) further showed that phosphopyruvate and nitrophenylphosphate could serve as phosphate donors in the transferase system and also that acid phosphatase could catalyse the reaction.

Recently Green and Meyerhof (1952) studied the kinetics of the transphosphorylation reaction with acid semen phosphatase and intestinal alkaline phosphatase and found the highest rates of transfer were given with acetyl phosphate and lowest with phosphopyruvate. Semen phosphatase catalysed a higher rate of transference than intestinal phosphatase. Adenosine triphosphate (ATP) was shown to participate in transphosphorylation to glycerol in the presence of alkaline phosphatase to a smaller extent than did the other phosphate

donors and was found to be an even less effective donor in the presence of semen phosphatase. The rate of transphosphorylation did not appear to be solely dependent upon the free energy of hydrolysis of the phosphate donor and the authors suggested that the relative affinities of the donors for the enzyme and the activity of the enzyme substrate complex are more important. With semen phosphatase, but not with intestinal phosphatase, certain phosphate donors produce glyceryl phosphate in excess of the theoretical equilibrium concentration, acetyl phosphate being the most effective phosphate donor in this respect.

THE SPECIFIC PHOSPHATASES OF LIVER.

The phosphomonoesterases discussed so far have a wide range of specificity but there exist in many mammalian tissues however, phosphatases attacking single or specific types of substrate.

Levene and Medigreceanu (1911) found that certain tissue extracts attacked mono-nucleotides with the production of inorganic phosphate. Reis (1934) described the presence in heart muscle of a highly specific 5'-nucleotide phosphatase, which was described as 5'-nucleotidase. Reis (1937a & b) later found this enzyme to be present in nervous tissue, lungs, testes, foetal membranes and retina. Snake venom was found

to be a very potent source of this enzyme by Gulland and Jackson (1938) and Mann (1945) demonstrated its presence in bull seminal plasma. Reis (1950 and 1951) studied the distribution of 5'-nucleotidase in human tissues and found the highest activity in thyroid, testes and aorta wall. Adenosine-5'-monophosphate was found to be hydrolysed much more readily than phenyl phosphate or β-glycerophosphate at pHs around 7.0 in all tissues with the exception of prostate gland and intestinal mucosa. The 5'-nucleotidase of snake venom has been separated from diesterase by adsorption chromatography using cellulose columns (Hurst and Butler, 1951).

Hepple and Hilmoe (1950 and 1951) achieved a 50-fold purification of the enzyme from bull seminal plasma utilising ammonium sulphate and alcohol fractionation, heating to 60° for 20 minutes and adsorption of the enzyme on aluminium gel. The purified enzyme split inosine-5'-monophosphate, nicotinamideriboside-5'-monophosphate, uridine-5'-monophosphate, cytidine-5'-monophosphate and ribose-5-phosphate. Experimental data indicated that all these substrates were attacked by the same enzyme and that adenosine-3'-monophosphate was not attacked. Mg^{++} ions were found to be essential for full activity and could not be replaced by Ca^{++} or Mn^{++} ions. The decrease in activity in the absence of Mg^{++} ions was dependent upon the electrolyte present in the medium, for

example, 0.01 M sodium fluoride caused 70% inhibition and borate buffer about an 85% inhibition.

Novikoff, Podber and Ryan (1950) have shown that 5'-nucleotidase is associated with the nuclear and mitochondrial fractions of the rat-liver cell.

The first suggestion that hexose phosphates were hydrolysed by specific phosphatases came from the work of Fantl and Rome (1945). These authors found the shape of the pH-activity curves for the enzymic hydrolysis of the hexose phosphates differed from those obtained when glycerophosphate or phenyl phosphate was used as substrate, no peak due to alkaline phosphatase activity being observed with the sugar phosphates. The existence of a specific glucose-6-phosphatase was proposed and it was suggested that the hydrolysis of the other hexose phosphates could be explained by an enzyme system involving this enzyme and phosphoglucomutase or hexose monophosphate isomerase. De Duve, Berthet, Hers and Dupret (1949) separated an enzyme specific for the hydrolysis of glucose-6-phosphate from acid phosphatase in liver by precipitation at pH 5.0 - 5.5. The specific enzyme was differentiated by a very rapid inactivation at acid pHs. Its activity towards glucose-1-phosphate, fructose-6-phosphate and β-glycerophosphate was not more than 2 - 3% of its activity towards glucose-6-phosphate, the

acid phosphatase, on the other hand, being much more active on β-glycerophosphate than on the hexose monophosphates.

Swanson (1950) claimed the separation and partial purification of a glucose-6-phosphatase from liver. Her method involved isoelectric precipitation at pH 5.5, adsorption of impurities on aluminium hydroxide and ammonium sulphate precipitation. The purified preparation was free from phosphoglucomutase activity and did not hydrolyse glucose-1-phosphate. As purification proceeded, the diminution in the rate of splitting of fructose-6-phosphate paralleled the decrease in isomerase activity. Arsenite, iodoacetate histidine, cyanide and phlorhizin had no effect on its activity, while molybdate inhibited strongly and arsenate and fluoride inhibited to a lesser extent. Calcium and magnesium ions had no effect on the rate of hydrolysis of glucose-6-phosphate by the purified enzyme which showed optimal activity at pH 6.5. All fractions which were active in splitting glucose-6-phosphate were also active in splitting glycerophosphate and the pH optimum with this latter substrate was also found to be 6.5.

The intracellular distribution of glucose-6-phosphatase was studied by De Duve, Berthet and Hers (1950) and they found that the glucose-6-phosphatase activity of liver and kidney was strongly bound to the microsomal fraction.

Since the glucose-6-phosphatase of Swanson had no activity on glucose-1-phosphate, the mechanism of hydrolysis of this latter compound is of great interest. Fleury, Courtois, Anagnostopolous and Desjobert (1950) claimed that the purified alkaline phosphatases of kidney, liver and bone and purified human prostate phosphatase were able to hydrolyse glucose-1-phosphate more or less rapidly. Broh-Kahn and Mirsky (1948) had investigated various possible mechanisms for the breakdown of glucose-1-phosphate and these workers conclude that the most probable route of hydrolysis of this compound is via a conversion to glucose-6-phosphate. Since the glucose phosphates of liver are the ultimate sources of blood glucose, the pathway of glucose-1-phosphate breakdown is of great physiological importance.

Phosphoglucomutase, the enzyme responsible for the conversion of glucose-1-phosphate to glucose-6-phosphate, was discovered by Cori and Cori (1936) and the effects of various metallic ions on mutase preparations of tissue extracts were studied by Cori, Colowick and Cori (1938). Mg^{++} and Mn^{++} ions were both shown to have an activating effect, the latter ion producing a greater effect than the former. Since the extracts still had activity after electrodialysis and 20-fold dilution, it was assumed that

the enzyme could act without the presence of metallic ions. The activating effect of Mg^{++} was inhibited by Na_2SO_4, NaCl, KCl and $CaCl_2$ at a concentration of 20 mM. The effect of Mg^{++} and Mn^{++} ions together was not additive. Citrate, veronal, glycerophosphate and sodium bicarbonate-carbonate buffers all inhibited when no accelerating ion was present.

Strickland (1949) studied in great detail the effect of metallic ions on mutase preparations. He observed maximum activity in the presence of two ions such as Mg^{++} and Cu^{++}.

Muscle phosphoglucomutase was obtained in crystalline form by Najjar (1948). The enzyme appeared to be quite stable when kept at a temperature of 63° for 3 minutes and this property was utilised in the purification. The pH optimum of the purified enzyme was at 7.5 and cysteine and magnesium both caused activation, each showing greatest activation at an optimal concentration. Sutherland (1949) showed that various metal binding agents such as cysteine, glutathione, 8-hydroxyquinoline and albumin all produced activation.

The existence of a coenzyme for phosphoglucomutase was first suggested by Leloir and his colleagues (Caputto, Leloir, Trucco, Cardini and Paladini, 1948). The coenzyme

was resistant to alkali but was destroyed by acid and appeared to follow inorganic phosphate in most schemes of fractionation. Kendal and Strickland (1938) had earlier shown that preparations of fructose diphosphate had an activating effect on phosphoglucomutase and this substance was at first believed to be the coenzyme since its properties were similar to those of the coenzyme discovered by Leloir and his colleagues with the one exception that the action of alkali on the coenzyme was different from its action on fructose diphosphate. However, the Leloir school believed that the fructose diphosphate preparations were contaminated with the coenzyme and from a consideration of their analytical data thought that glucose diphosphate would be the most likely substance to be the coenzyme. They showed that this was indeed the case (Leloir, Trucco, Cardini, Paladini and Caputto, 1948; Cardini, Paladini, Caputto, Leloir and Trucco, 1949) and a method for the separation of the coenzyme from fructose diphosphate was described.

A chemical synthesis of pure α - and β -glucose-1, 6-diphosphate was described by Posternack (1949) and the α - form was found to be active as the coenzyme.

An enzymatic synthesis of glucose diphosphate (G-1, 6-diP) was carried out by Paladini, Caputto, Leloir,

Trucco and Cardini (1949) using muscle or yeast enzyme preparations. The enzyme involved was a glucose-1-phosphate kinase and the reaction catalysed was:-

$$\text{G-1-P} + \text{ATP} \longrightarrow \text{G-1,6-diP} + \text{ADP}$$

where G-1-P = glucose-1-PO_4

ATP = adenosine triphosphate

ADP = adenosine diphosphate.

It showed a pH optimum at 6.8 and was activated by Mg^{++} or Mn^{++} ions.

Two additional enzymic syntheses of the coenzyme have been reported by Leloir and his colleagues. Leloir, Trucco, Cardini, Paladini and Caputto (1949), using extracts of E. Coli, obtained evidence of the following reaction:-

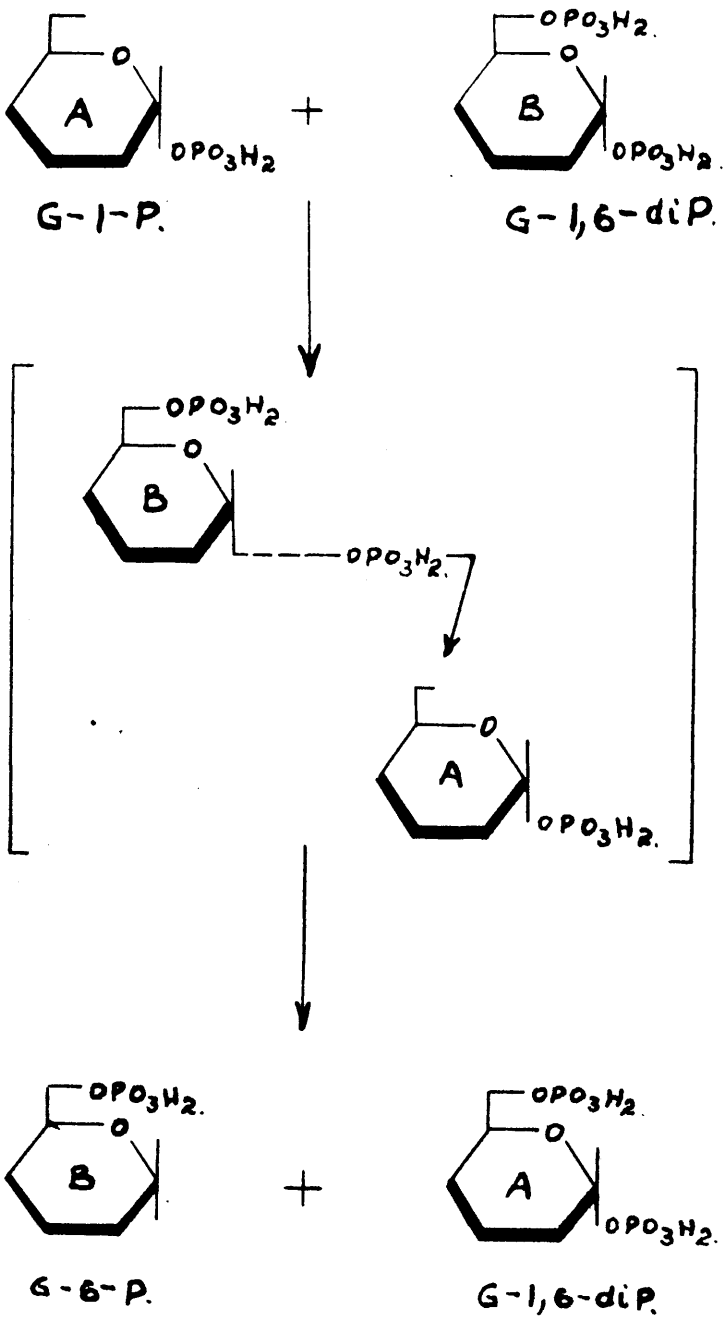
$$2\ \text{Glucose-1-}PO_4 \longrightarrow \text{Glucose} + \text{Glucose-1,6-diphosphate}$$

and a partial purification of the enzyme from cell-free extracts was reported. Adenosine triphosphate did not take part in the reaction and the enzyme was activated by cysteine and Mg^{++} ions and inhibited by fluorides. A small synthesis of the coenzyme, 0.1%, was found by Cardini (1951a) when he incubated glucose-1-phosphate with kidney alkaline phosphatase.

Paladini (1951) examined various rat organs for their content of glucose diphosphate and he reported the following

FIG. 1

Mechanism Of Phosphoglucomutase Reaction.
(After Sutherland et.al., 1949).



data:-

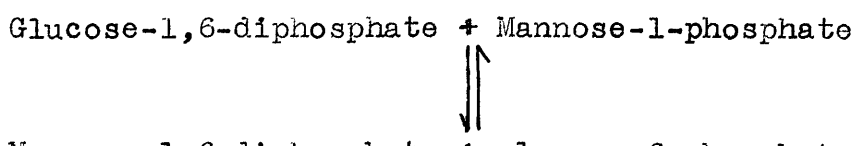
Organ	μ moles coenzyme per g. tissue
Muscle	0.006-0.10
heart	0.026
brain	0.027
liver	0.017
kidney	0.008
intestine	0.007
blood	0.013-0.130

All the glucose diphosphate in blood was found in the red blood cells. No significant changes were found in pancreatic or alloxan diabetes, but increases of about 100% were observed in muscle after injection of glucose, adrenaline or insulin.

The mechanism of the phosphoglucomutase reaction and the part played by the coenzyme was elucidated by Sutherland, Cohn, Posternack and Cori (1949) who found that when glucose-1-phosphate, labelled both with radioactive carbon and phosphorus, was incubated with unlabelled glucose-1,6-diphosphate and phosphoglucomutase, the glucose-1,6-diphosphate isolated during the reaction was found to be radioactive, both in the carbon and phosphate moieties. The scheme illustrated in Figure I was proposed to describe the reaction.

Although the coenzymic effect of glucose-1,6-diphosphate is apparent with dialysed yeast enzyme, the effect is more difficult to demonstrate with the muscle enzyme. However, when the latter is treated with acid ammonium sulphate, activation by the glucose diphosphate was observed (Cardini *et al.*, 1949). In muscle the coenzyme appears to be more firmly bound to the apoenzyme. Cardini (1951b) demonstrated that glucose diphosphate is also the coenzyme for jack-bean meal phosphoglucomutase.

Cori *et al.* (1938) found no evidence for the conversion of the 1-phosphate esters of mannose and galactose to the corresponding 6-phosphate esters by extracts of liver, muscle and yeast. Leloir (1951) showed, however, that with yeast and muscle extracts and with catalytic amounts of glucose diphosphate mannose-1-phosphate was transformed into mannose-6-phosphate. He suggested the following reaction takes place:-



The conversion was about 40 times slower with mannose-1-phosphate than with glucose-1-phosphate.

Phosphoglyceric acid mutase, the enzyme catalysing the interconversion of 2- and 3-phosphoglyceric acids, was

shown by Sutherland, Posternack and Cori (1949) to follow a similar reaction mechanism. In this case 2,3-diphosphoglyceric acid acts as coenzyme in a manner similar to that of glucose diphosphate in the phosphoglucomutase reaction.

The interconversion of ribose-1-phosphate and ribose-5-phosphate has also been found to resemble the phosphoglucomutase reaction. Schlenk (1949) first produced indirect evidence for the conversion of ribose-1-phosphate. Enzymes were later found in muscle by Sable (1952) and in liver by Abrams and Klenow (1951) which converted ribose-1-phosphate to an acid stable phosphate ester. The liver enzyme was found to be very sensitive to surface denaturation by frothing. Klenow (1953) found the liver enzyme to have a pH optimum of 7.5, to be heat stable at pH 5.0 and to be inhibited by various salts. These properties are identical with those of phosphoglucomutase and the possibility that this latter enzyme also catalysed the interconversion of the ribose monophosphates was therefore investigated by Klenow and Larsen (1952) and Klenow (1953). They found that with preparations of muscle and yeast mutase the ratio of the phosphoglucomutase activity to the phosphoribomutase activity was 100:1 in both cases and that this ratio did not change

during purification.

These workers also investigated the mechanism of the phosphoribomutase reaction. They found that glucose-1,6-diphosphate acted as coenzyme for preparations of yeast and muscle mutase. With the muscle enzyme, however, coenzymic activity was observed only in the presence of 8-hydroxyquinoline. It was also noted that the ribose-1-phosphate used as substrate had to be purified by ion-exchange chromatography since it contained an activator.

From an incubation mixture of mutase, ^{32}P labelled ribose-1-PO_4 and glucose diphosphate, a new labelled phosphate ester was isolated by ion-exchange chromatography, which had properties suggesting it to be ribose-1,5-diphosphate. This diphosphate was believed to be the activator observed in impure preparations of ribose-1-phosphate.

From an examination of the distribution of radioactivity in the new diphosphate, it was deduced that the reaction mechanism for the interconversion of the ribose monophosphates was as follows:

$$\text{R-1-P} + \text{G-1,6-P} \rightleftharpoons \text{R-1,5-P} + \text{G-6-P}$$

$$\text{R-1-P} + \text{R-1,5-P} \rightleftharpoons \text{R-5-P} + \text{R-1,5-P}$$

where:

R-1-P ≡ ribose-1-phosphate

R-5-P ≡ ribose-5-phosphate

R-1,5-P ≡ ribose-1,5-diphosphate

G-1,6-P ≡ glucose-1,6-diphosphate

G-6-P ≡ glucose-6-phosphate.

Gomori (1943) showed the presence in the kidneys and livers of many species of an enzyme which specifically hydrolysed the phosphate group in the 1-position of fructose-1,6-diphosphate. This enzyme differed from the non-specific alkaline phosphatase in being stable at pH 3.4, irreversibly inactivated in the presence of alcohol and acetone, activated by cyanide and inhibited by fluorides. The enzyme showed a pH optimum at 9.7 and had little activity in the absence of magnesium. A purified hexose diphosphatase preparation showed little activity towards β-glycerophosphate, phenyl phosphate, pyrophosphate and metaphosphate. Hers, Berthet, Berthet and De Duve (1951) found that the supernatant fraction of liver cell cytoplasm contained 96% of the total diphosphatase activity, while Pogell and McGilvery (1952) showed the enzyme to exist in an inactive form which was released by proteolysis. Although the phosphatase activity was found in the supernatant, the activating enzymes were found in an extract of the particulate fraction of the cell. Papain caused similar activation, but trypsin, rennin and pepsin had no effect.

The Physiological Rôle of the Phosphomonoesterases.

Despite the simplicity of the hydrolytic action of the phosphatases and the volume of work concerning them which has been published during the past forty-five years, the physiological function of the intracellular phosphatases remains a biochemical enigma. Specific phosphatases exist which hydrolyse naturally occurring phosphate esters such as 5'-nucleotides, glucose-6-phosphate and phosphoproteins, and which, moreover, act optimally at pHs nearer the physiological level than do the non-specific phosphomonoesterases. The importance of the synthetic and phosphotransferase activities *in vivo* is still a matter of conjecture.

At present theories concerning the function of the non-specific phosphatases are based on inferences drawn from chemical and histochemical studies of the activity of the enzymes in various tissues and of the changes in activity under varying physiological or pathological conditions.

An illustration of this is the suggested rôle of alkaline phosphatase in mineral salt deposition. Martland and Robison (1924) and Bodansky, Bakwin and Bakwin (1931) estimated the phosphatase content of bones and teeth and showed that the regions associated with organised mineral salt deposition had a high alkaline phosphatase content.

Further evidence was obtained by Fell and Robison (1929, 1930 and 1934), who showed that cultures of embryonic chick and rabbit bone showed a high phosphatase content at, or just before, the stage in their development when calcification began. A more recent theory of calcification involving alkaline phosphatase is that of Roche (1947) who suggested that glycogen, which has been shown by histochemical means to occur in osseus tissues during calcification (Kabat and Furth, 1941; Glock, 1940 and Horowitz, 1942), is broken down by bone phosphorylase and in this process blood inorganic phosphate is "fixed" in the formation of glucose-1-phosphate which, in turn, is hydrolysed by bone phosphatase at the site of mineral salt deposition.

The occurrence of high alkaline phosphatase activity in the striated border of the small intestine and in the brush borders of the convoluted tubules of the kidney (Bourne, 1943; Gomori, 1941) have led to the suggestion that the enzyme is concerned in processes of active absorption. Menten and Jansuch (1946) showed that alloxan diabetes is accompanied by a loss of kidney alkaline phosphatase and this was interpreted as evidence for alkaline phosphatase taking part in the resorption of glucose from the glomeruler filtrate.

Kochiakin (1947) studied the effect of the injection of male sex hormones into castrated mice. A decrease in the alkaline phosphatase and an increase in the acid phosphatase activity of kidney was observed while the liver and intestinal enzymes were not altered. The significance of these findings is not apparent.

As improved histochemical and microchemical techniques have become available, it has been possible to associate various biochemical reactions with different intracellular fractions. It is known that the glycolytic enzyme systems are to be found in the supernatant fraction of the cytoplasm (Hers, Berthet, Berthet and De Duve, 1951), while the aerobic oxidation systems (succinoxidase and cytochrome oxidase) have been shown to occur in the mitochondria (Schneider and Hogeboom, 1950). It has become obvious therefore that a knowledge of the intracellular distribution of the phosphatases may lead to some understanding of their function.

Novikoff, Podber and Ryan (1950) investigated the distribution of the phosphatases in water and 0.88 M sucrose dispersions of liver. They found that 40% of the acid phosphatase activity was associated with the mitochondria and most of the alkaline phosphatase activity was associated with the supernatant fraction. Berthet and De Duve (1951), working with dispersions of rat-liver in 0.25 M. sucrose, found

up to 68% of the acid phosphatase activity associated with the mitochondria, but their mitochondrial preparations, however, had no action on β-glycerophosphate until the mitochondria had been disrupted either by repeated freezing and thawing or homogenising in distilled water in a blender.

Much controversy has taken place concerning the occurrence of alkaline phosphatase in the nucleus. Dounce (1943) isolated rat-liver nuclei in dilute citric acid and found that only alkaline phosphatase of many enzymes examined showed higher activity in the nuclei on a dry weight basis than in the whole tissue. The histochemical technique of Gomori (1939) seemed to indicate that nuclei contained a high phosphatase activity, but, as already mentioned, this method can lead to erroneous results. Novikoff, Podber and Ryan (1950), working with 0.88 M sucrose homogenates of liver, found that the nucleus contained only about 15% of the total alkaline phosphatase activity; their nuclear fraction was, however, contaminated with some unbroken cells. Chromosomes isolated from thymus by Mirsky (1947) were shown to have alkaline phosphatase activity. In all these methods of isolation the fact that the nuclear phosphatase activity may have arisen by adsorption from other cell fractions during isolation must not be overlooked. Indeed, Novikoff (1952) showed that when nuclei were suspended in 0.70 M sucrose

solution along with purified alkaline phosphatase a considerable amount of the enzyme was adsorbed. Stern, Allfrey, Mirsky and Saetren (1952) showed that nuclei isolated by the Behrens technique, which gives clean preparations with little adsorption consequent upon the use of organic solvents, contained 12% of the activity of calf-liver and 28% of horse-liver.

From a consideration of the intensity of nuclear alkaline phosphatase activity, as demonstrated by Gomori's (1939) technique, and the rate of turnover of deoxyribonucleic acid phosphorus (DNAP), Brachet and Jeener (1948) suggested that the enzyme controlled the turnover of DNAP. Novikoff (1952) criticised this finding and pointed out that the turnover rate of DNAP in striated muscle was three times that in kidney, yet kidney nuclei stained intensely whereas muscle nuclei did not stain at all for alkaline phosphatase.

It has been pointed out by several workers that sites of active protein synthesis show a high alkaline phosphatase activity. Using histochemical techniques, Bevelander and Johnson (1945) found high activities in developing teeth, Bourne (1943) in bone and Johnson, Tutcher and Bevelander (1945) in hair, while Folley and Greenbaum (1947) found a progressive increase in mammary gland alkaline phosphatase during lactation.

The correlation of alkaline phosphatase activity with the synthesis of collagen type protein has been stressed by various workers. Bradfield (1946) showed that in vitamin C deficient guinea pigs there was a decrease in alkaline phosphatase activity along with a decreased collagen formation. Jeener (1947) found an increased alkaline phosphatase activity only in those cells which synthesise fibrous proteins in the vaginal wall, following injection of oestradiol in ovariectomised female mice. Gold and Gould (1951) found, however, that collagen fibres adsorb alkaline phosphatase strongly from solutions and the findings of Jeener may be explained by the newly formed collagen causing an increased local concentration of the enzyme by physical chemical factors rather than the phosphatase being instrumental in increased protein synthesis.

Oppenheimer and Flock (1947), Norberg (1949) and Goodlad, Mills and Smith (1951) have studied the behaviour of alkaline phosphatase in livers, regenerating after partial hepatectomy. The last-mentioned group using DNAP as a reference standard (Davidson and Leslie, 1950a & b) found an increase in alkaline phosphatase activity 2-3 days after operation which coincided with increases in ribonucleic acid phosphorus (RNAP) and protein concentration. Data were also presented to show that in the liver of the growing rat the

rate of accumulation of RNAP is the same as that of alkaline phosphatase. From these findings it might be suggested that there is a relation between alkaline phosphatase and ribonucleic acid synthesis or turnover.

Lowe and Salmon (1951) showed that male rat liver has a higher alkaline phosphatase content than female liver although this difference was not observed in regenerating livers. Rosenthal, Fahl and Vars (1951) showed that dietary protein depletion caused an increase in the alkaline phosphatase content of rat liver although there was a diminution in total protein.

From the above data it is not possible to draw any definite conclusion concerning the physiological rôle of alkaline phosphatase, while the position concerning the acid phosphatases is even less clear.

The present work was undertaken with the aim of gaining further information concerning the enzyme systems in rat liver which hydrolyse phosphate esters and, in particular, to investigate some of the more conflicting aspects of the subject such as the possible multiple nature of the non-specific acid and alkaline phosphatases and the mechanism of hydrolysis of the hexose-1-phosphates.

GENERAL METHODS.

Preparation of Tissue Extracts.

Male albino rats of 175-300 g. body weight were used throughout. Animals were killed by stunning and exsanguination, the livers were removed, washed with water and blotted with filter paper. In the preliminary work on pH activity curves of unfractionated rat liver a 1 in 10 homogenate (Potter and Elvehjem, 1936) in distilled water was used.

Assay of Phosphatase Activity.

A general method, applicable to all substrates, was based upon the β-glycerophosphate method of Bodansky (1933) where the inorganic phosphate liberated was separated from interfering substances by the method of Delory (1939) and subsequently determined by the method of Allen (1940). The details of the method are as follows:-

0.5 ml. buffer solution, 0.1 ml. substrate solution, 0.1 ml. water (or a solution of activator or inhibitor) and 0.3 ml. enzyme solution, usually a $^{1}/_{50}$ tissue homogenate, were incubated for a specific time, usually 1 hour, at 38° after which the reaction was stopped by the addition of 2 ml. of 5% trichloracetic acid. Precipitated protein was removed by centrifugation and 2 ml. aliquots were made alkaline with 0.880 ammonia (phenolphthalein), 1 ml. 2.5% $CaCl_2$ was added followed by 1 ml. 0.5% suspension light $MgCO_3$. The

tubes were shaken at intervals for 30 minutes and precipitates were centrifuged and washed once with 1 ml. 5% NH_4OH. The tubes were drained for 2-3 minutes in an inverted position, the precipitate dissolved in 1 ml. 2.5N H_2SO_4 and colour developed by the addition of 0.4 ml. 1% amidol (2:4 diaminophenyl hydrochloride) in 20% sodium metabisulphite, 0.2 ml. 8.3% ammonium molybdate and 3.4 ml. water. After standing 10 minutes the colours were estimated within 30 minutes using the Hilger Spekker absorptiometer (Ilford 608, red filters) or on the Unicam SP 600 spectrophotometer at 635 mμ. Phosphate was determined from calibration curves for the range 5-40 μgP. Controls were employed to determine the inorganic phosphate in the tissue and that formed by autolysis during incubation. In this the substrate was added after the addition of the trichloracetic acid.

The unit of activity under such condition is defined as: 1 unit = 1 μgP liberated per hour.

When phenyl phosphate was used as substrate, liberated phenol was estimated by a method similar to that of King (1946). 0.5 ml. buffer, 0.1 ml. 0.05M disodium phenylphosphate and 0.1 ml. water (or a solution of activator or inhibitor) were brought to 38° and 0.3 ml. enzyme preparation, usually a 1 in 100 homogenate, also at 38° added. After incubating for 20 minutes at 38°, the reaction was stopped by

the addition of 2 ml. of a 1 in 3 dilution of Folin-Ciocalten phenol reagent. Precipitated protein was removed by centrifugation and 2 ml. supernatant were added to 4 ml. 10% Na_2CO_3 and the mixture incubated for a further 30 minutes at 38°. After the addition of 2 ml. of water and mixing the blue colour was estimated in the Hilger Spekker absorptiometer (Ilford 608, red filter) or the Unicam SP 600 spectrophotometer at 650 mμ.. Liberated phenol was determined from calibration curves for the range 20-100 μg. phenol. Tissue blanks were determined by addition of substrate after the phenol reagent. There was no measurable breakdown of substrate in the absence of enzyme, at any pH used in the present work. The unit of activity under these conditions is defined as: 1 unit = 1 μg. phenol liberated per hour.

The assay method using p-nitrophenyl phosphate was a modification of the procedure of Bessey, Lowry and Block (1946). This technique is a highly sensitive and rapid method for the determination of acid phosphatases. 0.5 ml. buffer solution, 0.1 ml. 0.025M p-nitrophenyl phosphate and 0.1 ml. water (or solution of activator or inhibitor) brought to 38° and 0.3 ml. of enzyme preparation, usually a 1 in 400 liver homogenate, also preheated to 38° was added. Digests were incubated at 38° for 15 minutes, the reaction stopped by the addition of 1 ml. 0.4M NaOH followed by 6 ml. water and

protein precipitate removed by centrifugation. The yellow colour was estimated using the Hilger Spekker absorptiometer (Ilford 601, violet filter) or the Unicam SP 600 spectrophotometer at 400 mμ. and p-nitrophenol liberated determined from a calibration curve. The calibration curve only showed a linear relationship between optical density and p-nitrophenol concentration over the range 5-30 μg. per ml. of solution. Assays were therefore arranged so that the amount of p-nitrophenol liberated was within these limits. The colour was found to remain stable overnight. Controls were carried out where the substrate was added after the NaOH. The unit of activity in this instance was defined as:
1 unit = 1 μg. p-nitrophenol liberated per hour.

Assay of Phosphoglucomutase Activity.

Phosphoglucomutase activity was determined by the decrease in acid labile P on incubation with glucose-1-phosphate in the presence of cysteine and Mg^{++}. The method was based on that of Najjar (1948). 0.3 ml. enzyme solution, 0.5 ml. buffer, 0.1 ml. 0.05 M glucose-1-phosphate, 0.1 M with respect to cysteine, and 0.1 ml. 0.01 M $MgSO_4$ were incubated at 38° for 15 minutes. All solutions were preheated to 38°. The reaction was stopped by the addition of 1 ml. 5N H_2SO_4 followed by 3 ml. water. The precipitate was removed by centrifugation and 0.5 ml. of supernatant heated at 100° for

10 minutes. Total inorganic phosphate was estimated by the method already described. The initial acid labile P was determined by carrying out the above series of reactions, except that the substrate was added after the H_2SO_4. The decrease in acid labile P is equivalent to the amount of acid stable glucose-6-phosphate formed.

1 unit of phosphoglucomutase activity = 1 µg. acid stable P formed per hour under the above conditions.

Preparation of Substrates.

α-Glucose-1-phosphate (G-1-P) was prepared by the phosphorolysis of starch using potato juice as described by Hanes (1940) and was purified by ion-exchange chromatography as described by McCready and Hassid (1944). The following digest was prepared:

a) 450 ml. freshly prepared crude potato juice.

b) 38 g. starch made into paste in cold water and poured with stirring into 2 l. of boiling water and held at this temperature for 20 minutes and cooled.

c) 1063 ml. of M phosphate buffer pH 6.8.

d) 10 ml. toluene.

After standing at room temperature for 48 hours protein was precipitated by heating to 100° and inorganic phosphate by adding 1 mole of magnesium acetate and ammonia to pH 8.5.

Protein and phosphate were filtered off and the supernatant was run through a cation exchange column, Zeokarb 215 in H^+ form, until pH of effluent rose to 4.0. The effluent was then passed through an Amberlite IR 4 column in the $(NH_4)^+$ form which adsorbed the glucose-1-phosphate. The column was washed with 1.5 l. of distilled water to remove impurities and the G-1-P eluted with 40% NH_4OH until the pH of the effluent rose to 11.0. To the effluent 25 g. of potassium acetate were added and the pH adjusted to 12.0 with 10% KOH. 1.5 volumes of methanol were added and the dipotassium salt of G-1-P was allowed to crystallise out overnight at 4^o. The crystals were filtered off, washed with methanol and ether and finally dried <u>in vacuo</u>. Yield, 10 g.

Analysis: Total P: 7.95% (found); Required for

$C_6H_{11}O_6PO_3K_2.2H_2O$ 8.33%

Inorganic P: negligible.

7 minute hydrolysable P: 7.66% (found); 8.33% (theoretical)

Optical rotation: $[\alpha]_D^{25} = +77.2$ (c = 2.06 in water).

∴ By P analysis product was 92% pure.

<u>Glucose-6-phosphate (G-6-P)</u> was prepared from G-1-P by a partially purified preparation of rabbit muscle phosphoglucomutase prepared according to Najjar (1948). A rabbit was anaesthetised with nembutal, the vessels of the neck severed and the blood allowed to drain freely. The muscles of the

limbs and back were excised and minced and subsequent stages in the purification are shown in Figure II. The following digest was set up:

a) 11 g. enzymatically prepared dipotassium salt of G-1-P

b) 0.336 g. $MgSO_4 12H_2O$

c) 1 g. cysteine

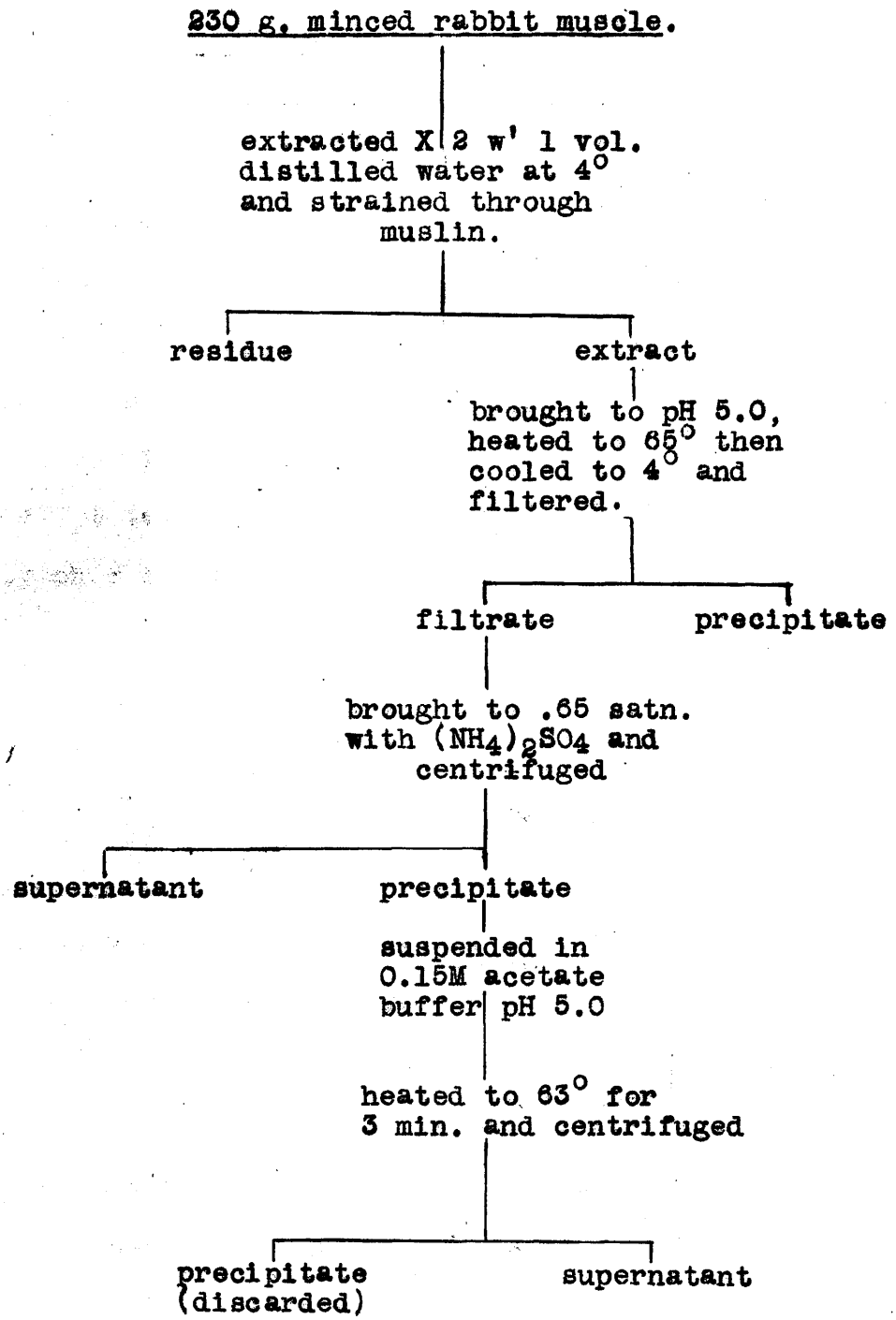
d) 20 ml. phosphoglucomutase solution

e) 20 ml. veronal acetate buffer (Michaelis, 1931) pH 7.2

f) distilled water to 1 l.

The mixture was incubated overnight at 37°, the reaction was stopped by heating to boiling and the precipitate removed by filtration. The filtrate was passed through a Zeokarb 215 cation exchange column, the hexose monophosphates were adsorbed from the effluent on an Amberlite IR 4 column, washed with 2 l. of water and eluted with 250 ml. 5% NH_4OH. The eluate was evaporated in vacuo with an acid trap. This serves to remove most of the ammonia. Evaporation was continued until volume was reduced to 50 ml. The solution was found to be neutral and 5N HCl was added to make the solution N. The G-1-P present was destroyed by boiling for 10 minutes. The solution was neutralised to phenolphthalein by the addition of a hot, saturated solution of $Ba(OH)_2$ and the precipitated $Ba_3(PO_4)_2$ removed by centrifugation. G-6-P was

Figure II.


230 g. minced rabbit muscle.
extracted X 2 w' 1 vol. distilled water at 4° and strained through muslin.
residue
extract
brought to pH 5.0, heated to 65° then cooled to 4° and filtered.
filtrate
precipitate
brought to .65 satn. with $(NH_4)_2SO_4$ and centrifuged
supernatant
precipitate
suspended in 0.15M acetate buffer pH 5.0
heated to 63° for 3 min. and centrifuged
precipitate (discarded)
supernatant

precipitated as the barium salt by the addition of absolute ethanol to the supernatant to give a final concentration of 50%. After standing 1-2 hours in the ice-box, the barium salt was filtered, washed in succession with 50% ethanol, absolute ethanol, and ether and dried *in vacuo*. Yield, 5.5 g.

Analysis: Total P = 7.25% (found): 7.84% (theoretical)

10 minute hydrolysable P - nil

inorganic P - nil

$[\alpha]_D^{20}$ = +17.6 (water): $[\alpha]_D^{25}$ = +17.4 (Levene and Raymond, 1931)

Purity on P basis = 93%.

α-Galactose-1-phosphate (Gal-1-P). Pentaacetyl galactose was prepared according to the method of Krahl and Cori (1949) for the preparation of pentaacetyl glucose. The method involves the acetylation of galactose by anhydrous sodium acetate and acetic anhydride. The product after recrystallisation from 95% ethanol was white and crystalline and had a melting point of 142° (uncorrected). 44 g. of the pentaacetyl derivative were obtained from 50 g. of anhydrous galactose.

Acetobromogalactose was prepared by the method of Ohle, Marecek and Bourjau (1929). 44 g. pentaacetyl galactose were allowed to react with 200 g. of a 50% solution of HBr in glacial acetic acid. 26 g. of product were obtained. M.pt. 84-85° (uncorrected). Ohle *et al.* quote the melting

point as 85°.

Acetobromogalactose was phosphorylated with trisilver phosphate in dry benzene suspension and Ba galactose-1-phosphate isolated as described by Kosterlitz (1939). The trisilver phosphate was prepared according to the method of Lipmann and Tuttle (1944).

The crude Ba salt was dissolved in water and passed through a Zeokarb 215 cation exchange column. The effluent was passed through an Amberlite IR 4 column which adsorbed the galactose-1-phosphate. The column was well washed with water and the gal-1-P eluted with 5% NH_5OH. The potassium salt was precipitated from the eluate by adding potassium acetate, adjusting pH to 12, and adding 1.5 volumes of ethanol.

Analysis:

The crystals were dissolved in water and the total P, acid labile P and inorganic P content of the solution were determined.

Total P = 8.600 mg./ml.

Inorganic P = 0.58 mg./ml.

Acid labile P = 8.02 mg./ml.

∴ Solution was assumed to contain 258 μ moles/ml.

Gal-1-P.

The only sugar present after hydrolysis was shown to be galactose by paper chromatography in ethyl acetate/pyridine/water.

Inosinic Acid. Inosine-5'-phosphate (I-5'-P) was prepared from rabbit muscle by the method of Marmur, Schlenk and Overland (1951).

Approximately 350 g. muscle were removed from a rabbit, killed by exsanguination under nembutal anaesthesia, and passed through a coarse mincer and then through a fine mincer. The pulp was stirred with half its volume of warm water to give a final temperature of 30°. The mixture was then incubated for three hours at room temperature with constant stirring to bring about the deamination of adenylic acid. The reaction was stopped by the addition of trichloracetic acid and the mixture centrifuged. Inorganic phosphate was removed from the supernatant as $Ba_3(PO_4)_2$ and the nucleotide fraction precipitated by the addition of mercuric acetate. After decomposition of the mercury salts with H_2S the barium salt of inosinic acid was obtained, by evaporation *in vacuo* to a small volume, adjusting the pH to 8.5 with hot, saturated $Ba(OH)_2$ solution and standing overnight in the cold room.

Yield: 150 mg.

Analysis: The product gave a peak of maximum absorption at 248 mμ at pH 7.0.

$= 10.28 \times 10^3$; theoretical $\lambda_{max.}$ for $C_{10}H_{11}N_4O_8PBa + 7.5\ H_2O = 11.5 \times 10^3$

∴ Purity = 91%.

Disodium p-nitrophenylphosphate was prepared according to the method of Bessey and Love (1952).

200 ml. of pyridine, previously dried over KOH, containing 30 ml. of redistilled $POCl_3$, were added, drop-wise, with stirring, to 27.8 g. p-nitrophenol, dissolved in 100 ml. dry pyridine, the temperature being maintained at 0°. After allowing reactants to stand for 30 minutes, the reaction mixture was poured with stirring into 400 ml. of ice-water and the pH adjusted to 7 by the addition of NaOH. The water and pyridine were removed by vacuum distillation and the residue extracted with boiling 87% ethanol. The required product crystallised on cooling, was washed with cold 87% ethanol, absolute alcohol and finally ether. The light-yellow powder was dried in vacuo.

Yield: 5 g.

Purity, from P analyses = 93%.

The final product was stored at 0°. Before use the solution of the substrate was extracted with ether to remove

any p-nitrophenol which had been formed by decomposition.

The other substrates employed in the present work were commercial preparations.

β-glycerophosphate was supplied by The General Chemical and Pharmaceutical Co.Ltd.

α-glycerophosphate was supplied by British Drug Houses, Ltd.

Muscle adenylic acid, adenosine-5'-phosphate was supplied by Messrs. L. Light & Co.Ltd.

Adenosine-3'-phosphate was supplied by Messrs. L. Light & Co.Ltd.

Disodium phenylphosphate was supplied by the British Drug Houses, Ltd.

Estimation of Phosphorus.

The P content of the above preparations was determined by the method of Allen (1940). Samples having a P content between 50-150 ug. were digested with 1.2 ml. 10N H_2SO_4 until the contents were colourless. A few drops of H_2O_2 were occasionally added to accelerate digestion and heating was continued for a further 15 minutes after clearing. The flask was allowed to cool and 6.4 ml. of water, followed by 2 ml. amidol reagent (1 g. 2:4 diaminophenylhydrochloride in 100 ml. of a 20% solution of sodium metabisulphite made up fresh

every 5 days), 1 ml. of an 8.3% solution of ammonium molybdate and a further 15 ml. of water were added. After standing 10 minutes the intensity of the blue colour was estimated in the Hilger Spekker absorptiometer (Ilford 608, red filter) or the Unicam, SP 600 spectrophotometer at 635 mμ. This last reading was taken before 30 minutes had elapsed after addition of the molybdate.

Estimation of Acid labile P.

1 ml. of the substrate solution which was calculated to have a suitable phosphate content was heated at a 100° with 1 ml. of 2N H_2SO_4 for 10 minutes. After cooling a 1 ml. aliquot was further acidified with 1.5 ml. of N H_2SO_4 and inorganic P present estimated by adding 0.4 ml. of a 1% solution of amidol in a 20% solution of sodium metabisulphite, 0.2 ml. of an 8.3% solution of ammonium molybdate and finally 1.9 ml. of water. The solution was allowed to stand for 10 minutes and the blue colour estimated as before. The P content was subsequently determined from a calibration curve.

Acid labile P = Above figure - Inorganic P.

Determination of Inorganic P. Inorganic P was determined by a method based on that of Delory (1939). 1 ml. of solution was neutralised to phenolphthalein with 0.880 ammonia. 1 ml. of a 2.5% solution of $CaCl_2$ was added followed by 1 ml. of a 0.5% suspension of light $MgCO_3$ in water. The mixture

was allowed to stand for 30 minutes, the precipitate being disturbed frequently. The precipitate was then centrifuged down and washed with 1 ml. of 5% ammonia. The precipitate was dissolved in 1 ml. of 2.5N H_2SO_4, 0.4 ml. of a 1% solution of amidol in 20% sodium metabisulphite, 0.2 ml. of 8.3% ammonium molybdate and 3.4 ml. of water. The P content was calculated as above.

Estimation of Deoxyribonucleic Acid Phosphorus (DNAP) and Ribonucleic Acid Phosphorus (RNAP).

Tissue extracts were freed from acid soluble P and lipid P by the method of Schmidt and Thannhauser (1945). Phosphorus was estimated by the method of Allen (1940).

When the nucleic acid P of whole liver was being determined a homogenate was prepared and an aliquot, equivalent to 250 mg. whole liver used. With nuclei and cytoplasmic fractions, aliquots equivalent to 500 mg. and 250 mg. of liver respectively, were used. 0.5 volume of ice-cold 30% trichloracetic acid (TCA) was added to the aliquots and the precipitate removed by centrifugation at 1000 r.p.m. for 4 minutes at 0°. The supernatant was discarded and the precipitate was extracted twice with 5 ml. volumes of 10% ice-cold TCA, centrifuging between extractions to remove the remainder of the acid soluble P.

The lipid P was then removed from the precipitate by

treatment with 5 ml. portions of:

a) Absolute ethanol (x 2)

b) 1:3 chloroform-ethanol mixture

c) 1:3 ether ethanol mixture (x 2)

d) ether.

DNAP and RNAP were then separated by alkaline digestion. 1 ml. of N NaOH was added per equivalent of 100 mg. original tissue and incubated at 37° for 15-20 hours. 2.5N HCl was added (0.5 ml. per 1 ml. NaOH) and the solution made up to a known volume. Aliquots, with a reasonable P content, were removed and deoxyribonucleic acid (DNA) precipitated by adding 30% TCA to a final concentration of 10%. The precipitate of DNA was centrifuged at 0° and washed twice with 1 ml. portions of 5% TCA, centrifuging between extractions. The P content of the combined supernatant and washings was estimated as RNAP (taking an aliquot if necessary). The precipitate was dissolved in 1 ml. N-NaOH and transferred with washing to a microkjeldhal flask and the P content determined as DNAP.

Protein Determination.

(a). Colorimetric method.

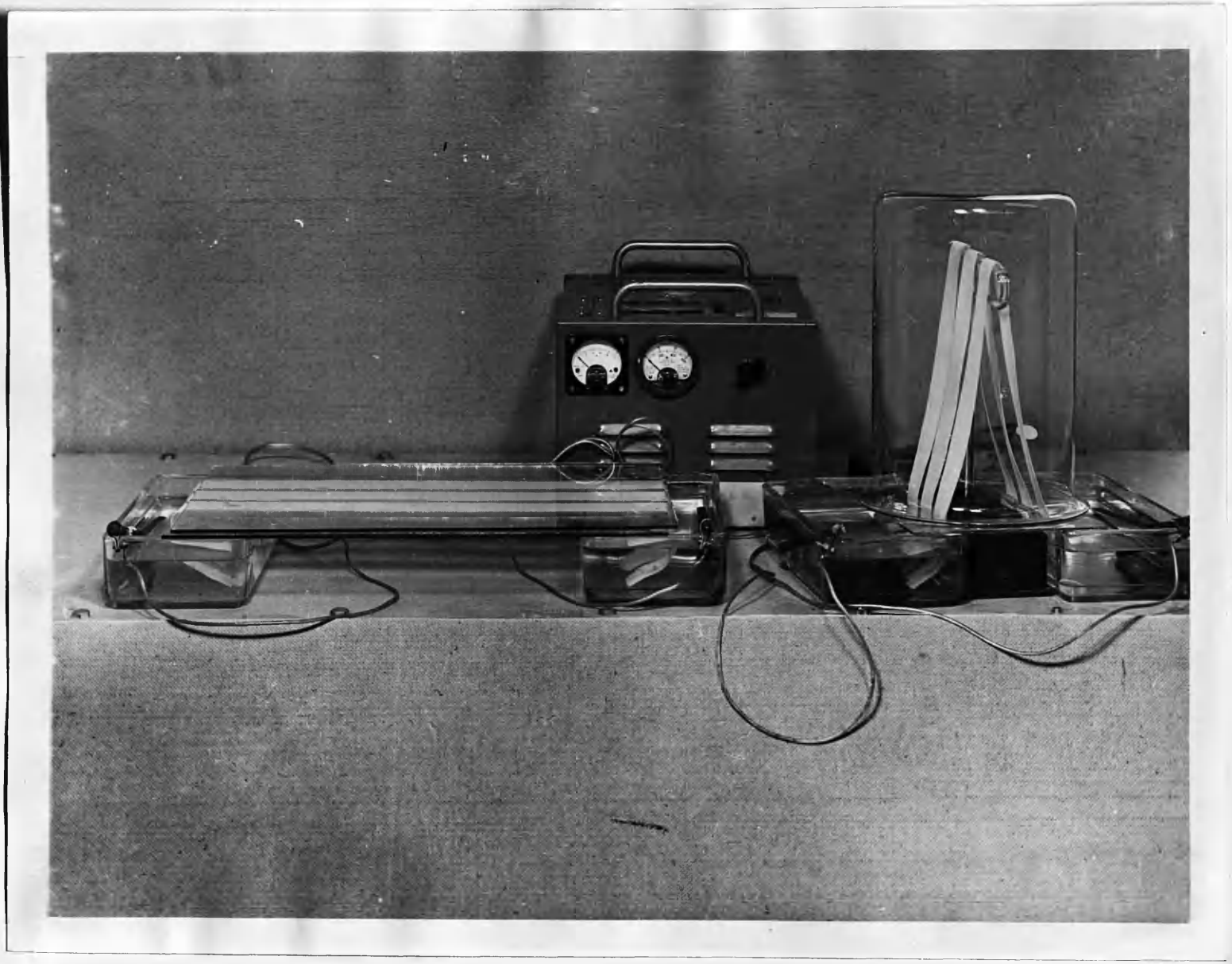
In preliminary studies on protein fractionation a rapid and sensitive method of determining protein was required. The method of Lowry, Rosebrough, Farr and Randall (1951)

appeared to meet this requirement. The principle of the method is a combination of the colour reaction of proteins with the biuret reagent and with the Folin and Ciocalteu phenol reagent.

The alkaline copper solution was prepared freshly every day by mixing 50 ml. 2% Na_2CO_3 in 0.1N NaOH with 1 ml. 0.5% $CuSO_4.5H_2O$ in 1% sodium potassium tartrate. 5 ml. of this solution were added to 1 ml. of protein solution containing 25-400 ug. protein and the mixture shaken well. The biuret colour was allowed to develop for 10 minutes at room temperature. 0.5 ml. of Folin and Ciocalteu phenol reagent, diluted to make it normal with respect to acidity, was then added very rapidly and the whole well mixed. After a further 30 minutes the blue colour was determined in the Unicam SP 600 spectrophotometer at 500 mμ. A calibration curve was prepared using crystalline egg albumin. A linear relationship between optical density and protein concentration was found for concentrations between 25 and 400 μg./ml.

(b) The Microkjeldhal N Determination was carried out by the method of Ma and Zuazaga (1942). The protein sample was digested with 1.5 ml. conc. H_2SO_4 using mercury as catalyst and digestion was continued for one hour after clearing and distillation carried out in the apparatus of Markham (1942).

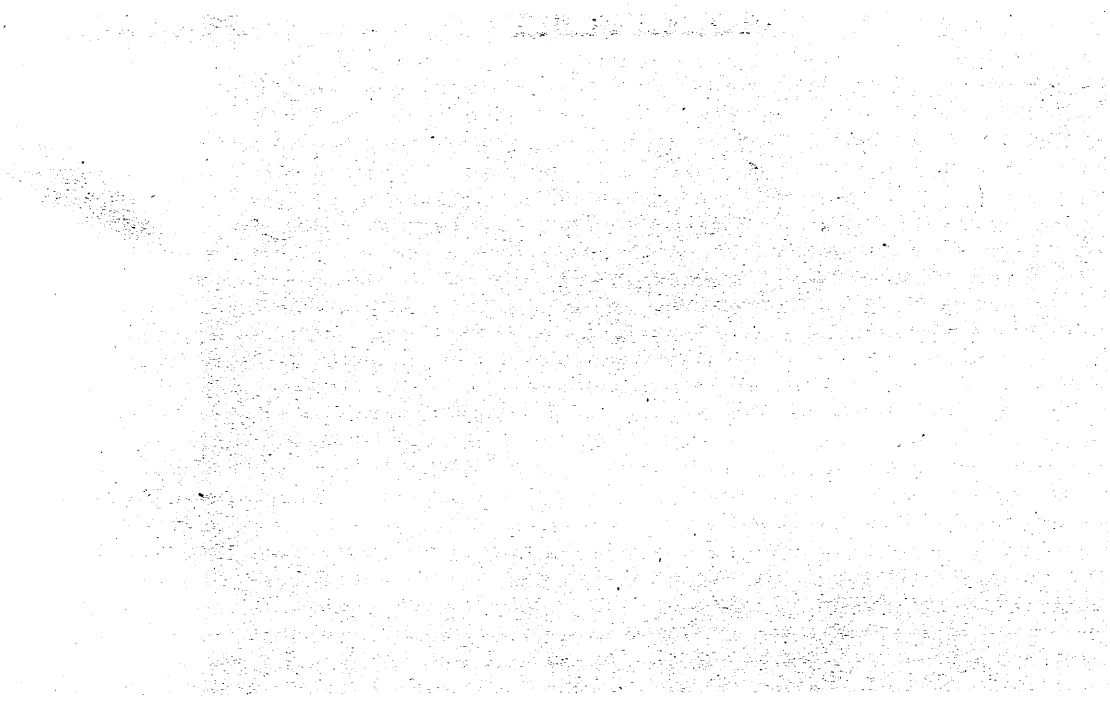
PLATE A.



Electrophoretic Analysis was carried out by the method of Tiselius (1937) using the Philpot-Svensson (Svensson, 1939) optical system (Adam Hilger & Co., London). The illumination was provided by a mercury vapour lamp and a green (546 mμ) filter and photographic records were made on Ilford Halftone panchromatic plates. The cell used was the double section of 3 ml. capacity.

Filter paper electrophoresis was carried out by the method of Mills and Smith (1951) which is based on the method of Durrum (1950). The apparatus used is shown in Plate A. All experiments were carried out in the cold room at a temperature of 0-4°. The enzyme solutions for analysis were applied to the middle of Whatman 3 MM filter paper strips, 55 cm. long and 3 or 7 cm. wide, by means of a micro-pipette so as to form a narrow transverse band. Protein was localised with bromophenol blue. After drying the paper was sectioned into 5 mm. strips, each section eluted in 1 ml. 0.01N NaOH and the intensity of the violet colour determined in the Hilger Spekker absorptiometer (Ilford 605, yellow-green filter) using 0.5 ml. micro-cells, optical density being plotted against migration. Electro-endosmosis was determined by the migration of a glucose spot. Location of the enzyme on the strips was effected by means of phenolphthalein phosphate as described by Mills and Smith (1951).

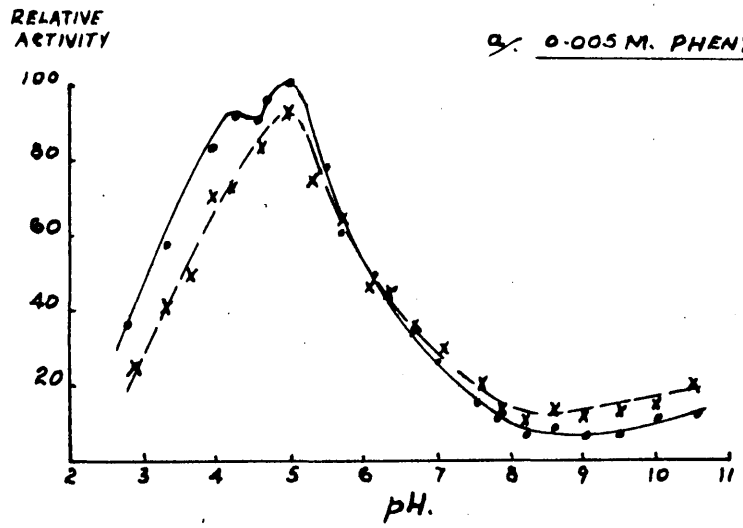
This method gives a rough indication of the position of the activity. For a more precise determination of the position of enzyme activity the undried strip was cut into 5 mm. sections and the activity of each section assayed using 0.005M phenyl phosphate in 1 ml. acetate buffer. Phenol was determined by the Folin-Ciocalteu method and enzymic activity plotted against distance of migration.

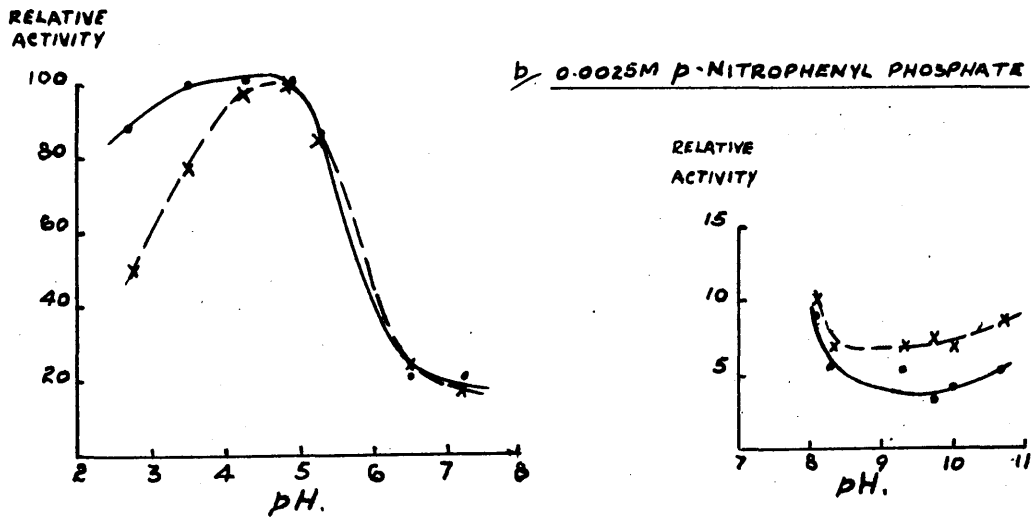


RESULTS.

FIG. 1. 1.

pH-Activity Curve Of Hydrolysis Of a) 0·005M. Phenyl Phosphate And b) 0·0025M. p-Nitrophenyl Phosphate By Rat Liver Homogenates





•————• ASSAYED WITHOUT ADDITION Mg^{++} IONS.

x------x ASSAYED IN PRESENCE OF 0·01M. $MgSO_4$.

1/100 Homogenate Used In a).

1/400 Homogenate Used In b) From pHs 2·8 - 7·2.

1/50 Homogenate Used In b) From pHs. 8·1 - 10·7.

SECTION I.

The influence of pH and other factors on the enzymic hydrolysis of phosphate esters.

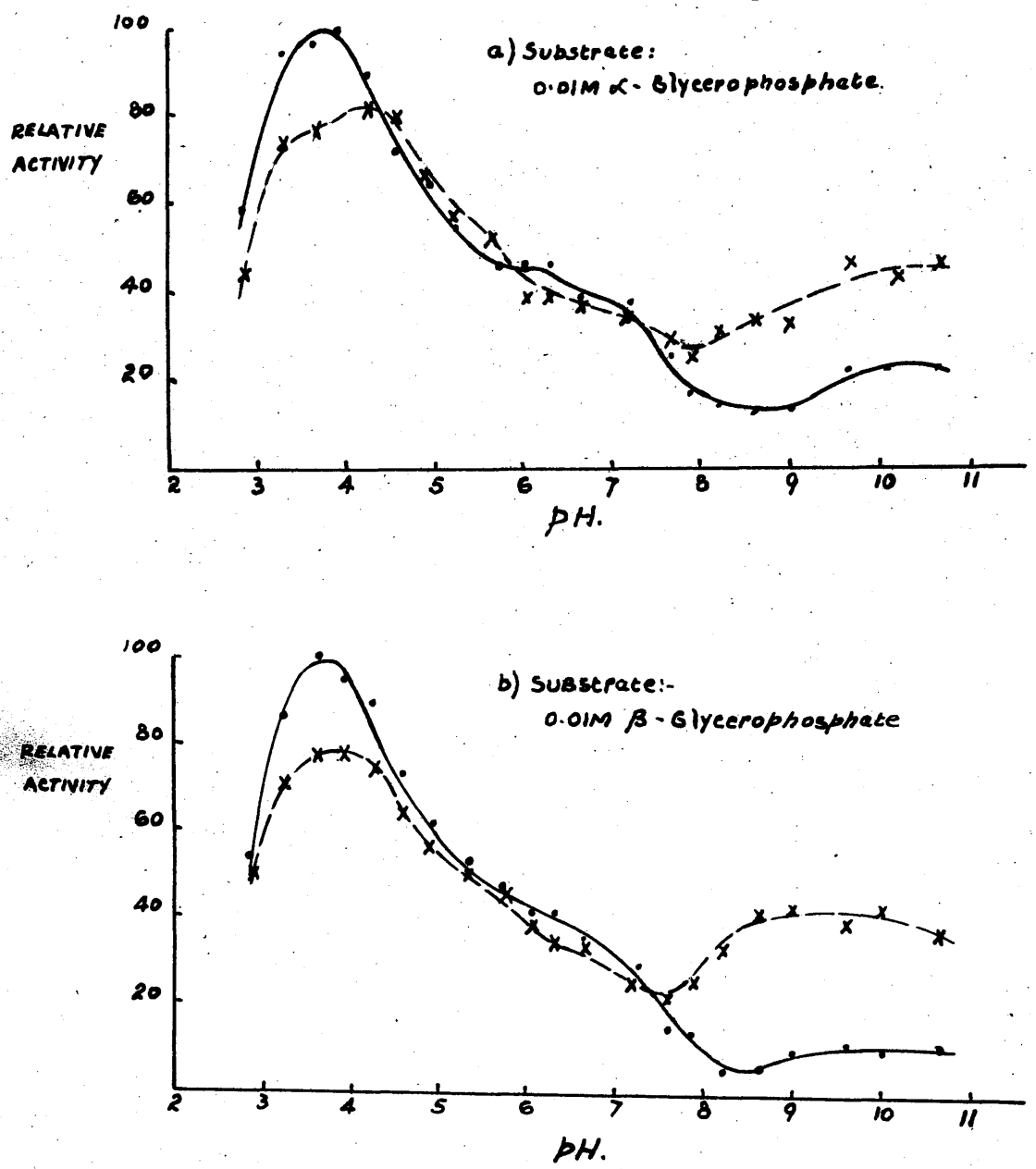
Many factors are known to influence the pH-activity curves of the phosphatases such as the type of substrate (King and Delory, 1939; Delory and King, 1943; Walker and King, 1950), the concentration of substrate (Ross, Ely and Archer, 1951), the type and ionic strength of buffer used (Aebi and Abelin, 1948; Zittle and Della Monica, 1950) and the presence or absence of activators or inhibitors.

In the present work the type of substrate and the effect of added Mg^{++} ions were the only variable factors studied since the main purpose was to investigate the specificity of the various phosphatases present in rat liver before proceeding with attempts at characterisation and examination of their properties. Fresh unfractionated rat-liver homogenates and veronal-acetate buffers were used throughout, the latter to obviate any changes in buffer type.

Figure 1.1 shows the pH-activity curves obtained with 0.005M phenyl phosphate and 0.0025M p-nitrophenyl phosphate. The same general picture was obtained with both substrates, the activity at acid pHs being much greater than that at alkaline pHs. At the low pHs Mg^{++} ions caused inhibition, from pH 5.0 to pH 7.0 they had little or no effect and

Fig. 1. 2.

pH - Activity Curve Of Hydrolysis Of α - And β - Glycerophosphates By Rat Liver Homogenates.



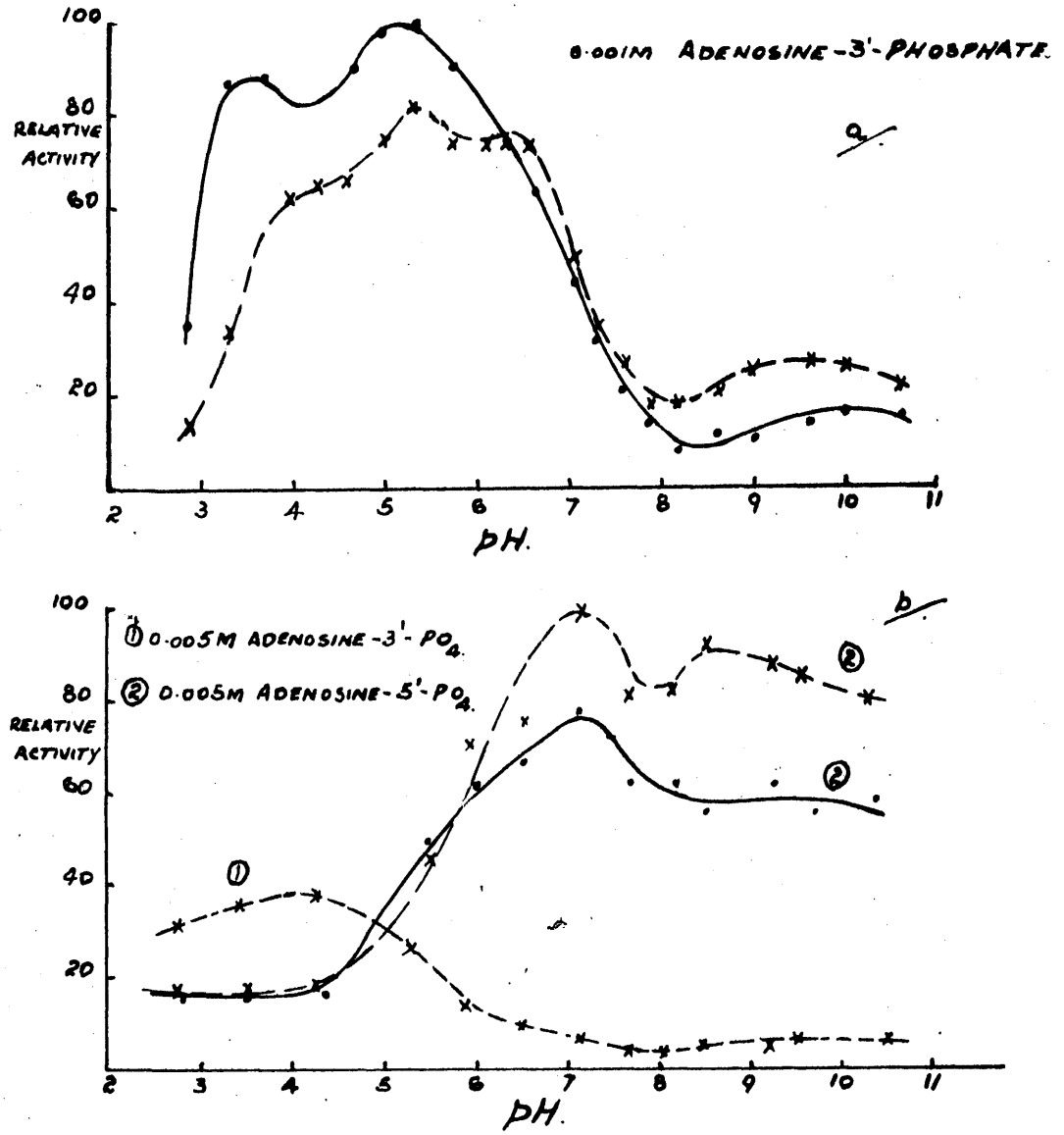
x------x Assayed In presence 0.01M $MgSO_4$.

•——• No $MgSO_4$. Added

1/50 Rat Liver Homogenates Used In Both Cases Assayed At 38° For 1 Hour

FIG. 1.3.

pH-Activity Curves Of Hydrolysis Of Adenosine-3'-Phosphate And Adenosine-5'-Phosphate By Rat Liver Suspensions.



x ----- x Assayed In Presence Of 0·01M $MgSO_4$.

• ——— • Assayed In Absence Of Added Mg^{++}.

Curves In FIG. 1.3.b. Obtained Using Same Rat Liver Preparation 1/30 Rat Liver Homogenate, Assayed For 75 Minutes At 38°

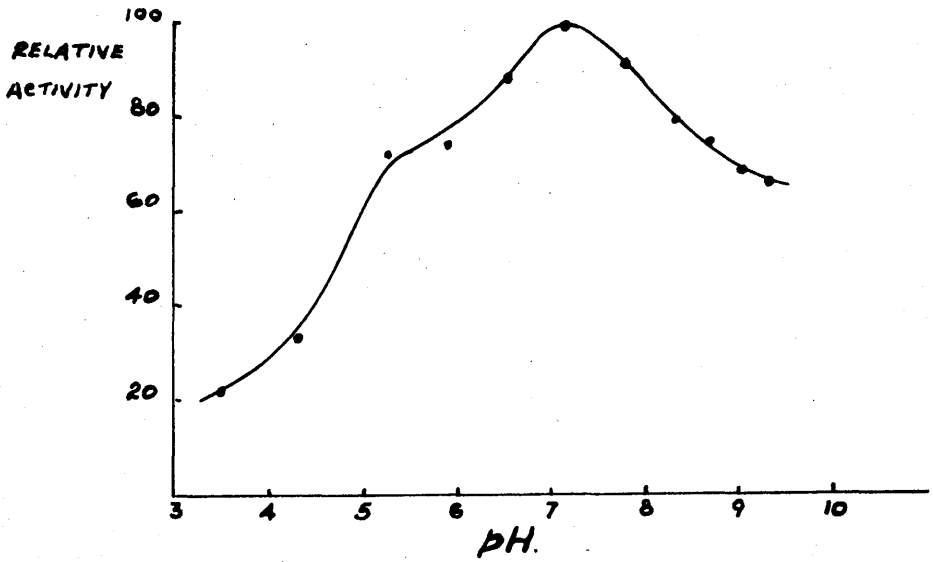
above pH 8.0 they caused activation.

α-and β-glycerophosphates (Figure 1.2) at a final concentration of 0.01M gave curves similar to those shown in Figure 1.1, but with both these substrates, the shape of the curve suggested that there might be another enzyme optimally active between pH6 and 7. Mg^{++} ions had a similar effect with these substrates as with phenyl and p-nitrophenyl phosphates.

The pH-activity curves for the hydrolysis of adenosine-3'-phosphate, adenosine-5'-phosphate and inosinic acid are shown in Figure 1.3 and 1.4. With adenosine-3'-phosphate the results were similar to those for the earlier substrates. Two peaks were found in the acid range, one with an optimum at pH 3.5, the other at about pH 5.3. Mg^{++} ions again had a similar effect, although in the presence of Mg^{++} ions a third peak at around pH 6.5 was observed. With the 5'-nucleotides, however, a very different picture was obtained. The activity between pHs 3 and 4 was only about half that obtained with the 3'-nucleotide whereas two marked peaks of activity were observed, one at pH 7.2 and the other between pH 8-9. Mg^{++} ions caused activation at optima. The maximum activity obtained with the 5'-nucleotide at pH 7.2 was 2.5 times the maximum obtained with the 3'-nucleotide which occurred at pH 4.0 (Figure 1.3.b). Inosinic acid (Figure 1.4) gave a

FIG. 1.4.

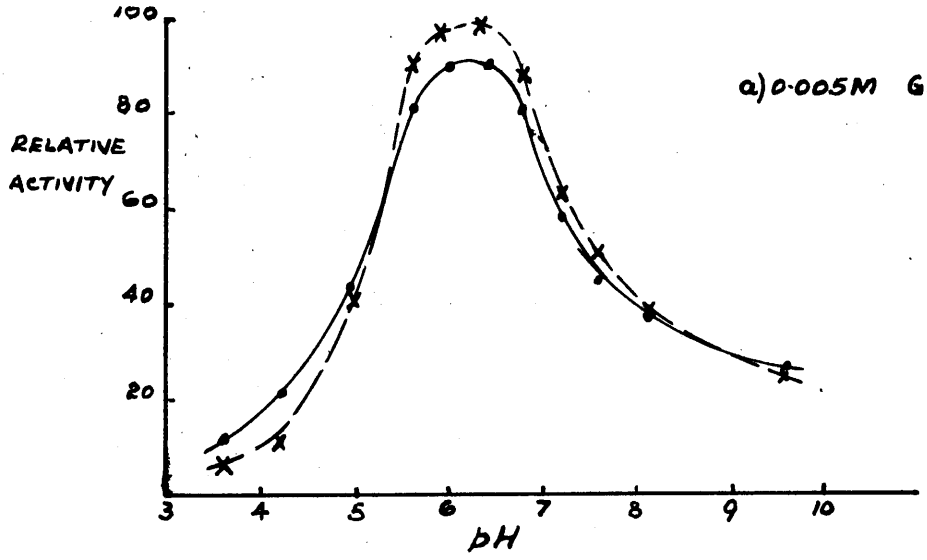
pH-Activity Curve Of Hydrolysis Of 0.005M Inosine-5'-Phosphate In Presence Of 0.01M. Mg^{++} By Rat Liver Homogenate

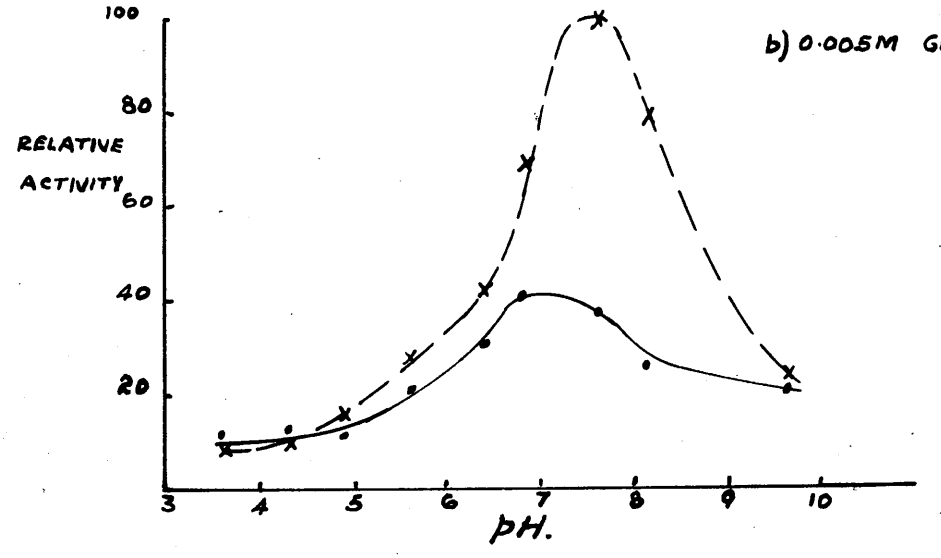


1/10a Rat Liver Homogenate Assayed For 30 Minutes At 38°

FIG. 1. 5

pH-Activity Curves Of Hydrolysis Of Glucuse-6-Phosphate And Glucose-1-Phosphate By Rat Liver Homogenates.





•———• Assayed In Absence Of Added 0·001M $MgCl_2$

———— Assayed In Presence Of 0·001M $MgCl_2$

1/50 Rat Liver Homogenate, Assayed For 1 Hour At 38°.

similar picture to that of adenosine-5'-phosphate. The activity between pHs 5 and 6 with the 5'-nucleotides was much greater than that between pHs 3 and 4, whereas with previous substrates the level of activities at these two pH ranges was of the same order.

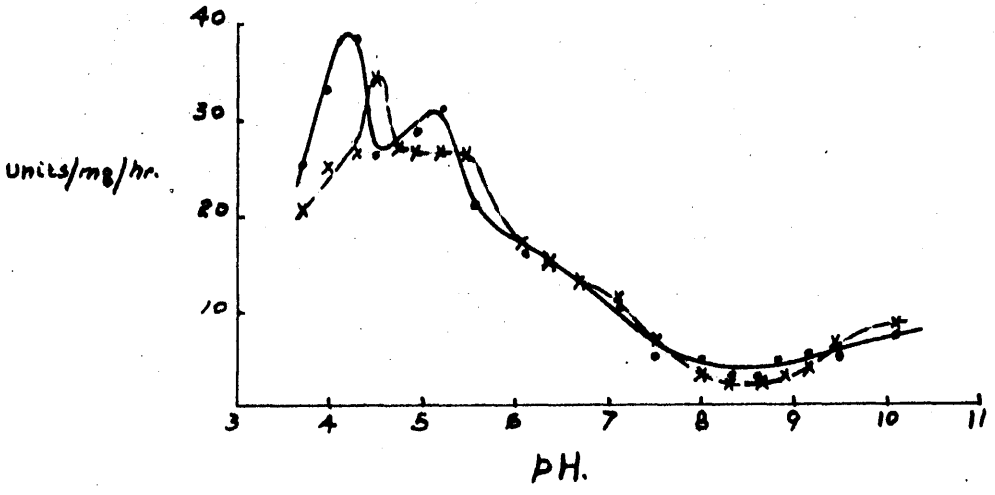
The hydrolysis curves with glucose-1-phosphate and glucose-6-phosphate were found to have only one maximum (Figure 1.5). The hydrolysis of glucose-1-phosphate is strongly activated by Mg^{++} ions and is optimal at pH 7.2, whereas Mg^{++} ions have little effect on the hydrolysis of glucose-6-phosphate, the optimal pH here being at pH 6.5.

It may be deduced from these results that rat liver contains two acid phosphatases showing optimal activity at about pH 3.5 and 5.0 respectively. The former enzyme is inhibited by 0.01M Mg^{++} ions whereas the latter appears to be unaffected. Both these enzymes appear very non-specific in the type of substrate they attack although it is difficult to assess their effect in hydrolysing the 5'-nucleotides and glucose phosphates due to the coexistence of powerful specific phosphatases for these substrates. There also appears to be some evidence for a glycerophosphatase acting optimally at pH 6.5.

With all substrates there appears to be evidence for phosphatases acting optimally in the range pH 9.0-10.5 and

FIG. 1. 6.

Effect Of Triton-X100 On pH-Activity Curve



pH Activity Curve 1/500 Homogenate Of Rat Liver In Water •——•, And 0.1% Triton X-100 x-----x. Substrate 0.005M Phenyl Phosphate Containing 0.001M Mg. Acetate. Assays Carried Out In Veronal-Acetate Buffers. Incubation 20 Minutes At 38°

which are strongly activated by Mg^{++} ions. This activating effect, however, was not so well marked in the case of the hexose phosphates.

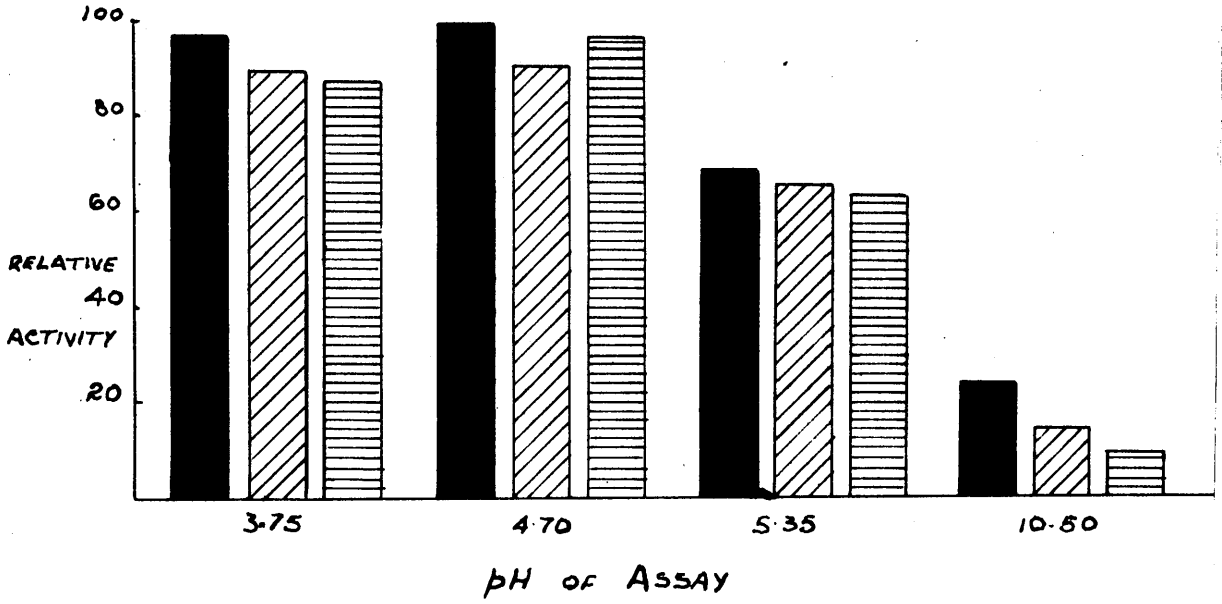
There would appear to be a relatively specific 5'-nucleotidase present in liver optimally active at pH 7.0-7.5 which has little activity towards 3'-nucleotides. With the 5'-nucleotides there is marked hydrolysis at alkaline pHs and it seems therefore that there is either a second specific 5'-nucleotidase or that the non-specific alkaline phosphatase has a high affinity for this type of substrate.

The hydrolysis of glucose-6-phosphate in homogenates is probably caused by the glucose-6-phosphatase isolated by Swanson (1950). The mechanism for the hydrolysis of glucose-1-phosphate appears to be of a different character and will be discussed in more detail in a later section.

It was considered that part of the enzymic activity in these crude liver homogenates might remain in an inactive or bound form and consequently the effect of homogenising in the presence of the detergent Triton X-100 was examined. 30% sucrose solution and 0.4M KCl were also used in place of water as homogenising medium. In the presence of Triton X-100 (Figure 1.6) there appeared to be some inactivation at the more acid pHs. Water appeared to be the most effective homogenising medium since homogenates prepared in sucrose

FIG. 1. 7

Effect Of Variation Of Homogenising Medium On Phosphatase Activity

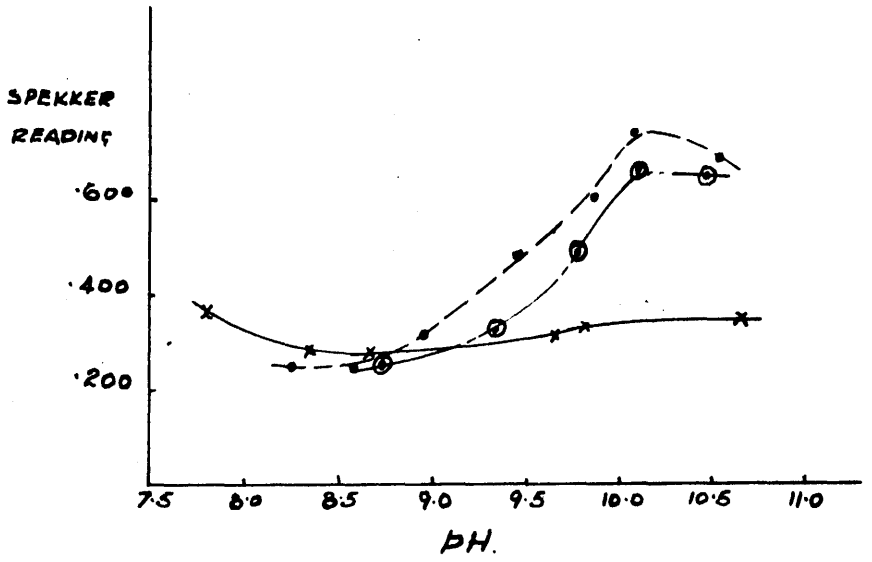


Relative Phosphatase Activity Of 3 Homogenates From Same Rat Liver Prepared In Water ■, 30% Sucrose Solution ▨, And 0·4M KCL ▤.

1/10 Homogenates Were Prepared With The Medium In Question And Diluted Further To 1/200 With Distilled Water

FIG. 1. 8.

Effect Of Varying Type Of Buffer On pH-Activity Curve Of Rat Liver Alkaline Phosphatase.



pH-Activity Curves Of 1/50 Rat Liver Homogenates.

Substrate: 0·005M Phenyl Phosphate, 0·01M with Respect To $MgSO_4$.

Incubated 30 Minutes At 38°

Michaelis' Veronal Acetate Buffer: x———x

Boric Acid - NaCl - NaOH: ·------·

Glycine NaCl - NaOH: ⊙—·—·—⊙

and KCl had much lower alkaline phosphatase activities (Figure 1.7).

Since veronal-acetate mixtures have a weak buffering capacity at high pHs, boric acid - NaCl-NaOH and glycine - NaCl-NaOH buffers were compared with veronal-acetate buffers over the pH range for alkaline phosphatase. Higher alkaline phosphatase activities were observed with the boric acid and glycine buffers than with the veronal-acetate buffers (Figure 1.8).

SECTION II.

The Intracellular Distribution of Rat-Liver Phosphatases.

A study of the intracellular distribution of the rat liver phosphatases was undertaken in the hope that it might lead to further characterisation of the various enzymes suggested by the results of Section I.

Novikoff, Podber and Ryan (1950), using rat-liver extracts, prepared in water and 0.88M sucrose, found that about 40% of the acid phosphatase activity was associated with the mitochondrial fraction and 50-60% of the alkaline phosphatase activity with the supernatant fraction of cytoplasm. Berthet and de Duve (1951), using the method of Schneider (1948) which involves differential centrifugation of extracts of tissue prepared in 0.25M sucrose, found 50-60% of the acid phosphatase activity in the mitochondria. Palade (1951) using 0.88 M sucrose solution found that only 40% of the acid phosphatase activity was present in the mitochondria and reported larger amounts in other fractions than did Berthet and de Duve. Berthet and de Duve assayed acid phosphatase at pH 5.0 using ABC buffers whereas Palade assayed at pH 4.5 using acetate buffers. The distribution of alkaline phosphatase was also studied by de Duve and his colleagues (Hers, Berthet, Berthet and de Duve, 1951) and they found that this enzyme was present to a large extent in

the microsome fraction of kidney and intestine. The microsome fraction, as separated by these workers, was shown to carry 70-80% of the glucose-6-phosphatase activity. Phosphoglucomutase, on the other hand, was recovered completely in the non-particulate cytoplasm.

Method of preparation of cellular fractions.

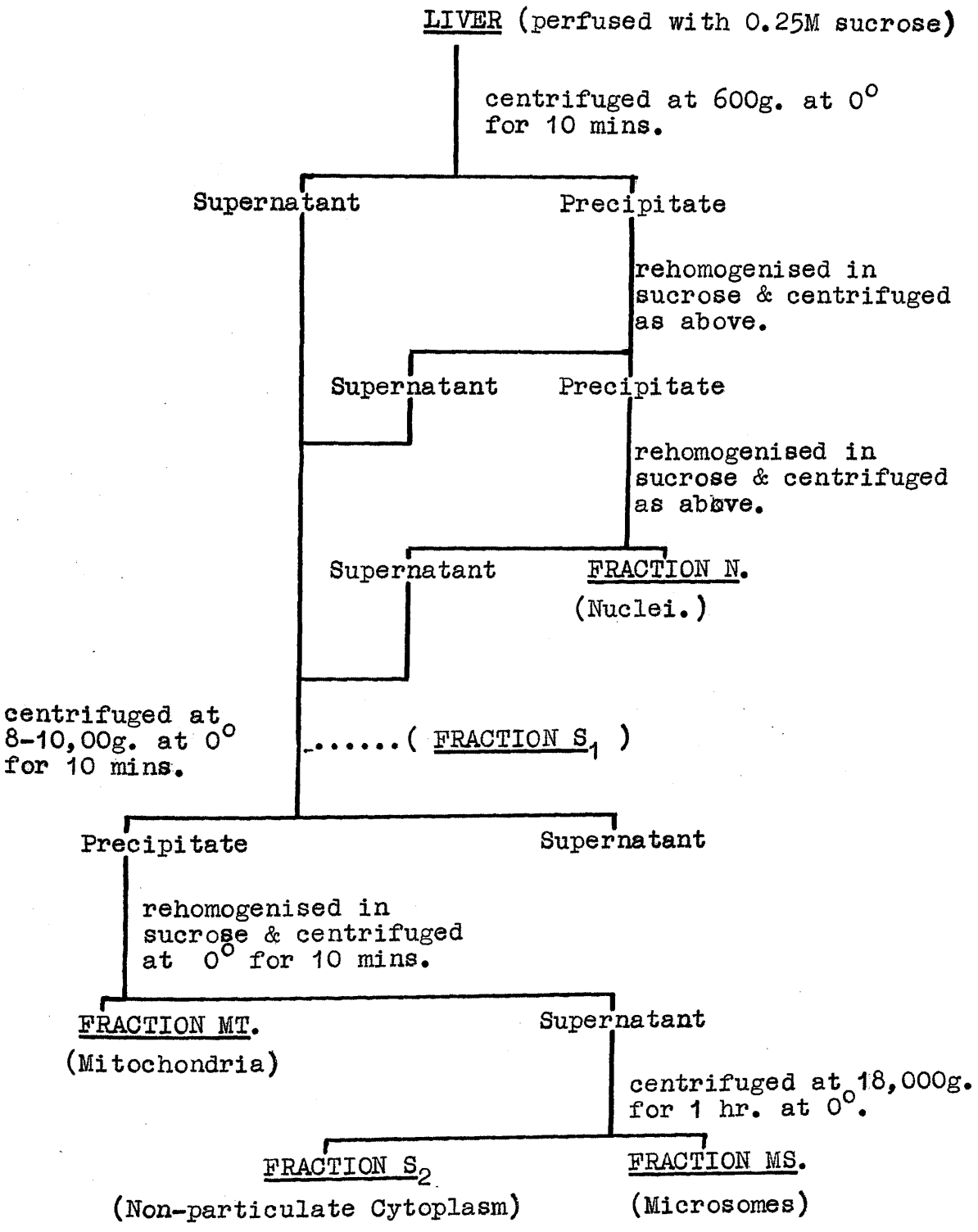
In view of the sensitivity of some enzymes to increases in ionic strength, e.g., phosphoglucomutase (Klenow, 1953), it was decided to employ 0.25M sucrose as the homogenisation medium. 0.88 M sucrose was not employed since some enzymes are inactivated by high concentrations of sucrose and also in view of the lack of suitable equipment for the centrifugal separation of microsomes in this high concentration of sucrose.

The technique adopted was essentially that of Schneider (1948). A rat was killed by decapitation and exanguination and the liver perfused *in situ* with ice-cold 0.25M sucrose solution. The liver was removed, washed with ice-cold sucrose solution and excess moisture dried with filter-paper. About 1 g. liver was homogenised in 9 volumes of ice-cold 0.25M sucrose solution for 2 minutes in a Potter-Elvehjem (1936) Pyrex glass homogeniser. The time of homogenisation was found to be very critical in that to ensure a preparation free from a significant number of unbroken cells homogenisation had to be continued for 4-5 minutes. Unfortunately there

was evidence that homogenising for this length of time brought about serious breakdown of cytoplasmic particles. It was necessary therefore to adopt a compromise between incomplete rupture of all cells and a breakdown of mitochondria.

The homogenate was centrifuged for 10 minutes at 2000 r.p.m. (600 g.) using the horizontal yoke (M.S.E. Major refrigerated centrifuge). The supernatant was removed with a Pasteur pipette and the residue rehomogenised in 5 ml. ice-cold 0.25M sucrose solution and recentrifuged for 10 minutes at 600 g. This washing process was repeated once more, the nuclei (Fraction N) suspended in 0.25M sucrose, a cell and nuclei count carried out and the suspension stored at -15°. An aliquot of the combined supernatant and washings was retained (Total cytoplasmic fraction - S_1). The rest of the cytoplasmic fraction was centrifuged at 8,500-10,000 g. for 10 minutes, the supernatant removed and the precipitate washed with 3 ml. of the sucrose solution. The suspension was recentrifuged as above, the supernatant removed and the residue suspended in sucrose solution and stored at -15° (the mitochondrial fraction - MT). The combined supernatants were spun at 18,000 g. for 1 hour and the supernatant was removed and stored at -15° (the non-particulate cytoplasmic fraction - S_2), while the residue was suspended in sucrose solution and stored at -15° (the microsomal fraction - MS).

Fig. 2.1. METHODS EMPLOYED IN PREPARING INTRACELLULAR FRACTIONS.



A flow sheet of the separation process is shown in Figure 2.1.

A count of nuclei and unbroken cells was carried out on fraction N in 3 ml. sucrose solution using a standard haemocytometer with 0.25M sucrose as diluting fluid. The results indicated a ratio of nuclei/unbroken cells of $^{11}/_{1}$ along with some mitochondria and fibrous cell debris.

Acid phosphatase activity was determined using 0.01M β-glycerophosphate as substrate in veronal-acetate buffers at pHs 3.8, 5.55 and 6.5.

Alkaline phosphatase activity was determined at pH 10.15 using the same substrate in a boric acid - KCl - NaOH buffer in the presence of Mg^{++} ions (10^{-2}M - final concentration).

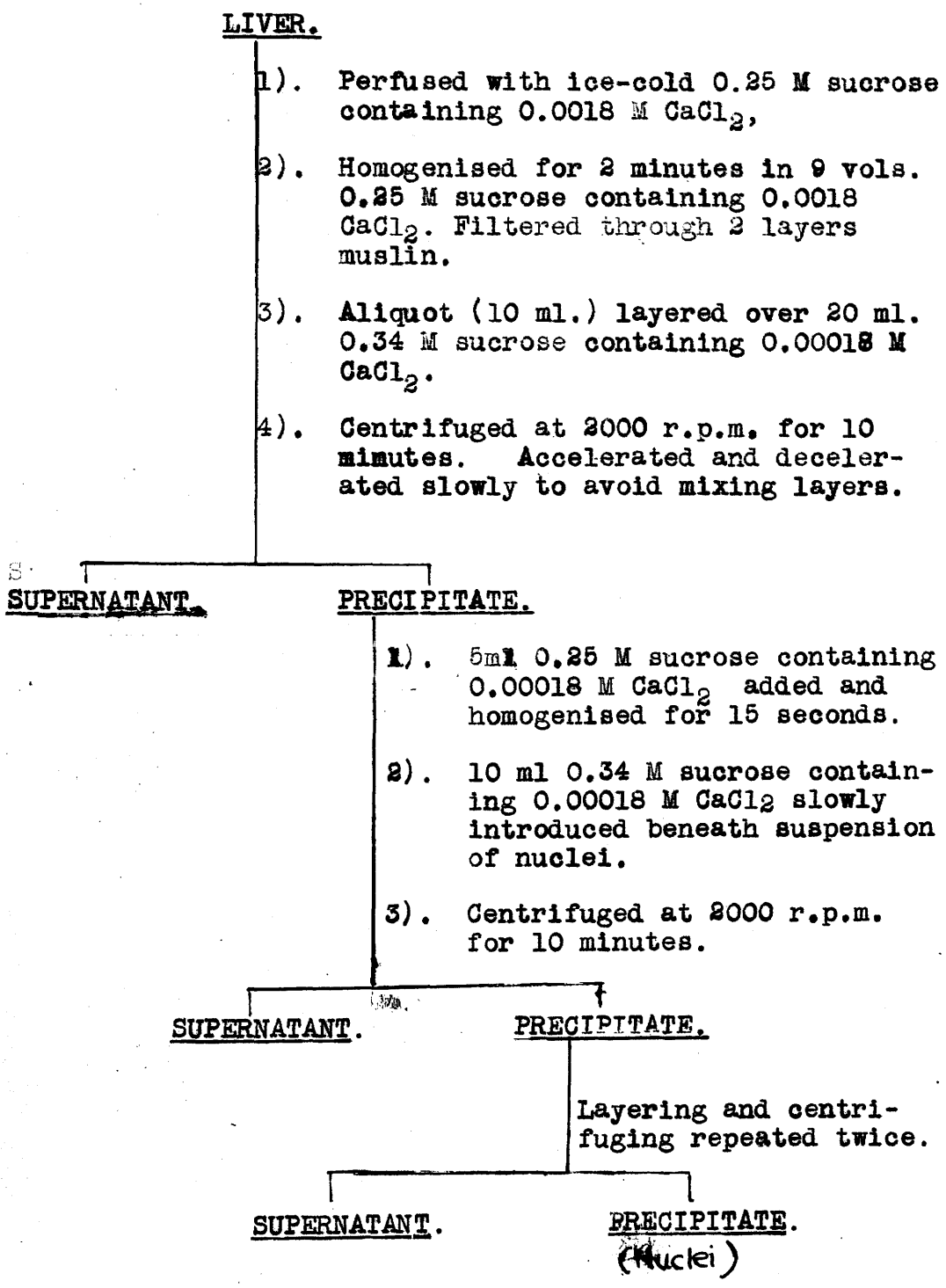
Glucose-6-phosphatase activity was assayed at pH 6.5 using a veronal-acetate buffer and 0.005M glucose-6-phosphate.

5'-Nucleotidase activity was assayed using 0.003M adenosine-5'-phosphate with Mg^{++} ions (10^{-2}M final concentration) at pH 7.2 in a veronal-acetate buffer, and at pH 10.15 in a boric acid-KCl-NaOH buffer.

Each fraction was frozen in a mixture of solid CO_2 and acetone and thawed rapidly. This was repeated twice in order to rupture completely the organised structures.

Assays were carried out on fractions N, MT, MS, S_1, S_2 and a reconstituted mixture of N + S_1 in equivalent proportions. This mixed fraction was used as an estimate of

Figure 2.2.



All manipulations carried out at 0°.

the total activity present.

The results are presented in Table 2.1. It will be seen that the distribution of the acid phosphatase activity was similar at the three pHs of assay with the greatest activity appearing in the mitochondrial fraction. Alkaline phosphatase activity was highest in fraction S_2 although fraction N showed a significant activity. Glucose-6-phosphatase activity was present in highest concentration in the mitochondrial fraction. The distribution of the 5'-nucleotidase activity was similar at both pH 7.2 and pH 10.15. Highest activity was obtained in fraction MS and fraction N again appeared to have significant activity. Recoveries of the non-specific phosphatases were good although there was an 18-28% loss with the specific enzymes.

Since it was observed that fraction N possessed significant phosphatase activity under certain conditions and in view of the contamination of this fraction with unbroken cells and mitochondria, it was decided to study the activities of nuclei isolated by the method of Hogeboom, Schneider and Striebich (1952). This procedure is outlined in Figure 2.2. Rat livers were perfused <u>in situ</u> with ice-cold 0.25M sucrose containing 0.0018M $CaCl_2$ and then removed and homogenised in 9 volumes of the same solution for two minutes using a Pyrex glass homogeniser (Potter and Elvejhem, 1936). The homogenate

Table 2.1.

Relative Phosphatase Activity of Various Fractions expressed as Percentage of that of Nuclear and Total Cytoplasm.

Fraction	Substrates and pH of Assay						
	0.01M β-glycerophosphate				.005M glucose-6-PO_4	.0025M adenosine-5'-PO_4	
	3.80	5.55	6.50	10.15	6.50	7.20	10.15
N + S_1	100	100	100	100	100	100	100
N	12	14	12	26	17	23	27
MT	40	40	42	7	33	14	9
MS	29	26	21	18	19	34	31
S_2	14	14	16	50	3	11	10
Recovery	95	94	91	101	72	82	77

was filtered through two layers of muslin and a 10 ml. aliquot was layered carefully over 20 ml. of ice-cold 0.34M sucrose containing 0.0018M $CaCl_2$ in a centrifuge tube and centrifuged at 2000 r.p.m. (600 g.) using the horizontal yoke. Acceleration and deceleration were carried out slowly to avoid mixing of the layers. The supernatant was removed with a Pasteur pipette and stored at 0°. The precipitate was rehomogenised for 15 seconds in 0.25M sucrose containing 0.00018M $CaCl_2$ and 10 ml. 0.34M sucrose containing 0.00018M $CaCl_2$ was introduced beneath the nuclear suspension and the whole centrifuged for 10 minutes at 600 g. The supernatant was removed and the washing procedure was repeated twice more. The supernatants were pooled and made up to a suitable volume. The nuclei were suspended in 0.25M sucrose/$CaCl_2$ solution so that the nuclear and cytoplasmic fractions were the same concentration with respect to sucrose and $CaCl_2$. Both fractions were frozen in a mixture of solid CO_2 in acetone and immediately thawed. This process was repeated three times to rupture the particulate structures.

Phosphatase assays were carried out as described above and DNAP and RNAP estimations carried out as an indication of the purity of the fractions.

The results of a typical experiment are presented in Table 2.2. Negligible amounts of acid phosphatase and

Table 2.2.

Relative Phosphatase Activities of Rat Liver Nuclei and Cytoplasm.

Fraction	Substrate and pH of Assay							DNAP ug/100 mg. Liver	RNAP ug/100 mg. Liver
	β-glycerophosphate				Glucose 6-PO_4	Adenosine-5'-PO_4			
	3.80	5.55	6.50	10.15	6.50	7.20	10.15		
Original	100	100	100	100	100	100	100	20.16	82.0
Nuclear	3	6	3	26	1	13	16	14.95	6.8
Cytoplasmic	84	98	83	80	58	68	66	5.21	65.6
Recovery	87	104	86	106	59	81	82	20.16	72.4

glucose-6-phosphatase were found in the nuclear fraction. The alkaline phosphatase and 5'-nucleotidase activity of this fraction must, however, be considered significant. From the distribution of DNAP it appeared that the nuclear fraction contained 75% of the nuclei originally present. The low RNAP of this fraction was an indication that it was free from significant contamination by unbroken cells and cytoplasmic particles which was confirmed by microscopie examination.

Further studies were carried out on the distribution of the phosphatases of the cytoplasmic fractions prepared by the original Schneider (1948) method as indicated above. In these studies the nuclear fraction was discarded and fractions MT, MS and S_2 prepared from the supernatant.

These fractions were assayed as described above and the results from three separate livers are shown in Table 2.3. The activities are expressed as percentages of the activity of the total cytoplasm, fraction S_1. In Figure 2.3 the results obtained with rat 2 are expressed in the form of a histogram.

In the 3 livers studied the same general pattern was observed as before. 45-60% of the acid phosphatase activity was recovered in the mitochondrial fraction, while the microsome fraction contained 20-38% of this activity. Over 50%

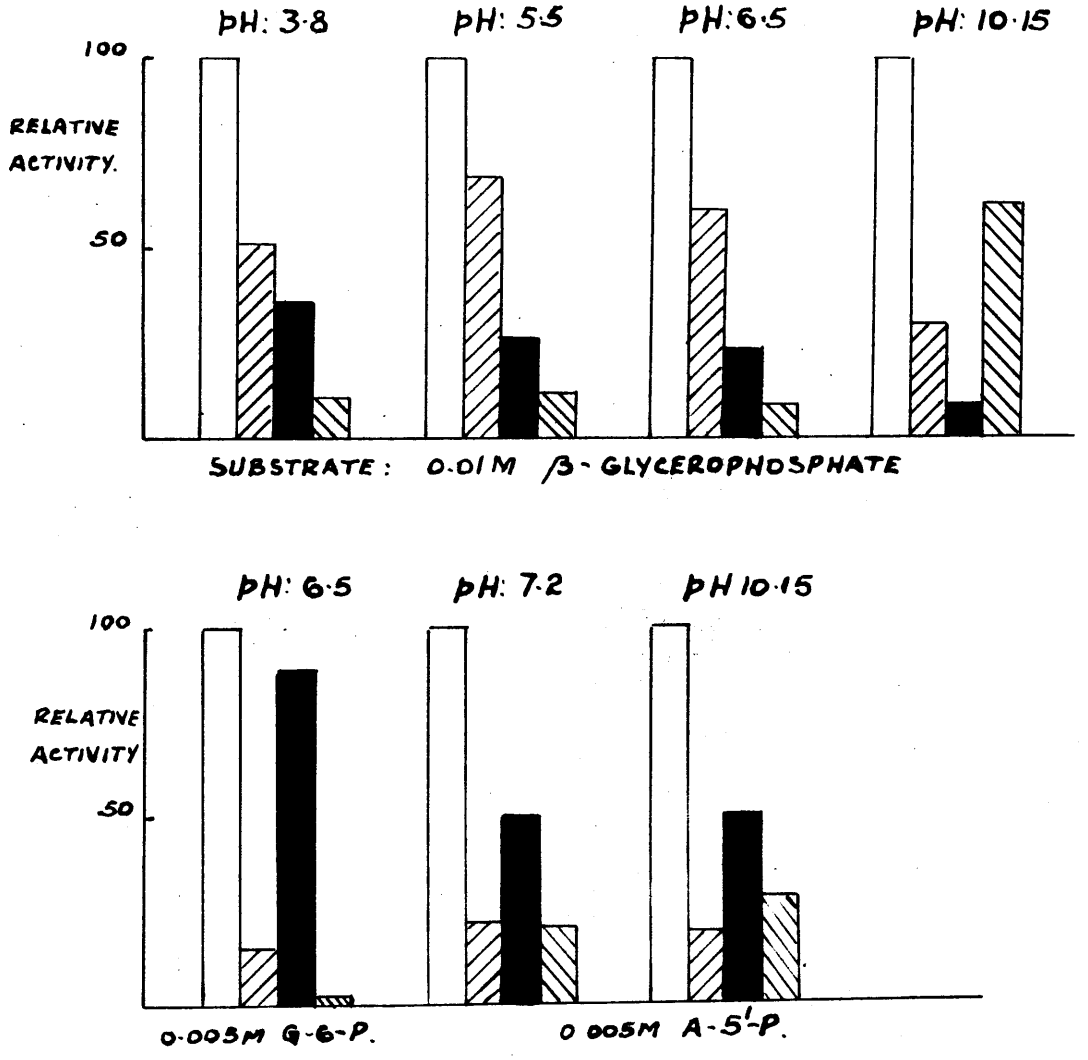
Table 2.3.

Relative Phosphatase Activity of Various Fractions of Rat Liver Cytoplasm.

Rat No.	Fraction	Substrates and pH of Assay						
		0.01M β-glycerophosphate				0.005M glucose-6-PO_4	0.005M adenosine-5'-PO_4	
		3.80	5.50	6.50	10.15	6.50	7.20	10.15
1	S_1	100	100	100	100	100	100	100
	MT	47	48	44	23	23	13	10
	MS	19	23	23	24	78	55	60
	S_2	15	10	8	53	0	18	19
	Recovery	81	81	75	100	101	86	89
2	S_1	100	100	100	100	100	100	100
	MT	51	68	60	30	15	22	19
	MS	37	27	23	9	88	50	50
	S_2	11	12	9	61	2	20	23
	Recovery	99	107	92	100	105	92	92
3	S_1	100	100	100	100	100	100	100
	MT	54	45	45	6	30	16	8
	MS	38	38	34	39	64	65	68
	S_2	20	20	21	55	6	18	23
	Recovery	112	103	100	100	100	99	99

FIG. 2.3

Relative Hydrolysis Of β-Glycerophosphate At pHs 3.8, 5.5 And 10.15, Glucose-6-Phosphate At pH 6.5 And Adenosine-5'-Phospate At pHs 7.5 And 10.15 By Different Cytoplasmic Fractions Of Rat Liver



of the alkaline phosphatase activity was recovered in the S_2 fraction with variable amounts in the other two fractions. Glucose-6-phosphatase activity was clearly concentrated in the microsome fraction and to a lesser extent in the mitochondrial fraction, the amount present in fraction S_2 being negligible. 5'-Nucleotidase also appeared to be concentrated in fraction MS, while fraction S_2 had a higher 5'-nucleotidase activity than glucose-6-phosphatase activity.

The results indicate that the distribution of acid phosphatase when assayed at the three pHs was essentially the same. There was also no evidence to indicate a different distribution of 5'-nucleotidase when the assay was carried out at pH 7.2 than when performed at pH 10.15.

SECTION III.

The Acid Phosphatases of Liver.

The only mammalian acid phosphatase which has been purified to any great extent is that of the prostate (London and Hudson, 1953) and relatively little work has been done on the intracellular acid phosphatases.

The existence of two acid phosphatases in liver was inferred from the results of studies on crude liver extracts (Roche, 1950) but the evidence was by no means conclusive. It was considered therefore that a more detailed study of the properties of rat liver acid phosphatase was of some importance.

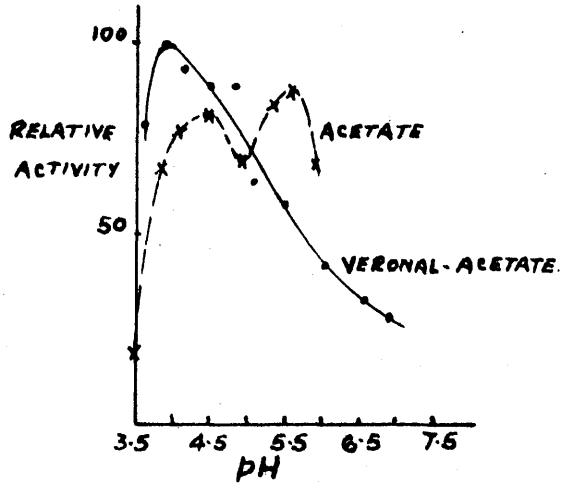
Abul-Fadl and King (1949d) studied the effects of various metallic ions and acid radicals on the hydrolysis of phenyl- and α - and β -glycerophosphates by crude extracts of prostate and erythrocytes at pH 5.0. Cu^{++} ions at low concentrations and formaldehyde were found to inhibit the erythrocyte enzyme strongly but to have little or no effect on the prostatic enzyme. Fluoride and Fe^{++} ions in acetate on the other hand were found to cause a marked inhibition of the prostatic enzyme but to have less effect on the erythrocyte enzyme. L-tartrate inhibited the prostate enzyme almost completely whereas it had little or no effect on the red cell enzyme. These workers found that human liver acid phosphatase was also strongly inhibited by this substance. Gordon (1952)

Fig. 3. 1.

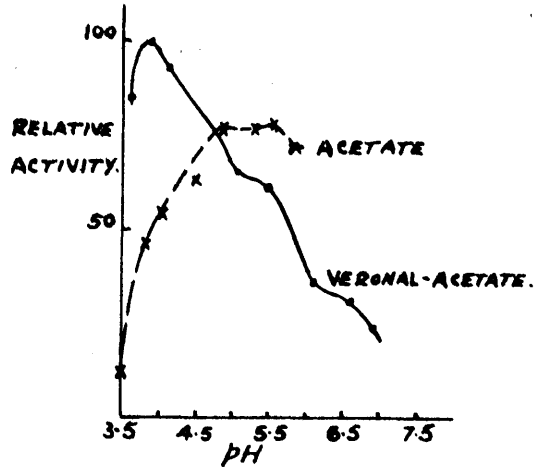
The Effect Of Type Of Buffer On pH Activity Curve Of Acid Phosphatase Of Rat Liver Homogenate.

a) Substrate :- Phenyl Phosphate

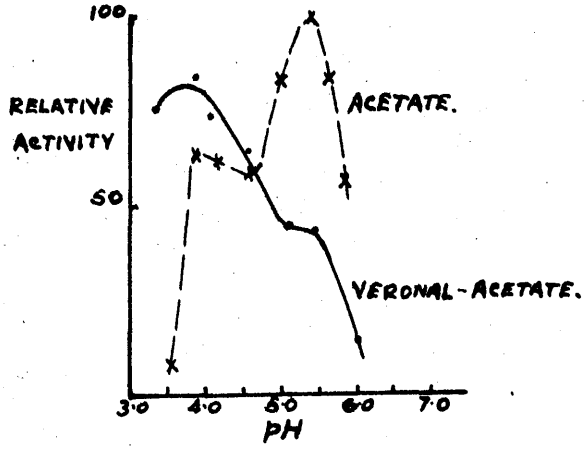
LIVER I



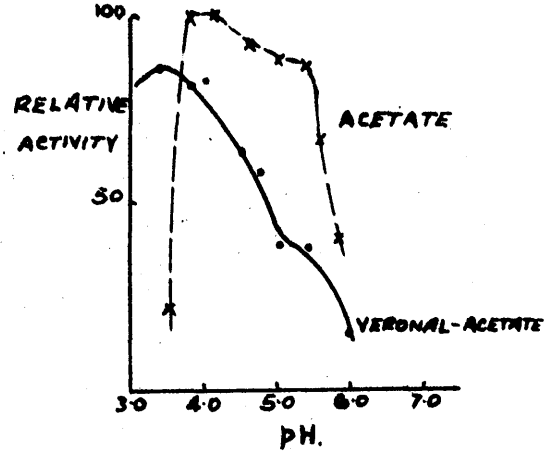
LIVER II



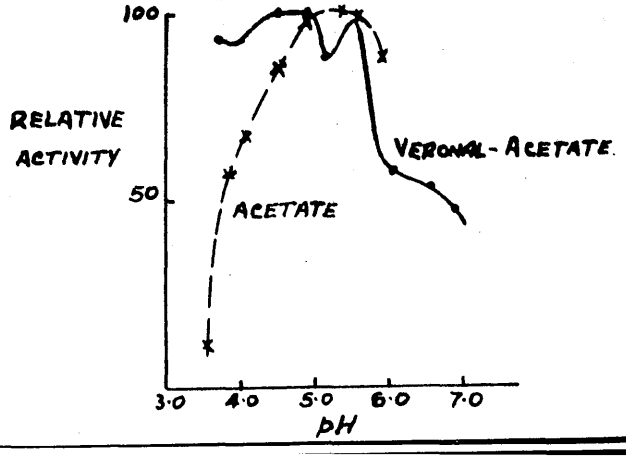
b) Substrate :- α- Glycero - Phosphate



c) Substrate :- β- Glycero - Phosphate



d) Substrate :- Adenosine - 3' - Phosphate



studied the effect of some of these substances on the acid phosphatase of rat adrenal cortex preparations and found Cu^{++}, formaldehyde and DL-tartrate to activate markedly at pH 5.2 whereas Fe^{++}, as in the case of the erythrocyte enzyme, had no effect.

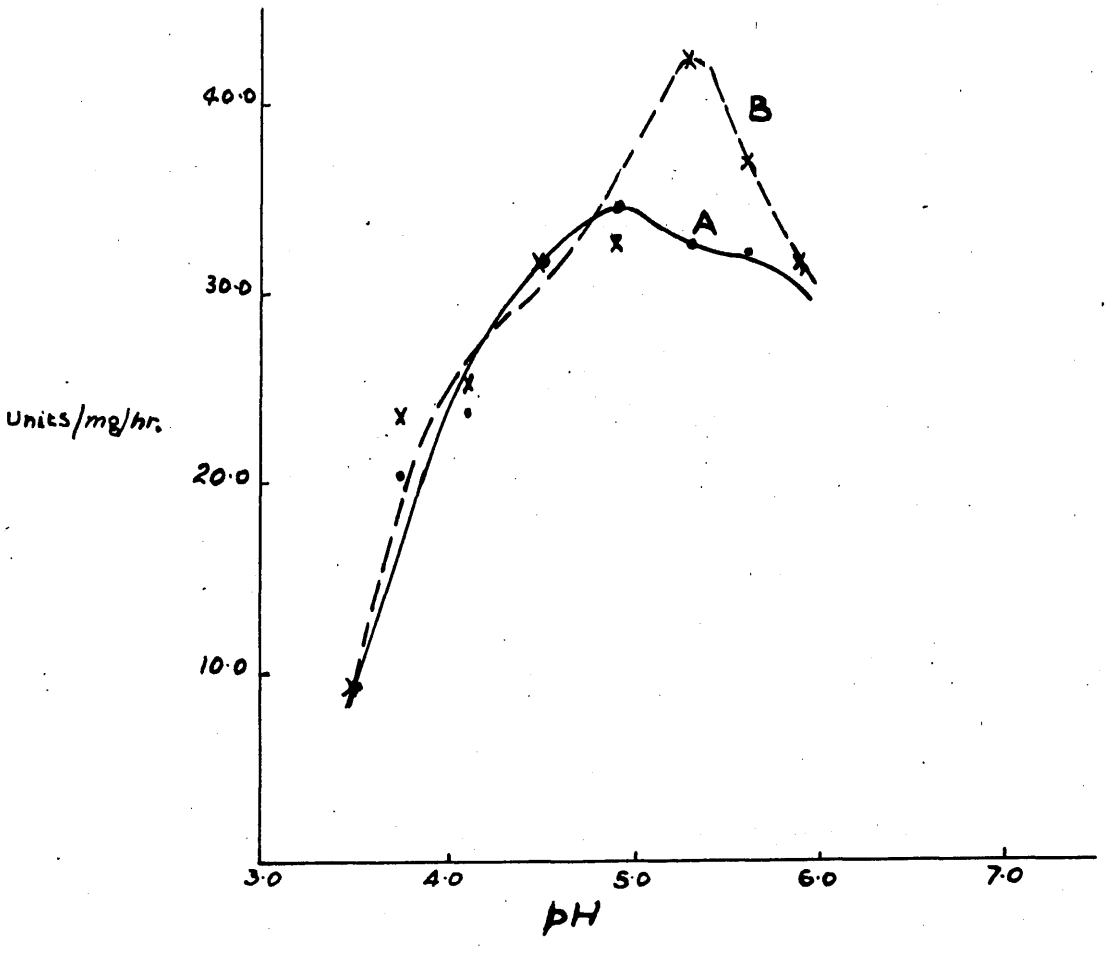
In the present work the effects of some of these substances were studied on the phosphatase activity of unfractionated rat liver homogenates over a wide pH range on the acid side of neutrality in an attempt to obtain more evidence for the multiple nature of the enzyme system present.

Effect of Various Substances on Acid Phosphatase Activity of Rat Liver Homogenates.

In preliminary experiments it was observed that the shape of the pH-activity curves obtained using sodium acetate-acetic acid buffer mixtures differed from those obtained when veronal-acetate buffers were used. Accordingly two series of buffers covering the same pH range and having the same final ionic strength, I = 0.172, were prepared using sodium acetate-acetic acid mixtures and veronal-sodium acetate-HCl mixtures respectively. The results of assays carried out in these buffer systems with phenyl phosphate, α - and β-glycerophosphate and adenosine-3'-phosphate are shown in Figure 3.1. In all cases higher activities at the most acid pHs were obtained with the veronal buffers, the converse being true with acetate

FIG. 3.2.

The Effect Of Storing At - 15° On Acid Phosphatase Activity Of Rat Liver Homogenate.



A. ORIGINAL SOLUTION

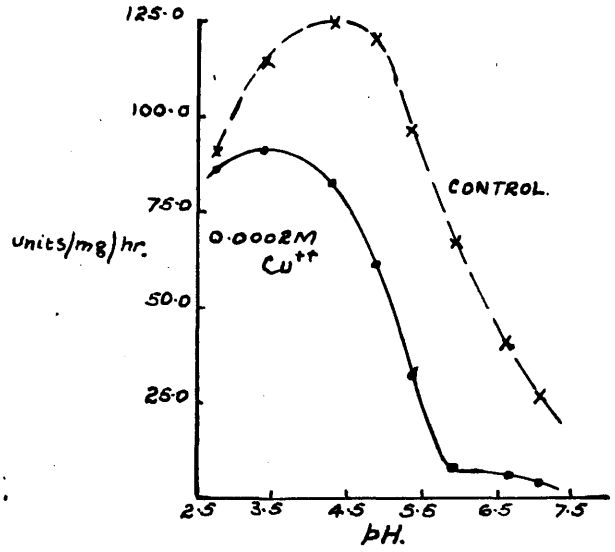
B. ORIGINAL SOLUTION AFTER STORING OVERNIGHT AT - 15°

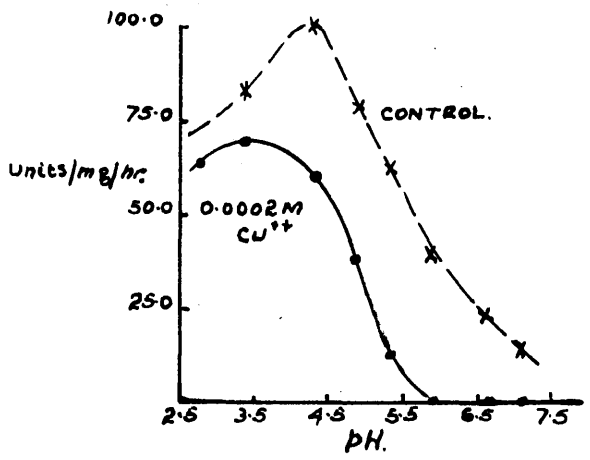
0·005M PHENYL PHOSPHATE USED AS SUBSTRATE

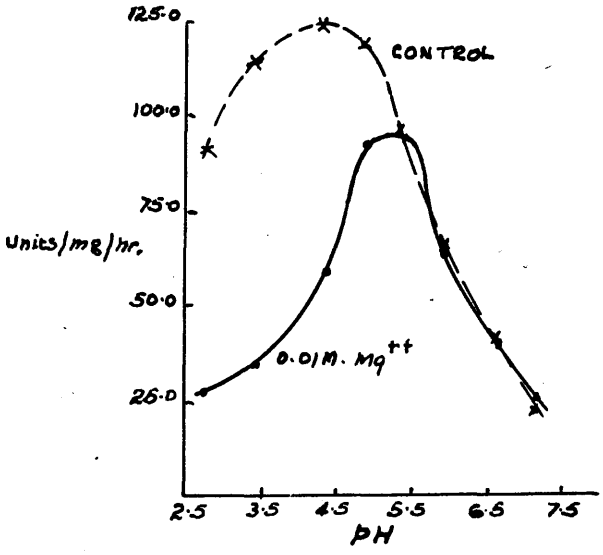
FIG. 3.3

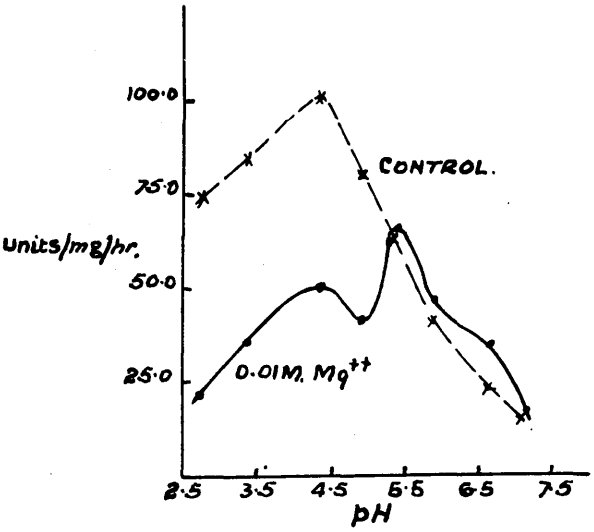
The Effect Of Cu^{++}, Mg^{++} And Tartrate On The Hydrolysis Of 0.0025M p-Nitrophenyl Phosphate By Rat Liver Acid Phosphatase

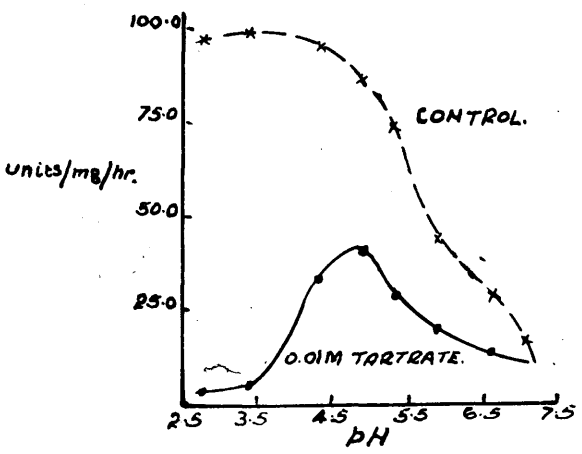
(Experiments Carried Out On Homogenates Prepared From Two Different Livers)











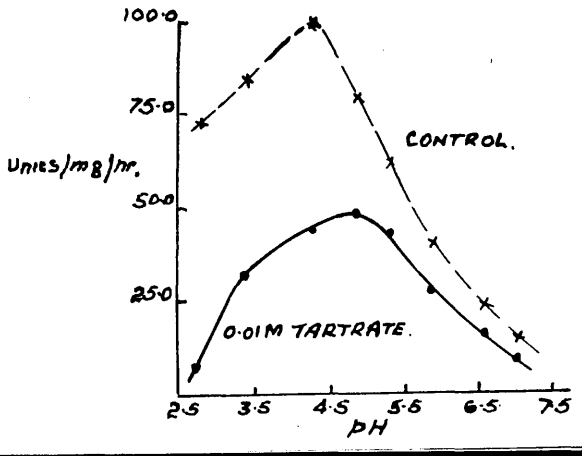
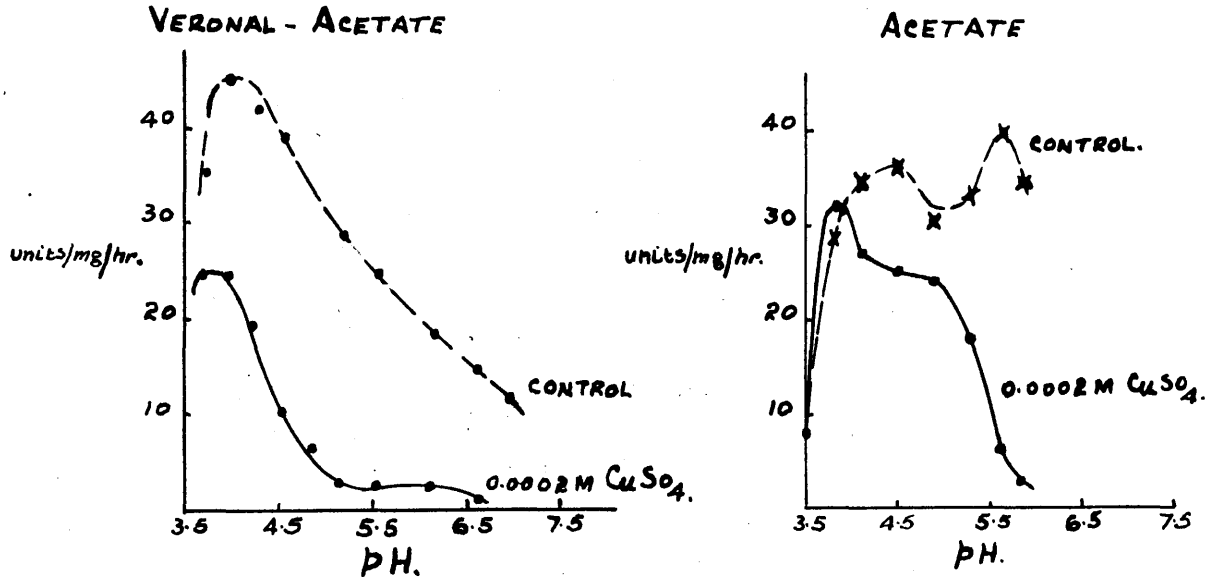


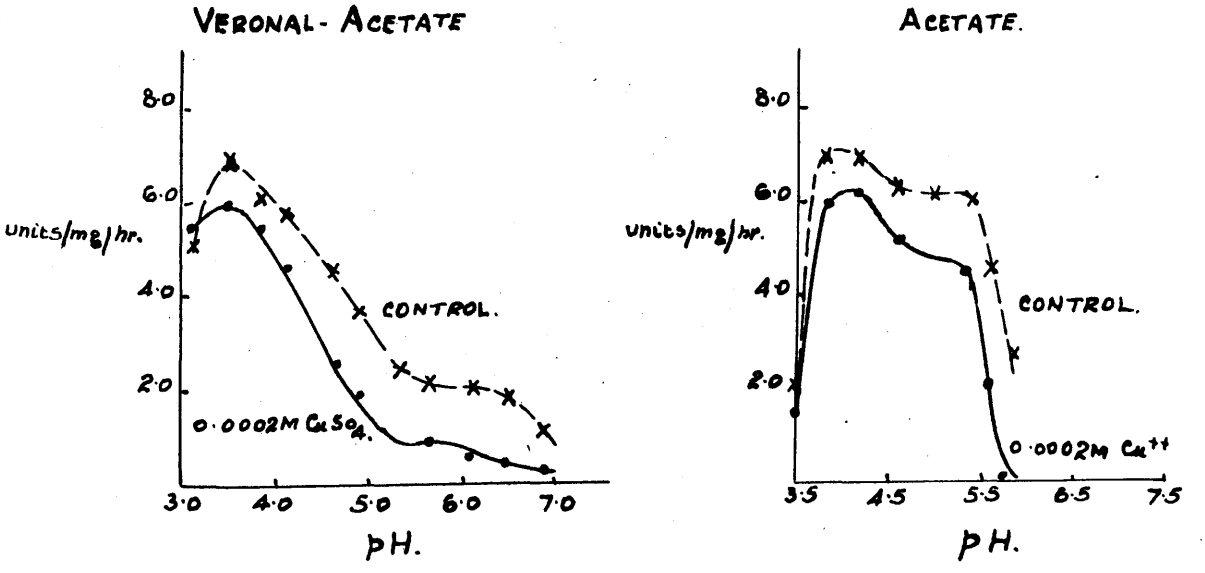
FIG. 3.4.

The Effect Of Cu^{++} On The pH-Activity Curves Of Acid Phosphatases In Rat Liver Homogenate In Acetate And Veronal-Acetate Buffers.

a) SUBSTRATE - 0.005M PHENYL - PHOSPHATE.



b) SUBSTRATE - 0.02M β-GLYCERO-PHOSPHATE



buffers.

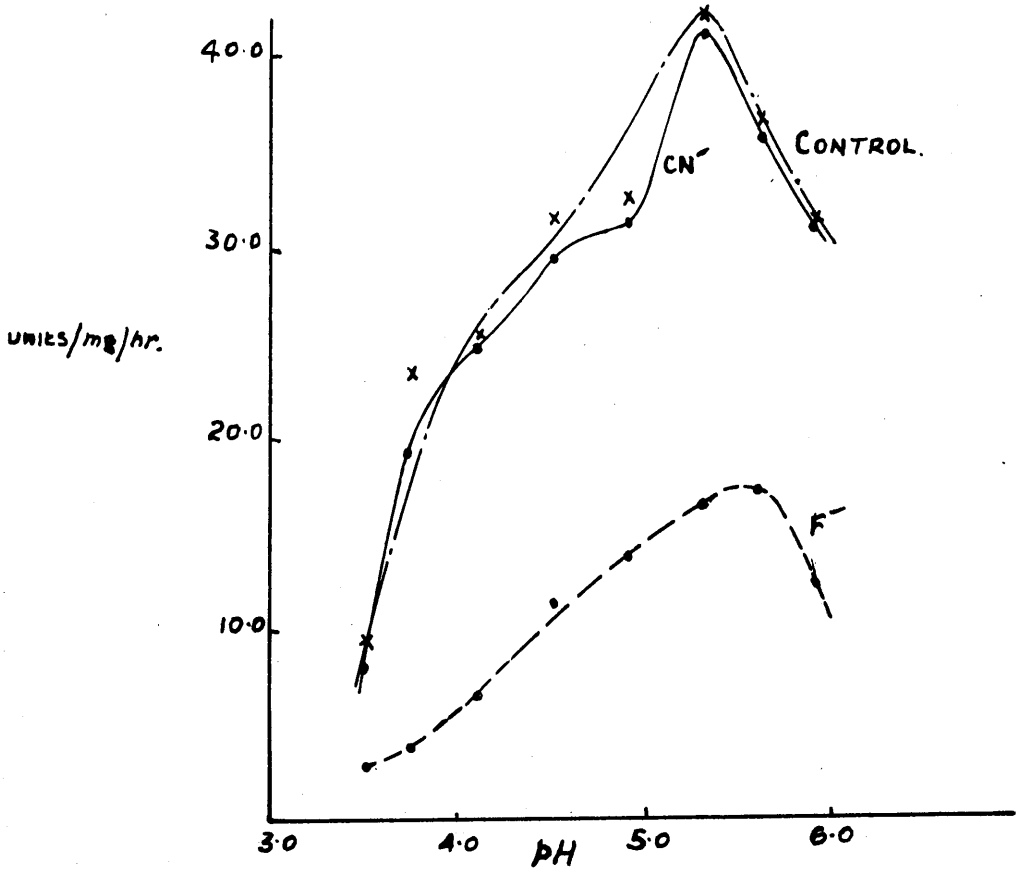
The effect of storing homogenates at -15° was also studied (Figure 3.2). Although the activity at pHs below 4.0 appeared to undergo little change, there appeared to be a significant activation around pH 5.0-5.5.

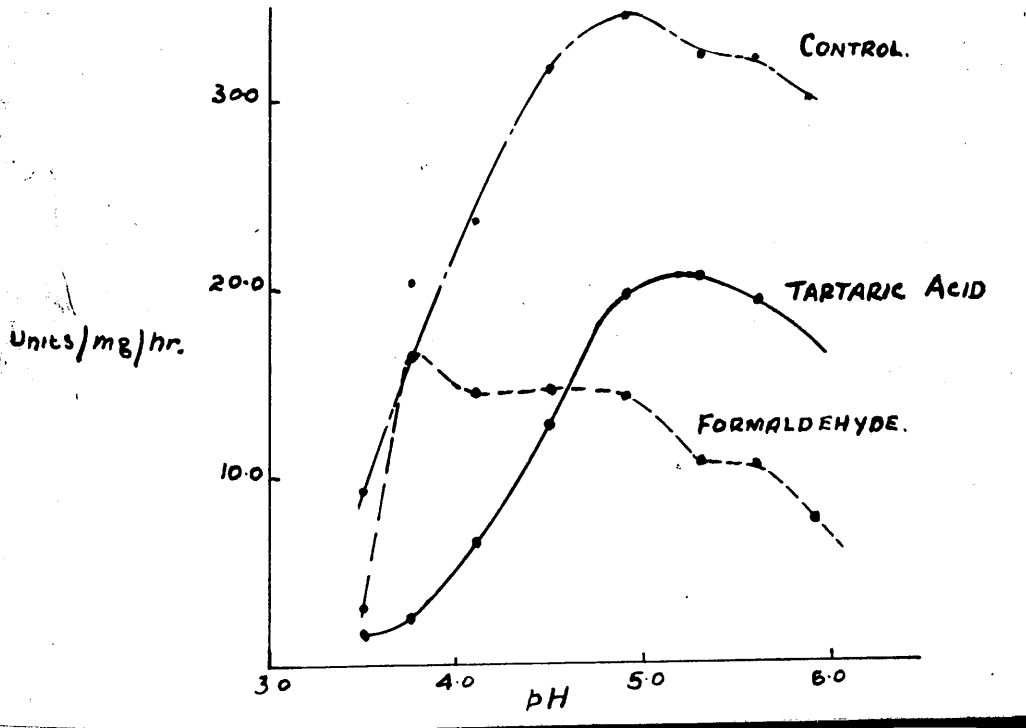
The effects of 0.002M $CuSO_4$, 0.01M $MgCl_2$ and DL-tartrate solutions on the hydrolysis of p-nitrophenyl phosphate by homogenates prepared from two different rat livers are shown in Figure 3.3. The tartrate solutions were adjusted to the required pH before adding to the enzyme digest. In this work veronal-acetate buffers were used in order that a wider pH range might be covered although this buffer appears to cause some inhibition at higher pHs. Cu^{++} ions, even at the low concentration employed, caused a very marked inhibition at pHs above 4.0 although little change in the activity was observed at lower pHs. This effect was also observed with other substrates and also when acetate buffers were employed instead of veronal-acetate buffers (Figure 3.4). Mg^{++} ions, on the other hand, caused a marked inhibition at the more acid pHs and did not appear to affect the activity at pHs above 5.5. The inhibition in the presence of tartrate was considerable below pH 4.0, but was not so marked at higher pHs, this substance having a similar effect on the hydrolysis of phenyl phosphate in acetate buffers (Figure 3.5). Figure 3.6 shows the effect

FIG. 3.5.

The Effect Of Cyanide, Tartrate, Fluoride And Formaldehyde On Acid Phosphatase Activity Of Rat Liver Homogenate In Acetate Buffer

(0.005M Phenyl Phosphate Used As Substrate)





of 0.5% formaldehyde, 0.01M NaF and 0.01M citrate on the hydrolysis of p-nitrophenyl phosphate by rat liver acid phosphatase. The greatest degree of inhibition with formaldehyde was observed between pHs 3.4-4.9 whereas using phenyl phosphate as substrate and assaying in acetate buffers, marked inhibition with formaldehyde was observed at all pHs below 4.0 (Figure 3.5). Fluoride caused considerable inhibition at all the pHs studied. Two maxima were observed in the pH-activity curve when this inhibitor was present, one between pH 3.5 and 4.5 and the other at pH 5.5. The effect of this substance on the enzymic hydrolysis of phenyl phosphate by rat liver homogenates in acetate buffers was also studied (Figure 3.5). Again there appeared to be considerable inhibition over the entire pH range studied although the shape of the pH-activity curve in the presence of the inhibitor showed only one maximum at pH 5.5. Citrate ions caused a marked inhibition at pHs below 4.5 but had no effect on the activity at higher pHs. The effect of saccharic acid (in the form of its 1,4-lactone) on the phosphatase activity was also studied using p-nitrophenylphosphate as substrate (Figure 3.7). A 10^{-2}M solution of potassium hydrogen saccharo-1,4-lactone was prepared by dissolving 0.124 g. potassium hydrogen saccharate in 50 ml. water and the solution slowly brought to the boiling. The solution was cooled and the volume readjusted to 50 ml. The results of this experiment

FIG. 3. 6.

The Effect Of Formaldehyde Sodium Fluoride, And Citrate On The Hydrolysis Of p-Nitrophenyl Phosphate By Acid Phosphatase Of Rat Liver Homogenates. (Experiments Carried Out On Homogenates Prepared From Two Different Livers)

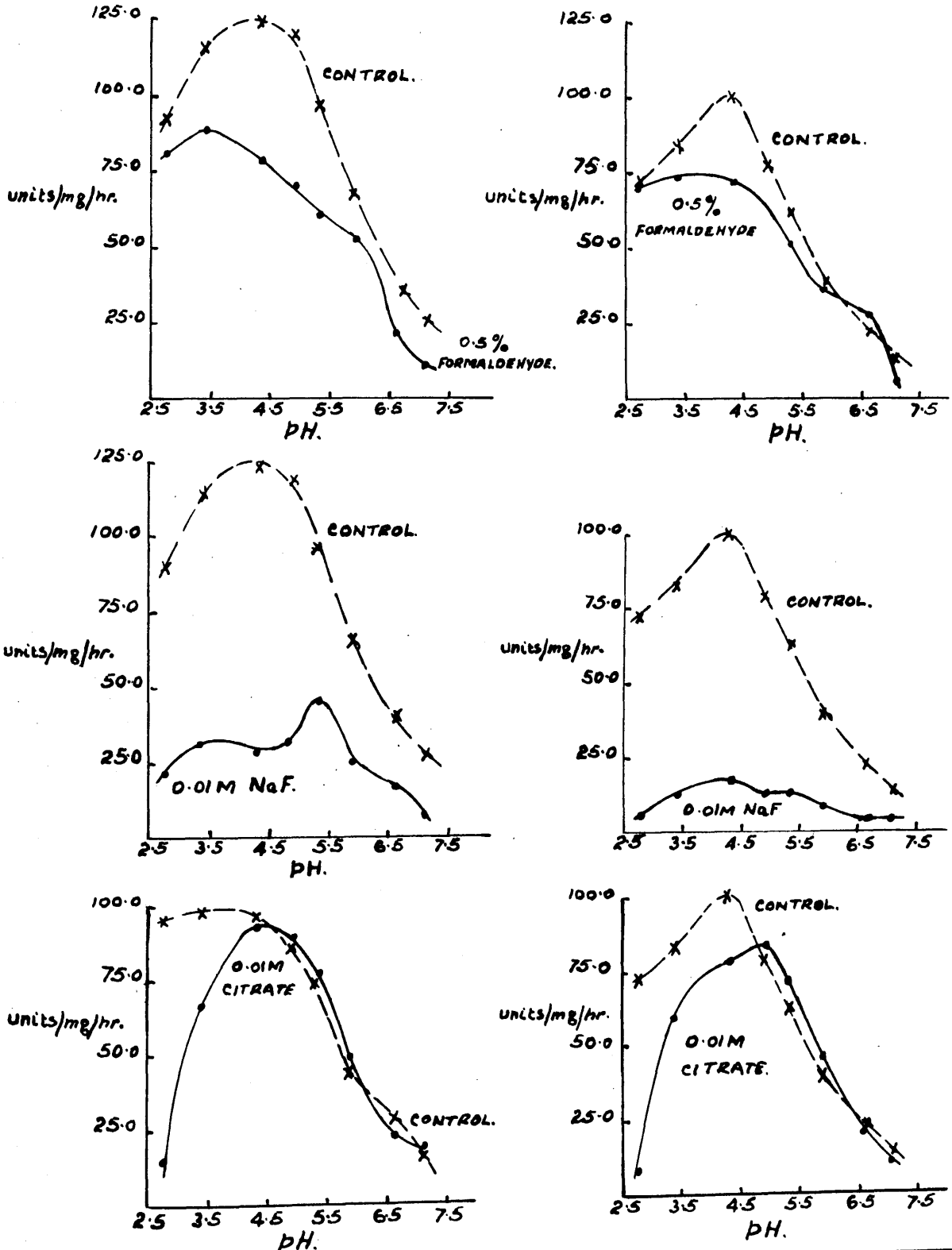
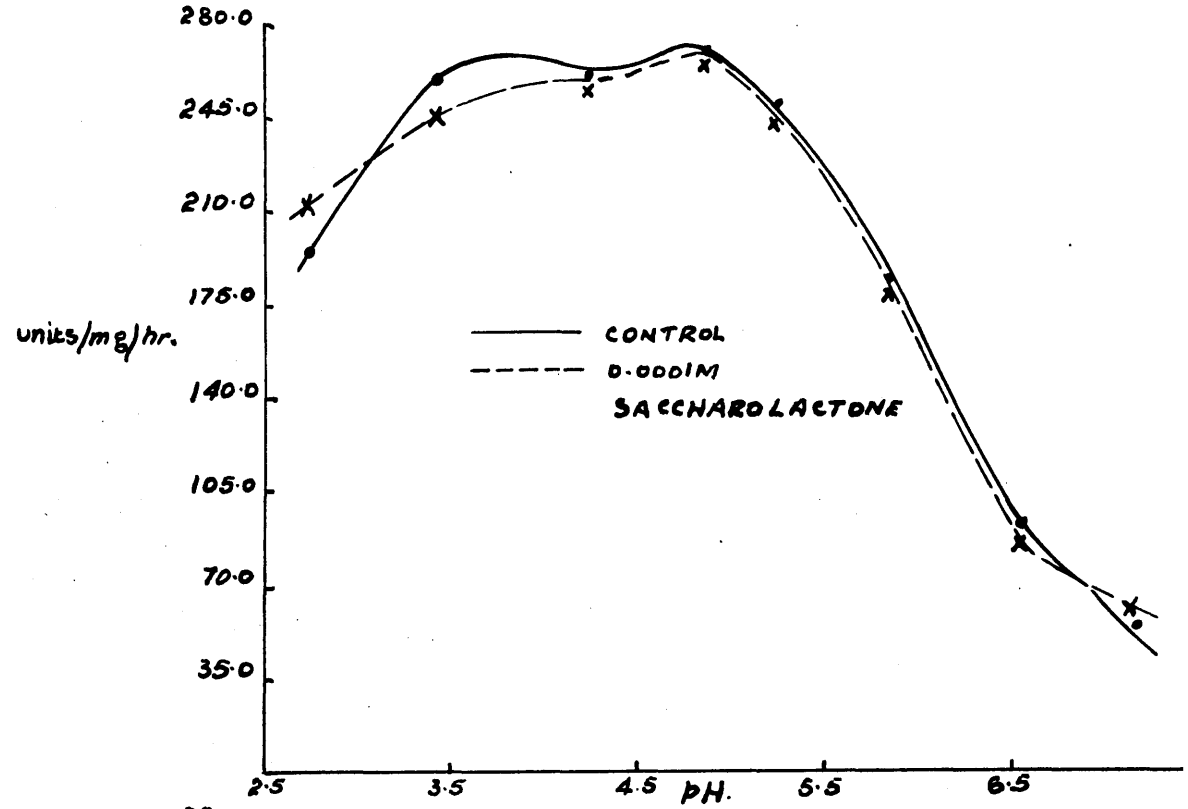
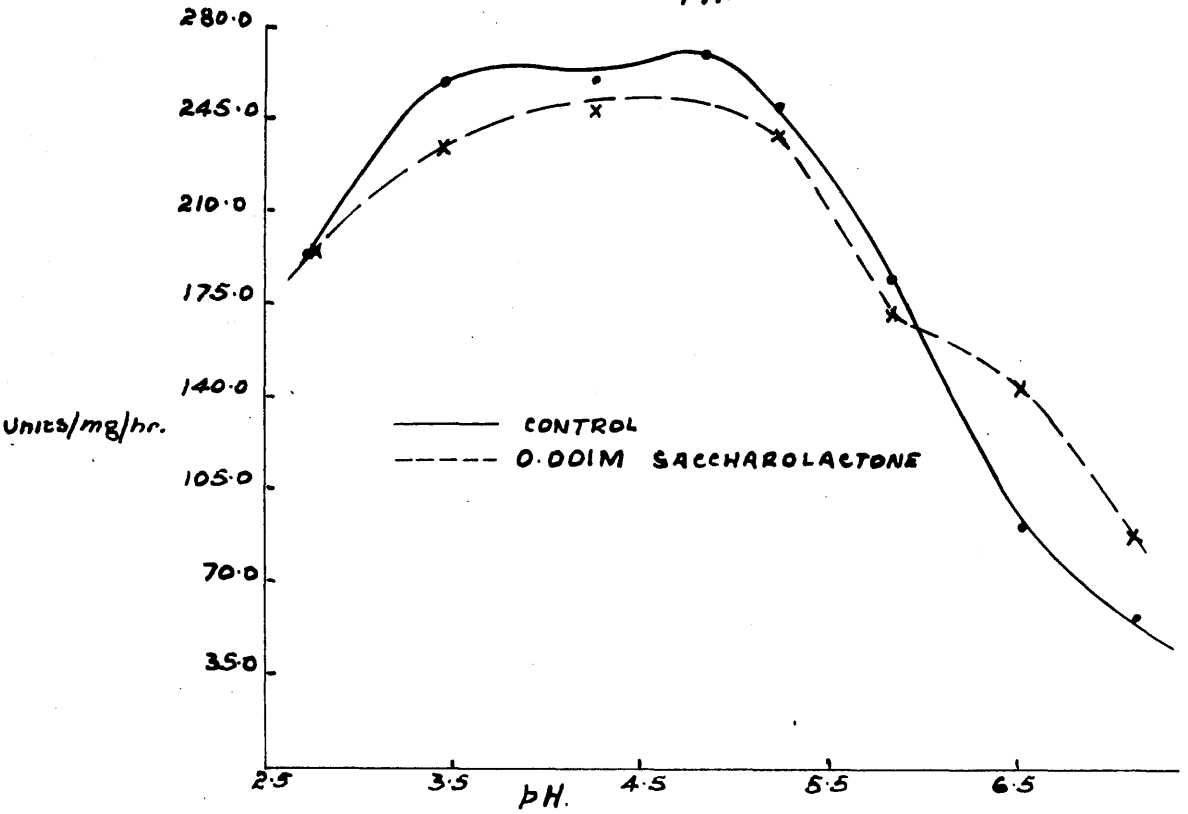


FIG. 3. 7.

The Effect Of Saccharate On Hydrolysis Of p-Nitrophenyl Phosphate By Rat Liver Acid Phosphatase.





are shown in Figure 3.7. Little alteration in activity at any of the pHs was observed with the 0.0001M solution. The activation shown at the higher pHs in the presence of 0.001M saccharo-lactone may have been due to inadequate pH control.

Experiments were carried out, the results of which indicated that none of the above systems interfered with the method of estimation of p-nitrophenol. A series of tubes was set up containing:-

0.5 ml. buffer at pHs
0.1 ml. of test substance at 10 times its final concentration
0.1 ml. of a solution of p-nitrophenol
0.3 ml. water.

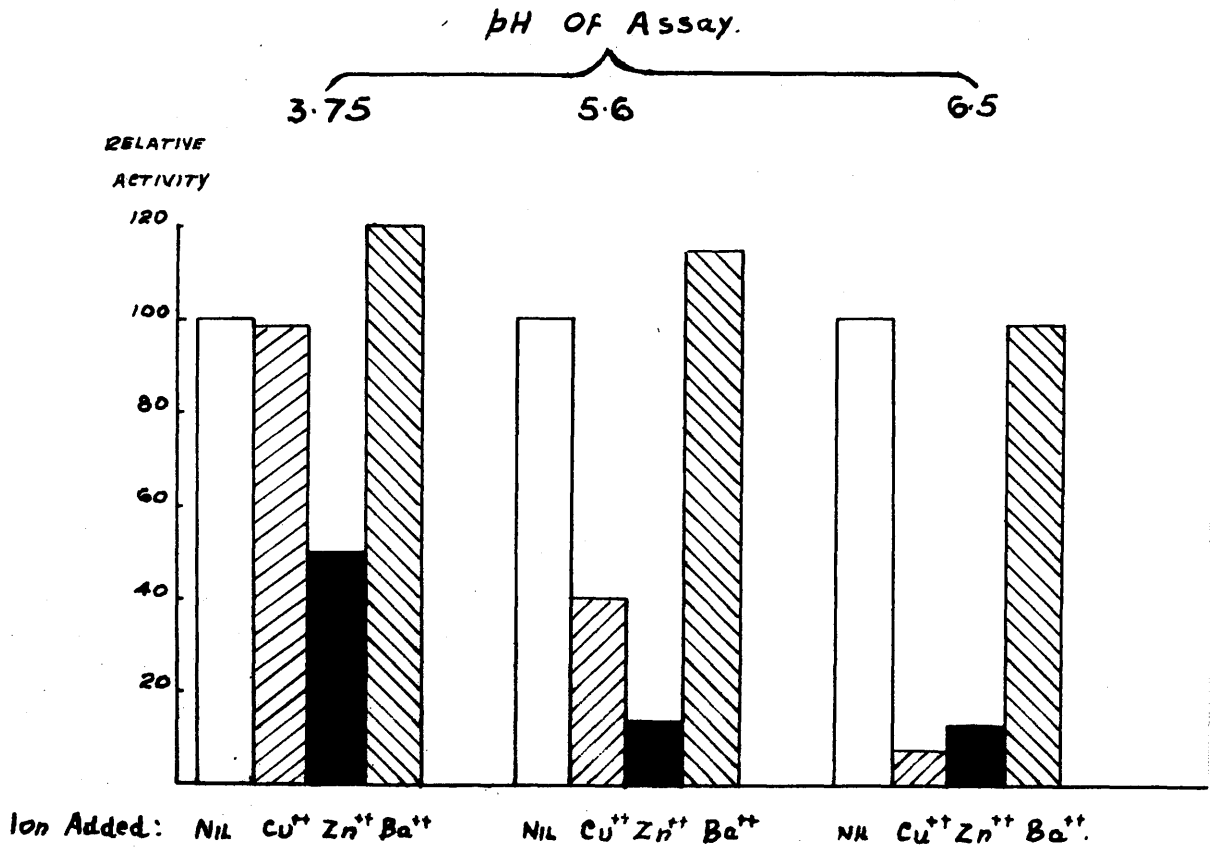
The tubes were incubated for 15 minutes at 38° and the p-nitrophenol was estimated by the method used in the enzyme assay. The recovery of p-nitrophenol was 98% - 102%.

0.01M NaCN was found to have little effect on the hydrolysis of phenyl phosphate by rat liver homogenates in acetate buffers (Figure 3.5).

The effect of Zn^{++} and Ba^{++} ions on rat liver acetate phosphatase activity was studied using phenyl phosphate as substrate at pHs 3.5, 5.55 in acetate buffers and at pH 6.5 using a veronal-acetate buffer. In this case the liberated PO_4''' was measured to avoid any interference due to the metal ions with the method of estimating phenol. The results are summarised in Figure 3.8 and it is seen that Zn^{++} ions inhibited at all pHs whereas Ba^{++} ions were shown to cause a slight activation

FIG. 5. 0.

The Effect Of ·0002M Cu^{++}, ·01M Zn^{++} And ·01M Ba^{++} ions On Hydrolysis Of Phenyl Phosphate By Acid Phosphatases Of Rat-Liver Homogenates



Final Concentrations Of Ions:

Cu^{++}	0·0002 M.
Zn^{++}	0·01 M.
Ba^{++}	0·01 M.

at the two higher pH levels.

The results of these studies would appear to indicate the presence in rat liver homogenates of at least two acid phosphatases, one of which was optimally active at pHs 3.5-4.0 and specifically inhibited by Mg^{++}, tartrate and citrate, and another optimally active at pH 5.0-5.5 and was specifically inhibited by veronal and Cu^{++} ions.

Studies on the Fractionation and Purification of the Acid Phosphatases of Rat Liver.

The preceding studies have pointed to the existence of two acid phosphatases in rat liver. These results may however be influenced by the binding of many of the substances tested by other proteins present in the homogenate which might vary with pH and cause alterations in activity due to entirely non-specific effects.

Attempts were made therefore to fractionate and purify these acid phosphatases in order to examine their properties in a more purified form.

In 1950 E.J. Cohn, et al. published a method for the separation of the protein components of human plasma utilising the interactions of different proteins with divalent cations in presence of organic solvents and this method has been further adapted for the separation of liver proteins (Cohn, Surgenor and Hunter, 1951). In view of the failure

of previous workers to achieve any fractionation by the more conventional methods of salting out by neutral salts and precipitation by organic solvents it was decided in the present work to investigate the possibilities of using metallo-protein complexes.

London and Hudson (1953) claimed a 1000-fold purification of the acid phosphatase of human prostate and the initial steps of their method were used in the present work.

Preparation of enzyme in soluble form.

All phosphatase assays were carried out in 0.2M acetate buffer, employing 0.005M phenyl phosphate as substrate. 91 g. liver obtained from 11 rats were homogenised in a Waring Blendor and extracted with 300 ml. of 0.2M acetate buffer pH 5.0 for 24 hours at 0°, yielding, after centrifugation, 290 ml. of a red opalescent solution containing about 40% of the original activity (Table 3.1). This solution (A) was dialysed against distilled water at 0° for 21 hours during which time a precipitate settled leaving a clear, brown supernatant (solution B). The precipitate and supernatant were separated by centrifuging at 0°. A further loss in activity was found on dialysis and the loss in activity did not justify this step.

Precipitation of phosphatase activity with $(NH_4)_2SO_4$.

Most of the acid phosphatase present in the supernatant after dialysis was precipitated by the addition of ammonium sulphate to 75% saturation and equilibration at 0° for 8 hours. The precipitate was separated by centrifugation at 0°, dissolved in about 50 ml. distilled water and dialysed for 14 hours against 2 l. of distilled water at 0°. A precipitate which formed was removed by centrifugation at 0° and the supernatant (solution C) was found to contain all the activity present in solution B.

Precipitation of enzymic activity by acetone in the presence of 0.02M zinc acetate.

To 1 ml. samples of enzyme solution (in the preliminary stages the dialysed extract was used) were added 1 ml. 0.12M zinc acetate solution varying amounts of 80% acetone and water to 6 ml. All manipulations were carried out in an ice-salt bath at -5 to -7° and the 80% acetone, which was introduced slowly, was precooled to -10°. The mixtures were allowed to equilibrate at -7° for 15 minutes and centrifuged at 3000 r.p.m. for 15 minutes at -7°. The supernatants were removed and the precipitates freed from acetone under vacuo. The precipitates were suspended in water, diluted to 10 ml. and assayed for phosphatase activity at pH 5.3. The activity associated with each precipitate is shown in

Table 3.1.

Partial Purification of Rat Liver Acid Phosphatase.

Assays carried out at pH 5.3.

	Units/ ml.	Total volume ml.	Total units	Purity	Percent-age yield
Liver Homogenate	584	-	5305×10^3	334 (712)	1.00
Soln.A: (Extract with .2M acetate buffer pH 5.0)	7600	290	2205×10^3	(1008)	41.4
Soln.A-dialysed:					
Soln.B-sup^n.	3624	309	1120×10^3	(856)	21.1
Precipitate		-	572×10^2	-	1
Soln.C. Fraction Pptd.by 75% $(NH_4)_2SO_4$	4525	250	1131×10^3	(1325)	21.3
Precipitate which came down while dialysing free from $(NH_4)_2SO_4$			59×10^2	-	.1
Soln.D. 0-15% Acet-one	3400	11.5	170×10^3	395 (850)	3.2
Soln.E 15-40% Acetone	9500	15	711×10^3	2892 (4130)	13.4

Purity expressed in terms of activity per mg. protein (obtained by microkjeldahl N determination). Figures in parenthesis represent purity when protein estimations carried out by colorimetric method.

FIG. 3.9.

Precipitation Of Acid Phosphatase By Acetone In Presence Of 0.02M Zn^{++} ions

Assays Carried Out At pH 5.3 In Acetate Buffers. 0.005M Phenyl Phosphate As Substrate

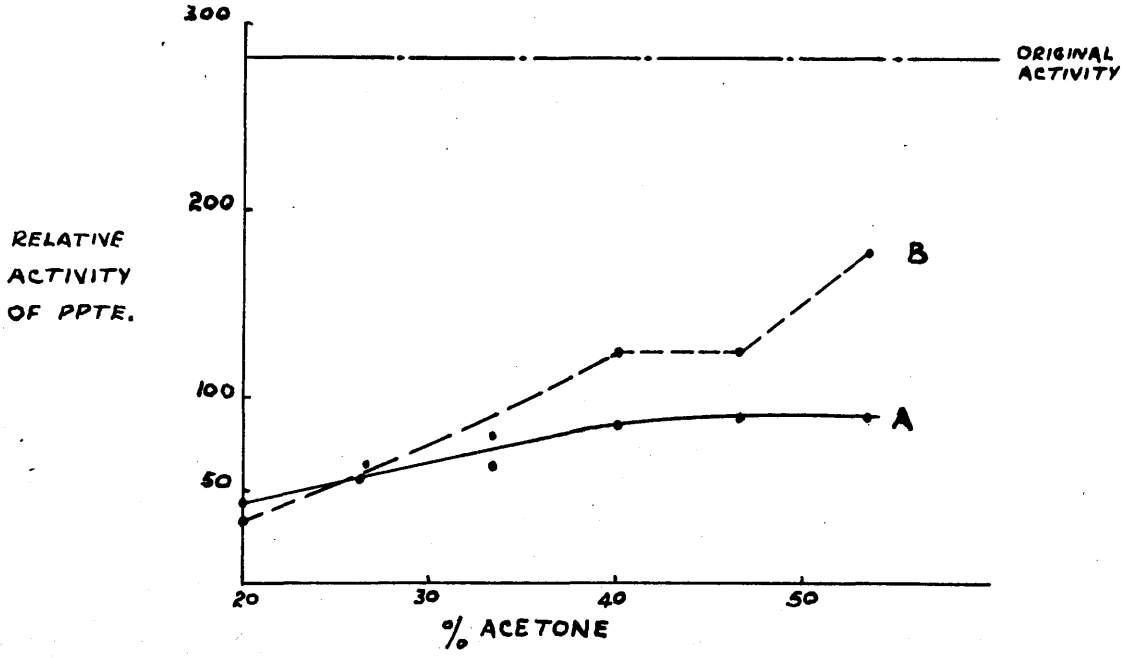


FIG. 3. 10.

Precipitation Of Acid Phosphatase And Protein From Solution C By Acetone In The Presence Of .015 M. Zn^{++}

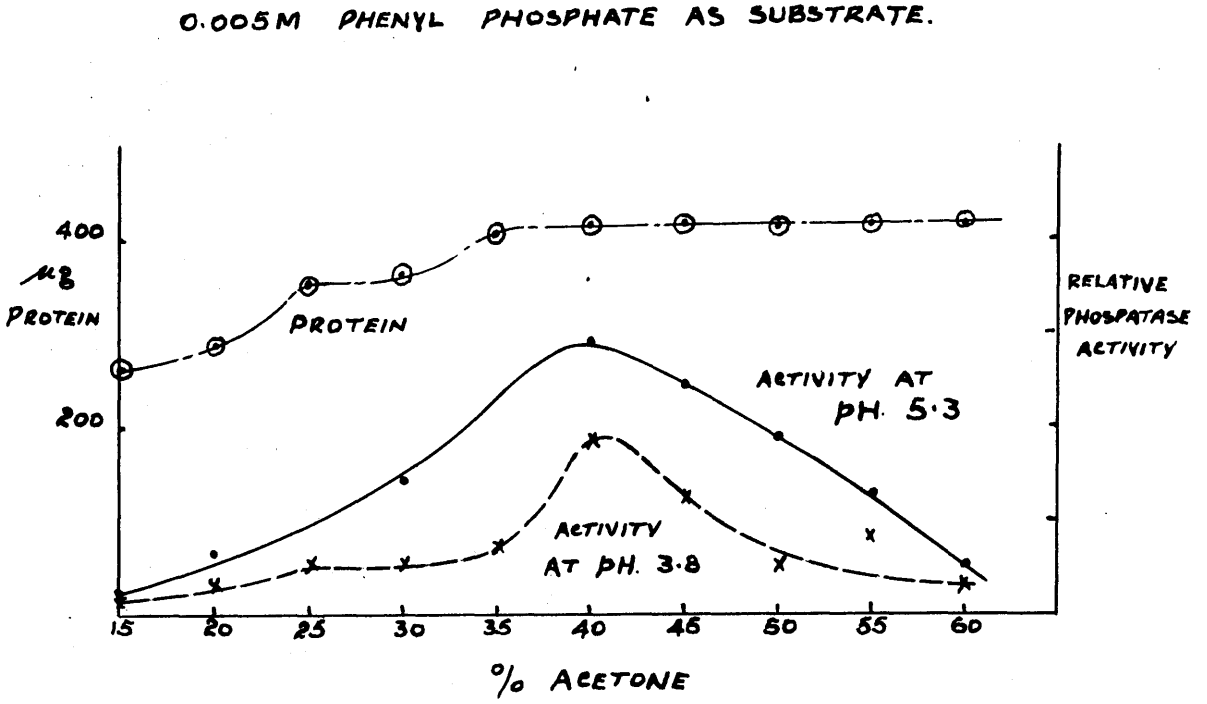


Figure 3.9 - curve A. The low activities recovered were rather disappointing and it was considered that a possible explanation might be Zn^{++} ion inhibition (Figure 3.8). In later experiments therefore the precipitates were suspended in 4 ml. 0.1M citrate buffer at pH 5.0 (as suggested by Cohn, et al., 1950), dialysed overnight at 0°, diluted to 10 ml. and centrifuged. The supernatants were assayed for phosphatase activity and the results are shown in Figure 3.9 - curve B. 63% of the original activity was precipitated at 53% acetone.

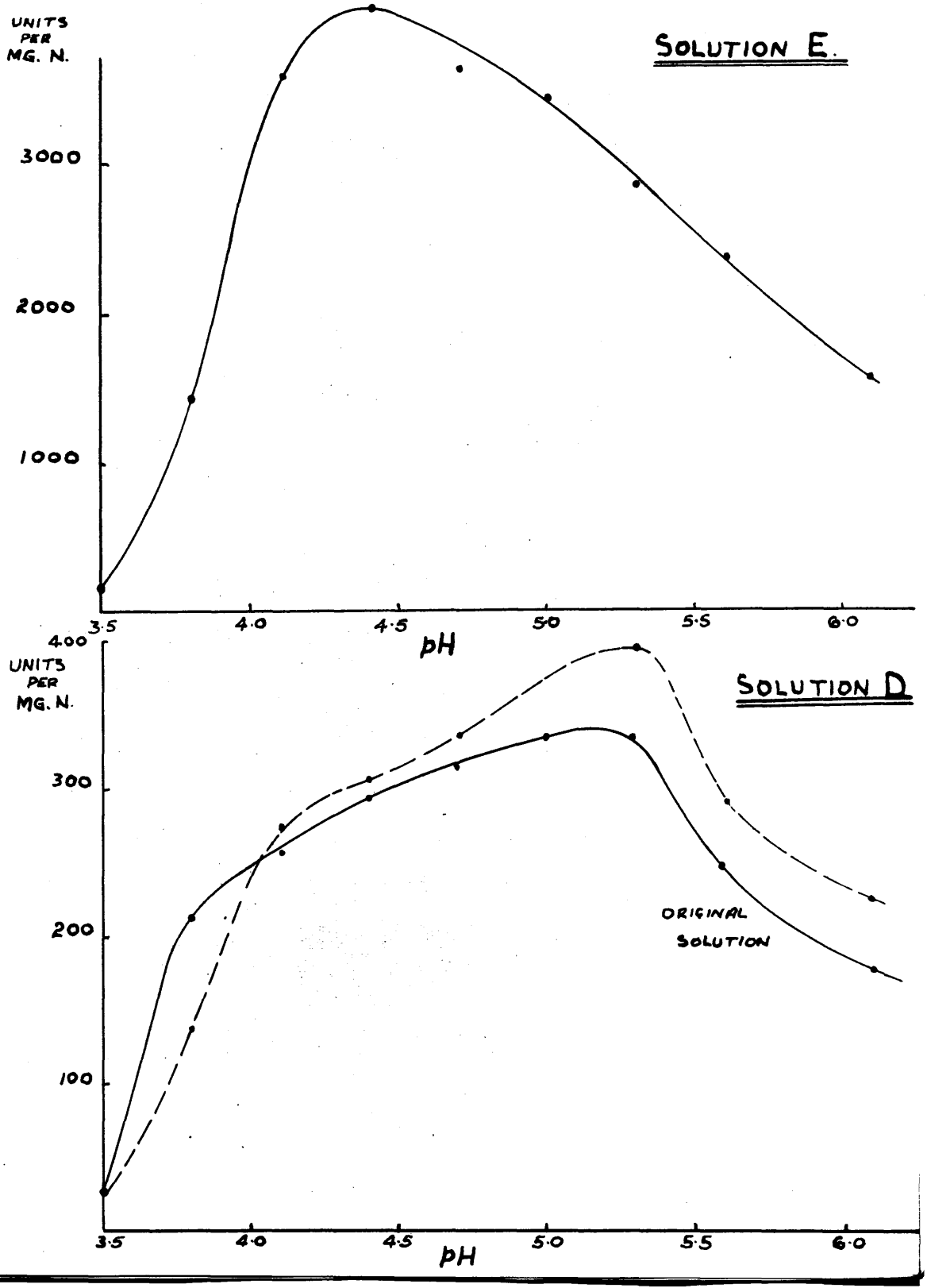
This experiment was repeated on solution C and in this case the protein content of the precipitates was estimated by the method of Lowry, Rosebrough, Farr and Randall (1951). The results are shown in Figure 3.10. Assays were carried out at pHs 3.8 and 5.3. It was apparent that at concentrations of acetone of above 40% considerable inactivation occurred. From these results it was decided to fractionate the main bulk of the preparation between 15 and 40% acetone at a Zn^{++} ion concentration of 0.015M. 0.218 g. zinc acetate dihydrate was added to 50 ml. solution C and the inactive precipitate removed at 0°. The solution was cooled to -7° and 80% acetone at -10° added to a final concentration of 15%. After equilibration at -7° for 15 minutes the precipitate was removed at -7°, dissolved in 8 ml. 0.1M

FIG 3.11

pH-Activity Curves Of Solutions D and E

Assays Carried Out In Acetate Buffer

Using 0.005M Phenyl Phosphate As Substrate



citrate buffer pH 4.7 and dialysed against 1 l. distilled water overnight at 0° (solution D). The supernatant was further precipitated at -7° by the addition of Zn^{++} ions and 80% acetone to final concentrations of 0.015M and 40% respectively. After equilibration for 15 minutes at -7° the precipitate was removed by centrifugation and treated as for the 15% acetone precipitate (solution E).

The pH-activity curves of these preparations are shown in Figure 3.11, activities being expressed as units/mg. N. Solution D, the 15% acetone precipitate, showed maximum activity at pH 5.6 and the shape of the curve suggested the presence of another enzyme acting optimally at a much lower pH. A single peak of activity was observed at pH 4.4 in the case of solution E which was the fraction precipitated between 15-40% acetone. The pH-activity curve of solution D was very similar to that of the original solution. From these results it appeared likely that two enzymes were indeed present in the original solution and that solution E contained a larger amount of the enzyme optimally active at a lower pH.

Quantitative details of the purification procedure are shown in Table 3.1.

Solution E assayed at either pH 3.8 or 5.3 showed an 8-fold increase in purity over the original, whereas the activity of solution E, assayed at pH 4.4 showed a purity

of 13.8 times that of the original.

One point of interest arose during this work. The method of Lowry, Rosebrough, Farr and Randall (1951) for protein estimations using egg albumin as standard was adopted at first since it was claimed to be more rapid and much more sensitive than methods based on nitrogen determinations. The protein nitrogen content of the original homogenate and solutions D and E were determined by the method of Ma and Zuazaga (1942) as a control on this method and there was found to be no correlation between the two techniques as shown in the following table:

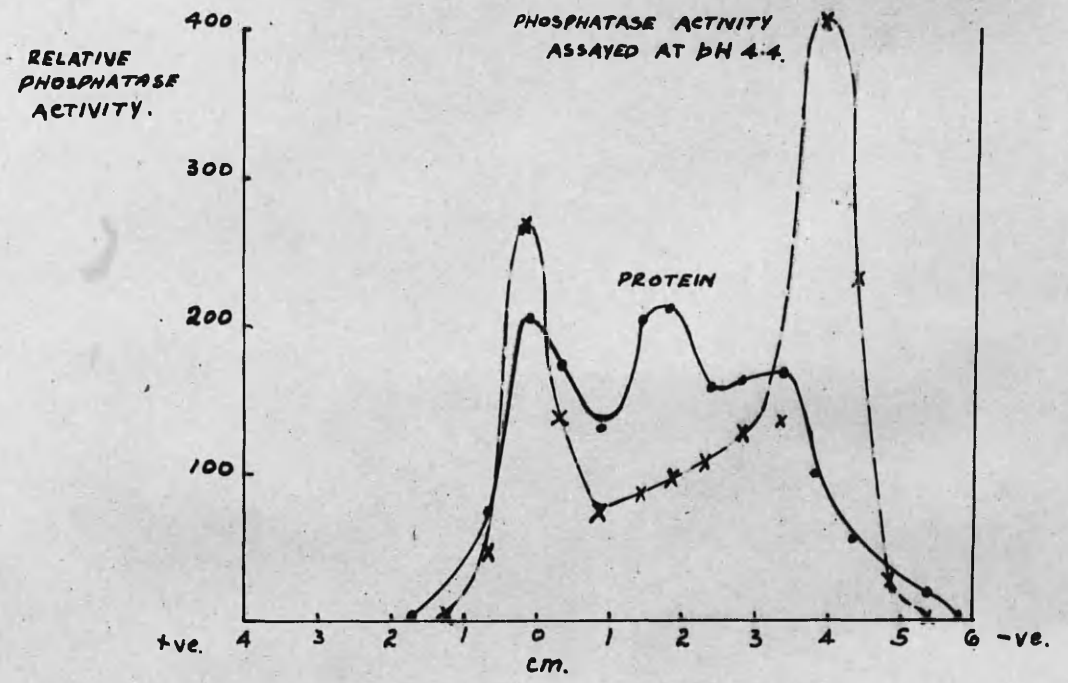
Solution	mg.protein/ml. Method I	mg.protein/ml. Method II	Ratio I/II
Original	1.75	0.82	2.16
D	8.62	4.00	2.15
E	3.28	2.310	1.42

I = N Estimation
II = Colorimetric Estimation.

The absolute values obtained by the colorimetric method are also much lower than those obtained by N determination.

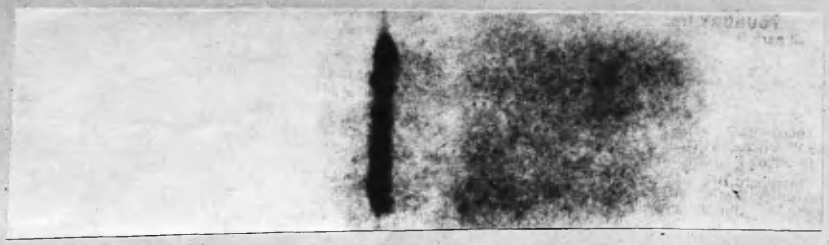
FIG. 3. 12

A. Migration Of Acid Phosphatase Activity Of Solution E On Filter Paper.



(ACETATE BUFFER pH 4·5; I = ·02; 4·2 V/cm. 13 hr. 40 mn.)

B.

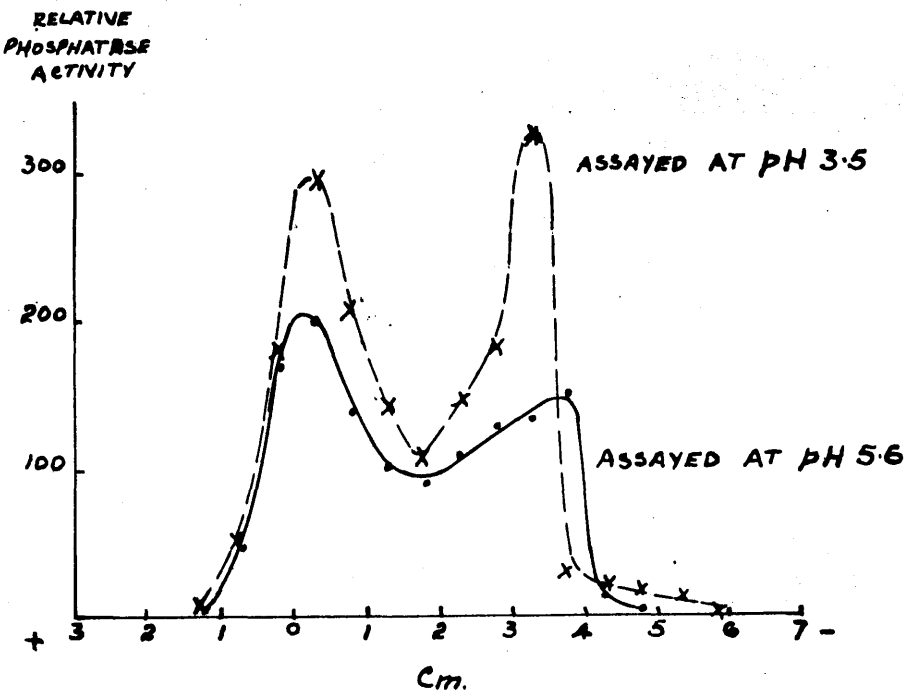


STRIP STAINED FOR PROTEIN.

ENDOSMOSIS DETERMINED BY MIGRATION of GLUCOSE SPOT.

FIG. 3. 13.

Migration Of Acid Phosphatase Of Solution E On Filter Paper



(ACETATE BUFFER pH 4·5, I = 0·02, 4·5 V/cm. 17 hr.)

Application of Electrophoresis to Phosphatase Fractionation.

Figure 3.12 (A) shows the electrophoretic pattern of the phosphatase activity and protein concentration of solution E as determined by paper electrophoresis in acetate buffer pH 4.5, I = 0.02 with assay for phosphatase activity at pH 4.4. The significance of the peak of phosphatase activity and protein at the origin is difficult to interpret since it was apparent (Figure 3.12.B) that a fraction of the protein was strongly adsorbed at the origin. Nevertheless a peak of very high phosphatase activity was seen to be associated with the fastest moving protein component. In a repeat of the experiment with phosphatase assays at pHs 3.5 and 5.6 two peaks of activity were again obtained, one of which was again associated with protein at the origin (Figure 3.13). The leading peak of phosphatase activity was much more pronounced when the strips were assayed at pH 3.5 than at pH 5.6.

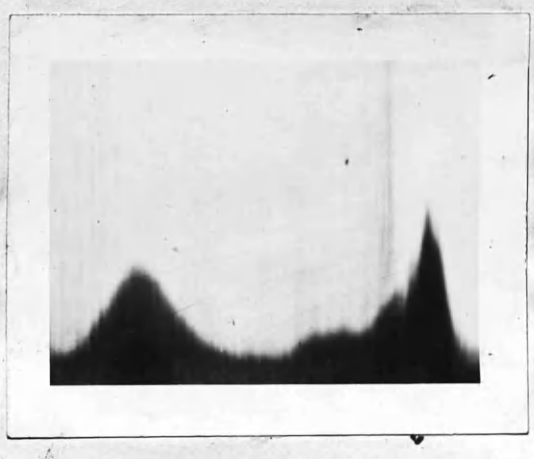
The fact that a large proportion of the phosphatase activity was associated with the leading protein component suggested that it might be possible to separate a protein fraction, rich in acid phosphatase, by electrophoresis in the Tiselius apparatus.

Solution E was freeze-dried and the solid residue dissolved in 5 ml. of acetate buffer, pH 4.25, I = 0.02. The solution was dialysed overnight against the buffer and

Figure 3.14.

Electrophoretic Analysis (Tiselius Method) of Solution E.

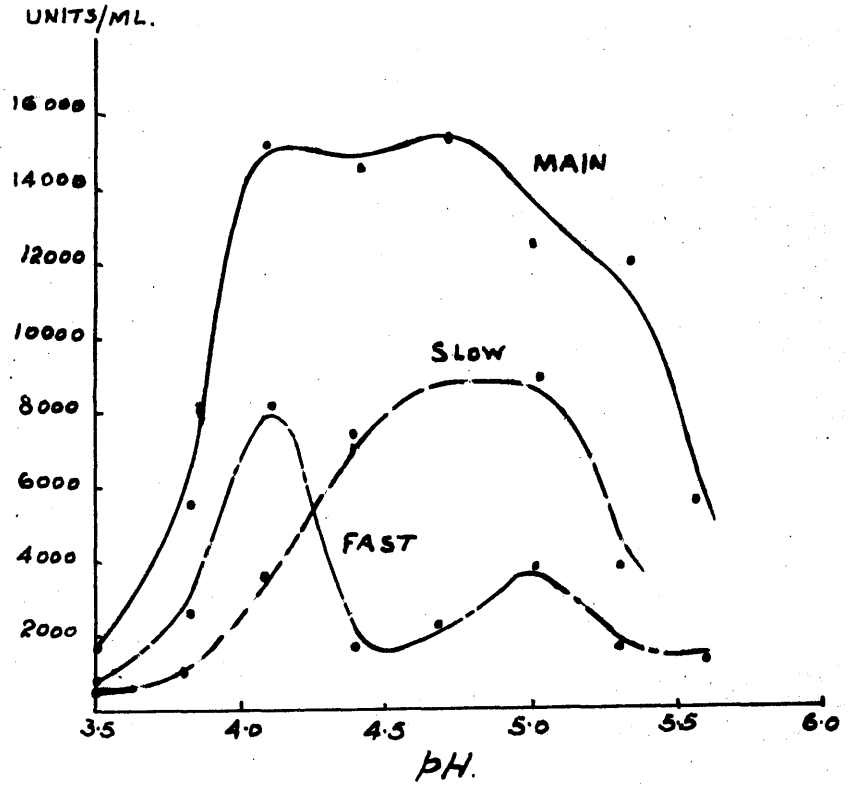
Acetate Buffer, pH 4·25, I = 0.02.



Ascending.

FIG. 3. 15

pH - Activity Curves Of Fast, Slow And Main Fractions Separated By Free Electrophoresis Of Solution E.



the small precipitate which formed was removed. Figure 3.14 shows the electrophoretic pattern seen. Owing to the low ionic strength a large boundary anomaly was obtained. The protein fractions associated with the leading boundary, the slower moving components and the "main" protein fraction were separated and collected at the completion of the run.

pH-activity curves were determined for these three fractions and the results are shown in Figure 3.15. A striking difference in the shape of the three curves was found. The fast fraction showed a well-defined optimum at pH 4.1 with a secondary peak of activity at pH 5.0. The slower moving fraction showed a broad maximum of activity between pHs 4.4 and 5.0. It is concluded from the data that a phosphatase with optimal activity at around pH 4.0 is concentrated largely in the faster moving fraction, whereas another enzyme, optimally active at about pH 5.0, appeared to be associated with the slower moving fraction. The form of the pH-activity curve of the main fraction appeared to be a summation of the curves of the other two fractions. The activity of the fast fraction assayed at pH 4.1 was 3,680 units/mg. protein, which represents a 14.3-fold purification over the starting material.

The enzymic activity appeared to be very unstable in these purified preparations since they lost all activity on storing overnight at -15°.

SECTION IV.

The Alkaline Phosphatases of Rat Liver.

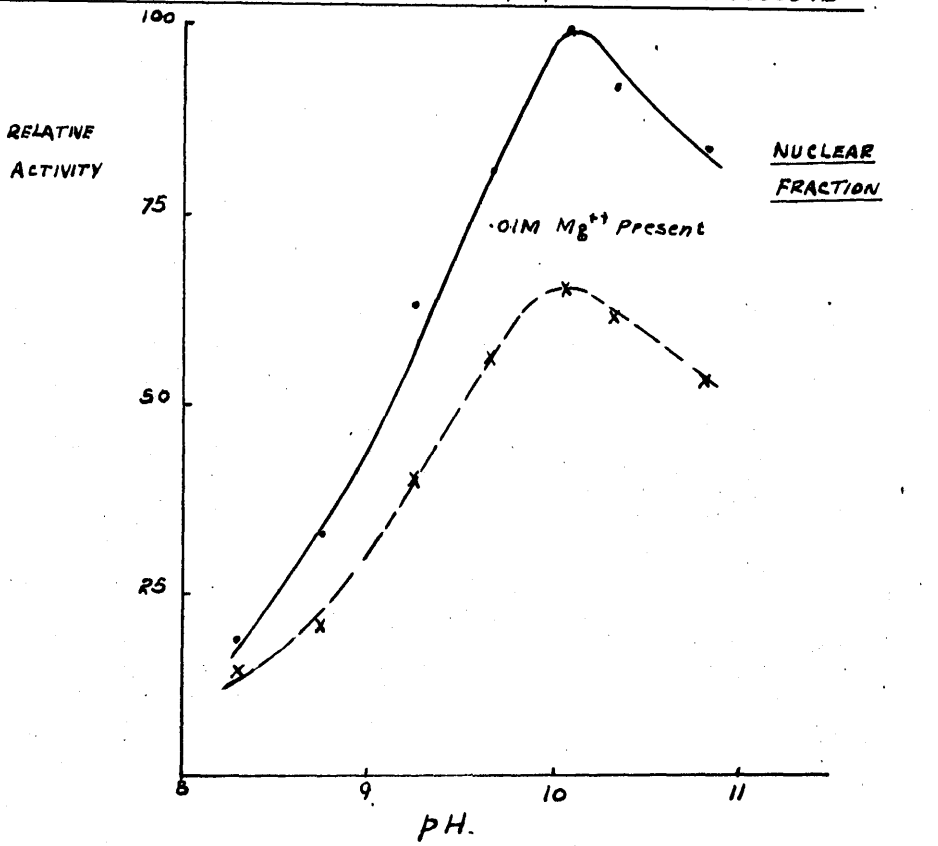
Evidence for the existence of two intracellular alkaline phosphatases which differed in their sensitivity towards Mg^{++} and CN^{-} ions was first advanced by Cloetens (1939a & b), who claimed a separation of the two types in liver tissue. From an examination of the pH-activity curve, Mg^{++} sensitivity and CN^{-} inhibition of the liver alkaline phosphatase of rats which had been kept on a protein deficient diet, Rosenthal, Fahl and Vars (1952) came to a similar conclusion.

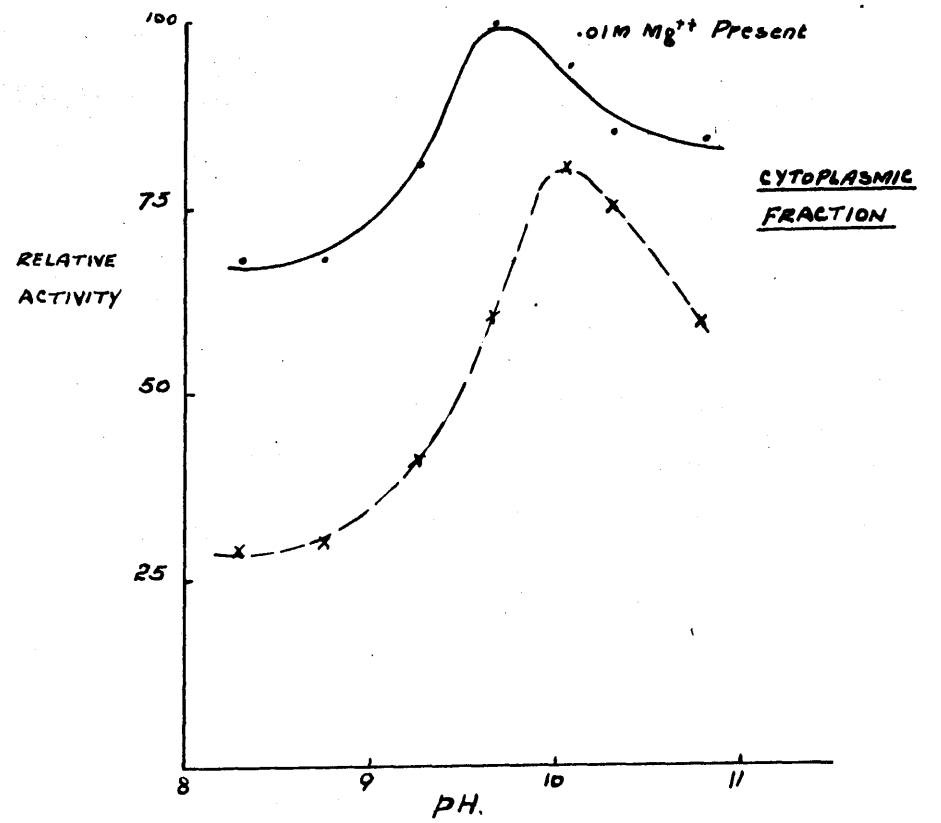
Giri, Prasad, Gouri Devi and Sri Ram (1952) claimed a separation of two kidney alkaline phosphatases by paper chromatography, but Levy and Mazia (1953) submitted a purified preparation of kidney alkaline phosphatase to electrophoresis on filter paper and failed to find evidence for the existence of more than one enzyme.

In the present work evidence was obtained which suggested that a considerable proportion of rat liver alkaline phosphatase was concentrated in the nuclear fraction. Novikoff (1952) demonstrated that the alkaline phosphatase activity of the nuclear fraction increased to a much greater extent than did that of any other cell fraction in the processes of regeneration following partial hepatectomy. An

FIG. 4.1.

pH-Activity Curves Of Nuclear And Cytoplasmic Fractions





investigation of some of the properties of nuclear and cytoplasmic alkaline phosphatase activity was therefore undertaken in order to examine the possibility that the enzymes in the two fractions were different and also to determine if any differences observed could substantiate previous claims for the existence of two enzymes. Fractionation studies of extracts of whole tissue were also carried out in a study of the nature of the enzymic activity.

Properties of Nuclear and Cytoplasmic Enzymes.

Nuclear and cytoplasmic fractions of rat liver were prepared according to the method of Hogeboom, Schneider and Striebich (1952), the final sucrose and $CaCl_2$ concentrations being made the same in both samples.

1). The effect of pH and Mg^{++} ion concentration.

pH-activity curves of these nuclear and cytoplasmic fractions were determined using β -glycerophosphate and the results are presented in Figure 4.1 along with the effect of 0.01M Mg^{++} ions. Two very marked differences between the curves were observed. Firstly the ratio of the activity at pH 10.05 to that at pH 8.30 was much greater in the case of the nuclear fraction than it was with the cytoplasmic fraction. Secondly, while Mg^{++} ions appeared to activate to a similar extent at all pHs with the nuclear fraction, the activation

FIG. 4.2.

The Effect Of Varying Substrate Concentration On Nuclear And Cytoplasmic Alkaline Phosphatase.

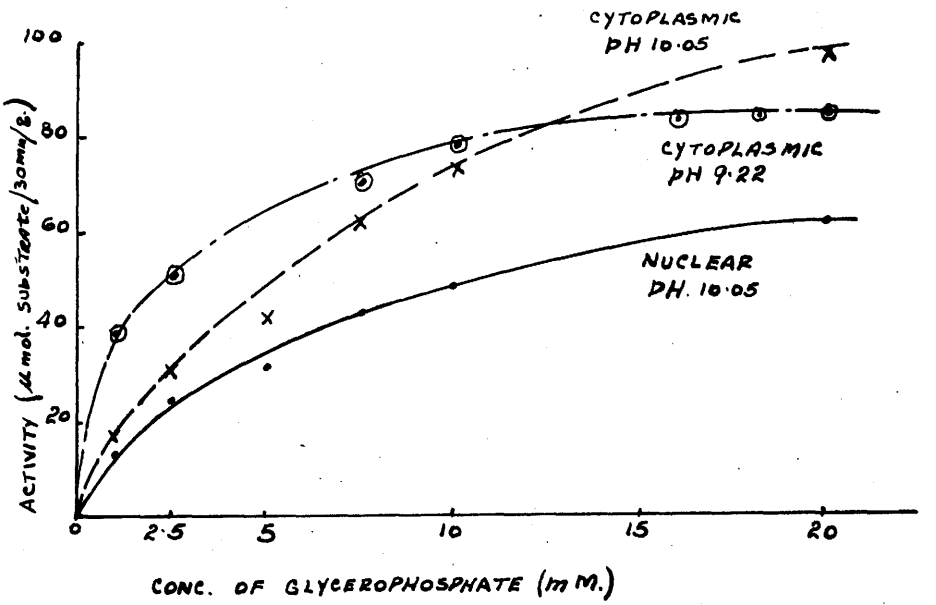
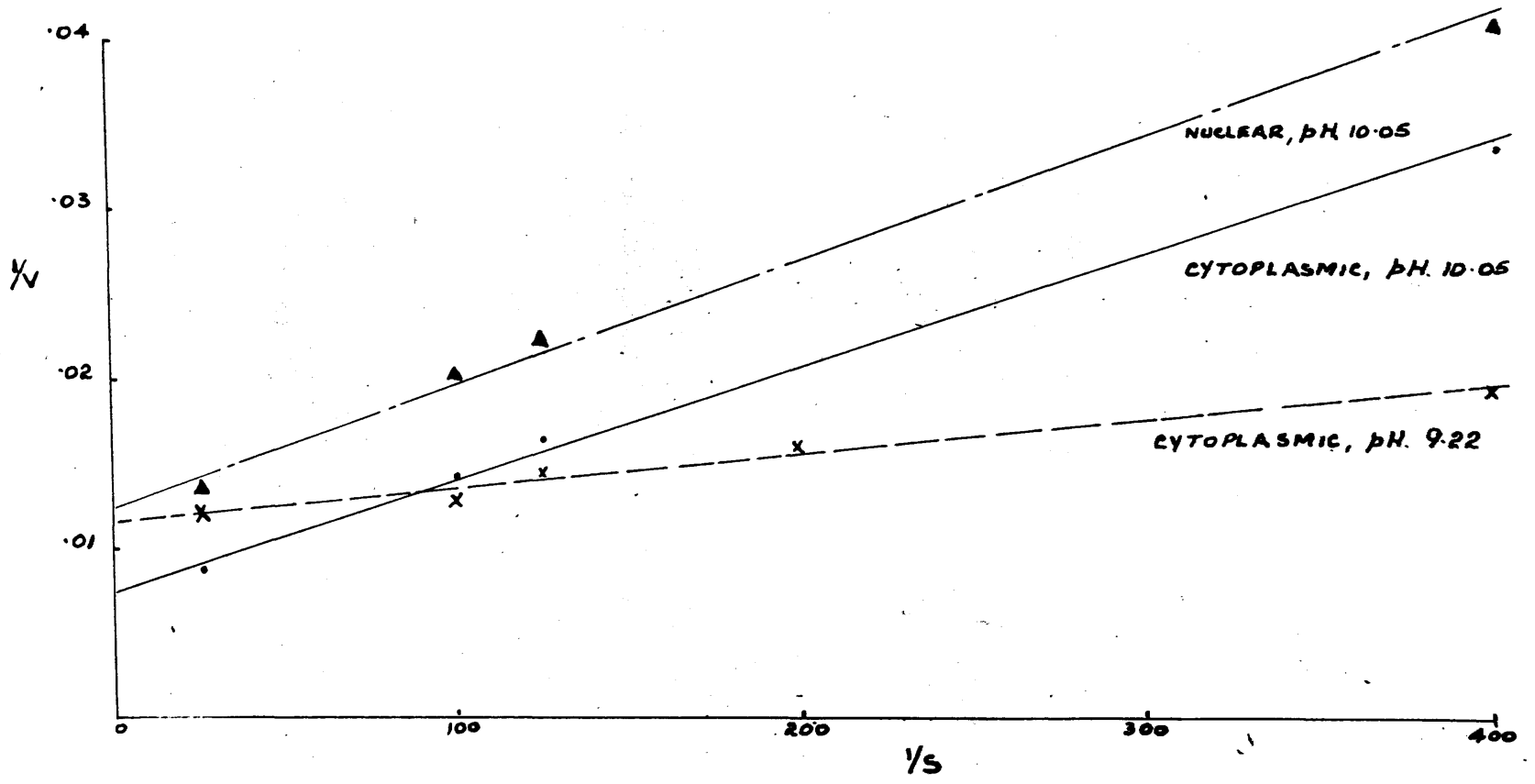


Fig. 4. 3.

Plot Of Reciprocal Of Reaction Velocity (1/v) Against The Reciprocal Of The Substrate Concentration (1/s)



produced by this ion with the cytoplasmic fraction was much greater at lower pHs than at pHs 10.05 and over. From these curves it appeared that while only one phosphatase was present in the nucleus, there was some evidence to suggest the presence of two enzymes in the cytoplasm.

2). The effect of substrate concentration.

The effect of variation of the substrate concentration (β-glycerophosphate) on the nuclear phosphatase activity assayed at pH 10.05 and in the cytoplasmic fractions assayed at pHs 9.22 and 10.05 is shown in Figure 4.2. The curves obtained for the nuclear fraction and the cytoplasmic fraction assayed at pH 10.05 were very similar, whereas that obtained in the case of the cytoplasmic activity assayed at pH 9.22 is of an entirely different character. In all three curves it was found that a substrate concentration of 0.02M produced almost maximum activity. The results were analysed according to the method of Lineweaver and Burk (1934) and from these curves (Figure 4.3) the following values of K_s were obtained:-

Fraction	pH of Assay	K_s
Nuclear	10.05	5.63×10^{-3}
Cytoplasmic	10.05	8.9×10^{-3}
Cytoplasmic	9.22	2.04×10^{-3}

3). <u>The activation energies of the alkaline phosphatase activity of the nuclear and cytoplasmic fractions.</u>

Arrhenius in 1889 studied the relationship between the rate of a chemical reaction and the temperature at which the reaction occurs. He found that the data in most cases fitted the relationship:-

$$\frac{d \ln k}{dT} = \frac{A}{RT^2} \quad \text{-------- (1)}$$

where k = reaction rate
T = absolute temperature
R = gas constant
A = constant

From theoretical considerations based on statistical mechanics this relationship has been derived in a more exact form:-

$$\frac{d \ln k}{dT} = \frac{E}{RT^2} \quad \text{-------- (2)}$$

where E = energy of activation of the molecules.

Equation (2) on integration yields:-

$$\log k = \frac{-E}{2.303R} \left(\frac{1}{T} + \text{constant}\right) \text{ ----- (3)}$$

The energy of activation can thus be determined by plotting the logarithm of the reaction rate against the reciprocal of the absolute temperature. The slope of this line multiplied by 4.58 gives the value of E in calories per mole.

It has been demonstrated that the empirical relationship of Arrhenius can be applied to enzymic reactions and Sizer (1943) discussed its application in greater detail. The value of the activation energy is a constant for a given enzyme and is independent of the degree of purity of the enzyme (Sizer, 1943; Smith, 1952).

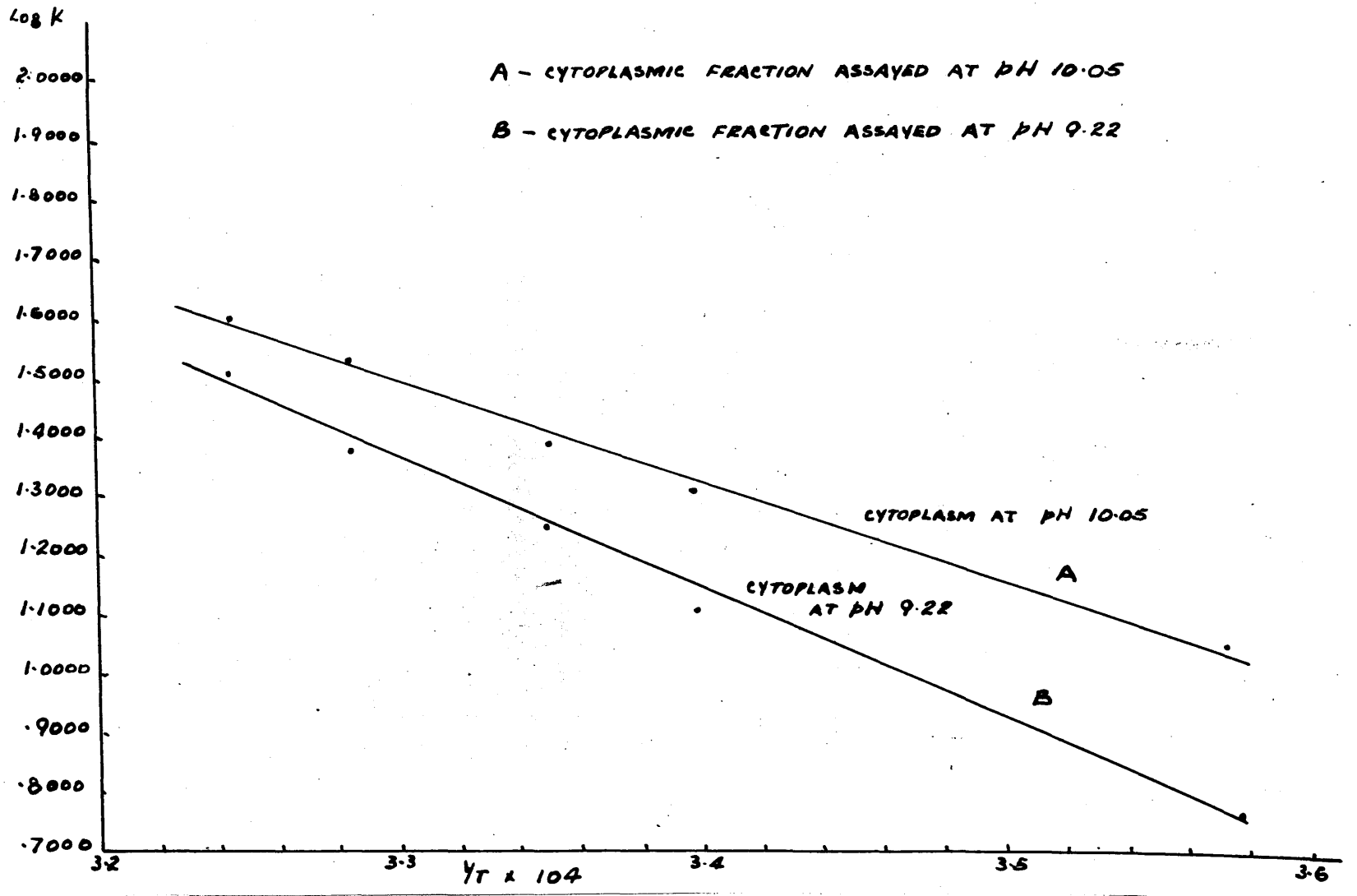
Assays were carried out using β-glycerophosphate as substrate at a final concentration of 0.02M to ensure optimal activity, at pHs 9.22 and 10.05 in the case of the cytoplasmic fractions and at pH 10.05 in the case of the nuclear fraction and at 7.5°, 22.5°, 26.6°, 32.5° and 36.8°. A regression line of log k against $^1/_T$ was calculated statistically for the three series of assays and the gradient of this line was used for the calculation of the activation energies. The following values of the activation energies were found:-

Fraction	pH of Assay	Activation Energy Cals./mole
Nuclear	10.05	8,760
Cytoplasmic	10.05	7,540
Cytoplasmic	9.22	9,900

The difference between the gradients of the two lines obtained with the cytoplasmic fraction was on the border-line of significance at the 5% level, but there was a considerable scatter in the case of the nuclear fraction and no conclusions

FIG. 4. 4.

Plot Of Log Reaction Rate Against Reciprocal Of Absolute Temperature ($1/T$)



will be drawn from this result.

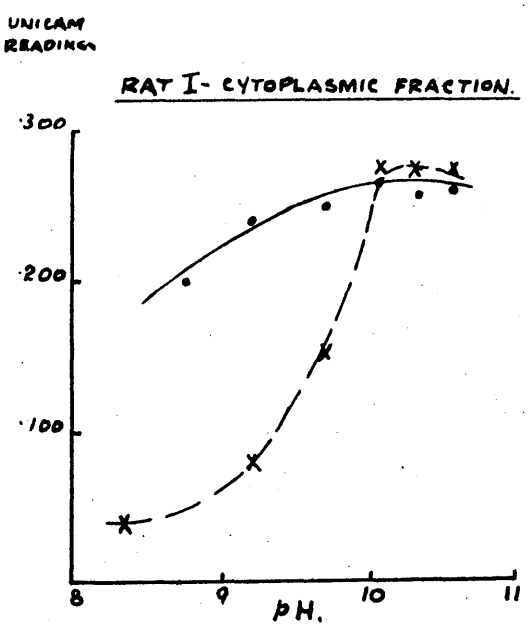
Figure 4.4 shows the curves obtained by "fitting by eye" and it is obvious that no great deviations are present. The gradients of these lines are 1695 for the cytoplasmic fraction at pH 10.05 and 2210 for the cytoplasmic fraction at pH 9.22, figures which compare very favourably with the values obtained by the statistical method which were 1648 and 2161 respectively. Owing to the wide scatter it was found to be very difficult to fit a line to the results of the experiment with the nuclear fraction. The relatively low statistical significance of the difference between the two cytoplasmic figures is largely due to the small number of results.

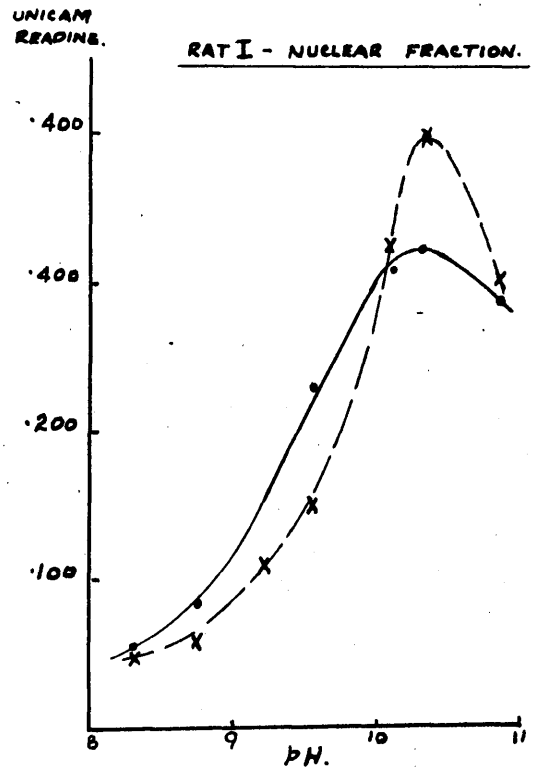
4). pH-activity curves of alkaline phosphatase activity in nuclear and cytoplasmic fractions of regenerating liver.

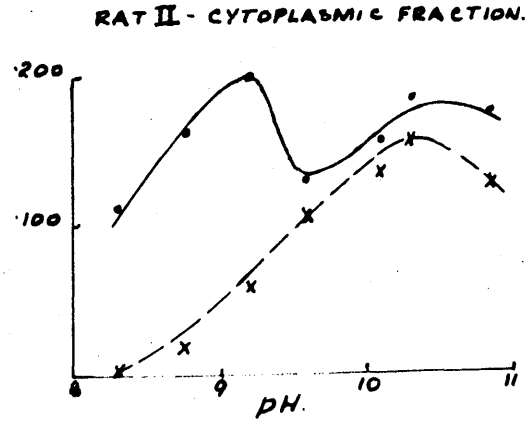
Rats were partially hepatectomised by the method of Higgins and Anderson (1931) and sacrificed three days after the operation. Nuclear and cytoplasmic fractions were prepared as above and pH-activity curves determined on both fractions. Although the purpose of this study was to investigate any qualitative difference which might have arisen, there was no evidence of any great general increase in activity as had been found previously (Goodlad, Mills and Smith, 1951) and the 5-fold increase in activity of the nuclear fraction found by Novikoff (1952) was not observed.

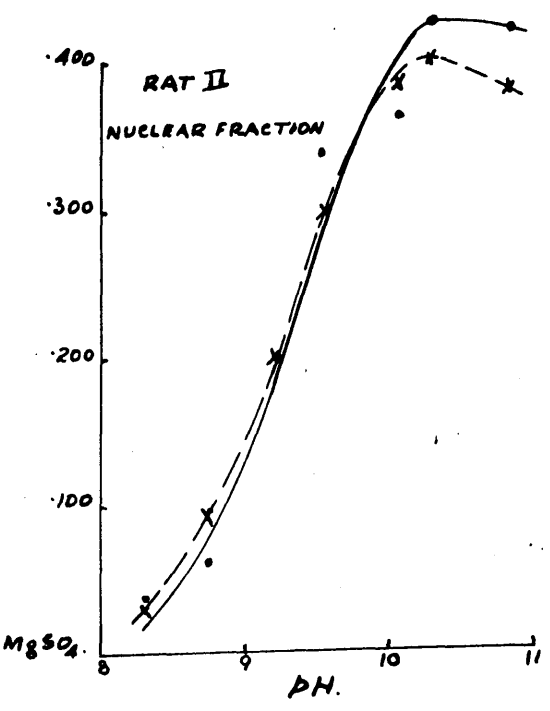
FIG. 4.5.

pH-Activity Curves Of Nuclear And Cytoplasmic Alkaline Phosphatase In Regenerating Rat Livers.









0·02M β-Glycerophosphate used As Substrate

Glycine - NaOH Buffers.

x- - - - - x Assayed Without Added Mg^{++}

•——— • Assayed In Presence Of 0·01M $MgSO_4$.

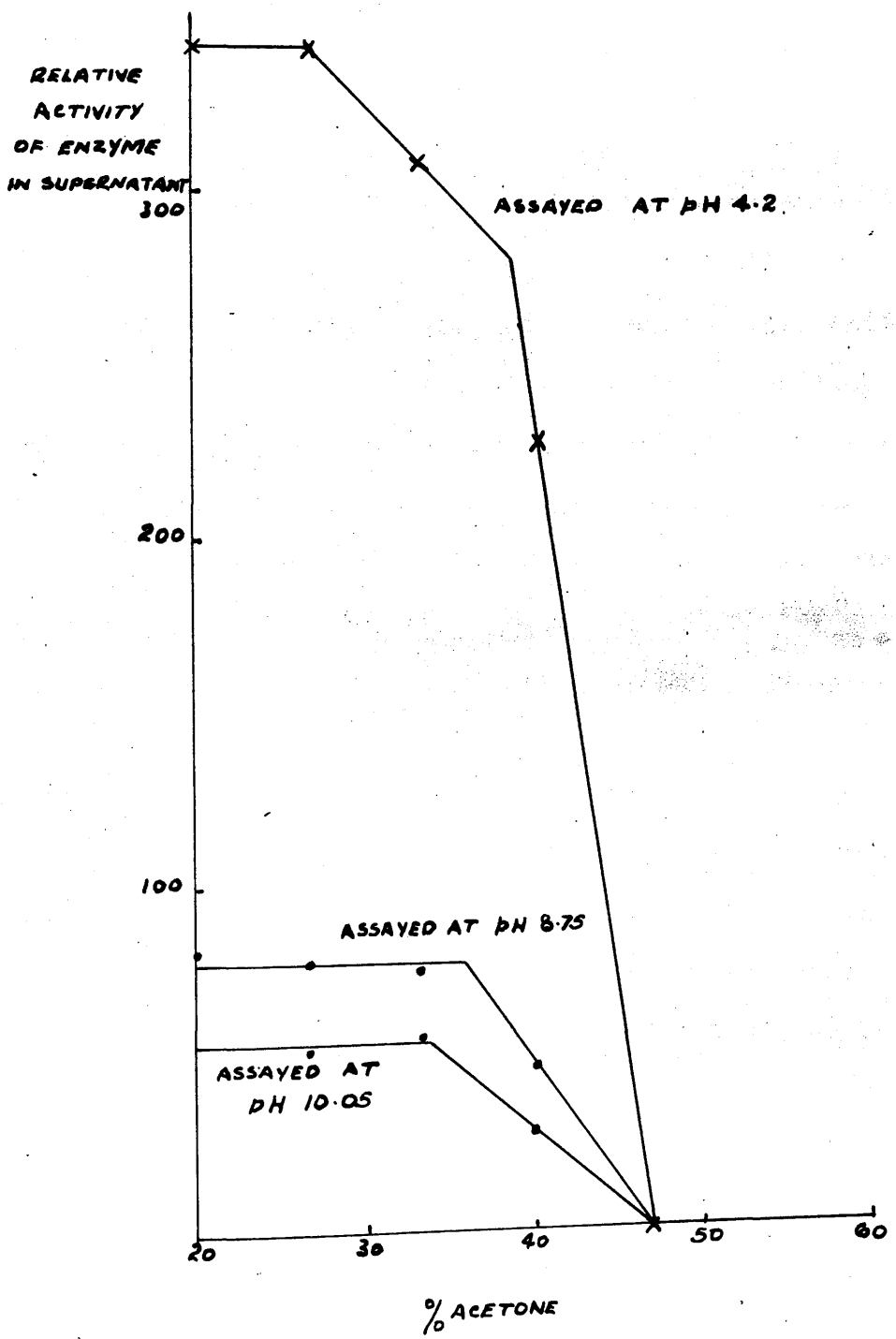
The results are shown in Figure 4.5 and once again they suggest the presence of two enzymes in the cytoplasmic fraction differing in their sensitivity towards Mg^{++} ions, while there appeared to be only one enzyme in the nuclear fraction.

Studies on the Purification of Alkaline Phosphatase.

85 g. liver from 7 male rats were homogenised in an equal volume of water in a Nelco blendor. An equal volume of 25% acetone containing 10% of a mixture of equal parts of toluene and ethyl acetate was added and the whole allowed to autolyse at room temperature for two days. The precipitate was centrifuged and discarded. 80% acetone, pre-cooled to -10^{o}, was added to the supernatant which had been cooled in an ice-bath to -5^{o} to give a final concentration of acetone of 60%. The mixture was kept at -5^{o} for 15 minutes, then centrifuged at this temperature. The precipitate was washed twice with acetone and allowed to dry in a vacuum desiccator, 100 mg. of the powder extracted 3 times with 5 ml. portions of distilled water and the extracts were combined and made up to a total volume of 20 ml. It was observed that Mg^{++} ions caused a considerable activation of the phosphatase activity of the 60% acetone precipitate at both pH 9.22 and 10.05 and that the precipitate had also

FIG. 4. 6.

Solubility Of Acid And Alkaline Phosphatase Activity Of Rat Liver Present In Fraction Precipitated With 60% Acetone.



considerable acid phosphatase activity (Table 4.1).

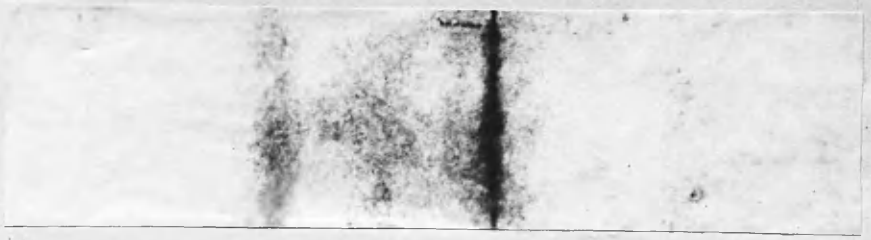
Acetone precipitations were carried out on 2 ml. portions of this solution at -5° by the addition of water and precooled 80% acetone to the final desired acetone concentration. The precipitates were spun down, dissolved in 5 ml. distilled water and the solutions were assayed at pHs 4.2, 8.75 and 10.05 using 0.02M β-glycerophosphate as substrate in the presence of Mg^{++} ions (final concentration = 0.01M). Solubility curves of the phosphatase activities are shown in Figure 4.6. A break in the solubility curve of the acid phosphatase was observed which might have been due to the presence of two acid phosphatases in the extract. The alkaline phosphatase activity was completely precipitated between 33%and 47% acetone. From the solubility curve no evidence for the existence of two alkaline phosphatases could be obtained.

100 mg. of the initial acetone dried powder was extracted with water as above and fractionally precipitated with acetone. The fraction precipitating between 36% and 50% acetone was collected, dried in a vacuum desiccator, and the dried precipitate (39.4 mg.) dissolved in 3 ml. water.

60 µl. portions of this solution were subjected to electrophoresis on Whatman 3 MM filter paper (3 cm. broad) for 16 hours 40 minutes at 4.1 volts/cm. in veronal buffer at pH 9.22 (temperature = 4°). The position of the alkaline

Figure 4.7.

Paper Electrophoresis of Partially Purified Rat-Liver Alkaline Phosphatase.



a) Strip Stained for Protein.

b) Strip Stained for Phosphatase Activity.

c) Endosmosis Determined by Migration of Glucose Spot.

60µl. Phosphatase Solution Applied.

Veronal Buffer, pH 9.22; 4.1 V/cm.;

16 hours 40 min. at 4°.

phosphatase activity at pH 9.22 was determined by the contact method using phenolphthalein phosphate as substrate. A similar strip run in parallel was developed for protein. The results are shown in Figure 4.7. Only one area of alkaline phosphatase activity was observed.

Table 4.1.

Relative Activity at pHs 4.2, 9.22 and 10.05 and Effect of Mg^{++} ions on activities of Rat liver preparation prepared by precipitating autolysate with 60% acetone.

.02M β-glycerophosphate used as substrate.

Final concentration of Mg^{++} ions when added = 0.01M.

pH of Assay	Relative Activity	
	In Absence added Mg^{++}	In Presence added Mg^{++}
4.2	100	-
9.22	4	24
10.05	3	20

SECTION V.

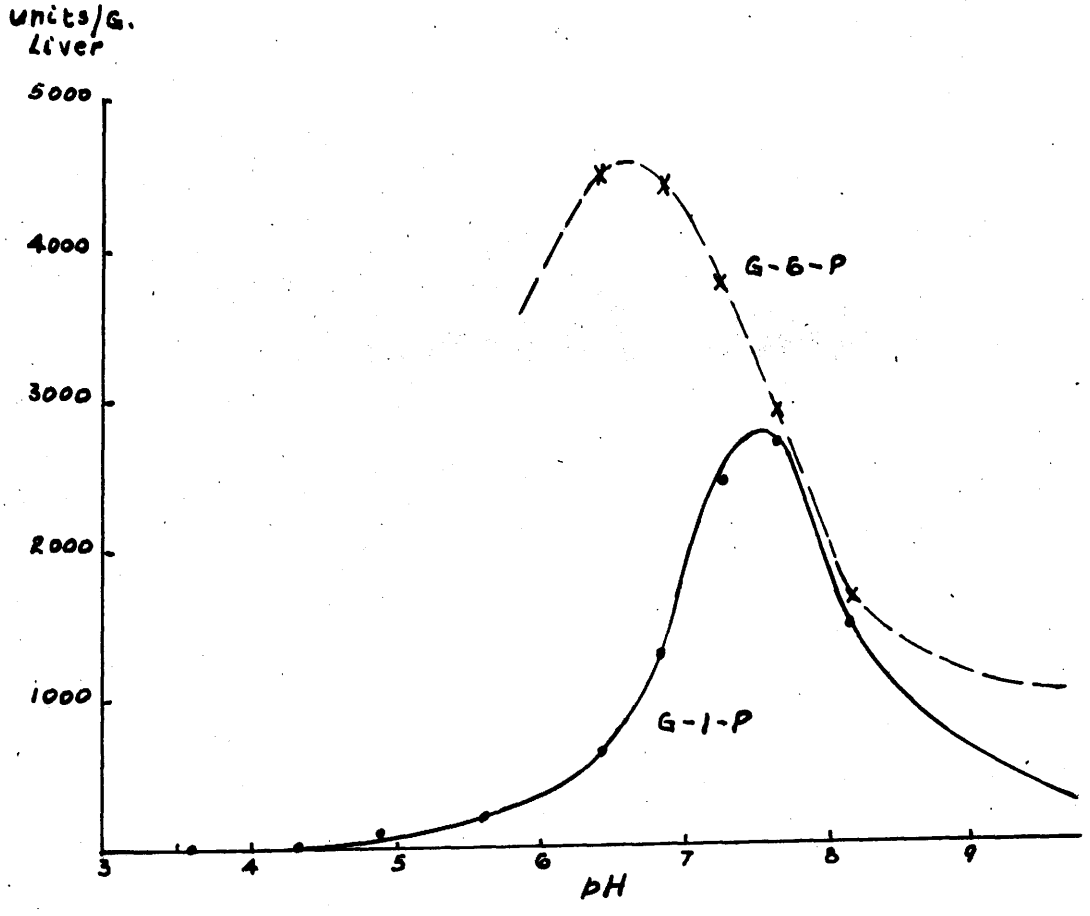
The Hydrolysis of Hexose Monophosphates by Rat Liver Extracts.

In 1945 Fantl and Rome suggested that the hexose monophosphates were hydrolysed by a different enzyme system from the non-specific phosphomonoesterases in liver extracts and later Broh-Kahn and Mirsky (1948) and De Duve, Berthet, Hess and Dupret (1949) suggested that liver contained a phosphatase, specific for the hydrolysis of glucose-6-phosphate. The separation and purification of this enzyme were undertaken by Swanson (1950) and her final purified product showed optimal activity for the hydrolysis of glucose-6-phosphate at pH 6.5, while it had no activity on glucose-1-phosphate and low activities towards other phosphate esters.

Glucose-1-phosphate is known to be formed during glycolysis and the mechanism of its breakdown is therefore of much significance in studies on general carbohydrate metabolism. Broh-Kahn and Mirsky (1948) followed the course of the loss of acid-labile P and increase in inorganic P when glucose-1-phosphate was incubated with liver extracts and deduced that a preliminary conversion to the stable 6-ester occurred. They then studied the action of various substances known to inhibit the phosphogluco-mutase reaction and found that they also inhibited the breakdown of glucose-1-phosphate. Their experiments did not prove conclusively,

FIG. 5.1.

The Hydrolysis Of Glucose-6-Phosphate And Glucose-1-Phosphate By The Same Rat Liver Homogenate.

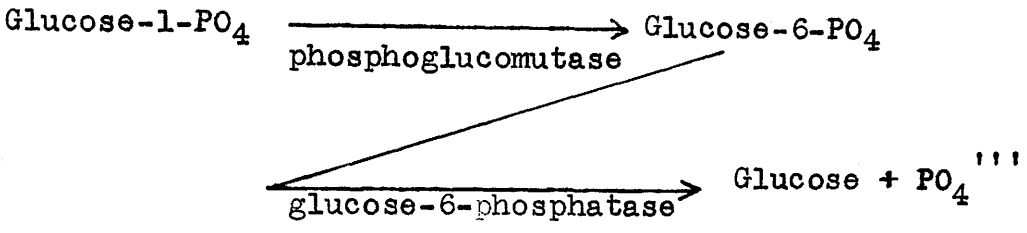


FINAL CONCENTRATION OF SUBSTRATE = 0·005M.

FINAL CONCENTRATION OF Mg^{++} = 0·001M.

VERONAL-ACETATE BUFFERS.

however, that glucose-1-phosphate was split completely by the pathway:-



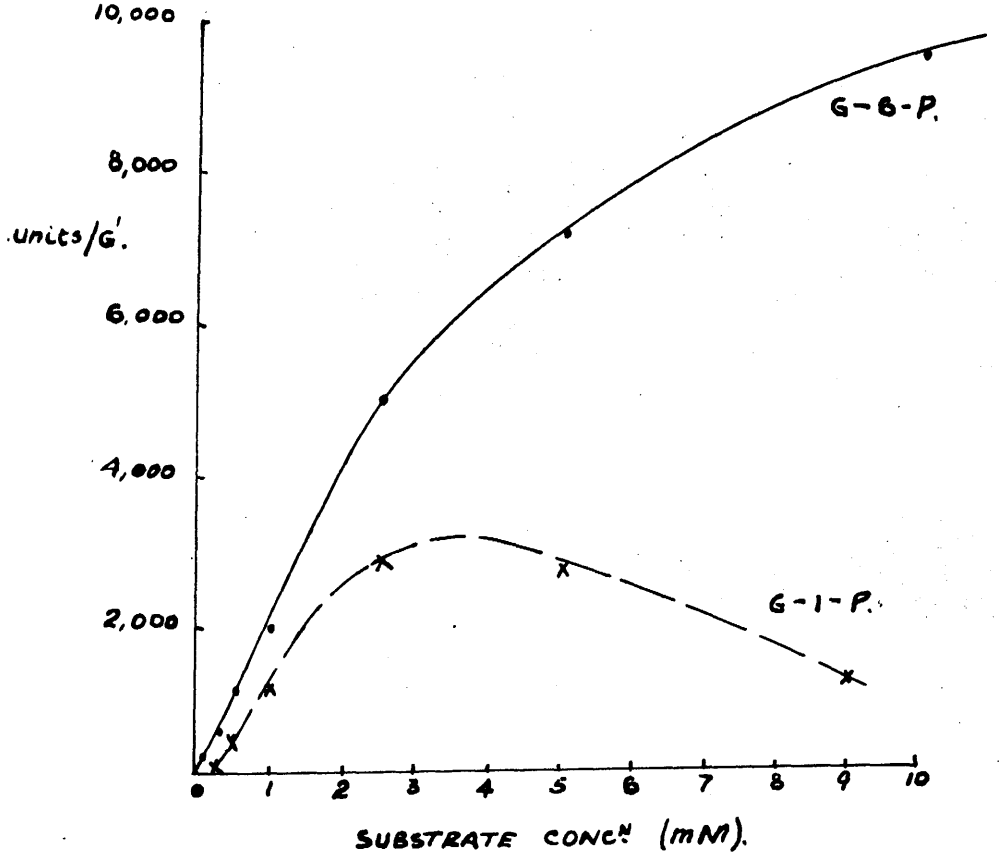
It was felt that this question was worthy of further consideration.

It will be recalled that the hydrolysis of glucose-1-PO_4 appeared to occur with a slightly higher pH optimum than did that of glucose-6-phosphate and to be strongly activated by Mg^{++} ions (Fig.1.5). It was found from a study of the hydrolysis of the two substrates, using the same liver homogenate, that there was indeed a significant difference (Fig.5.1). The optimal pH of glucose-6-phosphate hydrolysis was at pH 6.5 while that of glucose-1-phosphate was at pH 7.5. The hydrolysis of glucose-1-phosphate at pHs below 5.6 was negligible.

The effect of variation of the substrate concentration on the hydrolysis of the two substrates was found to differ. It was observed (Fig.5.2) that with higher concentrations of glucose-1-phosphate, substrate inhibition occurred, whereas there was no evidence for substrate inhibition with glucose-6-phosphate. This is further evidence in support of the view

Fig. 5. 2.

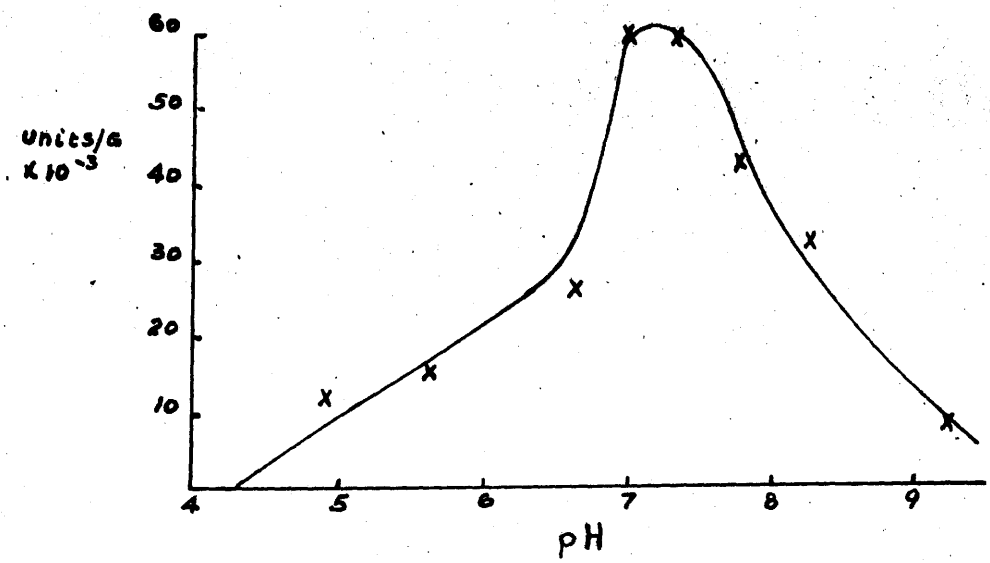
The Effect Of Substrate Concentration On The Hydrolisys Of G-1-P And G-6-P By Rat Liver Homogenates.



HYDROLYSIS OF GLUCOSE - 6 - PHOSPHATE STUDIED AT pH 6·5
HYDROLYSIS OF GLUCOSE - 1 - PHOSPHATE STUDIED AT pH 7·2
VERONAL - ACETATE BUFFERS.

FIG. 5. 3.

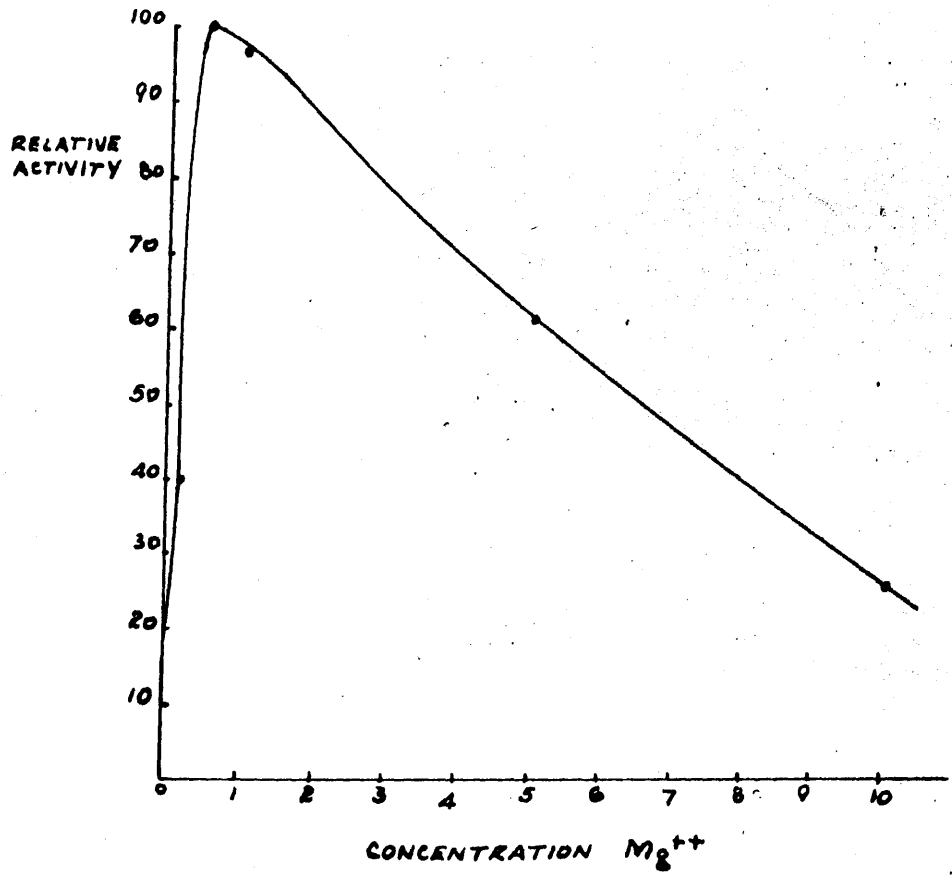
pH- Activity Curve Of Rat Liver Phosphoglucomutase Of Supernatant Fraction Of Cytoplasm.



FINAL CONCENTRATION G-1-P = 0.005 M.
FINAL CONCENTRATION Mg^{++} = 0.001 M.
FINAL CONCENTRATION CYSTEINE = 0.001 M.
VERONAL-ACETATE BUFFERS.

FIG. 5.4.

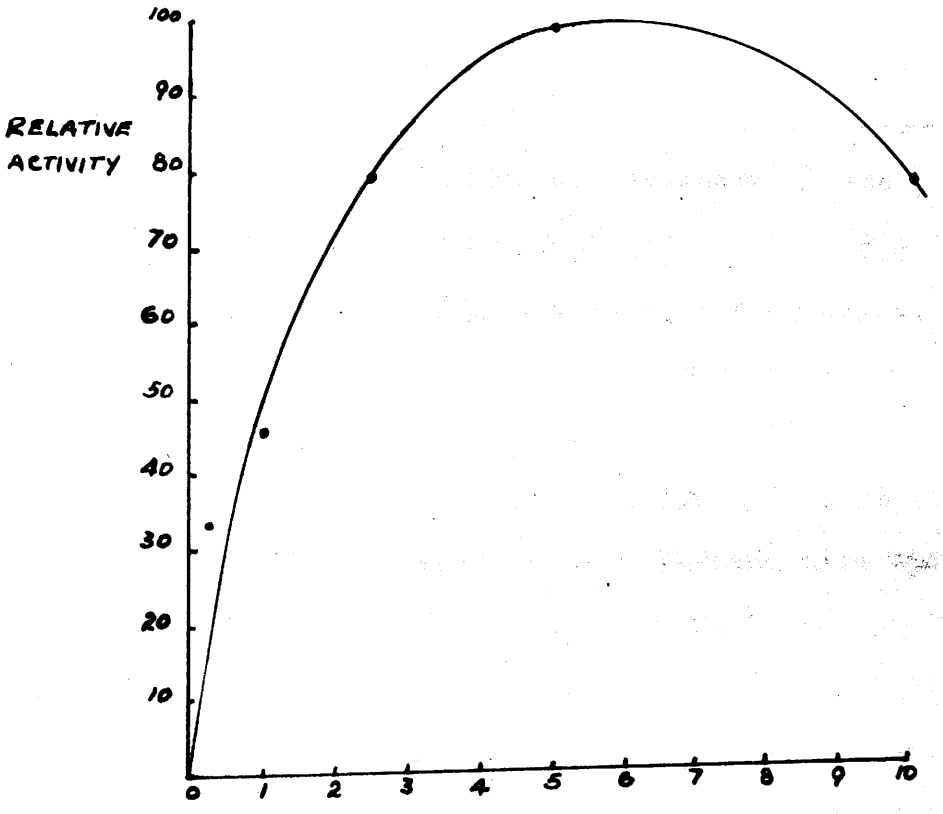
The Effect Of Varying Manganesium Ion Concentration On Activity Of Liver Phosphoglucomutase



ASSAYS CARRIED OUT AT pH 7.7
VERONAL-ACETATE BUFFERS,
FINAL CONC. G-1-P = 0.005M;
0.001M CYSTEINE PRESENT IN ALL CASES.

FIG. 5. 5.

Effect Of Variation Of Substrate Concentration On Phosphoglucomutase Activity Of Non-Particulate Cytoplasm Of Rat Liver



that different mechanisms are involved in the hydrolysis of the two substrates.

It was decided that an investigation of some of the properties of liver phosphoglucomutase might be of some help in the elucidation of this problem. The preparation of phosphoglucomutase used was the non-particulate supernatant fraction of liver cytoplasm separated in .25M sucrose solution, which has no glucose-6-phosphatase activity. The pH-optimum of this preparation was found to be about -7.2 (Fig.5.3). Mg^{++} ions were necessary for activity of the enzyme. Increased activation was observed at concentrations up to .001M, thereafter, increase in Mg^{++} ion concentration decreased the activity (Fig.5.4). These findings could account for the higher optimum pH of hydrolysis of G-1-P and also the activation produced by Mg^{++} ions. The effect of varying the concentration of G-1-P on the phosphoglucomutase activity is shown in Fig.5.5. Substrate inhibition was observed with higher concentrations of substrate and the curve is comparable with Fig.5.2.

Thus it would appear from these results that the hydrolysis of glucose-1-phosphate at pH 7.2 - 7.7 is a two stage process involving phosphoglucomutase and presumably the specific glucose-6-phosphatase.

These experiments, however, do not exclude other

secondary mechanisms and in view of this possibility, a study of the intracellular distribution of glucose-1-phosphatase activity was undertaken in an attempt to examine the likelihood of other pathways.

Rat liver cytoplasm was fractionated in 0.25M sucrose solution according to the method already described. The various fractions were assayed singly and in various combinations for their ability to hydrolyse glucose-1-phosphate and the results of two such experiments are summarised in Table 5.1.

The main point of this experiment was the demonstration of the low hydrolysis of glucose-1-phosphate by single intracellular fractions. When microsomal and supernatant fractions were recombined, however, practically the entire activity was recovered, while about 40% of the original activity was present in a mixture of the mitochondrial and supernatant fractions. The activities with the individual fractions observed in the preparation from Rat II are of such low order as could be explained by contamination.

The fact that boiling either the supernatant or the microsomal fraction before adding it to an unboiled sample of the microsomal or supernatant fraction respectively caused complete loss of activity, indicates that the increased activity achieved on combining the two fractions was not due to the addition of some activator or activators, and strongly

Table 5.1.

The Hydrolysis of Glucose-mono-phosphates by Various Fractions of Rat Liver Cytoplasm.

G-1-P = Glucose-1-phosphate. G-6-P = Glucose-6-phosphate.

Fraction	Relative Activity		Fraction	Relative Activity	
	G-1-P	G-6-P		G-1-P	G-6-P
MT+MS+S	100	100	MT+MS+S	100	100
MT	12	14	MT	6	23
MS	19	80	MS	8	78
S	23	14	S	4	0
MT + MS	4	93	MT + MS	4	92
MS + S	97	82	MS + S	85	49
MT + S	37	28	MT + S	40	21
			MS + S*	17	-
			MS*+ S	9	-

* Fraction boiled before assay.

Substrate concentrations : 0.005M

0.001M Mg^{++} present in case of G-1-P

pH of Assay: G-1-P - 7.2 } Veronal-acetate buffers.

G-6-P - 6.5 }

suggests the essential nature of one or more enzymes in each fraction.

Hers, Berthet, Berthet and De Duve (1951) found that phosphoglucomutase was present only in the non-particulate supernatant fraction of liver. The distribution of glucose-6-phosphatase was also studied in the various fractions in this work and found to be present in the microsomal fraction.

Using these two facts, the observed hydrolysis of glucose-1-phosphate can best be explained in terms of a two-stage enzymatic system: a conversion of the 1-phosphate to the 6-phosphate by phosphoglucomutase and subsequent hydrolysis of this compound by the specific glucose-6-phosphatase. Since the mitochondrial fractions contained significant amounts of this latter enzyme, the glucose-1-phosphatase activity of the combined mitochondrial and supernatant fractions is understandable.

The significance of the low recovery of glucose-6-phosphatase activity in the combined microsomal and supernatant fractions prepared from the liver of Rat II is not known.

No evidence for the existence of a secondary pathway of any significance for the breakdown of glucose-1-phosphate was apparent from these results.

The glucose-1-phosphatase activity of the nuclear fraction was not studied in this series of experiments, but

it is believed to be of negligible amount (Dr. Mills, personal communication).

Colowick and Cori (1938) failed to detect any hydrolysis of galactose-1-phosphate by liver extracts, and in view of the above results, it was decided to find if this could be verified. A hydrolysis of galactose-1-phosphate by fresh unfractionated rat liver homogenates was observed although of a much lower order of activity than had been observed with glucose-1-phosphate:

	ug.P split/mg.liver/hr. at pH 7.6
Glucose-1-PO_4	2.735
Galactose-1-PO_4	0.410

The final substrate concentrations were 0.005M and assays were carried out in the presence of 0.001M Mg^{++}. Thus the galactose-1-PO_4 was hydrolysed, but only about $^1/_7$ - $^1/_8$ as rapidly as glucose-1-PO_4.

Discussion.

pH-Activity Curves of Unfractionated Homogenates.

The pH-activity curves obtained with phenyl, p-nitrophenyl- and α- and β -glycero-phosphates are similar to those obtained by previous workers. Davies (1934) found two optima in the pH-activity curve of liver using β -glycerophosphate as substrate, as did Belfanti, Contardi and Ercoli (1935a) working with dialysed liver extracts, these workers finding optima at pHs 5.0 and 9.0, the latter being the more pronounced. The same general picture was also obtained by Bamaan and Riedel (1934) with ox and pig liver preparations.

The inhibition by Mg^{++} ions in the very acid range is indicative of the presence of a type III phosphatase as was suggested by Roche (1950). Evidence based on different degrees of activation or inhibition by substances at different pHs is not in itself sufficient to prove the existence of more than one enzyme. This is particularly true when the enzyme preparation is as crude as those studied here.

The suggestion of a third acid phosphatase, optimally active around pH 6.5, is interesting in view of the work of Swanson (1950) on purified glucose-6-phosphatase. She found that her purest preparations always possessed considerable glycerophosphatase activity which was optimal at pH 6.5. The point arises therefore, whether glucose-6-phosphatase

itself possesses glycerophosphatase activity or whether there exists a separate phosphatase acting optimally at the same pH. This point was further investigated in the studies on the intracellular distribution of the enzymes, the results of which are discussed below.

The acid phosphatase activity with those substrates showing definite optima in acid and alkaline pH ranges was always much greater than the corresponding alkaline phosphatase activity. This difference was most marked in the case of p-nitrophenyl phosphate, although it should be pointed out here that Green and Meyerhof (1952) found that p-nitrophenol inhibited intestinal alkaline phosphatase.

The alkaline phosphatase of liver appears to be activated by Mg^{++} ions, a finding in line with that of Bamaan and Riedel (1935), Belfanti, Contardi and Ercoli (1935), Rosenthal, Fahl and Vars (1952) and Cohn, et al. (1951).

The hydrolysis of adenosine-3'-phosphate is of a similar pattern to that of the glycerophosphates, the shape of the pH-activity curve in the acid range being suggestive of the existence of the three enzymes already discussed. Both the 5'-nucleotides examined (adenosine-5'-phosphate and inosine-5'-phosphate) showed curves of a different nature. The existence of a specific 5'-nucleotidase in liver with optimal pH at 7.2 has already been suggested by Reis (1951). High activity with both these substrates was also observed

in the alkaline pH range. This point will be discussed later.

Glucose-6-phosphate was hydrolysed with a single pH optimum at 6.5 due to the probable existence of a specific glucose-6-phosphatase (Swanson, 1950). The nature of the shape of the pH-activity curve with this substrate makes it difficult to decide the extent of hydrolysis by the non-specific acid and alkaline phosphatases. Glucose-1-phosphate was also hydrolysed with a single optimum which occurs at a more alkaline pH than did the optimum shown by glucose-6-phosphate. Mg^{++} ions showed a very much greater activating effect with G-1-P than with G-6-P. These findings support the work of de Duve et al. (1949), Swanson (1950) and Broh-Kahn and Mirsky (1948) who suggest that G-1-P is hydrolysed by a different pathway from G-6-P.

The Intracellular Distribution of Rat Liver Phosphatases.

In considering the results of experiments reported in Section II, several important factors must be considered.

The first is the possibility of an enzyme inhibitor or activator being concentrated in one particular fraction, a factor which will give a distorted picture of the enzyme distribution. To eliminate this factor to some degree, the activities of the individual fractions should be summed and the total compared with that of the original tissue homogenate.

In the present case good recoveries of activity were found in most of the experiments reported, the low recoveries of glucose-6-phosphatase activities shown in Tables 2.1 and 2.2 probably being due to the great lability of this enzyme (cf. Swanson, 1950).

Another disturbing factor is the possible adsorption of enzymes specifically on one type of particle. Wherever possible purified samples of the enzyme under examination should be added to suspensions of each particulate fraction and the amount of adsorption determined. This was not found practical in the present study. Novikoff (1952) found that rat-liver nuclei, suspended in 0.70 M sucrose adsorbed purified intestinal phosphatase and this enzyme was not removed even after six washings with 0.88M sucrose. Separations carried out in non-polar solvents, as in the Behren's technique for isolating nuclei, would considerably reduce the possibilities of adsorption. In general, however, this technique would be expected to result in considerable enzyme inactivation, although Stern, Allfrey, Mirsky and Saetern (1952) have published data for the activity of certain enzymes in nuclei separated by this technique.

Another important consideration is the extent to which larger sized particles are ruptured and smaller particles agglutinated during the preparative procedures. Mitochondrial

fractions might be contaminated with clumps of agglutinated microsomes and the microsome fraction, in its turn, might contain fragments of disrupted mitochondria. This difficulty was encountered in the present work during early experiments on the distribution of enzymic activity in nuclei, mitochondria, microsomes and supernatant separated from homogenates prepared in 0.25M sucrose. The purity of the nuclear fraction was followed by microscopic examination and it was found that 4-5 minutes homogenising was required to ensure that no unbroken cells remained. In these cases the microsomal and supernatant fractions contained 75% of the acid phosphatase activity. One such typical distribution is shown in the following table:

pH of Assay	Fraction	Relative Phosphatase Activity
4.05	N	2
	MT	15
	MS	28
	S	47

Shorter periods of homogenisation altered the distribution pattern, the nuclear and mitochondrial activities increasing with a corresponding fall in the activity of the supernatant fraction (Table 2.1). In view of this, nuclear activities

were studied using the method of Hogeboom, Schneider and Striebich (1952), an essential feature of which is the use of $CaCl_2$ which makes the separation of microsomes and mitochondria difficult. Consequently no attempt was made to separate the cytoplasmic fraction further in this series of experiments. The distribution of the activities in cytoplasm was studied using the original Schneider (1948) technique with homogenisation of the tissue for periods of less than two minutes and a discarding of the nuclear fraction.

Aggregation of microsomes with consequent contamination of the mitochondrial fraction may explain the significant glucose-6-phosphatase and 5'-nucleotidase content of the mitochondria in Table 2.3. Breakdown of the mitochondrial fraction may explain the acid phosphatase content of the microsomal and supernatant fractions. The overall results favour the view that acid phosphatase is predominantly located in the mitochondria and glucose-6-phosphatase and 5'-nucleotidase in the microsomes. The supernatant contains a significant amount of the total 5'-nucleotidase activity (Table 2.3). The fact that this fraction contains only traces of glucose-6-phosphatase activity would indicate that the 5'-nucleotidase present in this fraction is not due to breakdown of microsomes. A similar argument favours the view that at least 50% of the alkaline phosphatase activity of the homogenates occurs naturally in the non-particulate cytoplasm and is not derived

from the breakdown of cytoplasmic particles.

In 1951 Berthet and de Duve indicated that the acid phosphatase of rat liver mitochondria existed in an inactive form. A study of the various methods for the conversion of the inactive to an active form (Berthet, Berthet, Applemans and de Duve, 1951) led the authors to suggest that the mitochondria possessed a membrane which was impermeable to the substrate, in this case β-glycerophosphate. In order to avoid any such difficulty in the present work each fraction was disrupted by freezing and thawing.

During this work it has been assumed that cytoplasm contains only two main types of particles, the mitochondria and microsomes, and conditions of centrifugation were so chosen to give two fractions; one rich in the former type of particle, and the other in the latter. Chantrenne (1947), employing a method of differential centrifugation in electrolyte solutions, separated mouse liver cytoplasm into five fractions which, he claimed, were enzymatically and chemically heterogeneous. Keller (1951) claimed the separation of three microsome fractions which differed in the rate at which they replaced leucine in protein. The most complete evidence for the heterogeneity of cytoplasmic particles is that of Novikoff, Podber, Ryan and Noe (1953) which appeared after the completion of the present work. These workers studied the content of DNA, RNA, nitrogen and various enzymic activities in as

many as 10 different cytoplasmic fractions prepared from rat liver by differential centrifugation in 0.88M sucrose. Morphological differences in the particles present in the various fractions were observed by means of phase-contrast microscopy.

This work led to two important general findings. Firstly there appeared to be a distinct continuity between "mitochondria" and "microsomes" both morphologically and enzymatically. The smallest "mitochondria" appeared similar in size to "microsomes". No fraction was entirely free from any of the enzymes studied. Secondly the distribution of enzymes in the microsomes as a class differed sufficiently from that in the mitochondria to warrant the idea of two general types of cytoplasmic particles.

Among the enzymes studied by these authors were acid and alkaline phosphatase and 5'-nucleotidase. They report that a fraction, which was shown by phase-contrast microscopy to contain a mixture of small sized mitochondria and large numbers of "dense" and "less dense" microsomes, contained a considerable amount of the acid phosphatase activity. A microsomal fraction containing a mixture of both "dense" and "less dense" microsomes possessed even higher activity of this enzyme. A second microsomal fraction, consisting mainly of "less dense" microsomes, had a much lower activity as had the main mitochondrial fraction. Earlier work by these authors (Novikoff, Podber and Ryan, 1950) had shown that

acid phosphatase activity, assayed at pH 5.0 was concentrated largely in the mitochondrial fraction, although the separation of fractions was not carried to such a high degree as in their later work. Berthet and de Duve (1951), who employed 0.25M sucrose as separating medium, found the mitochondrial fraction to contain 56% of the phosphatase activity, whereas an "intermediary" fraction and a microsomal fraction contained only 10% and 7% respectively of the total, figures which compare reasonably well with those found in the present work.

Alkaline phosphatase was shown by Novikoff et al. (1953) to be concentrated largely in the supernatant fraction while 5'-nucleotidase activity was found mainly in the two microsomal fractions in similar amounts and also in the nuclear fraction. These findings are in agreement with those found in the present work.

The differences found in the distribution of acid phosphatase may be explained by assuming that this enzyme is concentrated to a high degree in some type of particle intermediate in size between a mitochondrion and a microsome which, in the present work, had separated largely with the mitochondria and also to some extent with the microsomes. The supernatant fractions prepared by these authors contained 36% of the acid phosphatase activity compared with less than 20% found in the present work. An important question which

arises is whether we can compare results obtained by the use of isotonic (0.25M) sucrose and those involving hypertonic sucrose solutions. Novikoff et al. report that succinoxidase and adenosine triphosphatase activities were concentrated to a large extent in their mitochondrial fractions. Thus it cannot be argued that the larger acid phosphatase activities which these workers found in the smaller sized particles and supernatant were due entirely to breakdown of mitochondria. It appears essential, therefore, in studies on the distribution of enzymes employing these methods that the problem of solubility, extraction and adsorption of enzymes must be seriously considered. De Duve has suggested that the acid phosphatase activity of rat liver is concentrated largely in the mitochondrial fraction and that the conditions of separation produce a distorted picture of the intracellular distribution due to extraction of the enzyme. On the other hand, de Duve and his colleagues have shown the acid phosphatase in their isolated mitochondria was very firmly bound and only with very drastic conditions, which brought about complete destruction of the particles, was it possible to produce the enzyme in a soluble form.

One aspect of the present study was an examination of possible differences in intracellular distribution of the three acid phosphatases whose existence was suggested by the

results of Section I. No significant difference in distribution of these phosphatase activities was however found. Thus if three separate acid phosphatases are present they must exist in similar types of particles. The distribution of glycerophosphatase activity, assayed at pH 6.5, was of interest in view of the finding of Swanson (1950) that purified glucose-6-phosphatase preparations had glycerophosphatase activity with an optimum pH at 6.5, similar to that of the glucose-6-phosphatase activity. Although the microsomal fraction was found to contain most of the glucose-6-phosphatase activity, the glycerophosphatase activity of this fraction was no greater at pH 6.5 than at pHs 3.8 and 5.55. Thus there either exists a phosphatase with similar solubility characteristics and a similar optimum pH but differing in intracellular distribution to that of glucose-6-phosphatase, or glucose-6-phosphatase itself can hydrolyse β-glycerophosphate. As already mentioned in the Introduction, a phosphatase is known to exist in red blood cells with an optimal pH at 6.0.

Assays of 5'-nucleotidase were carried out at pHs 7.2 and 10.15 in an attempt to differentiate between the two 5'-nucleotidases suggested from pH-activity curves for the hydrolysis of adenylic acid by rat liver homogenates. No significant difference was observed in the activity of any fraction whether the assay was carried out at pH 7.2 or 10.15.

The distribution assayed at pH 10.15 differed significantly from that of the glycerophosphatase activity, assayed at the same pH, which suggests that these two activities are quite distinct.

Thus in the case of the acid phosphatases of rat liver two or three enzymes exist with a similar substrate specificity but with different pH optima and, apparently, with the same intracellular distribution. A similar situation would appear to exist with the 5'-nucleotidases. Clearly an understanding of the meaning of the pH-optimum of an enzyme, as established by in vitro experiments, is of fundamental importance to our understanding of the physiological significance of enzymic activities assayed in vitro.

The enzymic activities of nuclei have been studied, in the main, by two groups of workers. Dounce and his colleagues have attempted to prepare pure nuclei, by a method involving the use of citric acid, and have studied the enzymic content of these preparations without reference to the activities of the other particles. The Schneider group, on the other hand, have studied nuclei, prepared in sucrose solution, along with the other intracellular fractions. Their nuclear preparations, they themselves admit, are not as clean as those obtained by Dounce, but they emphasise that all cell fractions must be studied to obtain information on any one fraction. Although

both points of view are fundamentally sound there are difficulties in achieving both ends at the one time. In the present work an attempt at a compromise was undertaken by employing the method of Hogeboom, Schneider and Striebich for preparing nuclear and cytoplasmic fractions. Microscopic and chemical examination of the nuclei isolated by this method showed them to be relatively free from contamination by other cell fractions. The activity of this fraction was compared with that of the original homogenate and that of the total cytoplasm. The early data reported by the Schneider group pointed to the nucleus having such negligible amounts of many enzymes that the values obtained could be explained by contamination with other types of cellular particles, and suggested that the nucleus was enzymatically inert. Nuclei prepared by the method of Dounce, however, have been shown to contain significant amounts of certain enzymes such as cytochrome oxidase, a number of enzymes of the glycolytic cycle (Dounce, 1950) and protease activity (Dounce, 1952). Arginase was also found in approximately the same concentration in cell nuclei isolated at pH 6.0 as in the whole homogenate. Lan (1943) found D-amino acid oxidase and uricase sometimes present in a higher concentration in isolated nuclei than in the original homogenate. Nucleolytic enzymes have also been shown by several workers to be present in cell nuclei (von

Euler, Fischer, Hasselquist and Jaarma, 1945; Marshak, 1948; Brown and Laskowski, 1951).

As Dounce has commented: "If the nucleus were indeed inert enzymatically, serving only as a carrier of chromosomes and genes, it would be difficult to understand how these genes could exert an influence upon the whole cell during the resting state, as they apparently do."

In the present work evidence was obtained for the occurrence of significant amounts of alkaline phosphatase and 5'-nucleotidase in nuclei. Previous histochemical work had suggested the presence of alkaline phosphatase in nuclei but the histochemical methods employed have not proved too reliable and the view has been taken that alkaline phosphatase was absent from lever nuclei.

Dounce (1943) working with nuclei isolated in citrate at pH 6.0 found high concentrations of alkaline phosphatase in his preparation. Novikoff (1952), when studying changes in the distribution of alkaline phosphatase activity in various cell fractions of rat liver separated in 0.88M sucrose following partial hepatectomy, found that the alkaline phosphatase activity of the nuclear fraction increased by as much as 500% whereas the increases in the other fractions, and in the homogenate as a whole were of the order of 65% or less. This seems good evidence for the existence of a separate

nuclear alkaline phosphatase.

One criticism of these results suggested by Novikoff might also apply, to some extent, to the present findings. Histochemical techniques have indicated that the bile canaliculi contain large amounts of alkaline phosphatase activity and phase-contrast microscopy has shown that these are among the contaminating particles in the nuclear fraction. The nuclei of the sinusoid cells and Kupffer cells of liver and leucocytes have been shown histochemically to have an increased alkaline phosphatase activity in regenerating liver. Cell counts have shown that there is also a 40-fold increase in this type of cell in regenerating liver. Thus the alkaline phosphatase activity observed in the nuclear fraction may be due to the activity of contaminating bile canaliculi and nuclei of the sinusoidal cells.

Differences between the alkaline phosphatase activity of the nucleus and that of the cytoplasm will be discussed in a later section.

Earlier evidence for the occurrence of 5'-nucleotidase activity in the nucleus was obtained from histochemical studies using a modification of the Gomori (1939) technique (Novikoff, 1952 and Pearse and Reis, 1952). One difficulty encountered was the differentiation of specific 5'-nucleotidase activity from non-specific alkaline phosphate activity without

modifying the conditions to such an extent as to increase the chance of misleading results due to diffusion of calcium phosphate. In the present work the alkaline phosphatase activity at pH 7.5 has been shown to be negligible and could not account for the values of 5'-nucleotidase activity found at this pH in the nuclear fraction.

The Acid Phosphatases of Liver.

Evidence for the existence of both a type II and a type III phosphatase in liver tissue was of a very indefinite character and no report of the isolation or fractionation of a type III enzyme from any mammalian tissue has been reported (see Roche, 1950). It was therefore decided to investigate the nature of the acid phosphatase complex of liver in an endeavour to demonstrate more clearly the multiple nature of the system present.

Two methods of approach were adopted; firstly the effect of a series of substances on the pH-activity curve of fresh, unfractionated rat liver homogenates was studied and secondly an attempt was made to fractionate the two enzymes by acetone precipitation in the presence of zinc ions.

The results obtained in the first part of this study clearly indicated the possibility of there being a type II and a type III enzyme present in these homogenates. The

overall picture obtained was of one enzyme having a pH optimum between pH 3.5-4.0, while the other was optimally active at pH 5.0-5.5. The former enzyme appeared to be activated by veronal, while the latter appeared to be inhibited by this substance. Various metallic ions varied in their effect on the two enzymes. Mg^{++} appeared to inactivate the former but to have little effect on the latter. Cu^{++} ions, even at a concentration of 0.0002M, inhibited the latter but had no effect on the former. Ba^{++} ions activated both enzymes to a slight extent, whereas Zn^{++} ions caused considerable inhibition in both instances. Citrate inhibited the enzyme optimally active at low pHs but had no effect on the other. Fluoride caused considerable inhibition of both enzymes, while CN^{-} ions had little effect on either. DL-tartrate appeared to inhibit the enzyme with the lower optimum pH almost completely, whereas the inhibition produced at higher pHs was less marked.

Abul Fadl and King (1949d) carried out a similar study on the type II acid phosphatases of red blood cells and prostate while Gordon (1952) examined the effects of some of these substances on the enzyme in extracts of adrenal cortex. Table 3.2 compares some of these workers' data with that obtained for the type II acid phosphatase of liver in the present work.

Table 5.2.

Substance	Source of Enzyme			
	Liver	Prostate	Red Blood Cell	A. Cortex
.01M Mg^{++}	Nil	sl. Inhib.	sl. Inhib.	Nil
.0002M Cu^{++}	-85%	Sl. Inhib.	-95%	-86%
.01M CN^{-}	Nil	+12%	+8%	-
.01M DL-tartrate	-41%	+95%	Nil	-91%
.01M F^{-}	-53%	-96%	-8%	-
0.5% Formaldehyde	-68%	Nil	-100%	-30%

Results with red blood cell, prostate and liver enzyme obtained using .005M phenyl phosphate in acetate buffers; those with adrenal cortical enzyme obtained using β-glycerophosphate in acetate buffers.

From these results it was observed that none of the type II enzymes was exactly similar in its behaviour in the presence of all the substances. The question arises as to whether these differences are due to the differences in protein content, etc., of the tissue extracts due to their being prepared from different tissues or whether the enzymes themselves are different in nature. This question can only be settled when studies on more purified enzyme solutions are carried out. In this connection it is of interest to note that Anagnostopolous (1953), working with purified prostate phosphatase, found that L-tartrate caused a 95% inhibition.

Abul-Fadl and King found that human liver acid phosphatase over the pH range 4-6 was almost completely inhibited by .01M L-tartrate. It would be of interest to determine the effect of L-tartrate on rat liver to ascertain if a species difference in the type II acid phosphatase existed as well as the organ difference suggested above.

Various criticisms might be applied to the use of this method to demonstrate the presence of two enzymes in a tissue extract. Anagnostopolous (1953) studied the effects of certain organic acids on purified prostate phosphatase. He found, in the first place, that the shape of the pH-activity curve of his preparation varied according as to

whether the assay were carried out using β-glycerophosphate or phenylphosphate as substrate and Fig.3.16 is taken from his results.

Secondly, in the presence of added 0.01M citrate, the curve with phenylphosphate as substrate was similar to that obtained with β-glycerophosphate. This author does not argue that these results support the view that there are two acid phosphatases in the prostate, but explains his results as being due to the citrate enhancing the affinity of the enzyme for β-glycero-PO_4 at lower pHs. Similarly, in the present work it might be argued that the variation of the inhibition with pH shown by various substances might be due to their affecting the affinity of a single enzyme for its substrate to a different degree at different pH levels.

Another factor which must be considered when working with unfractionated tissue preparations is the possibility that the substance added might be adsorbed preferentially over a certain pH range by a protein or proteins present in the extract. Adsorbed substance would not be expected to interfere with the enzyme system and the variation of inhibition could be explained in terms of adsorption.

To obtain conclusive proof of the existence of two acid phosphatases an attempt to fractionate the two enzymes was undertaken.

The problem of separating protein fractions from complex mixtures of proteins is indeed very involved and the simplest method, utilising the salting out effect of neutral salts, depends on four variables - salt concentration, protein concentration, pH and temperature. The advantage of introducing a water-miscible organic solvent into the system increases the number of possibilities available for the separation of a given protein component. Although, in the present work, the final purification achieved was only 14-fold, it is the view of the present author that a much more highly purified preparation could have been obtained if various factors such as protein concentration, temperature of precipitation, etc., which were not studied had been taken into consideration.

It is obvious that a better method for isolating the enzyme from liver must be found. Preliminary experiments to obtain a clear enzyme solution were carried out by extracting with acetate buffer at a series of pHs between 3.5 and 7.0. Iso-electric precipitation at pH 5.0 gave the best results. The purification in terms of units/mg. protein obtained with this method was only a fraction of that achieved by London and Hudson (1953) in the purification of prostate phosphatase utilising a similar method. These workers found that extraction of prostate with acetate buffer

of pH 5.0 gave a 21-fold purification and on dialysing the extracts a further 2-fold increase in purity was obtained. 58% of the original activity was still present at this stage. These figures are to be compared with the 1.6-fold purification and the 80% loss in activity obtained with the same operations on rat liver. The low purity figures could possibly be explained as being largely due to the difference in the type of protein present in the two tissues. In the prostate a large amount of the protein would be in the form of insoluble fibrous protein and the low recoveries of the liver enzyme suggest that this enzyme was more labile than the prostate enzyme.

A fuller investigation of the precipitation of the phosphatase activity in the presence of Zn^{++} ions where the effects of varying the protein concentration, Zn^{++} ion concentration and temperature of precipitation is also worthy of consideration.

The limitations of methods such as that of Lowry, Rosebrough, Farr and Randall (1951) for determining proteins were exemplified in the present work. Despite the claim by these authors that their method was very convenient for following protein fractionation, this was not found to be the case. This is quite understandable from a consideration of their method, which consists of a combination of the biuret

colour reaction and the Folon-Ciocalteu phenol reaction. In comparing complex mixtures of proteins where the average tyrosine content does not vary appreciably, good correlation between this method and methods depending on N-determinations is to be expected. As the components of a protein mixture become separated it is reasonable to suppose that the various fractions will differ appreciably in tyrosine content and thus the chance of obtaining a good correlation between the protein content determined by this method and that based on N contents becomes less. The authors themselves showed that the same amount of different proteins gave different amounts of colour.

Paper electrophoresis, although a convenient and rapid method, was found to be unsuitable in the present instance to separate the two enzyme fractions which were demonstrated by free electrophoresis.

The final fractionation achieved by electrophoresis on the Tiselius apparatus is the most convincing indication that more than one acid phosphatase is present in rat liver. The pH optima of the fast fraction were distinctly different from those of the slow fraction and a summation of the pH activity curves of the fast and slow fractions accounts satisfactorily for the shape of the pH-activity curve of the main fraction, good evidence that an actual separation has

been achieved. The final fractions were not pure enough, however, to permit kinetic studies to be carried out to confirm the multiple nature of the acid phosphatases of liver.

The fact that two acid phosphatases have been shown to exist with different pH optima but the same apparent intracellular distribution, raises the question of their physiological function. Norberg (1951) found a difference in the shape of the pH-activity curve of rat liver acid phosphatase during processes of regeneration and it has been reported (Goodlad, Mills and Smith, 1951) that rat liver acid phosphatase activity, assayed at pH 5.5, showed a 50% increase in activity in rats which had been maintained on a low protein, low choline diet although the activity assayed at pH 3.75 showed no change.

It thus appears that the enzymes have different physiological functions.

The Alkaline Phosphatases of Liver.

Since the alkaline phosphatase activity of rat liver has been shown to alter markedly under various conditions such as partial hepatectomy and protein depletion, a complete understanding of the nature of the enzyme system present is obviously essential before an interpretation of the observed variations in activity can be made.

From the results of the study of the pH-activity curves of the nuclear and cytoplasmic fractions of normal rat liver it appeared that the nucleus contained a single enzyme showing optimum activity at pH 10.05. There appeared to be two enzymes present in the cytoplasm, one optimally active at pH 10.05 and the other somewhere around pH 8.5-9.50. The activating effect of Mg^{++} ions on the cytoplasmic enzyme optimally active at the lower pH distinguished it from the other enzyme in the cytoplasm and also from the nuclear enzyme. This latter enzyme appeared similar in many ways to the cytoplasmic enzyme which showed optimal activity at the higher pH.

The existence of two alkaline phosphatases in cytoplasm was suggested also from the pH-activity curves of nuclear and cytoplasmic fractions obtained from regenerating livers. Although no great quantitative change in activity was apparent, two well-defined peaks of activity were obtained with the cytoplasmic fraction of one rat liver (Fig.4.5). The failure to find a raised alkaline phosphatase activity in these livers may have been due to the fact that the rats were not examined until the third day after operation and, by this time, the rise in enzyme activity may have passed.

While the application of kinetic studies on such crude preparations as tissue homogenate is open to criticism

it should be borne in mind that the present work was mainly undertaken to ascertain the presence of more than one enzyme and not as an attempt to evaluate accurate enzyme kinetic data. Although the values of K_s for the nuclear activity at pH 10.05, the cytoplasmic activity at pH 10.05 and the cytoplasmic activity at pH 9.22 were all different, it is felt that too much weight should not be put on these results. The K_s value is determined by the velocity of combination of the enzyme with the substrate and this has been shown to vary markedly with pH in a number of cases (Dixon 1953; Van Slyke, 1942), although Smith (1952) found no variation in K_s values with pH for her purified glucuronidase fractions. It is worth noting in the present case that the difference between the behaviour of the cytoplasmic fraction when assayed at pH 10.05 and pH 9.22 is greater than the difference observed between the cytoplasmic and nuclear fractions at pH 10.05.

Sizer (1937) found that the activation energy of yeast invertase was constant over the pH range 3.2-7.9. The same author (Sizer, 1943) has reviewed the subject of the effect of temperature on enzymic reactions and cites numerous other examples where this constancy has been observed. He also states that the value of the activation energy is unaffected by the state of purity of the preparation. In

view of these facts it seems not unreasonable to accept the observed differences in activation energies observed at pHs 9.22 and 10.05 with the cytoplasmic fraction as evidence for the existence of two distinct enzymes.

The fractionation studies, however, failed to produce any indication of the complex nature of rat liver alkaline phosphatase. This fact in itself cannot be taken to mean that such a possibility does not exist. In the initial autolysis of the tissue there is a danger that one enzyme might have been inactivated. That this may have occurred in the present case is suggested by the increased activating effect of Mg^{++} ions at pH 10.05 observed in the preparation prepared by precipitating with 60% alcohol.

Paper electrophoresis failed to show the presence of two alkaline phosphatases in a purified kidney preparation (Levy and Mazia, 1953) while Giri, et al. (1952) claimed a separation of two phosphatases from an acetone dried powder of kidney. One factor which must not be overlooked in discussing results obtained from electrophoretic studies is the possible denaturing effect of the electric current on an enzyme. In this connection it was found by Smith (1952) that highly purified preparations of β-glucuronidase which showed multiple peaks of activity underwent a transformation during electrophoresis on filter paper or on the

Tiselius apparatus to give a single peak of activity. In the present work, also, paper-electrophoresis failed to show more than one enzyme.

The results of this experiment, however, did indicate that the alkaline phosphatase activity was associated with the fastest moving protein component which suggests that a method of purifying the enzyme might be achieved using electrophoresis on the Tiselius apparatus. Roche and Bouchilloux (1953) and Levy and Mazia (1953) have suggested that a similar process might be useful in the purification of the alkaline phosphatases of intestinal mucosa and kidney.

The Hydrolysis of Hexose Monophosphates.

Broh-Kahn and Mirsky (1948) listed 4 possible pathways for the hydrolysis of G-1-P:-

a) direct dephosphorylation by a specific phosphatase:

b) some glucose-1-phosphate dephosphorylated directly by a phosphatase, specific for this ester, while the rest might be converted into glucose-6-phosphate, which in turn is hydrolysed by glucose-6-phosphatase:

c) some G-1-P might be converted to G-6-P and the remaining G-1-P and the G-6-P might be dephosphorylated by the same enzyme:

d) all the G-1-P must be converted to G-6-P as a necessary step in its hydrolysis and the latter compound hydrolysed by a specific glucose-6-phosphatase.

The first pathway is not compatible with the fact that no individual cytoplasmic fraction of rat liver homogenates brought about a significant dephosphorylation of G-1-P but combinations of either the microsomal and supernatant fraction or, to a lesser extent, the mitochondrial and supernatant fractions brought about considerable hydrolysis. No fraction therefore appears to contain a specific glucose-1-phosphatase. This fact also makes the second and the third proposed mechanism highly improbable.

The fourth suggested route of hydrolysis is in keeping with the observed results and is indeed the only logical explanation.

This section of the work illustrates the need for caution in interpretation of results obtained from crude enzyme preparations. If only the results shown in Figures 5.1 and 2 and I.5 had been available, then it would have been quite justifiable to conclude that glucose-1-phosphate and glucose-6-phosphate are hydrolysed by two separate enzymes, differing in their pH-optima, sensitivity to Mg^{++} ions and activity in presence of varying substrate concentrations.

On the other hand, if the results of such an investigation are to have any physiological significance, then one must contrive to keep conditions as near as possible to those occurring naturally. It is still a matter of some conjecture whether even results obtained in fresh unfractionated homogenates can be taken as a true reflection of processes occurring in the liver cell. Nevertheless, the present findings will be discussed in relation to wider aspects of carbohydrate metabolism.

One of the most interesting facts about the intracellular distribution of the enzymes responsible for the hydrolysis of glucose-1-phosphate is the fact that a spatial separation of the components of the enzyme system was found to exist. The rate at which liver secretes glucose into the blood stream depends on two opposing mechanisms - glycogenesis and glycogenolysis. Hers et al. (1951) have suggested that the enzymes of the glycolytic cycle which they studied occurred more or less completely in the non-particulate cytoplasm and with phosphoglucomutase they were able to achieve 100% recoveries of the activity of the whole cell in this fraction. These workers also found that 70-80% of the glucose-6-phosphatase activity was present in the microsomal fraction of liver, a finding which has been confirmed in the present work. The question arises: does glucose-6-phosphate

which is formed during glycolysis in the non-particulate supernatant fraction have to "penetrate" the microsomes before hydrolysis? The existence of such a system suggests numerous possibilities. The control of glucose-6-phosphate breakdown and consequently the control of blood sugar level could be dependent upon the rate at which glucose-6-phosphate enters the microsome and it is tempting to suggest that a microsomal "barrier" exists. It is not intended here to discuss the possibilities of such a structure, but it is of interest to note that Berthet and De Duve (1951) found that liver acid phosphatase was concentrated in a mitochondrial-like particle which appeared to be impermeable to β-glycerophosphate. In fact the particles could be isolated in an isotonic solution of this substance and no hydrolysis was observed as long as the particles remained intact (Berthet, Berthet, Appelmans and De Duve, 1951). If such a system existed, it could be postulated that the hormonal control of blood sugar level might be effected by an alteration in the rate of entry of the glucose-6-phosphate to the microsome.

On the other hand, the possibility exists that glucose-6-phosphatase is synthesised or stored in the microsomes and is "secreted" into the non-particulate cytoplasm as required. Purified glucose-6-phosphatase has been shown to be an extremely unstable enzyme (Swanson, 1950) which is

inactivated at room temperature in a matter of minutes, and it may be rapidly destroyed in the supernatant and replenished from the microsomes as required. In this connection Moyson and Gavosto (1953) investigated the changes in the intracellular distribution of glucose-6-phosphatase of rat liver following shock. They found that there was a very significant increase in the amount of this enzyme in the microsomal fraction and also that a significant activity appeared in the supernatant fraction which had been devoid of activity in the control animals. The connection between hyperactivity of the glucose-6-phosphatase in the liver and the hyperglycaemic conditions of the animals was emphasised.

Although no attempt was made to study the properties of liver phosphoglucomutase in detail, it was found in the present work that the pH optimum and effect of Mg^{++} ions were similar to that observed in preparations from other sources (Najjar, 1948; Cardini, 1951). Preliminary studies on the purification of the enzyme showed it to be resistant to heating to 63° for 3 minutes and also that most of the activity could be precipitated between 40-50% saturation with ammonium sulphate.

The finding that galactose-1-phosphate was hydrolysed by a rat liver homogenate was of interest in view of the

failure of Cori, et al. (1938) to find any hydrolysis of this substance by liver extracts. The activity observed with this substrate was of a much lower order than that observed with glucose phosphate. This latter compound is never found free in liver whereas Kosterlitz & Ritchie (1943), after feeding large amounts of galactose to rabbits, was able to isolate galactose-1-phosphate from their livers. In view of the failure to find a specific glucose-1-phosphatase, the mechanism whereby the galactose-1-phosphate is hydrolysed is of importance.

The Waldenase system has not yet been reported as occurring in liver but the most probable route of galactose-1-phosphate hydrolysis is via the following pathway:-

Galactose-1-phosphate
↓ Waldenase
Glucose-1-phosphate
↓ Phosphoglucomutase
Glucose-6-phosphate
↓ Glucose-6-phosphatase
Glucose + PO_4'''

Clearly an investigation of the mechanism of hydrolysis of both 1- and 6-phosphogalactose by liver would be of interest in many respects. The question of the existence of a liver Waldenase system and the specificity of liver phosphogluco-

mutase and glucose-6-phosphatase are all of great importance in the construction of a clear picture of the processes of hexose metabolism in the animal organism.

Klenow (1953) (personal communication to G.T. Mills) has found muscle phosphoglucomutase in the presence of G-1, 6-diP to react with galactose-1-PO_4 at about $^1/_{100}$th the rate with glucose-1-PO_4.

These results suggest that the hydrolysis of galactose-1-PO_4 may follow a different pathway from that of glucose-1-PO_4. Since, in the present work, the rate of hydrolysis of galactose-1-PO_4 was much more than $^1/_{100}$ that of glucose-1-PO_4, it is probable that galactose-1-PO_4 is hydrolysed via the Waldenase system and not by a mechanism involving preliminary conversion to galactose-6-PO_4.

Summary.

1. pH-activity curves have been determined for the phosphatase activity of rat liver homogenates using as substrates, α- and β-glycerophosphate, phenyl phosphate, p-nitrophenyl phosphate, adenosine-3'-phosphate, adenosine-5'-phosphate, inosine-5'-phosphate, glucose-1-phosphate and glucose-6-phosphate.

The effect of Mg^{++} ions on these curves was also studied.

Evidence was obtained from these studies which indicated the presence in liver of three non-specific acid phosphatases, a non-specific alkaline phosphatase, specific 5'-nucleotidase and glucose-6-phosphatase activity.

2. The intracellular distribution of these enzymes was studied using the differential centrifugation methods of Schneider (1948) and Hogeboom, Schneider and Striebich (1952).

Alkaline phosphatase and 5'-nucleotidase were the only enzymes found in significant amounts in the nucleus. Non-specific acid phosphatase activity was present in large amounts in the mitochondria and to a lesser extent in the microsomes. The microsomal fraction contained large amounts of glucose-6-phosphatase activity and 5'-nucleotidase activity. More than 50% of the alkaline phosphatase activity

was found in the non-particulate cytoplasm.

3. The effect of various inhibitors and activators on the acid phosphatase activity of rat liver homogenates was studied over a wide pH range and the results confirmed the existence of at least two acid phosphatases.

4. Further evidence for the existence of two acid phosphatases was obtained from fractionation studies involving acetone precipitation in the presence of Zn^{++} ions and electrophoresis in the Tiselius apparatus.

5. The alkaline phosphatase activity of nuclear and cytoplasmic fractions was further studied in normal and regenerating rat livers, and from the effect of pH and Mg^{++} ions it was concluded that two enzymes were probably present in the cytoplasm and only one in the nucleus. The presence of two alkaline phosphatases in the cytoplasm was also suggested from determinations of the activation energy at different pHs.

Fractionation and electrophoretic studies, however, failed to indicate the presence of more than one alkaline phosphatase.

6. The hydrolysis of glucose-1-phosphate by whole tissue extracts and intracellular fractions was studied along with

some of the properties of liver phosphoglucomutase.

The results indicate that the mechanism of hydrolysis of G-1-P is by a two stage process involving a preliminary conversion to glucose-6-PO_4 and subsequent hydrolysis of this compound by glucose-6-phosphatase. No evidence for any secondary pathway was obtained.

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