

The work described in this thesis was carried out during the tenure of a Carnegie Research Fellowship in Biochemistry at the Kaiser Wilhelm Institut für Zellphysiologie, Berlin-Dahlem.

THE ISOLATION AND PROPERTIES OF  
THE ENZYME URICASE.

I wish to express my thanks to Professor Otto Warburg for his encouragement and advice during the course of the work and for his generous provision of laboratory facilities and materials.

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A Thesis submitted for the degree of  
Doctor of Medicine

by

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PART I.

INTRODUCTION.

THE CHEMICAL CONSTITUTION OF ENZYMES.

THE ISOLATION OF ENZYMES.

Since the time of Berzelius (1825) it has been known that living cells produce substances which act very much in the same way as inorganic catalysts, and for such catalysts produced by living cells the name "enzyme" was suggested by Kühne in 1867.

For a time a violent controversy raged between the supporters of Pasteur, who maintained that reactions caused by enzymes, such as alcoholic fermentation, were essentially vital "processes" which could only be caused by living cells, and the supporters of Liebig, who maintained that such reactions could occur without living cells. The controversy was eventually settled by Buchner (1897) who showed that fermentation could be caused by a cell free extract of yeast. The production of such cell free enzyme preparations stimulated chemists and physiologists to attempt to

isolate enzymes and to determine their chemical nature, and this work has culminated in the preparation of a number of enzymes in crystalline form.

Enzymes have for many years been considered to be proteins or to be inseparably associated with proteins, although, since pure preparations of enzymes were not obtained until a dozen years ago, the evidence for the identity of enzymes and proteins was necessarily indirect. Of recent years this view has been strengthened and confirmed by the preparation of a number of enzymes in a highly purified form and in some cases by the crystallisation of the enzymes. All these purified enzymes are proteins.

Some doubt of the protein nature of enzymes was cast by Willstätter (1928) who, as the result of a long series of researches, culminating in the preparation of several enzymes in a highly purified form but in very dilute solutions, had come to the conclusion that enzymes were not proteins, since his purified preparations did not give protein tests. However, as Northrop (1939) points out, enzymes are extremely active bodies, and their activity may be detected in solutions which are far too dilute to give the

relatively crude chemical tests. For example bacteriophage may be detected biologically in a solution containing only  $10^{-15}$  Gm. per ml. - a concentration so minute that tests for any chemical compound are negative.

#### ENZYMES AS PROTEINS.

Although crystalline and highly purified enzymes all give positive protein tests and give elementary analysis figures similar to those of proteins, the possibility still remains that enzyme and protein might not be identical. The enzyme activity might for example be due to minute amounts of a more powerful substance adsorbed on the protein surface or contaminating the protein.

Northrop realised this difficulty and attempted to overcome it in the case of crystalline pepsin (Northrop (1935, 1939)).

Unfortunately, although enzymes have been prepared in crystalline form, the criteria of purity which can normally be applied to crystalline organic compounds cannot be applied to enzymes. For example, proteins decompose without melting, and melting point

determinations cannot therefore be made. Even the fact that crystals have been obtained is no criterion of purity, as was first shown by Sørensen (1925) who found that egg albumin which had been repeatedly recrystallised, consisted of more than one component and was in fact a solid solution.

These difficulties can be overcome by various methods. Northrop recrystallised pepsin many times and found no change either in enzyme activity or in composition (nitrogen content, ash, etc.) and no indication that the material could be separated into fractions by repeated recrystallisation.

The most delicate test for the homogeneity of a crystalline protein, however, is a solubility test based on the phase rule. If we have a two phase system consisting of a saturated solution of a substance in equilibrium with the solid phase, then the composition of the solution is fixed and is independent of the quantity of solid present. On the other hand, if we are dealing with a three (or more) phase system in which the solid consists of more than one phase, the composition of the solution will vary with the

amount of solid present. This principle was used very successfully by Landsteiner and Heidelberger (1924) to detect the difference between haemoglobins of closely related species in cases where even immunological tests were not decisive.

When a test of this type is applied to pepsin it is found that in the presence of excess protein the solubility and composition of the solution become independent of the amount of solid phase present, and there is therefore no doubt that pepsin is neither a mixture nor an adsorption complex. The possibility of it being a solid solution is not completely excluded but is rendered remote.

Another method of testing the homogeneous nature of pepsin is by the use of the ultracentrifuge. Pepsin solutions are found to show uniform sedimentation and to give a sharp boundary (Philpot and Eriksson-Quensel (1933)). Since proteins of the same molecular weight would tend to sediment out at the same rate this method is not absolutely conclusive but it nevertheless provides confirmatory evidence.

Diffusion experiments have also been employed. In



the case of pepsin it has been found that the rate of diffusion as determined by protein analysis or nitrogen estimations is the same as that determined by enzyme activity. Enzyme and protein therefore diffuse together.

Moreover the temperature coefficient for the inactivation of the enzyme is the same as that for the denaturation of the protein, and the conditions for inactivation by heat and alkali are the same for both enzyme and protein. Exposure to ultraviolet light or to the beta and gamma rays of radium causes inactivation of pepsin with a corresponding loss of native protein. There appears also to be a definite relationship between the absorption spectrum of pepsin and the inactivating efficiency of light at various wavelengths.

Tests such as those described for pepsin have also been applied to other enzymes and the protein nature of enzymes is now generally accepted.

Whether or not one accepts the theories of Wrinch regarding protein structure, there can be no doubt that the surface of a protein has a definite pattern or

design which is characteristic of that particular protein and it is this pattern which is responsible for the specificity of the enzyme. The substrate will be adsorbed on a particular area of the enzyme surface, in the same way as, to use Emil Fischer's simile, a key fits the lock.

The first report of the preparation of an enzyme in crystalline form was made in 1926, when Sumner crystallised urease, and three years later Northrop (1929) crystallised the enzyme pepsin. Both these crystalline enzymes are proteins.

Since then fourteen enzymes have been prepared in crystalline form and others have been prepared in a highly purified state although not actually crystallised.

The following enzymes, together with three precursors, have been crystallised:-

- |         |                                                |
|---------|------------------------------------------------|
| Urease  | by Sumner (1926)                               |
| Pepsin  | by Northrop (1929)                             |
|         | and pepsinogen by Herriott and Northrop (1936) |
| Amylase | by Caldwell Bocher and Sherman (1931)          |
| Trypsin | by Northrop and Kunitz (1932)                  |
|         | and Trypsinogen by Kunitz and Northrop (1934)  |

"The yellow enzyme" by Theorell (1934)

Chymotrypsin by Kunitz and Northrop (1935)

and chymotrypsinogen by Kunitz and  
Northrop (1935)

Carboxypolypeptidase by Anson (1935)

Catalase by Sumner and Dounce (1937)

Ficin proteinase by Walti (1937)

Papain by Balls, Lineweaver, and Thompson  
(1937)

Lysozyme by Abraham and Robinson (1937)

Protein of acetaldehyde reductase by Negelein  
and Wulff (1937)

Tyrosinase by Dalton and Nelson (1938)

Protein constituent of the oxidation system of  
1:3 glyceraldehydephosphoric acid  
by Warburg and Christian (1939)

The following enzymes have not been crystallised  
but have been isolated in a very pure state:-

A number of flavoproteins, by various workers.

Xanthine oxidase by Ball (1939).

Polyphenol oxidase by Kubowitz (1937).

Laccase by Keilin and Mann (1939a).

Carbonic anhydrase by Keilin and Mann (1939b).

Since the experimental work described in this thesis deals with the isolation and properties of an enzyme, a very brief account will first be given of those enzymes which have already been isolated and of their chemical constitution as far as is known.

#### THE HYDROLYTIC ENZYMES.

The first enzymes to be crystallised were hydrolytic enzymes and enzymes of this group have been very thoroughly investigated, chiefly by Northrop and his associates at the Rockefeller Institute in New York.

UREASE. The first crystallisation of an enzyme was accomplished in 1926, when Sumner succeeded in crystallising urease, the enzyme which hydrolyses urea to ammonium carbonate (Sumner (1926, 1932)). The process which he employed was extremely simple. Jack bean meal was stirred with 31.6% acetone and the mixture was filtered in the ice chest overnight. Crystals of urease appeared in the filtrate. Recrystallisation could be carried out repeatedly without alteration in the properties. The crystals were of typical protein nature. Even the most highly purified crystals, however, have been shown in the ultracentrifuge to contain

15% impurity (Summer, Gralen, and Eriksson-Quensel (1938)). The molecular weight of urease is 483,000.

PEPSIN. The next advance was made in 1929, when Northrop (1929, 1932) was able to show that the proteolytic enzyme pepsin of the stomach could also be obtained in crystalline form. The starting material was crude commercial pepsin which was worked up by a process of fractional precipitation with acid after solution in alkali. The crystallisation of pepsin by allowing strong solutions at 45° to cool was a relatively simple process, and the yield of crystalline product was extremely good. A similar method could be employed to isolate pepsin from gastric juice (Northrop (1933)).

A suggestion that the pepsin molecule is built up on the basis of a closed cyclol structure with 288 amino acid residues as units has been put forward by Wrinch (1937). Its molecular weight is 36,000 (Philpot and Eriksson-Quensel (1933)).

PEPSINOGEN. The inactive precursor of pepsin, pepsinogen, has also been isolated and crystallised by

Herriott and Northrop (1936) from the gastric mucosa by a process of ammonium sulphate fractionation.

TRYPsin, CHYMOTRYPsin and their Precursors. A similar process of ammonium sulphate fractionation was also employed by Northrop and Kunitz (1932) in the isolation and crystallisation of trypsin, the proteolytic enzyme of the pancreas, using as starting material either commercial pancreatin or fresh pancreas tissue. From fresh pancreas, also, the same authors were able two years later (Northrop and Kunitz (1934)) to prepare the precursor of trypsin, trypsinogen, in crystalline form.

The other chief proteinase of the pancreas, chymotrypsin, and its precursor, chymotrypsinogen, have also been isolated and crystallised by a modification of the ammonium sulphate fractionation process used in the extraction of trypsin (Kunitz and Northrop (1935)).

CARBOXYPEPTIDASE. The digestion of proteins, begun in the stomach by the action of pepsin and continued by trypsin and chymotrypsin, is carried on in

its later stages by the peptidases. One of these, carboxypeptidase, attacks peptide chains at the end, carrying the carboxyl group and liberates an amino acid, which, in the intact substrate, carries a free carboxyl group. Carboxypeptidase has recently been crystallised by Anson (1937) who used as his starting material the fluid which exudes when frozen pancreas is thawed. This fluid is acidified to remove slimy colloidal material which can then be easily filtered off, and the filtrate on dilution with water yields a precipitate of carboxypeptidase which can then be crystallised by dissolving in barium hydroxide and acidifying.

The crystals are insoluble in water but dissolve in dilute salt solutions. Carboxypeptidase is therefore a globulin.

PAPAIN. From the coagulated latex of the green papaya fruit *Carica papaya*, Balls, Lineweaver, and Thompson (1937) have isolated crystals of the protease, papain, by a process of fractionation with ammonium sulphate, and more recently Balls and Lineweaver (1939) have shown that the presence of free -SH groups in the enzyme molecule is essential for its activity. The

importance of these groupings has also been demonstrated in the case of other enzymes, for example in the case of succinic dehydrogenase, which on incubation with oxidised glutathione (GSSG) loses its activity but regains it on treatment with reduced glutathione (GSH). As the result of experiments of this type, Hopkins and his associates (1938) have suggested that succinic dehydrogenase contains sulphydryl groups which are essential for enzymatic activity. When they are oxidised, activity vanishes.

Such sulphydryl groupings which are essential for activity are the nearest approach to active groupings or prosthetic groups which are found in the proteolytic enzymes.

FICIN PROTEINASE. Another very similar enzyme, which also contains sulphydryl groups, is ficin, which has been isolated and crystallised from the latex of *Ficus* by Walti (1937).

AMYLASE. Pancreatic amylase has been purified to a very high degree by a process of adsorption on alumina gel and subsequent elution, and such a highly active



preparation has been induced by Caldwell, Booher and Sherman (1931) to crystallise from buffered aqueous alcohol solutions. The crystals are typical proteins.

The crystallisation of amylase has finally put an end to the suggestion of Willstätter (1928) and his associates that amylase was not a protein. Willstätter's solutions were so dilute that, while they still retained powerful enzyme activity, they were not able to give positive protein tests.

PROSTHETIC GROUPS. It is generally believed that the activity of an enzyme is due to one particular grouping in the protein molecule, the prosthetic group, which actually reacts with the substrate and which is chemically relatively simple in nature. The rest of the molecule, the protein component, acts as a carrier to this prosthetic group and is responsible for the specificity of the enzyme. An enzyme can therefore be divided into two parts - the prosthetic group, and its colloidal carrier or protein.

In some cases, e.g. in the case of the hydrolytic enzymes, the prosthetic group must be regarded as one particular area situated on the protein surface and

inseparably bound to it, but in other cases, particularly in the case of the oxidising enzymes, it has been found possible to split the enzyme into its two component parts. Separately these are quite inactive, but when they are brought together the enzyme is reformed and full activity is restored. As we shall see, the degree of binding between protein and prosthetic group, varies greatly in different enzymes.

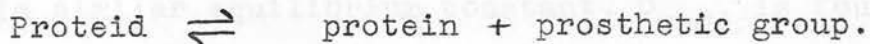
#### OXIDISING ENZYMES.

The second great group of enzymes comprises the oxidising enzymes which have been investigated chiefly by Warburg and his assistants at the Kaiser Wilhelm Institut für Zellphysiologie at Berlin-Dahlem. As the result of the isolation and investigation of a number of these enzymes, Warburg (1938) has been led to formulate a general theory regarding the chemical constitution of enzymes.

#### WARBURG'S THEORY of the Chemical Constitution of Enzymes.

According to this theory all enzymes are what

Warburg calls dissociating proteids which can split up reversibly into a protein carrier and a prosthetic group thus:-

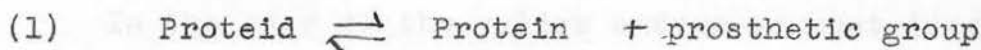


A coenzyme, according to Warburg, is simply a prosthetic group leading an independent existence owing to the preponderance of the reaction to the right.

The specificity of the enzyme depends upon the protein component and is controlled by the configuration of the surface of the protein molecule.

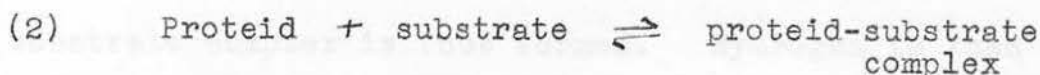
Different proteins may combine with the same prosthetic group to form different enzymes. This is well exemplified in the case of the flavoproteins, or alloxazine nucleotides, and in the case of the pyridine proteids.

The prosthetic group in its turn contains active groups which take part in the reaction with the substrate. The protein part of the enzyme also combines reversibly with the specific partner with which the prosthetic group of the enzyme reacts in the living cell. There are therefore two sets of equilibria and two equilibrium constants thus:-



$$D_{\text{ox.}} = \frac{(\text{protein})(\text{prosthetic group})}{(\text{proteid})}$$

(a similar equilibrium constant,  $D_{\text{red}}$ , is found for the reduced form of the enzyme).



$$d_{\text{ox}} = \frac{(\text{proteid})(\text{substrate})}{(\text{proteid-substrate complex})}$$

(a similar equilibrium constant,  $d_{\text{red}}$ , is found for the reduced form of the enzyme).

To take an example, the "yellow enzyme" of Warburg and Christian is what Warburg calls an alloxazine proteid. It consists of a protein bound to a prosthetic group, lactoflavin phosphate, which contains an active group, the alloxazine ring system.

The proteid can be fairly easily split up, for example, by dialysis, into the prosthetic group which is diffusible, and the protein which is non-diffusible. Moreover the proteid can also combine with the substrate of the enzyme, the dihydrotriphosphopyridine-nucleotide giving the protein substrate complex.

In the case of the yellow enzyme we must imagine that what happens is this:- The dihydrotriphosphopyridinenucleotide is adsorbed on the protein surface of the enzyme, by virtue of the particular configuration of the surface, in the neighbourhood of the prosthetic group, lactoflavin phosphate. The proteid substrate complex is thus formed. Hydrogen is then transferred from the substrate to the active group of the prosthetic group, i.e. to the alloxazine ring. The oxidation product of the substrate, i.e. triphosphopyridinenucleotide then splits off leaving the reduced form of the enzyme in which the prosthetic group contains the dihydroalloxazine ring. The reduced enzyme then reacts with oxygen to which the hydrogen is transferred with the formation of hydrogen peroxide, while the original oxidised form of the enzyme is reconstituted.

Euler has also developed a similar conception of the nature of enzymes with a difference in nomenclature. He calls the complete enzyme (Warburg's proteid) a holodehydrogenase. It can split up into two parts, an apodehydrogenase corresponding to Warburg's protein and

a codehydrase corresponding to the prosthetic group, (von Euler and Albers (1936)). In this scheme, however, the term apodehydrogenase is sometimes applied to the complete enzyme and sometimes to the protein component only.

It is unfortunate that two different systems of nomenclature should have come into being. According to the Warburg scheme the enzyme is the proteid, i.e. the protein plus prosthetic group no matter whether the prosthetic group is so tightly bound to the protein that it cannot be separated as in the case of succinic dehydrogenase (in which the active groups appear to be -SH groupings), or whether the enzyme and prosthetic group are normally closely bound but can be separated with some difficulty, as in the case of the amino acid oxidase (alloxazine adenine proteid  $O_2$  amino acid), or whether the protein and prosthetic group are only loosely bound as in the case of the pyridine proteids, e.g. alcohol dehydrogenase. According to the scheme of nomenclature adopted by Euler and the Cambridge school, the enzyme is sometimes the protein plus prosthetic group as in the case of succinic dehydrogenase and amino acid oxidase, but at other times the term enzyme

is reserved for the protein alone as in the case of alcohol dehydrogenase, and all other dehydrogenases in which the binding between protein and prosthetic group or coenzyme is but slight. Such inconsistency is to be regretted, since even in those cases where protein and prosthetic group are loosely bound, a definite compound between them is undoubtedly formed, and the dissociation constants for such compounds are known.

As Warburg's nomenclature appears to be more logical and consistent, it has been adhered to throughout this thesis.

Of the enzymes which play a part in the oxidation reduction systems of living cells, two main groups have been isolated. One of these groups comprises the enzymes which contain a heavy metal such as iron or copper as their active group, and which are therefore inhibited by such substances as potassium cyanide, while the other group consists of enzymes formed by the combination of protein with dyestuffs of the flavin group, and known therefore as flavoproteins.

ALLOXAZINE PROTEIDS OR FLAVOPROTEINS."THE YELLOW ENZYME" of Warburg and Christian.

As the result of a study of the oxidation reduction systems of red blood cells and of the oxidation of hexose monophosphate (Robison ester) by cytolysed blood, Warburg and Christian (1932) discovered in yeast an enzyme to which, on account of its colour, they gave the name of "the yellow enzyme". This substance can exist in two forms, an oxidised form which is yellow and a reduced form which is almost colourless. The yellow enzyme catalyses the oxidation of the dihydro forms of the di- and tri-phosphopyridinenucleotides, being itself converted thereby to the reduced form of the enzyme. It, in its turn, is re-oxidised by molecular oxygen, or in the living cell more probably by cytochrome (Theorell (1936)). The yellow enzyme has sometimes been referred to as merely a hydrogen carrier, but since it catalyses specifically the oxidation of the pyridine nucleotides, it can legitimately be regarded as an enzyme.

Its structure may be considered in some detail, since it was the study of this enzyme which led to great



advances in our knowledge of enzymes in general.

The yellow enzyme has been prepared in pure crystalline form by Theorell (1934, 1937). The most important step in its preparation from the "Lebedewsaft" of yeast is a purification by cataphoresis on a large scale. At a pH of 4.1 - 4.5 the enzyme migrates to the cathode, is collected and is then precipitated with ammonium sulphate, dissolved in acetate buffer, and dialysed overnight in the presence of a carefully adjusted amount of ammonium sulphate against buffer solution, when crystallisation of the enzyme occurs.

The pure enzyme exhibits all the properties of crystalline proteins. Its molecular weight is 73,000. It can exist in two forms, an oxidised form which is yellow and a reduced form which is almost colourless. When the enzyme is dialysed in salt free aqueous solution against N/50 hydrochloric acid, it splits up into two parts, a colourless protein which remains in the dialysing sac and a yellow dye which diffuses through the membrane. This dye has been proved to be one of a group of pigments known as flavins, and is in fact lactoflavin phosphate, or 6:7-dimethyl-9-dl-ribitylisoalloxazine-5'-phosphoric acid. Lactoflavin itself has,

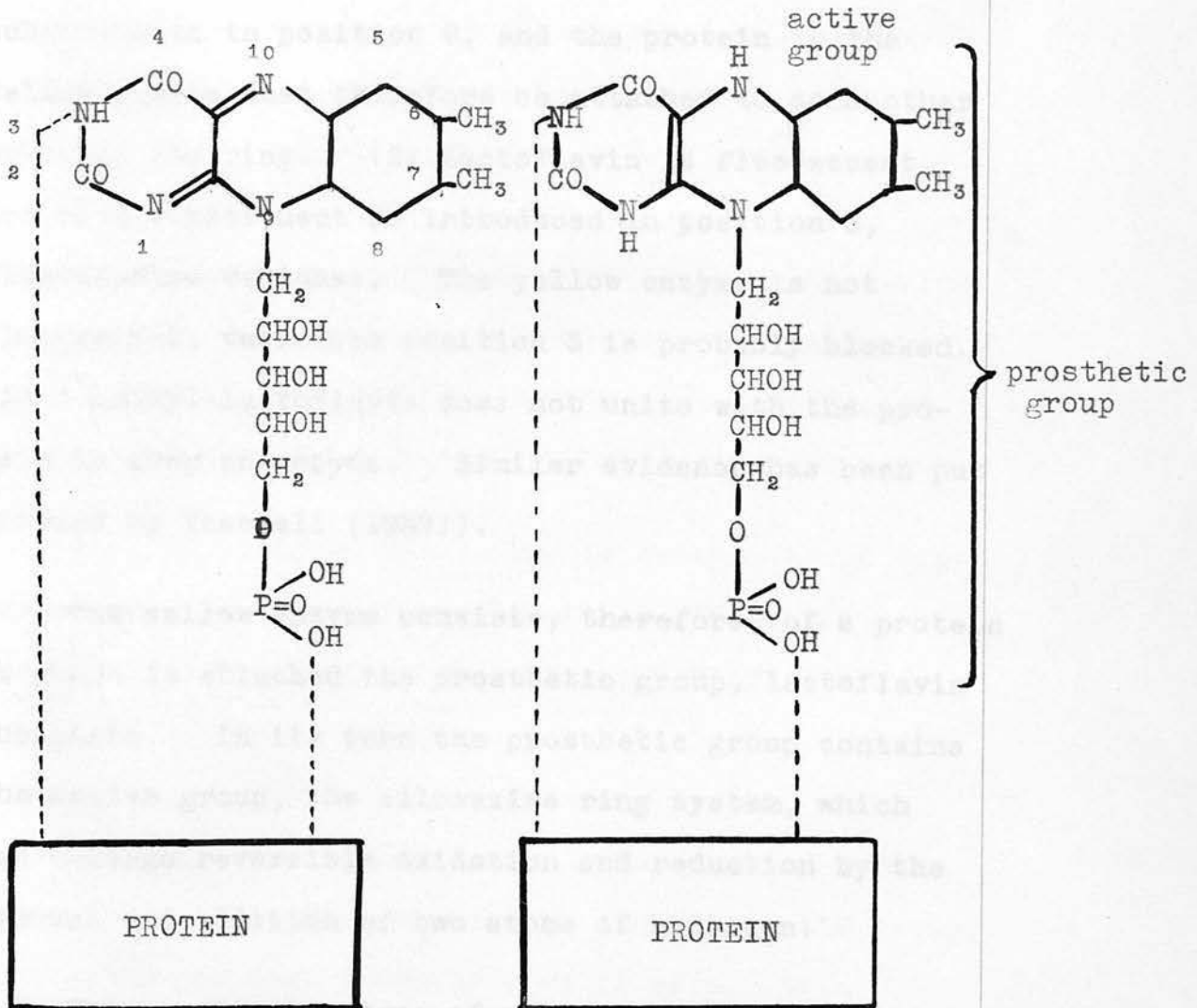
of course, been known for some time as one of the vitamins of the vitamin B<sub>2</sub> group.

Separately neither the protein nor the dyestuff has any activity, but when they are brought together the enzyme is reformed and full activity is restored. Moreover when the protein is brought into contact with synthetic lactoflavin phosphate (synthesised by Kuhn, Rudy, and Weygand (1937)) the enzyme is formed. This is the first instance in which an enzyme has in part been synthesised. The corresponding lactoflavin phosphate containing arabinose instead of ribose forms a yellow enzyme which is much less active than the naturally occurring enzyme.

Kuhn and Boulanger (1935) have shown that lactoflavin phosphate is bound to the protein component at two points. It is bound at the phosphoric acid grouping, as might be expected on general grounds, but it is also bound to the N atom in the 3 position in the alloxazine ring.

The evidence for this is as follows. (1) The redox potential of the yellow enzyme is much higher than that of lactoflavin. The redox potential of

## "The yellow enzyme"

Alloxazine-proteid<sub>0</sub><sub>2</sub> dihydropyridine

Oxidised form.

Reduced form.

lactoflavin is not altered by the presence of different substituents in position 9, and the protein in the yellow enzyme must therefore be attached to some other point in the ring. (2) Lactoflavin is fluorescent but if a substituent is introduced in position 3, fluorescence vanishes. The yellow enzyme is not fluorescent, therefore position 3 is probably blocked. (3) 3-methyl-lactoflavin does not unite with the protein to form an enzyme. Similar evidence has been put forward by Theorell (1937)).

The yellow enzyme consists, therefore, of a protein to which is attached the prosthetic group, lactoflavin phosphate. In its turn the prosthetic group contains the active group, the alloxazine ring system, which can undergo reversible oxidation and reduction by the removal and addition of two atoms of hydrogen.

This particular type of proteid has been termed by Warburg an alloxazine proteid. The complete enzyme or proteid has itself been isolated, it has been split into protein and prosthetic group each of which has been very fully investigated, and the mechanism of the combination of the two components has been studied.

AMINO ACID OXIDASE.

Since the discovery of the original yellow enzyme a number of similar flavoproteins have been investigated. One of these is the amino acid oxidase, discovered by Krebs in 1933, which oxidises the non-naturally occurring optical isomers of the amino acids.

Warburg and Christian (1938a) have isolated this enzyme from sheep kidney and have used a simple and ingenious method to split it into its component parts.

If a solution of the enzyme is brought to pH 2.8 and treated with ammonium sulphate to 20% saturation, the protein component precipitates out leaving the prosthetic group in solution. The isolation of the free protein so produced has been carried out by Negelein and Brömel (1939a), while Warburg and Christian (1938a) have concentrated on the prosthetic group. This group has been shown to consist of a flavin-adenine-dinucleotide, i.e. each molecule can be hydrolysed into one molecule of alloxazine derivative, one of adenine, two of ribose, and two of phosphoric acid.

The amino acid oxidase is, therefore, in Warburg's terminology, an alloxazine adenine proteid, consisting of a protein bound to the prosthetic group, the

alloxazine adenine dinucleotide.

Half the protein is combined with the prosthetic group when the concentration of free prosthetic group is  $2.5 \times 10^{-7}$  M.

#### A SYNTHETIC ENZYME.

By taking the protein component of the original yellow enzyme and combining it with the alloxazine adenine dinucleotide, Warburg and Christian (1938b) have prepared an alloxazine adenine proteid which has not yet been isolated from natural sources. A completely new enzyme has thus been produced in the laboratory. It functions in the same way as the old yellow enzyme, transferring hydrogen from the reduced pyridine nucleotides to molecular oxygen.

Although this enzyme has not been found in nature, it is probable that it does occur, but that in the process of extraction and isolation it loses adenine to give the original yellow enzyme.

HAAS' ENZYME.

A fourth enzyme of this group has been isolated by Haas (1938) in which the prosthetic group is alloxazine adenine dinucleotide, but in which the protein component is different from any yet known. The enzyme is reduced by the pyridine nucleotides and is reoxidised by methylene blue (and also to a certain extent by oxygen but at a much slower rate than is the case with the old yellow enzyme).

XANTHINE OXIDASE.

Possibly the most interesting and at the same time the most complex of the flavoproteins is xanthine oxidase. This enzyme has recently been isolated from cream by Ball (1939) using a most ingenious method of purification. The pure enzyme, which is golden brown in colour, can be partially split by dialysis into protein and prosthetic group. The prosthetic group appears to be an alloxazine adenine dinucleotide of a rather complex nature, since, although it can replace the alloxazine adenine dinucleotides of the amino acid oxidase and of Haas' enzyme, the converse is not the

case and the prosthetic groups of these enzymes do not form xanthine oxidase when combined with Ball's protein.

#### OTHER FLAVOPROTEINS.

Although the yellow enzyme can bring about the oxidation of the reduced pyridine nucleotides in yeast, in animal tissues this oxidation is brought about by an enzyme which has been named "diaphorase" by von Euler et al (1938) and "coenzyme factor" by Dewan and Green (1938). This enzyme has been isolated from heart muscle by Straub (1939) who has shown that it consists of a protein bound to the same flavin adenine dinucleotide which acts as prosthetic group to the amino acid oxidase.

A similar enzyme has been isolated by Corran and Green (1938) from milk. Its prosthetic group is also flavin adenine dinucleotide and it is able to catalase the oxidation of the pyridine nucleotides by methylene blue or cytochrome. It has no xanthine oxidase activity, but Ball (1939) suggests that Corran and Green's flavoprotein is probably xanthine oxidase which has been partially inactivated in the process of extraction.



Table 1a.

Flavoproteins. Yellow enzymes. Alloxazine  
proteids.

Warburg [1938b]

Theorell [1939b]

1. Alloxazine proteid<sub>O<sub>2</sub></sub> dihydropyridine  
The "yellow enzyme" of Warburg and Christian.
2. Alloxazine adenine proteid<sub>O<sub>2</sub></sub> amino acids  
Amino acid oxidase. [Warburg and Christian]
3. Alloxazine adenine proteid<sub>O<sub>2</sub></sub> dihydropyridine  
Synthetic enzyme. [Warburg and Christian]
4. Alloxazine adenine proteid<sub>methylen blue</sub> dihydropyridine  
Haas' enzyme.
5. Alloxazine adenine proteid<sub>O<sub>2</sub></sub> xanthine  
Xanthine oxidase [Ball]
6. Alloxazine adenine proteid<sub>cytochrome c</sub> dihydropyridine  
Corran and Green's enzyme.
7. Alloxazine adenine proteid<sub>cytochrome</sub> dihydropyridine  
Diaphorase. Coenzyme factor. [Straub]

### Pyridine proteid

formed by the combination of protein with  
triphosphopyridinenucleotide.

These flavoproteins have recently been discussed and classified by Warburg and Christian (1938b) who have suggested a system of nomenclature in which the substances oxidised and reduced by the enzyme, are written as indexes after the name of the proteid. For example, the amino acid oxidase is written

alloxazine-adenine-proteid<sub>O<sub>2</sub></sub>, amino acids

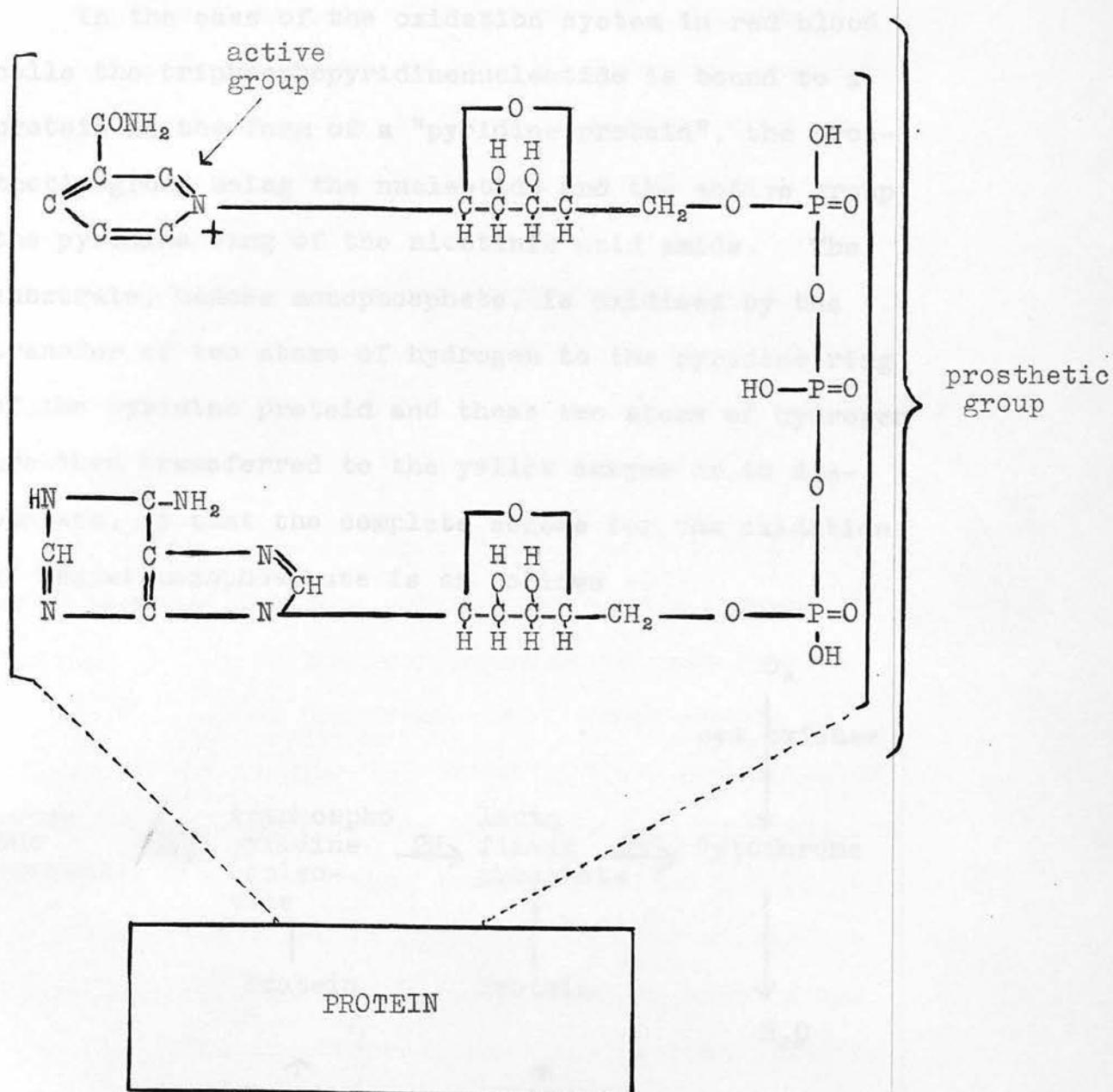
A list of the known flavoproteins is given in Table 1a.

### PYRIDINE PROTEIDS.

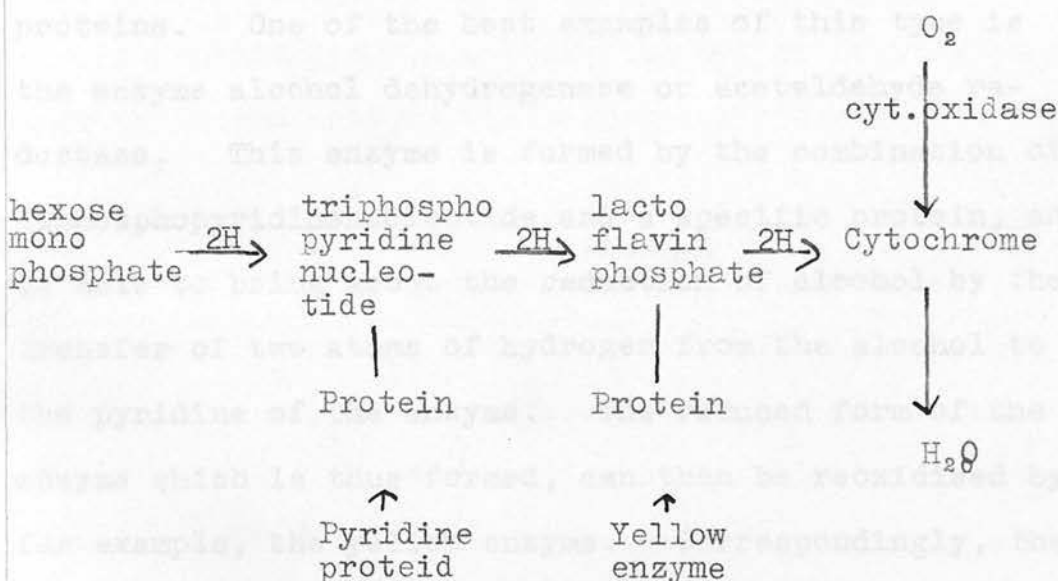
In the course of their investigation of the oxidation systems of red blood cells, Warburg, Christian and Griese (1935) isolated from horse erythrocytes a substance which they called triphosphopyridinenucleotide and which on hydrolysis yields one molecule of nicotinic acid amide, one of adenine, two of ribose and three of phosphoric acid. It is sometimes also referred to as coenzyme II. A similar substance, diphosphopyridinenucleotide (coenzyme I), containing one molecule of phosphoric acid less, is identical with cozymase.

## Pyridine proteid

formed by the combination of protein with  
triphosphopyridinenucleotide.



In the case of the oxidation system in red blood cells the triphosphopyridinenucleotide is bound to a protein in the form of a "pyridine proteid", the prosthetic group being the nucleotide and the active group the pyridine ring of the nicotinic acid amide. The substrate, hexose monophosphate, is oxidised by the transfer of two atoms of hydrogen to the pyridine ring of the pyridine proteid and these two atoms of hydrogen are then transferred to the yellow enzyme or to diaphorase, so that the complete scheme for the oxidation of hexose monophosphate is as follows -



The phosphohexonic acid produced by the oxidation of the hexose monophosphate is then oxidised (by the removal of two H atoms) by a similar system in which the triphosphopyridine nucleotide is attached to a different protein to form a new pyridine proteid. The complete oxidation of hexose monophosphate is thus achieved by a series of enzymes all pyridine proteids, containing the same prosthetic group, triphosphopyridine-nucleotide, attached to different proteins.

Pyridine proteids can therefore be formed by the combination of pyridine nucleotides with a number of proteins. One of the best examples of this type is the enzyme alcohol dehydrogenase or acetaldehyde reductase. This enzyme is formed by the combination of diphosphopyridinenucleotide and a specific protein, and is able to bring about the reduction of alcohol by the transfer of two atoms of hydrogen from the alcohol to the pyridine of the enzyme. The reduced form of the enzyme which is thus formed, can then be reoxidised by, for example, the yellow enzyme. Correspondingly, the reduced form of the enzyme can reduce acetaldehyde to alcohol.

In this case, therefore, we have a pyridine proteid of which the prosthetic group is diphosphopyridine nucleotide and the active group, pyridine. The protein of the enzyme has been isolated and crystallised by Negelein and Wulff (1937) who have also investigated in detail the combination between protein and prosthetic group. The hydrogenated form of the prosthetic group combines about three times more firmly with the protein than the non-hydrogenated form. They have also shown that half of the protein component is combined with the prosthetic group when the concentration of free prosthetic group is about  $1/50,000$  M. Protein and prosthetic group are therefore not so tightly bound here as in the case of the alloxazine proteids.

The most recent case of the isolation and crystallisation of an enzyme comes again from Warburg's laboratory. Warburg and Christian (1939) have isolated and crystallised the particular protein which combines with diphosphopyridine nucleotide to form the pyridine proteid which brings about the oxidation of 1:3-glyceraldehyde phosphoric acid. (See also Negelein and Brömel (1939b).)

Table 1b.

Metal proteids.

1. Copper proteids.

METAL PROTEIDS.

(Table 1b)

A number of enzymes is now known in which the active group is a metal. The best known are copper proteids, iron proteids, and a zinc proteid. Included in this group also is a number of metal protein complexes which are not strictly speaking enzymes.

1. COPPER PROTEIDS.

Kubowitz (1937, 1938) has recently isolated from potatoes an enzyme variously known as potato oxidase, phenoloxidase, polyphenoloxidase, and catecholoxidase, which is able to bring about the oxidation of a number of phenols such as pyrocatechol.

This enzyme is inhibited by cyanide, by hydrogen sulphide, and by carbon monoxide and appears therefore to be a heavy metal complex. Kubowitz found that his pure preparations contained copper and suggests that the enzyme is a copper proteid. The copper is tightly bound to the protein and cannot be removed by prolonged dialysis although acid will remove it. Added copper does not increase the activity of the enzyme nor is

## Table 1b.

## Metal proteids.

## 1. Copper proteids.

Polyphenol oxidase.

Laccase

Tyrosinase

Haemocyanin

Haemocuprein

Hepatocuprein

Ascorbic acid oxidase [?]

## 2. Iron proteids

Catalase

Peroxidase

Warburg's "sauerstoffübertragendes  
Ferment"

Cytochrome

## 3. Zinc proteid

Carbonic anhydrase

## 4. Manganese proteids

Arginase

Dihydroxymaleic acid oxidase

## 5. Magnesium proteid

Enzyme of photosynthesis.



copper alone active. The importance of the copper is shown by the fact that the copper content bears a linear relationship to the activity of the preparation but an even more convincing proof of its importance can be given. If polyphenol oxidase is treated with potassium cyanide and dialysed for several days, the copper is removed as a diffusible copper cyanide complex, leaving a completely inactive copper free protein. On the addition of copper to this protein, full activity is restored.

The method of isolation of the enzyme from potatoes is an extremely long and elaborate process and the final product contains 0.2% of copper.

In this case the copper may be regarded as the prosthetic group, or at least the active group of the enzyme and Kubowitz considers that the copper oscillates between the cuprous and the cupric forms, being reduced to the cuprous form by the substrate and being reoxidised to the cupric form by oxygen. The occurrence of this change of valency may be demonstrated by exposing a large amount of enzyme in a manometer to carbon monoxide. No gas is taken up. If now

substrate is added, the copper is reduced from the cupric to the cuprous form, which takes up one molecule of carbon monoxide for each two atoms of copper. On the addition of cyanide the copper carbonyl complex is decomposed with the formation of a more stable copper cyanide complex and a volume of carbon monoxide equal to that originally taken up is evolved.

Whether the copper is directly bound to the protein or whether it forms part of a complex as iron does in haematin is unknown.

The substrate usually employed for this enzyme is pyrocatechol, which is oxidised to orthoquinone. As is the case with many of these enzymes, the product of the reaction acts as a poison to the enzyme and steps must therefore be taken to remove it as rapidly as it is formed. This can be done by the addition of hexose-monophosphate and a pyridine proteid, by the action of which the orthoquinone is reduced to pyrocatechol as soon as it is formed. The coupling of two enzyme systems in this way is not uncommon in nature.

Polyphenol oxidase has also been isolated from a different source, mushrooms, by Keilin and Mann (1938)

who obtained an enzyme similar to that of Kubowitz but with a slightly greater copper content.

LACCASE. An enzyme belonging to the same group as polyphenol oxidase is laccase, which has been isolated from the latex of *Rhus succedanea* by Keilin and Mann (1939a). It catalyses the oxidation of polyphenols, e.g. pyrocatechol, and of diamines such as p-phenylenediamine, but not p-cresol or tyrosine. The crude enzyme also catalyses the oxidation of ascorbic acid directly. The purest preparation contains no iron or manganese but it contains 0.154% copper and the copper content of different preparations is proportional to the activity. The pure enzyme is blue but the colour is reversibly discharged on the addition of ascorbic acid or quinol. Like other <sup>copper</sup>proteids, it is inhibited by cyanides, hydrogen sulphide and sodium azide, although not by carbon monoxide.

TYROSINASE. Another related enzyme is tyrosinase, which has been isolated from an aqueous extract of *Lactarius piperatus* in the form of crystals containing 0.25% of copper, by Dalton and Nelson (1938).

HAEMOCYANIN. A number of copper proteids are known which are not strictly speaking enzymes but which play a part in biological oxidations. One of these, for example, is haemocyanin, which contains 0.17 - 0.26% copper. Kubowitz (1938) has shown that the copper may be removed by dialysis with cyanide, as in the case of polyphenoloxidase.

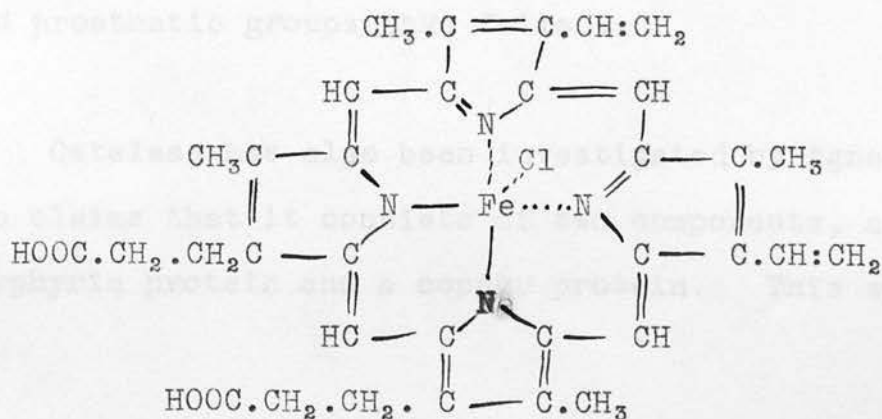
HAEMOCUPREIN and HEPATOCUPREIN. Keilin and Mann (1938) have recently isolated from the red blood cells of the ox, crystals of a copper proteid containing 0.34% Cu which they have termed haemocuprein. It is blue in colour but the colour is discharged irreversibly on treatment with sodium hydrosulphite. A similar, but almost colourless compound, named hepatocuprein, has been isolated from liver tissue. The precise significance of these compounds is not known.

ASCORBIC ACID OXIDASE. Stotz, Harrer and King (1937) have put forward evidence that ascorbic acid oxidase is a copper proteid but this has not yet been conclusively proved.

## 2. IRON PROTEIDS.

Although haemoglobin and its derivatives might strictly speaking be classified among the iron proteids, they will not be discussed here.

CATALASE. The enzyme catalase, which brings about the decomposition of hydrogen peroxide to water and oxygen, has been the subject of extensive investigations. Stern (1935) has shown that the addition of acetone containing hydrochloric acid to concentrated preparations of catalase results in the precipitation of a colourless protein and the formation of a bluish supernatant fluid which, on removal of acetone, yields haemin identical with that obtained from blood. Catalase therefore appears to be an iron proteid, in which the iron is present in the form of haematin. In a case of this sort the prosthetic group of the enzyme would be haemin and the active group iron.



More recently, however, the purification of catalase has been carried further and crystals of the enzyme have been obtained from beef liver by Sumner and Dounce (1937) and from horse liver by Dounce and Frampton (1939). The enzyme is extracted from the liver tissue by dilute aqueous dioxane, precipitated by the addition of more dioxane, dissolved in water and precipitated in crystalline form by the careful addition of ammonium sulphate.

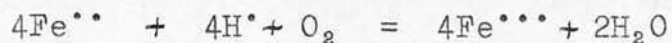
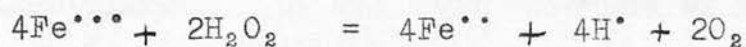
Sumner and Dounce (1939) have recently carried out an extensive investigation of crystalline catalase with a view to elucidating its structure. Catalase contains about 0.1% of iron which is present in two side chains. The one side chain is haemin, while the second, which is responsible for half the iron content of catalase, is split off as a blue substance of unknown composition on the addition of acetone and acid. All attempts to split catalase reversibly into protein and prosthetic groups have failed.

Catalase has also been investigated by Agner (1938), who claims that it consists of two components, an iron porphyrin protein and a copper protein. This was

denied by Sumner and Dounce (1939) who found only a trace of copper in their purest preparations, and Agner (1939) has recently stated that he has been able to remove copper from his preparations without altering the activity.

The molecular weight of catalase is about 248,000 (Sumner and Gralen (1938)).

Keilin and Hartree (1938) have investigated the mode of action of catalase and have found that, as is the case with most metal proteids, the iron undergoes a valency change in the course of the reaction with substrate, being reduced from the ferric to the ferrous state by hydrogen peroxide, and being subsequently reoxidised to the ferric state by oxygen thus:-



PEROXIDASE. Although peroxidase has not yet been isolated, there is nevertheless some evidence that

it is an iron proteid containing haemin. A suggestion to this effect was made by Kuhn, Hand, and Florkin in 1931, but doubt was later cast by Elliot and Keilin (1934). Later, however, Keilin and Mann (1937) found that part of the haemin in horse radish extracts could combine with hydrogen peroxide, and this haemin is proportional to the peroxidase activity. These workers therefore consider that peroxidase is a haemin derivative.

WARBURG'S RESPIRATION CATALYST. One of the most important enzymes in cellular respiration is the enzyme chiefly associated with the name of Warburg and termed by him the "sauerstoffübertragendes Ferment" although it is sometimes also referred to as the "Atmungsferment". It has been reviewed by Warburg in his Nobel Prize Dissertation (1925) and by Reid (1932).

This enzyme has never been isolated, but it has been very fully investigated and there is little doubt that it is an iron proteid. Warburg (1938) now refers to it as the "autoxidisable iron proteid".

Its function in nature is to act as a link between



Cytochrome c.

Theorell (1936)

cytochrome and molecular oxygen and it is now considered by many workers (e.g. Keilin and Hartree (1939)) to be identical with the enzyme cytochrome oxidase (formerly known as indophenol oxidase).

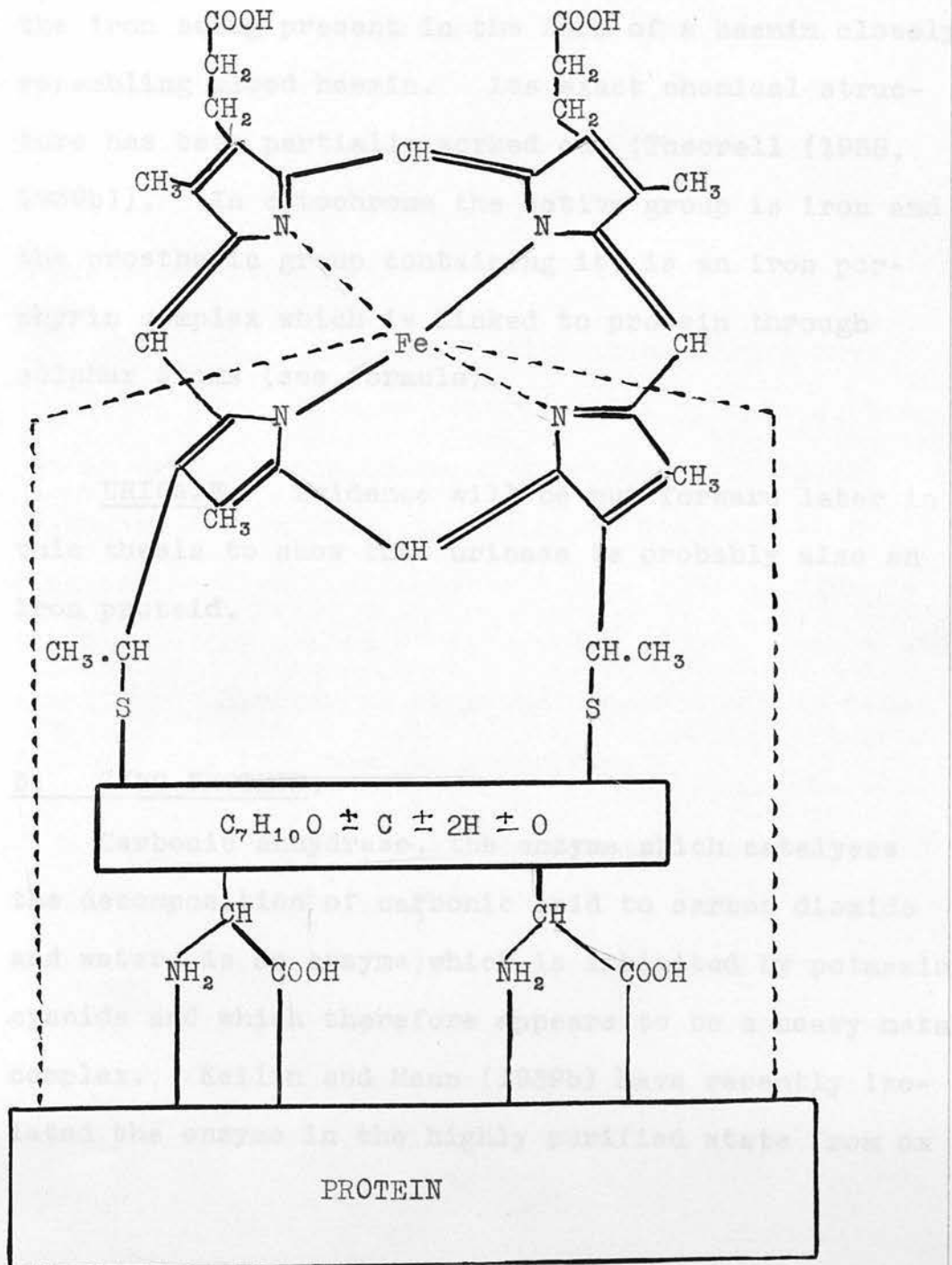
CYTOCHROME. Although cytochrome is not strictly speaking an enzyme, the important part which it plays in biological oxidations justifies its inclusion in the category of iron proteids.

Cytochrome is a mixture of three closely related haemochromogen-like substances which are capable of existing in oxidised and reduced forms. It acts as a respiratory pigment, linking the metabolites in the cell with molecular oxygen, being oxidised from ferro-cytochrome to ferri-cytochrome by molecular oxygen under the influence of the enzyme cytochrome oxidase, and being reduced again by metabolites in the cell in conjunction with their specific dehydrogenases. For example it links up the succinic acid cycle of Szent-Györgyi (1937) with oxygen, and it is responsible for the oxidation in the cell of the reduced form of the yellow enzyme (Theorell (1936)).

One of the three components, Cytochrome c, has

## Cytochrome c.

Theorell [1938]



been isolated in a very highly purified form from heart muscle by Theorell (1935, 1936). It has a molecular weight of 16,500 and contains 0.34% of iron, the iron being present in the form of a haemin closely resembling blood haemin. Its exact chemical structure has been partially worked out (Theorell (1938, 1939b)). In cytochrome the active group is iron and the prosthetic group containing it, is an iron porphyrin complex which is linked to protein through sulphur atoms (see formula).

URICASE. Evidence will be put forward later in this thesis to show that uricase is probably also an iron proteid.

### 3. ZINC PROTEID.

Carbonic anhydrase, the enzyme which catalyses the decomposition of carbonic acid to carbon dioxide and water, is an enzyme which is inhibited by potassium cyanide and which therefore appears to be a heavy metal complex. Keilin and Mann (1939b) have recently isolated the enzyme in the highly purified state from ox

blood and have found that it contains 0.31 - 0.34% of zinc, but that it is free from other metals. There is a close parallelism between the enzyme activity and the zinc content in different fractions. This is the only known case of a zinc proteid.

#### 4. MANGANESE PROTEIDS.

ARGINASE. Edlbacher and Pinösch (1937) have suggested that arginase consists of a protein carrier and an active group containing manganese. Liver arginase, for example, on prolonged dialysis at 0° becomes inactive (Edlbacher and Baur (1938)), but is reactivated by traces of manganese, nickel, cobalt and vanadium. Similarly, dialysed preparations of yeast arginase are activated by manganese.

Somewhat similar results have also been obtained by Hellerman and Perkins (1935-36) who found that arginase was activated by a number of metal ions including cobalt, nickel, and manganese. These workers have also found that urease, which does not of course hydrolyse arginine, will do so after the addition of



cobalt, nickel or manganese.

DIHYDROXYMALEIC ACID OXIDASE. Recently Theorell and Swedin (1939) have prepared from the juice of leaves of *Rumex acetosa* the enzyme, dihydroxymaleic acid oxidase, in the pure state. When this enzyme is dialysed it loses much of its activity, but is reactivated by small amounts of manganese. The activity of the undialysed enzyme is not increased by manganese. The purified enzyme, also, on the addition of pyridine and sodium hydrosulphite showed a protohaemochromogen spectrum with a strong band at 557  $m\mu$  and a weak one at 525  $m\mu$ . It is therefore probable that this enzyme, in addition to containing manganese, is a haemin derivative. This possibility is strengthened by the fact that a model enzyme which will oxidise dihydroxymaleic acid can be prepared by treating cytochrome c with manganese but not with iron, chromium, nickel, cobalt or copper.

## 5. MAGNESIUM PROTEIDS.

It has been known for a long time that chlorophyll contains magnesium, but C. S. French (1938) has now brought forward evidence that, in the cell, chlorophyll is bound to protein, and that the complex so formed is necessary for photosynthesis. It is probable, therefore, that photosynthesis is brought about by a magnesium proteid.

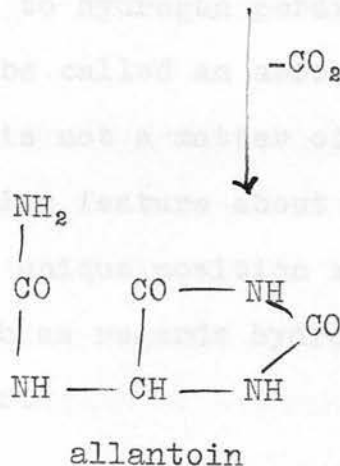
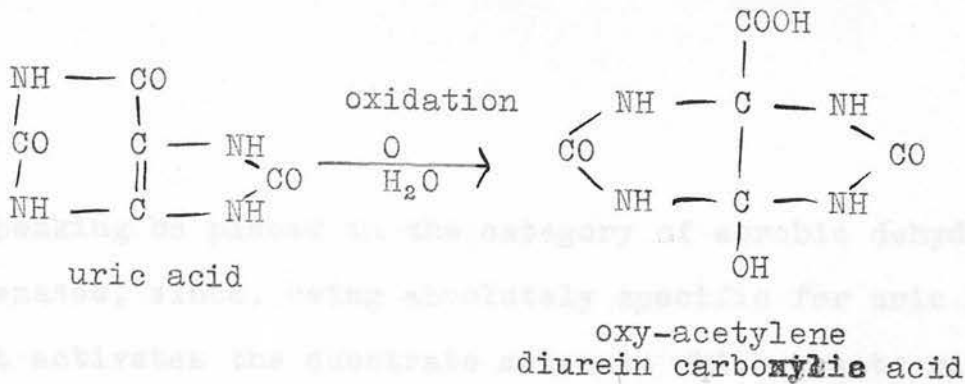
Uricase is the enzyme which brings about the oxidation of uric acid to allantoin, and intermediate product being oxy-acetylene-diuricic carbonylic acid which later undergoes decarboxylation [Saxler and Feinzel (1932)].

PART II.

THE HISTORY AND LITERATURE OF URICASE.

Of the oxidising enzymes, one of the most interesting and at the same time one of the most difficult to extract is the enzyme uricase. Mainly on account of its insolubility, comparatively few attempts have been made to purify and isolate it, and most of these attempts have resulted in only a slight purification of the substance.

Uricase is the enzyme which brings about the oxidation of uric acid to allantoin, and intermediate product being oxy-acetylene-diurein carboxylic acid which later undergoes decarboxylation [Schuler and Reindel [1932] ].



Uricase is usually classified in the group of enzymes known as aerobic oxidases. These are enzymes which catalase the oxidation of the substrate in the presence of molecular oxygen only. Methylene blue or other dyes cannot replace oxygen as hydrogen acceptor. On the other hand Keilin and Hartree (1936) hold that uricase should strictly



speaking be placed in the category of aerobic dehydrogenases, since, being absolutely specific for uric acid, it activates the substrate molecule which reacts with molecular oxygen reducing it to hydrogen peroxide.

Whether uricase should be called an aerobic oxidase or an aerobic dehydrogenase is not a matter of great importance, but the interesting feature about uricase is that it occupies a rather unique position as an enzyme which is specific both as regards hydrogen donator and hydrogen acceptor.

The history of uricase dates from the year 1860 when Stockvis (1860), working in Amsterdam, noticed that uric acid was decomposed by finely ground animal tissue. This discovery seems to have aroused but little interest at the time and it was not until forty years later that the subject was reopened when Lauder Brunton (1905) confirmed Stockvis' results and showed that liver tissue was able to decompose uric acid. Liver tissue from fasting animals, on the other hand, proved to be inactive.

About the same time the subject was taken up by Schittenhelm (1905) who recognised that a ferment which

decomposed uric acid was present in certain tissues, particularly the kidneys, liver and muscle of the ox but not in the spleen, lungs and intestine. He attempted to extract and purify the ferment by a process which was surprisingly advanced for the period. Finely minced kidney was ground with water and sand and the extract treated with an alkaline solution of uranyl acetate which precipitated the enzyme. Subsequent extraction of the precipitate with dilute sodium hydroxide resulted in the formation of a solution of the enzyme.

Twenty years later, Schittenhelm again took up the problem (Schittenhelm and Crometska (1927)) without however making much progress.

In an attempt to extract the uricolytic enzyme from the dry powder obtained by treating minced tissue with alcohol, Croftan (1908) obtained three fractions, all of which he claimed were necessary for activity. One was a nucleoprotein, the second was an albumose, and the third was a solution of salts necessary to keep the first in solution. He also compared the uricolytic action of various tissues and found human liver and muscle to possess high uricolytic action.

On the other hand, Battelli and Stern (1909) who examined uricase in the course of a long series of investigations on enzymes in general, found that although uricase was present in most mammalian tissues, it was not present in those of man. Their attempts at extraction were much more thorough and more successful than those of previous investigators, and they were the first authors to emphasise the fact that uricase can only be extracted from tissues by the use of alkaline solutions. As source of enzyme they used ox kidney and horse liver either in the form of the fresh tissue or as a dry powder prepared by mincing the tissue in alcohol. After extraction with dilute ammonia, the enzyme was precipitated by the addition of alcohol and the precipitate centrifuged off and dried. The preparation so obtained was a powerful source of the enzyme. Similar experiments were also carried out by Wiechowski and Wiener (1909).

A modification of this procedure was adopted by Galeotti (1911) who extracted the minced liver of the dog ~~scyllium~~ with water and then precipitated the extract with acetone. The precipitate was extracted with dilute sodium chloride solution and a highly

active extract obtained. Battelli and Stern (1909), in addition, carried out extensive investigations on the distribution of uricase in various tissues and in various species. Similar experiments on a much more limited scale were also carried out by a large number of other workers, e.g. Almazia (1905), Brugsh and Schittenhelm (1907, 1908), Friedmann and Mandel (1908), Jones and Austrian (1906), Künzel and Schittenhelm (1908), Mendel and Mitchell (1907), Mendel and Wells (1909), Miller and Jones (1909), Preti (1909), Scaffdi (1909), Schittenhelm and Schmidt (1907), Schittenhelm (1905), Wells and Corper (1909), Wells (1909-10), Wiechowski and Wiener (1907), Wiener (1899), and more recently by Truszkowski and Gubermanowna (1933).

As the result of these experiments it was found that uricase was present in the liver and kidney of most mammals with the exception of man, e.g. in the ox, dog, pig, rabbit, guinea pig, sheep, cat and horse, but that it was absent in the case of one important mammal, man, and in the turtle, goose and mollusc. Other tissues contained little or no uricase. The muscles of the horse and ox contained small amounts, but not those of the sheep and dog. No uricase was

Uricolytic indices [Hunter and Ward, 1920]

Order and Species.

Index.

Mammalia.

found in the spleen (ox, dog, pig, and sheep), in the lung (ox, horse, dog and sheep), in the intestine (ox), in the blood (man, ox, dog, sheep and horse), in the pancreas (horse), in the brain (horse and dog), and in the leucocytes and bone marrow (dog).

From these results it will be evident, as might be expected, that there is a connection between the species distribution of uricase and the purine metabolism of the species.

In the course of purine metabolism, the purine bases are eventually oxidised to uric acid which, in the majority of mammals, is converted into allantoin prior to excretion, but in one or two cases, notably in man, uric acid cannot be further oxidised and is excreted as such. The extent of uric acid oxidation has been expressed by Hunter and Ward (1920) in the form of the so called "uricolytic index", which represents the ratio of allantoin nitrogen to the sum of uric acid nitrogen and allantoin nitrogen in the urine. The uricolytic indices of various mammals are given in Table 2.

A peculiar anomaly in purine metabolism was

TABLE 2.

## Uricolytic indices [Hunter and Ward, 1920]

Order and Species.	Index.
<b>Marsupialia</b>	
Opossum	79
<b>Rodentia</b>	
Rat	96
Mouse	98
Guinea pig	94
Rabbit	95
<b>Carnivora</b>	
Black bear	94
Badger	98
Cat	97
Dog	98
Dalmatian coach dog	32
<b>Ungulata</b>	
Cow	93
Horse	88
Sheep	80
Goat	92
Pig	98
<b>Proboscidea</b>	
Elephant	72
<b>Primates</b>	
Monkey	89
Chimpanzee	0
Man	2

discovered by Benedict (1916) and confirmed by Wells (1918) in the case of the Dalmatian dog, which excretes uric acid rather than allantoin when placed on a purine free diet, and which excretes subcutaneously injected uric acid quantitatively as such. When such dogs are crossed with other dogs (Onslow (1923)) this peculiarity disappears.

From an examination of the table of uricolytic indices it might be expected that those animals which have a low uricolytic index and which therefore excrete uric acid in preference to allantoin, e.g. man and the chimpanzee, would have little or no uricase in their tissues, and this has been found to be indeed the case. The absence of uricase from human liver and kidney has been demonstrated by a number of workers, including Battelli and Stern (1909), Miller and Jones (1909), Kleinmann and Bork (1933), and Ro (1931), and similar results have been obtained in the case of anthropoid apes by Wiechowski (1912) and by Wells and Caldwell (1914).

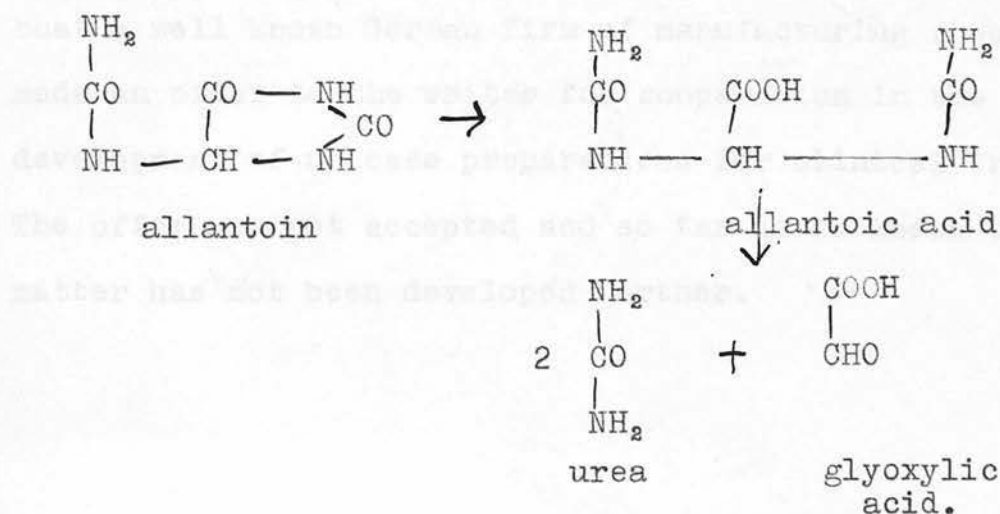
On the other hand the monkey, which excretes allantoin, possesses uricase in its tissues (Wells (1909-10)). The position of the Dalmatian dog is

again anomalous. Although it excretes uric acid its tissues contain uricase (Wells (1918), Klemperer et al. (1938)).

Birds of course excrete uric acid as such in large quantities and possess no uricase.

Of the reptilia, snakes and lizards excrete uric acid and possess no uricase, but the turtle may convert uric acid to allantoin.

In fishes and in many invertebrates, uricase is present, and uric acid is converted into allantoin. These creatures, however, possess other two enzymes which are able to break down allantoin - allantoinase, which converts allantoin to allantoic acid, and allantoicase, which converts allantoic acid to urea and glyoxylic acid (Fosse (1929, 1933), Fosse et al (1929, 1930)).





URICASE and GOUT.

Whether or not the absence of uricase from human tissues is responsible for man's liability to gout is a matter of great doubt. Although gout may be associated with a high uric acid content in the blood, there are other conditions, such as leukaemia, in which the blood uric acid may be markedly raised without symptoms of gout supervening.

At the same time it has more than once been suggested that the administration of uricase might be effective in the treatment of gout. The use of an enzyme as a therapeutic agent, however, especially an enzyme which would of necessity be given parenterally, is fraught with difficulties.

That the use of uricase as a therapeutic agent cannot be completely ridiculed is shown by the fact that a well known German firm of manufacturing chemists made an offer to the writer for cooperation in the development of uricase preparations for clinical trial. The offer was not accepted and so far as is known the matter has not been developed further.

Serious attempts to purify uricase, using modern technique, began to be made about ten years ago, but have all been hampered by the difficulty with which the enzyme can be made to go into solution. Grynberg (1931), for example, states that the enzyme is insoluble, but most workers have found it possible to prepare solutions by extracting the starting material with weak alkaline solutions. Felix, Scheel, and Schuler (1929), for example, extracted pig liver powder with 0.001 N sodium hydroxide solution, or preferably with 0.005 N secondary sodium phosphate and glycerol, and obtained an extract with powerful enzyme activity. Their claim that uricase consists of two enzymes, an oxidising and a decarboxylating enzyme, has not been confirmed by other workers. Schuler later (1932) used borate buffer at pH 9 as extracting agent at 37° and attempted to purify his extract by a variety of methods,

including precipitation with various reagents, salting out, digestion of accompanying proteins, dialysis, and adsorption methods, without however obtaining any marked degree of purification.

A more successful attempt was made by some Polish investigators - by Przylecki (1928) but chiefly by Truszkowski (1930, 1932, 1934), who has published a long series of papers on the subject. (See also Truszkowski and Gubermanowna (1935, 1936).)

The earliest experiments of the Polish investigators were made with minced frog tissue, but later ox kidney was found to be a much more convenient starting material. Minced ox kidney was thoroughly washed in a stream of cold water, and the insoluble material dried in an oven at  $37^{\circ}$  and ground to a powder in a mill. The powder was washed with alcohol and ether and was then extracted for 48-72 hours at room temperature with dilute sodium carbonate solution. The extract was then treated with 0.6% acetic acid till maximum turbidity was obtained, and the precipitate so formed was filtered off, washed with alcohol and ether, and dried at  $30^{\circ}$  in vacuo. The resulting powder was a highly active preparation of the enzyme.

A sample of his purest preparation was kindly sent by Professor Truszkowski to the present writer but its activity was found to be much less than that of preparations prepared as described later in this thesis.

Truszkowski states that uricase solutions are clear or slightly opalescent, straw-coloured liquids, with an amine-like odour, foaming on shaking. They give positive protein reactions. The enzyme is able to pass freely through Berkfeld filters and partly through Bechold filters. It is inactivated by digestion with proteolytic enzymes and by heating for five minutes at  $70^{\circ}$ . At  $60^{\circ}$  partial inactivation occurs but none at  $50^{\circ}$ . On standing, solutions retain their activity for about four days; after that time activity diminishes and is completely gone in thirty-six days.

A somewhat similar method of preparation was employed by Ro (1931) in an attempt to isolate uricase. He also used ox kidney which he extracted with water, a very inefficient method of extracting the enzyme. To the extract was added an equal volume of acetone and the precipitate so formed was dissolved in water and again precipitated with acetone. When this procedure had been repeated four times, a white, haemo-

extracted with normal saline solution and the extract globin free, precipitate resulted which was washed with alcohol and ether and dried at 37°. This powder was extracted with dilute sodium carbonate solution for thirty minutes at 30° and a highly active solution obtained.

Attempts were made to continue the purification, using various adsorbent materials such as animal charcoal, aluminium hydroxide, and kaolin but without success. Fractional precipitation with ammonium hydroxide was unsatisfactory.

Ro's uricase solution was pale yellow in colour, with a peculiar smell. It gave positive protein tests and negative sugar reactions. It had no optical activity and no absorption bands. It contained both copper and iron in small amounts (0.033 mg. of each per 100 ml.), but there was no relationship between copper or iron content and uricase activity. Activity was destroyed by heating to 70° for fifteen minutes and at a pH more alkaline than 11.3 and more acid than 2.3.

Instead of ox kidney, Kleinmann and Bork (1933) used pig liver which they converted into a dry powder with the aid of alcohol. The powder was then

extracted with normal saline solution and the extract was then treated with kaolin or kieselguhr, which adsorbed the enzyme. Elution was effected by the use of dilute sodium or ammonium hydroxide. Adsorption and elution were then repeated. The resulting enzyme solution was water clear and gave positive protein but negative carbohydrate tests. The enzyme could be precipitated from solution by the addition of nine volumes of alcohol.

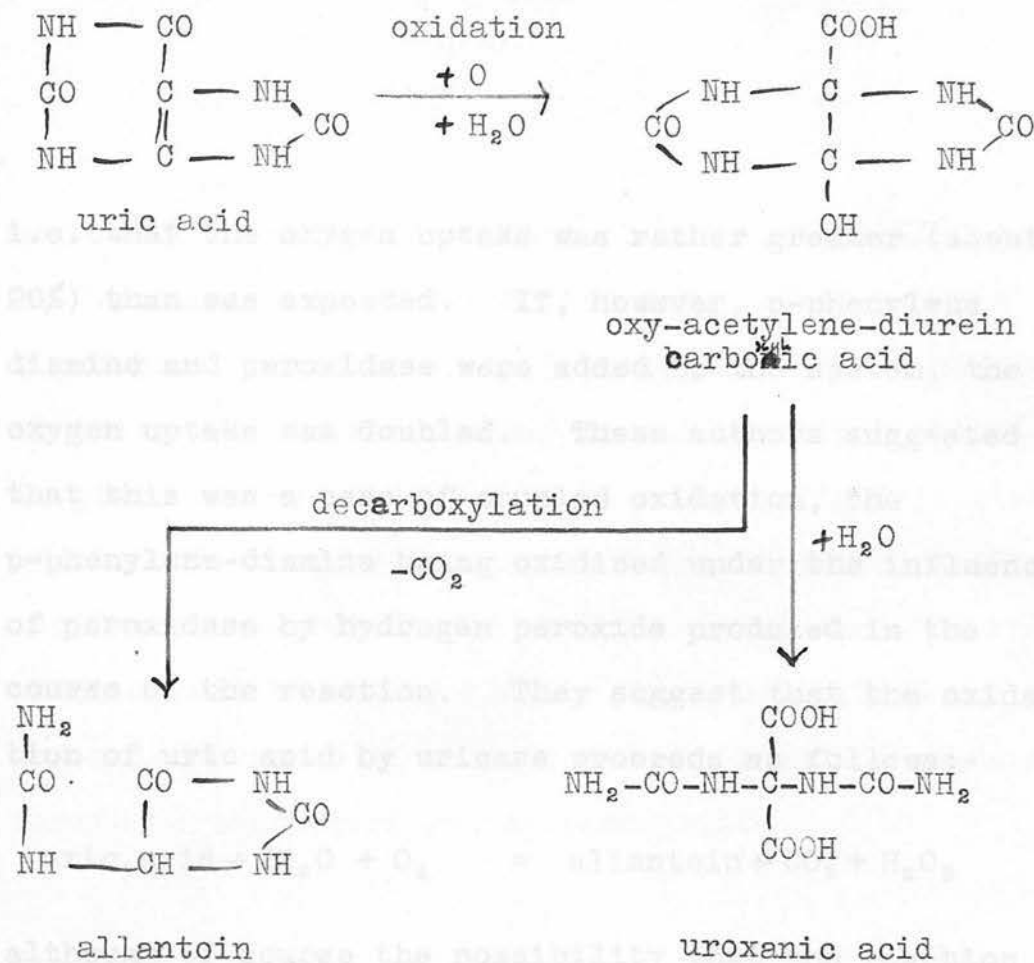
The degree of purification obtained by these means was 117 times.

That other factors besides uricase can bring about the oxidation of uric acid was shown by Gomolinska (1928), who showed that blood contains a uricolytic agent which is unaffected by heating and which appears to be identical with haemoglobin. Iron and copper were also shown by Kleinmann and Bork (1933) to have some uricolytic action.

#### THE COURSE OF THE REACTION.

In the course of their investigations on uricase, Battelli and Stern came to the conclusion that the

respiratory quotient for the oxidation of uric<sup>acid</sup>~~acid~~ was 2, i.e. that one atom of oxygen is taken up for each molecule of uric acid oxidised and that one molecule of carbon dioxide is thereby produced. A similar conclusion was reached by Grynberg (1931) in a study of the kinetics of the reaction. On the other hand Felix, Scheel and Schuler (1929) concluded that the reaction took place in two stages, the first stage being an oxidation and the second a decarboxylation, and Schuler (1932) postulated the existence of an intermediate product formed by oxidation without decarboxylation, which Schuler and Reindel (1932) later isolated as oxy-acetylene-diurein carboxylic acid (also described by Bilz and Schauder (1923)) in the form of its silver salt. The statement by Felix et al. (1929) that two enzymes are necessary for the oxidation of uric acid, an oxidising and a decarboxylating enzyme, is not now regarded as valid and it is considered that the first step in the break down of uric acid is its oxidation to oxy-acetylene-diurein carboxylic acid under the influence of uricase, while the second step is the breakdown of this unstable compound independently of enzyme action into allantoin and carbon dioxide.



An alternative process by which uric acid is broken down into one molecule of oxalic acid, one molecule of carbon dioxide and two molecules of urea is discussed by Kleinmann and Bork (1933).

Although earlier investigators had found that the R.Q. for the reaction was 2, Keilin and Hartree (1936) in a more recent investigation of the problem, found in many cases that a figure less than 2 was obtained,



i.e. that the oxygen uptake was rather greater (about 20%) than was expected. If, however, p-phenylene diamine and peroxidase were added to the system, the oxygen uptake was doubled. These authors suggested that this was a case of coupled oxidation, the p-phenylene-diamine being oxidised under the influence of peroxidase by hydrogen peroxide produced in the course of the reaction. They suggest that the oxidation of uric acid by uricase proceeds as follows:-



although of course the possibility that the reaction proceeds in stages through 4:5 glycol uric acid and oxy-acetylene-diurein carboxylic acid is not excluded and is even regarded as established.

#### THE EFFECT OF pH ON THE ACTION OF URICASE.

The influence of change of pH on the action of uricase has been studied by many workers, with very variable results, but all are agreed that the optimum pH is well to the alkaline side of neutrality. Some workers claim that two pH optima exist, at pH 8.9 and 9.9

according to Felix et al (1929), and at pH 8.85 and 10.0 according to Kleinmann and Bork (1933). This suggestion is supported by Truszkowski (1930) who finds optima at pH 7.5 and 9.4 but in a later paper (1936) he gives one pH optimum at 8.8. One optimum is also given by Ro at pH 9.3 and this is confirmed by Keilin and Hartree (1936) who, in a very careful study of the problem, came to the conclusion that the optimum was in the neighbourhood of pH 9.25 and who found no evidence for the existence of two optimum points.

#### THE SPECIFICITY OF THE ENZYME.

Uricase is an enzyme of very marked specificity. It oxidises uric acid only. Not only does it oxidise no other purine but it fails to oxidise methyl or ethyl derivatives of uric acid (Keilin and Hartree (1936)). In an investigation of 16 such derivatives, however, several were found to act as inhibitors to the enzyme, a fact which indicates that they react with the same active groupings as uric acid on the surface of the enzyme molecule and obstruct access of uric acid to these groupings. These compounds therefore behave as

competitive inhibitors. Inhibited by cyanide, the inhibi-

tion. Such derivatives of uric acid as uric acid 4:5 glycol, uric acid 4:5 dimethyl ether, and spiro-5:5 dihydantoin are not attacked by uricase although the 4-monomethyl derivative of uric acid may be converted to the corresponding methylated allantoin (Brüning, Einecke, Peters, Rabl and Viehl (1928)).

heavy metals. The effect of carbon monoxide, for example, was tried but even with a ratio of CO/O<sub>2</sub> of 8

#### EFFECT OF INHIBITORS.

with One of the most characteristic features of uricase is its inhibition by cyanides in small concentration, a phenomenon which was demonstrated by Foss et al. (1929, 1930) and also by Truszkowski (1930). The action of cyanides was investigated more fully by Keilin and Hartree (1936), who found that  $10^{-3}$  M KCN inhibits uricase by 94% and that as low a concentration as  $3 \times 10^{-5}$  inhibits it by 83%. That the inhibition was reversible was easily shown by placing paper soaked in concentrated KOH in the manometer vessels so as to absorb the HCN gradually. Another method depended on the fact that cyanide combines with methaemoglobin. When methaemoglobin solution was added from a side bulb

to a uricase solution inhibited by cyanide, the inhibition was abolished by the withdrawal of cyanide in combination with the methaemoglobin.

The fact that uricase is inhibited by cyanide suggests that the enzyme is a heavy metal complex, and Keilin and Hartree therefore investigated the action of other substances which are known to react with heavy metals. The effect of carbon monoxide, for example, was tried but even with a ratio of  $\text{CO}/\text{O}_2$  of 8, no inhibition was found nor was there any inhibition with M/250 sodium sulphide, M/10 sodium fluoride or 5% urethane. On the other hand about 40% inhibition was produced by M/500 sodium azide, M/50 hydroxylamine, M/250 5-nitro-4-chlor-2-aminophenol, and M/250 1-amino-2-naphthol-6-sulphonic acid. A lesser degree of inhibition was produced by 2-aminophenol-4-sulphonic acid and by 8-hydroxy quinoline. Pyrophosphate on the other hand slightly increased the activity of the enzyme in concentrations of between M/50 and M/25. An increase in activity of about 50% was also produced, as had been shown by Battelli and Stern (1909), by allowing the reaction to take place in pure oxygen instead of air.

PART III.EXPERIMENTAL.

In view of the fact that early attempts to purify uricase had met with but little success, it was considered that a fresh attempt at isolation in the light of the methods recently adopted in the case of other enzymes would be justifiable and it was therefore carried out. A trial of Ro's method proved it to be very unsatisfactory and quite unreliable. Truszkowski's method was more reliable but it did not carry the purification very far. A sample of his best preparation, kindly supplied by Professor Truszkowski, was found to have a specific activity of  $0.33 \mu\text{l. per mg. per min.}$  as compared with a value of  $85-90 \mu\text{l. per mg. per min.}$  for the purified enzyme prepared by the method which was finally evolved. Truszkowski's preparation was therefore not very highly purified and the enzyme could not be said to have been actually isolated.

All these earlier attempts at the purification of uricase were hampered by the fact that no satisfactory

test for the enzyme had been developed. Most workers added the enzyme preparation to a known amount of uric acid solution (in the form of sodium or lithium urate) and measured the amount of uric acid left in the mixture after a given time, i.e. the activity of an enzyme preparation was measured by the amount of uric acid which it decomposed in a given time, usually several hours. This method is obviously unsatisfactory. It is slow and clumsy and it makes the progress of the reaction difficult to follow.

The only workers to use a manometric method in the study of uricase were Keilin and Hartree (1936) who, however, made no attempt to isolate the enzyme but studied its properties in the form of a crude pig's liver powder.

Since the oxidation of uric acid is accompanied by the taking up of oxygen, the manometric method is obviously ideal for investigating the action of uricase, but the insoluble nature of uric acid and its salts renders it difficult to obtain a sufficient quantity of the substrate in the small volume of solution which the side cup of a manometer vessel will hold. Keilin and Hartree overcame this difficulty by using weighed

amounts of solid uric acid in dangling platinum cups, the contents of which were tipped into the manometric flask at a suitable moment. This method, however, is rather clumsy and an improvement was therefore sought.

#### THE MANOMETRIC TEST.

The first step in the investigation of uricase was the development of a convenient and rapid manometric test to determine the activity of enzyme preparations. Warburg manometers were chosen as being the most suitable type, but the question of adding the substrate from the side bulb of the manometer vessel caused some difficulty. Eventually it was found possible to prepare a sufficiently concentrated solution (M/30) of lithium urate, 0.4 ml. of which contained 2.24 mg. uric acid. The solution was prepared by dissolving 0.56 Gm. uric acid in 35 ml. boiling N/10 lithium hydroxide and making up to 100 ml. with water. Such a solution had a pH of about 9 and remained stable for about 24 hours. After that time the lithium urate tended to crystallise out and the solution was therefore prepared fresh daily.

The test was carried out at pH 9 (the optimum pH

for uricase) by using borate buffer as the medium for the reaction.

The details of the test as finally developed were as follows:-

The main chamber of the manometer vessel contained 1.0 ml. M/5 borate buffer pH 9 and 1.5 ml. enzyme preparation plus water. The central cup contained 0.2 ml. 10% KOH to absorb  $\text{CO}_2$  and the side bulb 0.4 ml. M/30 lithium urate. The gas space contained pure oxygen. The manometers were shaken in a thermostat at  $37^\circ$ , the substrate was tipped in from the side bulb after five minutes, and the oxygen uptake was measured over a period of thirty minutes.

The results of a preliminary experiment with an extract of pig liver powder are shown in Table 3, and graphically in Figs. I and II. It is seen that the oxygen consumption curve is a straight line over a period of thirty minutes and that the oxygen consumption bears a linear relationship to the amount of enzyme. The conditions of the test were therefore satisfactory for the measurement of enzyme activity.

The activity of a given enzyme was measured by determining the so-called specific activity. By this



Table 3.

The manometric test.

Oxygen uptake with different amounts of enzyme.

38°C.

pH 9.0

	Manometers		
	1.	2.	3.
Time after addition of substrate. Minutes.	Main manometer vessel.		
	1.0 ml. M/5 borate buffer pH 9.0	→	→
	0.52 ml. original extract	0.78ml.	1.04ml.
	0.98 ml. water	0.72ml.	0.46ml.
	Centre cup		
	0.2 ml. 10% KOH	→	→
Side bulb			
	0.4ml. M/30 lithium urate	→	→
Gas space			
	100% O <sub>2</sub>	→	→
10	8.1 μl.	12.2	17.6
20	16.2 μl.	26.6	34.7
30	25.6 μl.	40.0	50.5

Fig. 1.

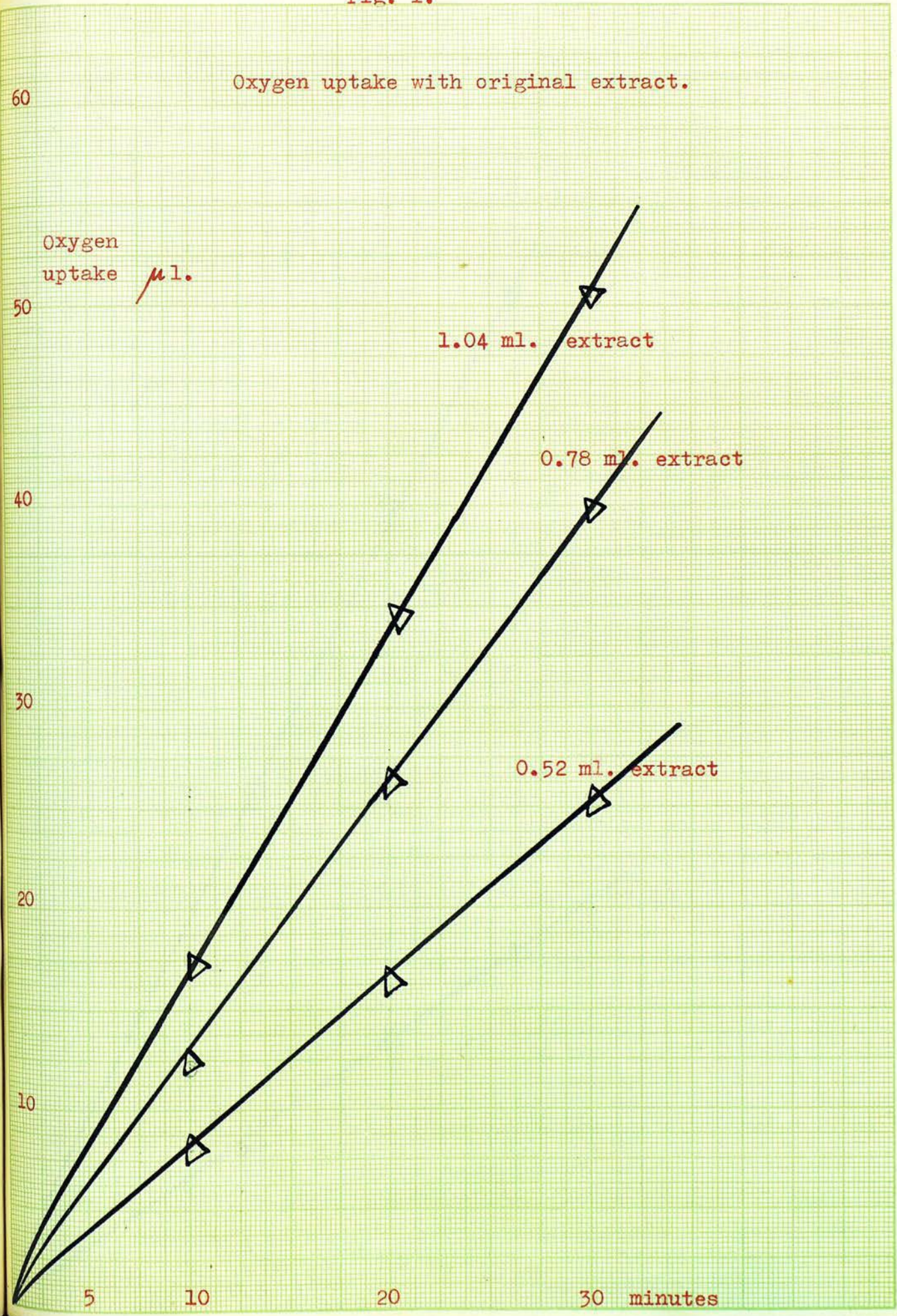
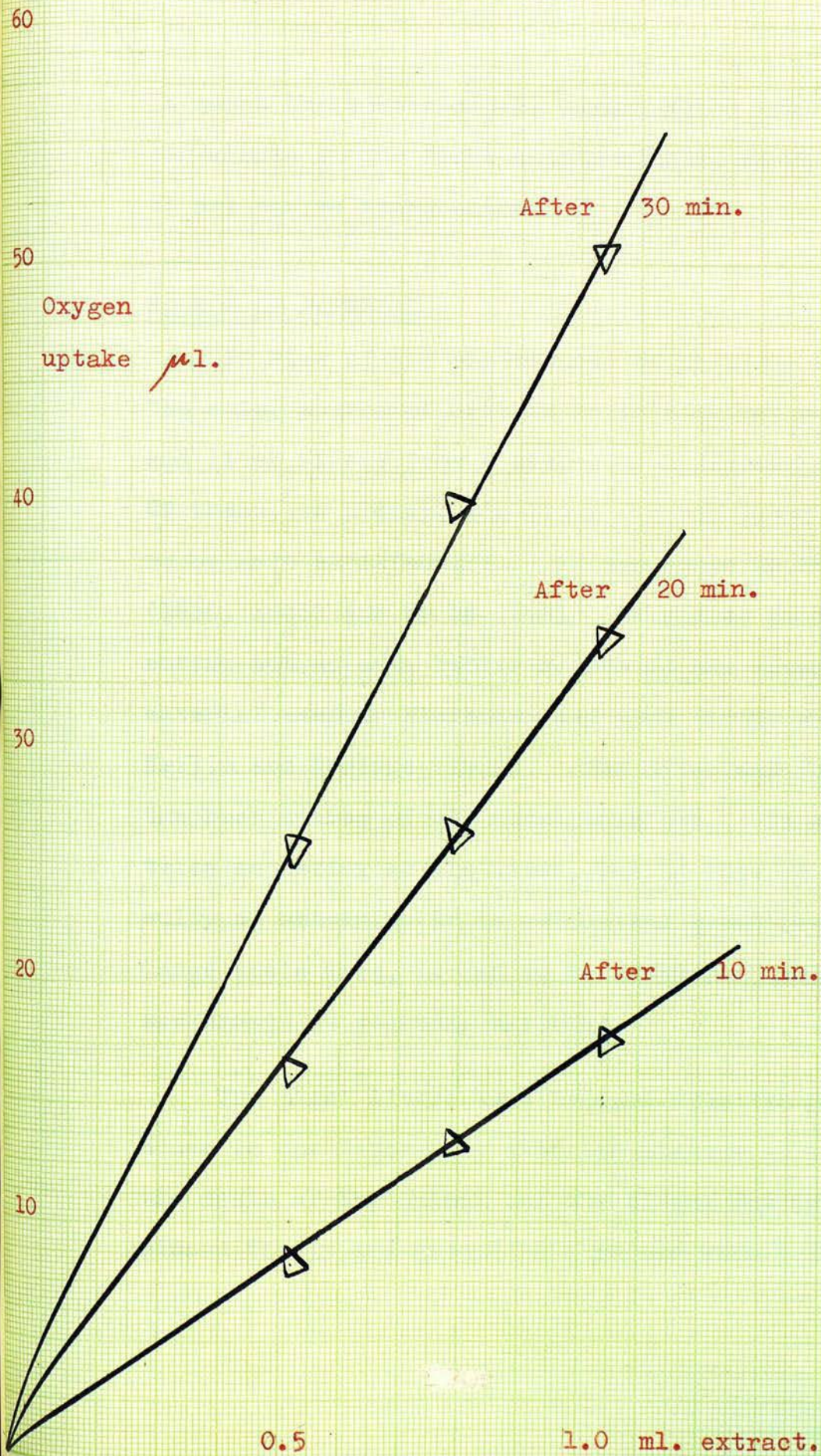


Fig. II

Oxygen uptake with original extract.



is meant the number of  $\mu$ l. oxygen which are taken up per minute in the first thirty minutes by one milligram of dry enzyme preparation.

#### SOURCE OF MATERIAL.

In view of the experiences of previous workers, the chief sources of uricase to be examined were kidney and liver tissue from a number of different mammals. Of these, ox kidney, pig liver and rat liver were found to be most satisfactory. As was shown by Truszkowski (1930, 1936) and by Ro (1931), ox kidney is rich in the enzyme but an even better source is pig liver, the material used by Kleinmann and Bork (1933) and by Keilin and Hartree (1936). Rat liver was found to be the best source of all those examined. It was about twice as active as pig liver, but in view of the difficulty of obtaining it in sufficient quantities for large scale work, it had to be abandoned, and pig liver was chosen as starting material.

The pigs' livers were brought from the slaughterhouse to the laboratory with the minimum of delay after the death of the animal and the tissue was immediately minced finely and ground in a mortar with 5 liters of

acetone to each kilo of minced tissue. The mixture was then centrifuged in large scale centrifuges each carrying 8 litres, and the solid material was shaken up with acetone, using half the volume of acetone originally employed. The mixture was again centrifuged, the acetone was poured off, and the solid mass was broken up and spread out in a thin layer on trays which were placed in a current of air. When the bulk of the acetone had evaporated, the material was transferred to vacuum desiccators in which it was dried overnight. It was then passed through a sieve to remove coarse particles. The resulting light brown powder was found to retain its activity for several months when stored in vacuum desiccators at room temperature. Its specific activity was of the order of 0.12 - 0.15  $\mu$ l. per mg. per minute. Occasionally a liver was found to yield a powder with a lower specific activity than this. As experience showed that such livers yielded poor extracts, the powders produced from them were discarded.

It is important to note that the acetone used in the preparation of the liver powders must be fresh and not redistilled material from acetone residues.

Redistilled acetone contains an impurity of unknown nature which has a deleterious action on uricase. The nature of the impurity is unknown, but a similar effect has been found by Kubowitz (private communication) in the case of polyphenol oxidase.

The mixture was then centrifuged in the cold

#### EXTRACTION OF THE ENZYME.

The first step in the process of the purification of uricase was to prepare a solution of the enzyme. As the enzyme is not easily soluble, this was a matter which presented some difficulty, and a large number of solvents was tried, including water, normal saline, phosphate buffers at different pH values, borate buffers, and dilute alkalies. The effect of varying the time of extraction, the temperature of the fluid and the ratio of fluid to solid was also investigated. Water and normal saline proved useless. Phosphate buffers were fairly successful, provided they were sufficiently alkaline, but their range did not extend far enough to the alkaline side of neutrality. Dilute alkalies such as N/100 ammonium hydroxide had not sufficient buffering power and were therefore discarded. Alkaline borate buffers, on the other hand, were able

to extract the enzyme with considerable success and were therefore employed.

At first the method of extraction was to grind the liver powder with M/10 borate buffer at pH 9 (using 30 ml. buffer per gm. powder) for one hour at 0°C. The mixture was then centrifuged in the cold room and a clear brown solution containing uricase was obtained. Later it was found that a more nearly complete extraction could be obtained by altering the pH of the buffer to 10 and by raising the temperature to 37° but, as this method resulted in the extraction not only of uricase but of a very large amount of inactive protein material, a further step was introduced and the liver powder was first of all extracted with ice cold phosphate buffer at pH 7.4. This extraction resulted in the removal of a large amount of inactive protein but only a negligible amount of enzyme. The residue was then extracted with borate buffer, which dissolved the uricase.

250 g. liver powder were stirred with 2.5 l. ice cold M/10 phosphate buffer at pH 7.4. After standing for 20 minutes at 0°, the mixture was centrifuged and the inactive reddish extract discarded. The solid

residue was then stirred with 5 l. M/10 borate buffer of pH 10 at 38°. After 20 minutes the mixture was rapidly cooled to 0° and centrifuged. The clear brownish extract contained the bulk (80%-90%) of the uricase. The specific activity of the material obtained by this extraction was 0.5  $\mu$ l. per mg. solid per minute.

By this process a solution of uricase was obtained which could be used as a starting point for further attempts at purification. It will be referred to as the "original extract".

This solution retained its activity for several weeks at 0°. It was always stored in the ice chest, and all further manipulations were carried out at 0° unless otherwise stated. Large scale manipulations were all carried out in the cold room, and all solutions and reagents used in bench experiments were kept in jars of ice. Enzyme solutions kept at room temperature rapidly lost activity.

#### PURIFICATION OF THE ENZYME.

Once a solution of uricase had been obtained, the usual methods adopted in the purification of enzyme



proteins could be applied. These include fractional precipitation with ammonium sulphate, precipitation by pH alteration, precipitation with organic solvents, precipitation with heavy metals, dialysis, heat denaturation and so on.

Fractionation with Ammonium Sulphate. Of these methods, the first to be attempted was fractionation with ammonium sulphate. Portions of original extract were treated with varying amounts of saturated ammonium sulphate solution and the precipitate so formed was centrifuged off and dissolved in buffer solution or, latterly, in water. (It was found that although uricase was insoluble in pure water, the ammonium sulphate precipitate carried with it sufficient salt to keep the uricase in solution.) The various solutions so produced were tested and it was found that the precipitate produced by the addition of ammonium sulphate to 50% saturation contained the bulk of the uricase. The solution produced by dissolving this precipitate in water was then fractionated with ammonium sulphate. At this second fractionation, 33% saturation was sufficient to precipitate the bulk of the enzyme. A

third fractionation was tried but proved valueless and even the second was eventually abandoned as it entailed considerable loss of activity.

It should be noted at this point that every step in the purification of an enzyme is accompanied by a marked loss of enzyme, no matter how great the purification produced by that step may be. Losses of 30% and even 50% must be regarded as quite usual in any particular step, provided the degree of purification - that is to say the amount of inactive accompanying protein removed by the step - is sufficient to justify it. As a result, the yield of highly purified enzyme is very small and large amounts of starting material may be used in the preparation of minute amounts of the highly purified product.

The Effect of Heat. The solution produced by dissolving the ammonium sulphate precipitate in water was found to be unstable and to keep for only a few days even at 0°. The effect of heating this solution at different pH values and to different temperatures was investigated. Even moderate degrees of heat resulted in considerable destruction of enzyme if the

pH were acid (pH 3) or alkaline (pH 10), but at pH 7.4 the solution could be heated to 55° without loss although at 65° destruction of the enzyme was almost complete. Heating to 55° resulted in the formation of a heavy flocculent precipitate of denatured protein which was completely inactive, and which could therefore be centrifuged off and discarded. The clear supernatant fluid contained all the activity.

By heating the solution to 55° at pH 7.4, therefore, a considerable degree of purification could be obtained by the removal of inactive protein. The nature of the precipitate depended to a certain extent on the amount of salts present in the solution on heating. In the presence of too little salt, the precipitate was gelatinous in character and difficult to centrifuge. In the presence of a little added ammonium sulphate, however, the precipitate was flocculent and centrifuged easily. A small amount (1/10 vol.) of saturated ice-cold ammonium sulphate was therefore added to the solution before heating. This amount was in itself too little to produce any precipitate, but it was sufficient to ensure that the precipitate produced on heating was flocculent in nature.

This step may be summarised as follows:-

To the solution of the ammonium sulphate precipitate was added 1/10 vol. saturated ice-cold ammonium sulphate, and the solution, which had a pH of 7.2-7.4, was then heated to 55° for five minutes, with vigorous stirring. It was rapidly cooled, and the precipitate of inactive material was centrifuged off and discarded. The resulting enzyme solution was clear and straw-coloured.

The specific activity of the material produced after purification by heat was 1.0 - 1.5  $\mu$ l. per mg. per min.

Dialysis. The enzyme solution produced by the previous step was dialysed to free it of salts. It was placed, along with a glass ball, in a cellophane tube which lay in a 5 ft. long glass tube, 2 in. in diameter, through which a current of ice-cold distilled water flowed slowly. The glass tube was mounted on a rocking table, with the result that the glass marble rolled to and fro in the sac and kept the contents stirred. Dialysis was therefore rapid. The whole apparatus was placed in the cold room.

When dialysis had proceeded for 12-15 hours, a heavy, yellowish-brown precipitate appeared. This precipitate contained the entire enzyme activity. The enzyme therefore appeared to be insoluble in pure water. It was centrifuged off and washed with ice-cold water on the centrifuge. No loss occurred on precipitation of the enzyme by dialysis.

When large amounts of material were being worked up, the volume of solution to be dialysed was often of the order of several litres, a rather large amount to handle with ease. The device was therefore adopted of concentrating the solution by ammonium sulphate precipitation before dialysing. To 5 l. enzyme solution an equal volume of saturated ammonium sulphate solution was added and the precipitate so produced was centrifuged off and dissolved in 1 l. ice-cold distilled water. The solution so obtained was then dialysed as described.

The precipitate produced on dialysis was of a very insoluble nature. It was treated with various solvents in an attempt either to dissolve the enzyme from the precipitate, leaving the bulk of the protein behind, or, alternatively, to dissolve out inactive protein,

leaving the enzyme for subsequent treatment. The latter process could be achieved to a certain extent by the use of M/100 acetate buffer at pH 4 which dissolved out a certain amount of inactive material, but the loss of activity on this treatment was too great to justify its use. Similar treatment with ammonium sulphate solution of various strengths were no more successful.

On the other hand, the known solubility of uricase in alkaline buffers suggested that a borate buffer solution might bring about the extraction, and this was indeed found to be the case. When the precipitate was ground in an ice-cold mortar, with cold borate buffer at pH 9, a large proportion of the solid material, including the enzyme, went into solution, leaving a dark brown insoluble residue which could only be completely removed by the use of a high speed centrifuge (15,000 r.p.m.). This process of extraction was later improved by adopting the principle used in the initial extraction of the liver powder, namely extraction first with phosphate buffer to remove inactive protein but not uricase, followed by extraction with borate buffer to dissolve the enzyme.

The precipitate produced on dialysis, therefore, after washing with ice-cold water, was rubbed in a cooled mortar with 400 ml. M/10 phosphate buffer at pH 7.4 at 0° and centrifuged on the high speed centrifuge. The extract, which contained much protein but little enzyme, was discarded. The residue was rubbed in a mortar with 250 ml. M/10 borate buffer pH 10 at room temperature and was centrifuged on the high speed centrifuge. The extraction with 250 ml. borate buffer was repeated, the extracts containing the bulk of the enzyme were combined and the residue consisting of brown insoluble protein was discarded.

A clear, pale yellow solution of uricase was thus obtained (Solution II). The material in this solution had a specific activity of 10 - 25  $\mu$ l. per mg. per min.

Attempt at purification by precipitation on acidification. Portions of Solution II were treated with varying amounts of 0.2N acetic acid. Between pH 10.0 and pH 7.2 no precipitate was produced. At pH 6.8 a slight precipitate appeared; between pH 5.4 and 4.6 the precipitate was heavy but at pH 4.1 it was again only slight. At pH 3.8 no precipitate was

produced. Each of these precipitates was centrifuged off and its activity determined. Greatest activity was found in the precipitates produced between pH 5.4 and 4.6, (see Fig. III). The isoelectric point of uricase is therefore about pH 5.0.

Although the precipitation of uricase was almost complete at pH 5.0, the degree of purification produced by this step was so slight that such a step was useless as an isolation procedure.

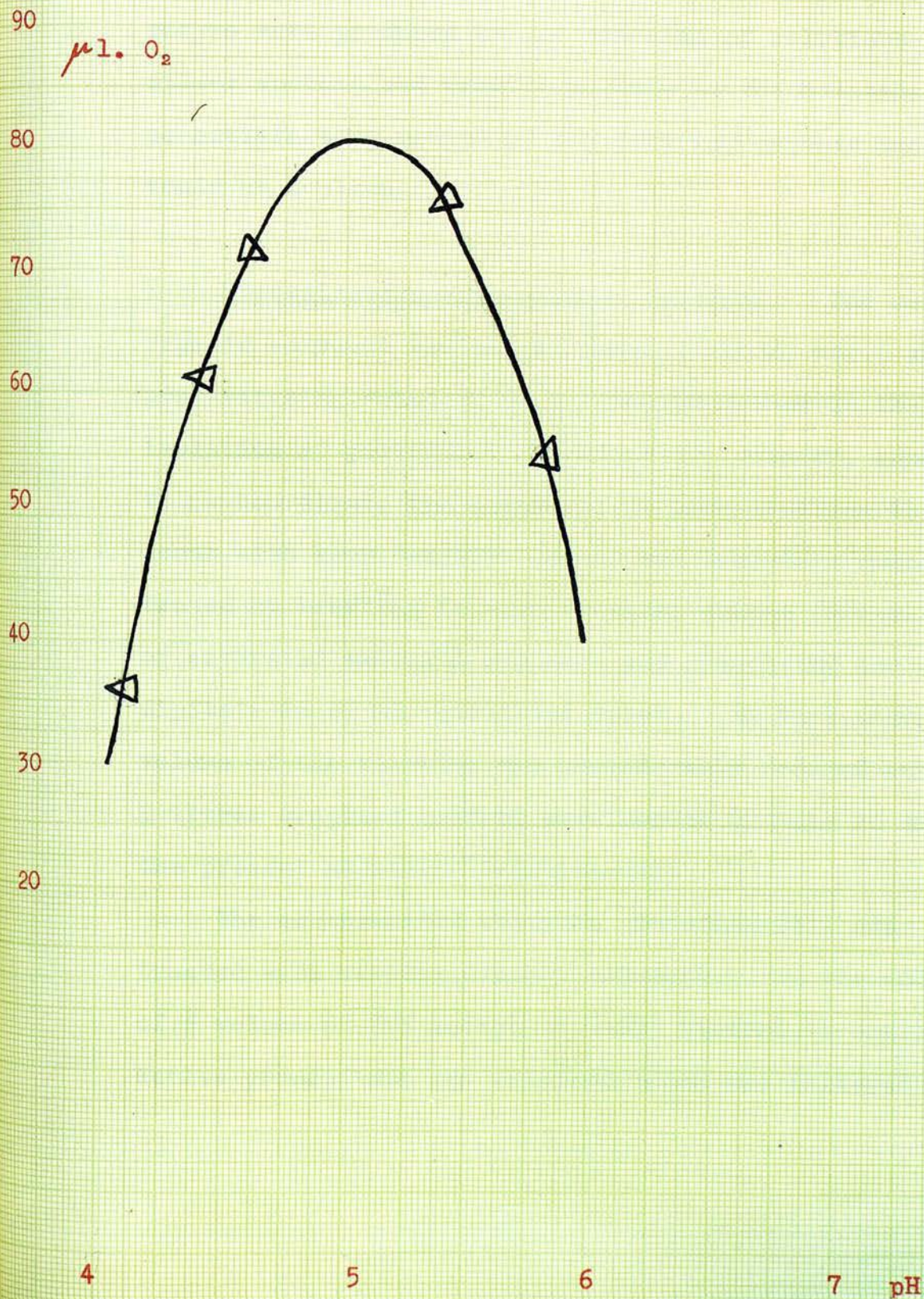
Attempts at purification by other means. In view of the success with which the use of heat had been employed earlier in the purification of the enzyme, an attempt was made to carry out a similar procedure with Solution II, but it was found that the now highly purified enzyme was much more heat labile, and exposure to 55° for five minutes at any pH resulted in almost total inactivation. Even exposure to 37° for two hours caused considerable inactivation without any impurity being thrown down. Heat purification at this stage was therefore abandoned.

Gentle heating in the presence of small amounts of organic solvents such as chloroform was also useless.



Fig. III

Enzyme content of precipitate at different pH values



Precipitation of the enzyme by heavy metals was next tried. Although precipitates containing the enzyme were produced by the addition of solutions of silver acetate and uranium acetate to the Solution II, no satisfactory method of purification by this means could be evolved.

Precipitation with Organic Solvents. The use of organic solvents such as acetone, alcohol, etc., as precipitating reagents is a standard method in the isolation of proteins. Solution II, however, appeared to be too dilute for satisfactory precipitation to occur with such reagents and it was therefore concentrated by treating it with an equal volume (500 ml.) of saturated ammonium sulphate and dissolving the precipitate in 60 ml. distilled water. By this means the volume of the solution was considerably reduced and 5 litres original extract were worked up into 60 ml. of the present solution (Solution III).

The concentrated solution (Solution III) was treated with various organic solvents. On the addition of  $\frac{1}{4}$  volume of 80% methyl or ethyl alcohol, a small, active precipitate was obtained which was filtered off,

washed with water and shaken with borate buffer which dissolved the uricase, leaving an inactive residue. By this procedure a considerable degree of purification was obtained but the loss was great and the method could not be depended upon to give consistent results. The method was therefore abandoned.

Acetone precipitation, however, gave rather different results. On the addition of 9/10 vol. ice-cold 80% acetone, a precipitate appeared after about 20 minutes. This precipitate was bulky but contained very little enzyme. It was filtered off and discarded. The supernatant fluid which contained the bulk of the enzyme was submitted to vacuum distillation at room temperature. The addition of two drops of octyl alcohol prevented frothing. During the distillation, a small amount of highly active precipitate appeared. It increased slightly in amount on standing overnight at 0°. It was centrifuged off on the high speed centrifuge and washed three times on the centrifuge with ice-cold distilled water. It was then rubbed in a mortar with 5 ml. M/10 borate buffer at pH 10. The enzyme dissolved, leaving a small inactive, brownish residue which was centrifuged off on the high speed

centrifuge and discarded. The supernatant liquid (Solution IV) was almost colourless. From it the free enzyme could be obtained by dialysing the solution against running distilled water at 0° overnight, when the free enzyme precipitated out as a white, flocculent precipitate of specific activity 85 - 90  $\mu$ l. per mg. per minute. This was the highest activity obtained.

Attempts were made to purify the enzyme (Solution IV) still further, using all the methods previously described, but without success. Enzyme solutions were also treated with hydrosulphite to convert the uricase to the reduced form in the hope that this would have different properties towards precipitating reagents, but no change in properties was observed.

Standard methods for crystallising proteins were also applied to uricase, but the enzyme could not be obtained in crystalline form.

The complete process for the purification and isolation of the enzyme may be summarised as follows:-

Pig's liver is finely minced and ground in a mortar with five parts of acetone. The solid material is centrifuged off and shaken with three parts of

acetone. After centrifuging, the liver powder is dried at first rapidly in a current of air and then overnight in vacuum desiccators, and is then finely powdered and passed through a sieve. The resulting light brown powder has a specific activity of 0.12 - 0.15  $\mu$ l. per mg. per min. and retains its activity for several months when stored in desiccators at room temperature. Samples showing a specific activity of less than 0.10  $\mu$ l. per mg. per min. are discarded.

250 g. liver powder are stirred with 2.5 l. ice-cold M/10 phosphate buffer pH 7.4. After standing for 20 min. at 0° the mixture is centrifuged, and the extract, which contains much protein but only little uricase is discarded. The solid residue is then stirred with 5 l. M/10 borate buffer pH 10 at 38°. After 20 min. the mixture is rapidly cooled to 0° and centrifuged. The clear extract contains the bulk of the enzyme and has a specific activity of 0.5  $\mu$ l. per mg. per min.

An equal volume of saturated ice-cold ammonium sulphate solution is added to this solution, and the resulting precipitate, which contains the enzyme, is centrifuged off at 0° and dissolved with shaking in

5 l. ice-cold distilled water. 1/10 vol. saturated ice-cold ammonium sulphate is then added (no precipitate being formed), and the solution, which has a pH of 7.2-7.4, is heated to 55° for 5 min. with vigorous stirring, whereby a heavy flocculent precipitate of denatured protein is formed. The solution is rapidly cooled to 0° and the precipitate is centrifuged off and discarded. The enzyme solution, which now has a specific activity of 1.0-1.5  $\mu$ l. per mg. per min., is treated with enough saturated ammonium sulphate solution at 0° to bring the degree of saturation to 0.5. The resulting precipitate, which contains the enzyme, is centrifuged off at 0° and dissolved in 1 l. of ice-cold distilled water. This solution is dialysed for 15 hr. in cellophane sacs against running distilled water at 0°. A brown precipitate which appears during the dialysis and which contains the enzyme is centrifuged off and washed with ice-cold water on the centrifuge. It is then rubbed in a cooled mortar with 400 ml. M/10 phosphate buffer pH 7.4 at 0° and centrifuged on the high speed centrifuge (15,000 r.p.m.). The extract, which contains much protein but little enzyme, is discarded. The residue is rubbed in a mortar with

250 ml. M/10 borate buffer pH 10 at room temperature and is centrifuged on the high speed centrifuge. The extraction with 250 ml. borate buffer is repeated, the extracts, which contain the bulk of the enzyme, are combined, and the residue, which consists of brown insoluble protein, is discarded.

The clear, pale yellow uricase solution, which now has a specific activity of 10-25  $\mu$ l. per mg. per min., is cooled to 0°, treated with an equal volume of saturated ice-cold ammonium sulphate solution, and the resulting precipitate, which contains the enzyme, is centrifuged off at 0° and dissolved in 60 ml. ice-cold water.

To the solution just enough (0.5-1.0 vol.) ice-cold 80% acetone is added so that a flocculent precipitate appears after 20 min. The precipitate is centrifuged off and discarded. Acetone is removed from the supernatant fluid by vacuum distillation at 20° with two drops octyl alcohol and the resulting slightly turbid solution, after standing overnight at 0°, is centrifuged on the high speed centrifuge. A small precipitate of very high activity is thus obtained, and is washed three times on the centrifuge with ice-cold

distilled water. It is then rubbed up with 5 ml. M/10 borate buffer pH 10 at room temperature, in which the enzyme dissolves, leaving a small inactive brownish residue which is centrifuged off at high speed and discarded.

The uricase solution is almost colourless. When it is dialysed against running distilled water at 0° the free enzyme is obtained. Specific activity is 85-90  $\mu$ l. per mg. per min. The yield of the purest preparation is about 5 mg. per 100 g. liver powder.



THE PROPERTIES OF PURIFIED URICASE.

Purified uricase is a white substance which gives all tests for proteins. It is quite insoluble in water, almost insoluble in phosphate buffer pH 7.4, but moderately soluble in alkaline solutions such as borate buffer pH 10. It contains 14.4% of nitrogen (micro Kjeldahl), a figure which is to be expected in the case of a protein. In alkaline solution, e.g. in borate buffer, the activity of the enzyme is retained for long periods and no loss occurs even after two months storage at 0°. If the preparation is stored in the form of a suspension in water, the activity diminishes more rapidly.

On the other hand the enzyme cannot be dried without great loss of activity. Preparations either of a suspension of the pure enzyme in water or of a solution of the enzyme in buffer were dried in a vacuum desiccator over phosphorus pentoxide at 0°, but such treatment resulted in such loss of activity that the possibility of storing purified preparations in dried form was excluded.

HEAVY METAL CONTENT.

The most interesting feature of the purified uricase preparation was the fact that it contained a considerable amount of iron. The purest preparation contained 0.15 - 0.20% Fe, a comparatively large amount, comparable with the amount found in crystalline catalase. Every care was taken to exclude the possibility that the iron might be present as an accidental impurity. Iron estimations were carried out only on specimens which had been thoroughly dialysed. All the glassware with which the purified preparations came in contact was specially cleaned to render it free from traces of heavy metal impurities. To this end it was cleaned thoroughly with strong, hot, chromic acid mixture and then washed thoroughly with distilled water without being touched by tap water.

Iron estimations were carried out by a modification of the method of Kennedy (1927). To the sample of enzyme in a long necked, round bottomed flask 1 ml. concentrated sulphuric acid and 0.3 ml. perchloric acid were added. The flask was heated over a small flame till bubbles ceased to appear and the flask was filled with white fumes. The mixture was then cooled and one

drop concentrated nitric acid and 5 ml. water were added. After further cooling, 2.5 ml. 20% potassium thiocyanate and 5 ml. amyl alcohol were added and the mixture thoroughly shaken. The amyl alcohol layer, which took up the red colour of the ferric thiocyanate, was pipetted off and centrifuged to clarify it. The reddish colour in the amyl alcohol was compared with that produced from a standard solution of iron treated in a similar way.

All the iron estimations were carried out by W. Lüttgens, microanalyst to the Kaiser Wilhelm Institut für Zellphysiologie, to whom grateful thanks are expressed.

The preparation was also tested for other heavy metals. Copper estimations were carried out by the catalytic manometric method of Warburg (1927), using quartz manometer vessels which had been specially washed with chromic acid and distilled water and then heated to a red heat in a furnace. The method depends on the ability of minute amounts of copper to catalyse the oxidation of cystein in the presence of oxygen. Only a trace of copper, however, was found in the enzyme.

Cobalt was estimated by the catalytic manometric method of Lüttgens (unpublished) which depends on the ability of minute amounts of cobalt to catalyse the decomposition of hydrogen peroxide. No cobalt was found in the enzyme. This method can also be adapted to the estimation of manganese since manganese inhibits the catalytic decomposition of hydrogen peroxide by added cobalt. No manganese was present in the enzyme.

The fact that uricase is inhibited by cyanides suggests that it is a heavy metal compound, and in view of the high iron content of the purified material, the question naturally arises as to the possibility of the catalytic activity of the enzyme being due to the iron. The fact that iron estimations were carried out only on carefully dialysed preparations which were allowed to come into contact only with specially cleaned glassware, and the consistency with which the value 0.15 - 0.20% was obtained in repeated preparations tempts one to think that the iron is more than a mere impurity.

It is possible, of course, that uricase might be

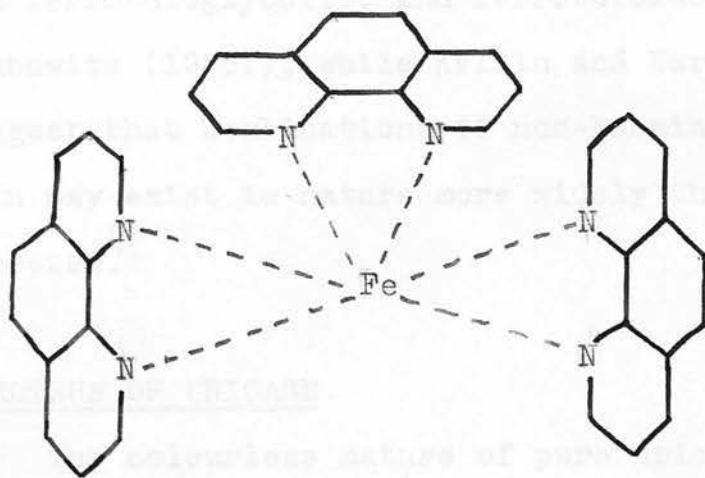
a protein containing iron in which the iron does not act as the active group of the enzyme, but this is unlikely.

A comparison of the iron content of preparations of different specific activity did not reveal a direct linear relationship between iron content and the activity since crude preparations all contained iron as an impurity, although in much smaller amounts than 0.2%. The fact that the iron content tends to increase rather than decrease on purification is itself suggestive.

The known iron proteid enzymes, e.g. catalase, are mainly haemin derivatives in which an iron porphyrin complex is attached to a protein. Such haemin derivatives are deeply coloured compounds. Since uricase is colourless it cannot be a haemin derivative. This point was verified by the failure of an attempt to prepare a haemochromogen by treating uricase with pyridine and alkaline hydrosulphite.

Confirmatory evidence that uricase contained iron not in the form of a haemin derivative was obtained by the use of  $\alpha\alpha'$  phenanthroline which, as shown by

Zuckerkindl et al. (1933, 1934), combines with iron in the ferrous state (but not with haemin iron) to form a red compound with the structure:



A similar reaction is given by  $\alpha\alpha'$  dipyridyl (c.f. Hill (1931)).

A sample of uricase treated with phenanthroline in alkaline solution developed no colour, but when the uricase was first reduced by the addition of a small amount of hydrosulphite a reddish colour developed, indicating the presence of non-haemin iron.

Organic compounds of iron in which the metal is not present in the form of haemin, are well known.

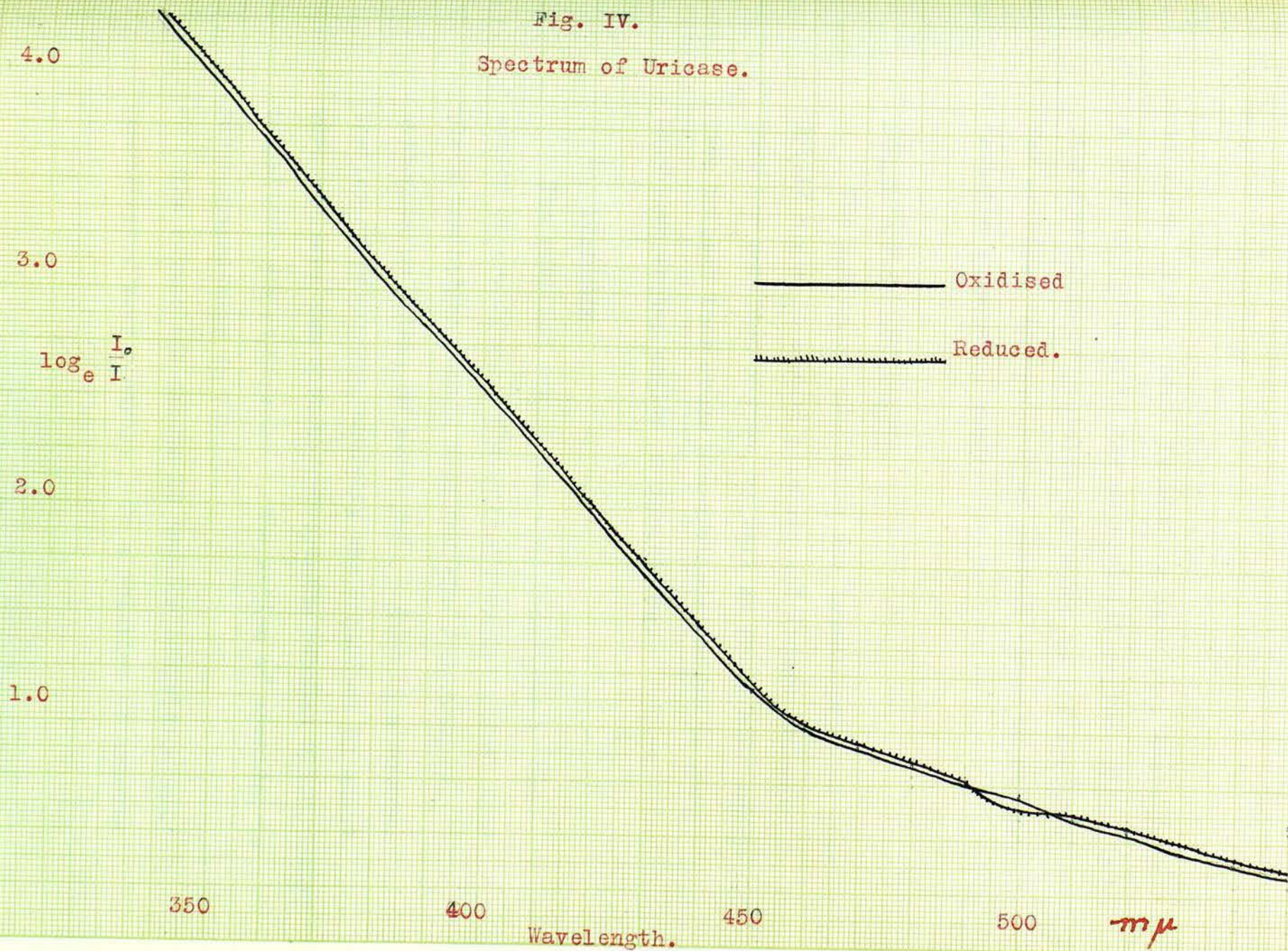
Such compounds usually contain sulphur as well as iron such as, for example, ferrocystein (Cremer (1928)), ferroglutathione (Kubowitz (1935) and Hartmann (1930)) and ferrothioglycollic and ferrothiolactic acids (Kubowitz (1935)), while Keilin and Hartree (1938) suggest that combinations of non-haemin iron with protein may exist in nature more widely than has been expected.

#### SPECTRUM OF URICASE.

The colourless nature of pure uricase rendered it improbable that the enzyme, in contrast to the deeply coloured haemin derivatives, would have a very characteristic absorption spectrum. The spectrum of a strong solution of uricase was, however, measured in the spectro-photometer described by Warburg and Negelein (1929). Uric acid was then run into the absorption trough in the manner described by Warburg and Christian (1932) with the intention of converting the enzyme from the oxidised to the reduced form in the same way as, for example, xanthine oxidase, may be reduced by the addition of substrate (Ball (1939)).

The spectrum of both oxidised and reduced uricase

Fig. IV.  
Spectrum of Uricase.





is shown in Fig. IV. It will be seen that apart from a very marked band in the ultraviolet region, which is characteristic of proteins and which is not specific for uricase, the spectrum of uricase shows no characteristic absorption bands.

#### THE ACTION OF INHIBITORS.

The action of inhibitors was investigated in some detail since it seemed possible and indeed probable that the purified enzyme, as well as being more sensitive to heat than crude preparations, might be more sensitive to the action of inhibitors.

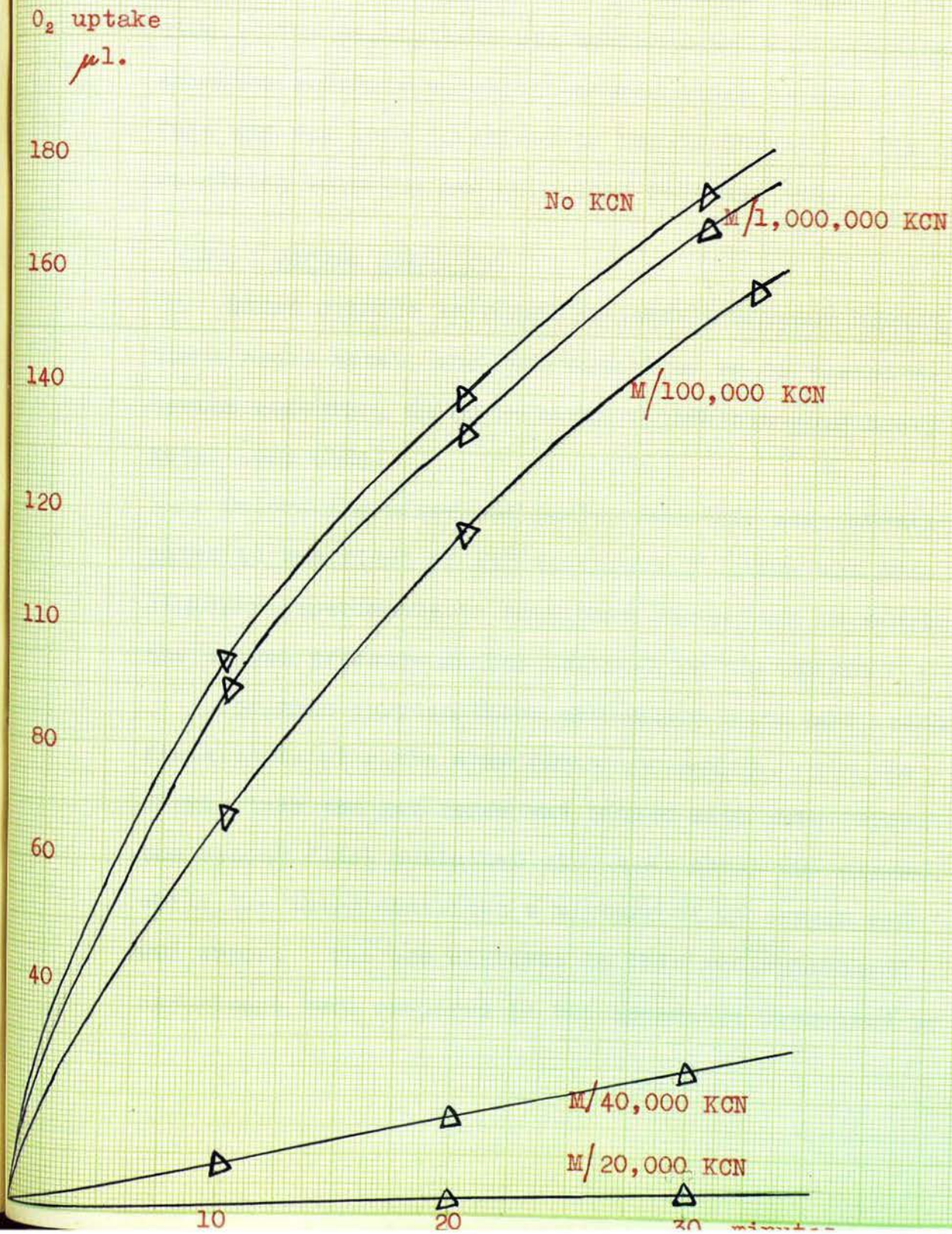
##### 1. Cyanides.

The purified enzyme was very sensitive to the action of cyanides.  $M/20,000$  KCN caused almost complete inhibition, while  $M/40,000$  KCN inhibited the action to an extent of about 80%. Even at a concentration of  $M/100,000$ , activity was inhibited by about 20%, but when the dilution of cyanide reached  $M/1,000,000$ , inhibition was negligible. The action of cyanides is shown graphically in Fig. V.

The reversible nature of cyanide inhibition was demonstrated very clearly. To a solution of uricase

Fig. V

Inhibition by potassium cyanide.



sufficient cyanide was added to cause complete inhibition. The enzyme was then precipitated by the addition of an equal volume of saturated ammonium sulphate solution. The precipitate was washed with half saturated ammonium sulphate solution several times on the centrifuge and was then dissolved in borate buffer. The resulting solution had recovered full activity.

## 2. Carbon monoxide.

Since cyanide is known to inhibit enzymes containing a heavy metal, the next inhibitor to be tested was carbon monoxide, which is known to inhibit some heavy metal compounds.

Before the effect of carbon monoxide was investigated it was first of all necessary to carry out preliminary experiments to ascertain the effect of varying the oxygen pressure on the activity of the enzyme.

For this purpose three manometers were used, each flask containing the same amount of enzyme. In the first flask the gas space was filled with pure oxygen, the second flask contained air, i.e. about 20% oxygen, while the third contained a mixture of 2% oxygen and 98% argon. The gas mixtures in this and <sup>the</sup> subsequent experiment were prepared in the apparatus described by

Warburg, Kubowitz and Christian (1931).

A record of such an experiment is shown in Table 4 and graphically in Fig. VI. It is seen that the velocity of the reaction is proportional to the partial pressure of oxygen.

In each experiment with carbon monoxide, therefore, it was necessary to have a control manometer in which the partial pressure of oxygen was the same as that in the manometer containing carbon monoxide. The manometric measurements were made in the dark room lest the action of CO be light sensitive. The velocity of the reaction using a mixture of 80% CO and 20% O<sub>2</sub> was found to be the same as that in air. The partial pressure of CO was therefore increased and a mixture of 98% CO and 2% O<sub>2</sub> was used, the control manometer containing 98% Argon and 2% O<sub>2</sub>. As is shown in Table 5 no inhibition of the enzyme occurs even when the ration CO/O<sub>2</sub> = 49/1. As was mentioned previously Keilin and Hartree (1936) found that at a ratio CO/O<sub>2</sub> = 8/1 no inhibition of crude uricase occurred.

### 3. Thiocyanate.

Potassium thiocyanate was found to have a rather

Table 4.

The effect of varying O<sub>2</sub> pressure.

Oxygen uptake in different gas mixtures.

38° C.

pH 9.0

	Manometers		
	1.	2.	3.
Time after addition of substrate. Minutes.	Main manometer vessel. 1.0 ml. M/5 borate buffer pH 9.0 0.1 ml. enzyme solution 1.4 ml. water Centre cup 0.2 ml. 10% KOH Side bulb 0.4 ml. M/30 lithium urate Gas space 100% O <sub>2</sub> 0% Ar	→ → → → → → 20% O <sub>2</sub> 80% Ar [air]	→ → → → → → 2% O <sub>2</sub> 98% Ar
10	82 μl.	24	4
20	131 μl.	44	9
30	-	60	14

Fig. VI

The effect on enzyme activity of alteration in the partial pressure of oxygen.

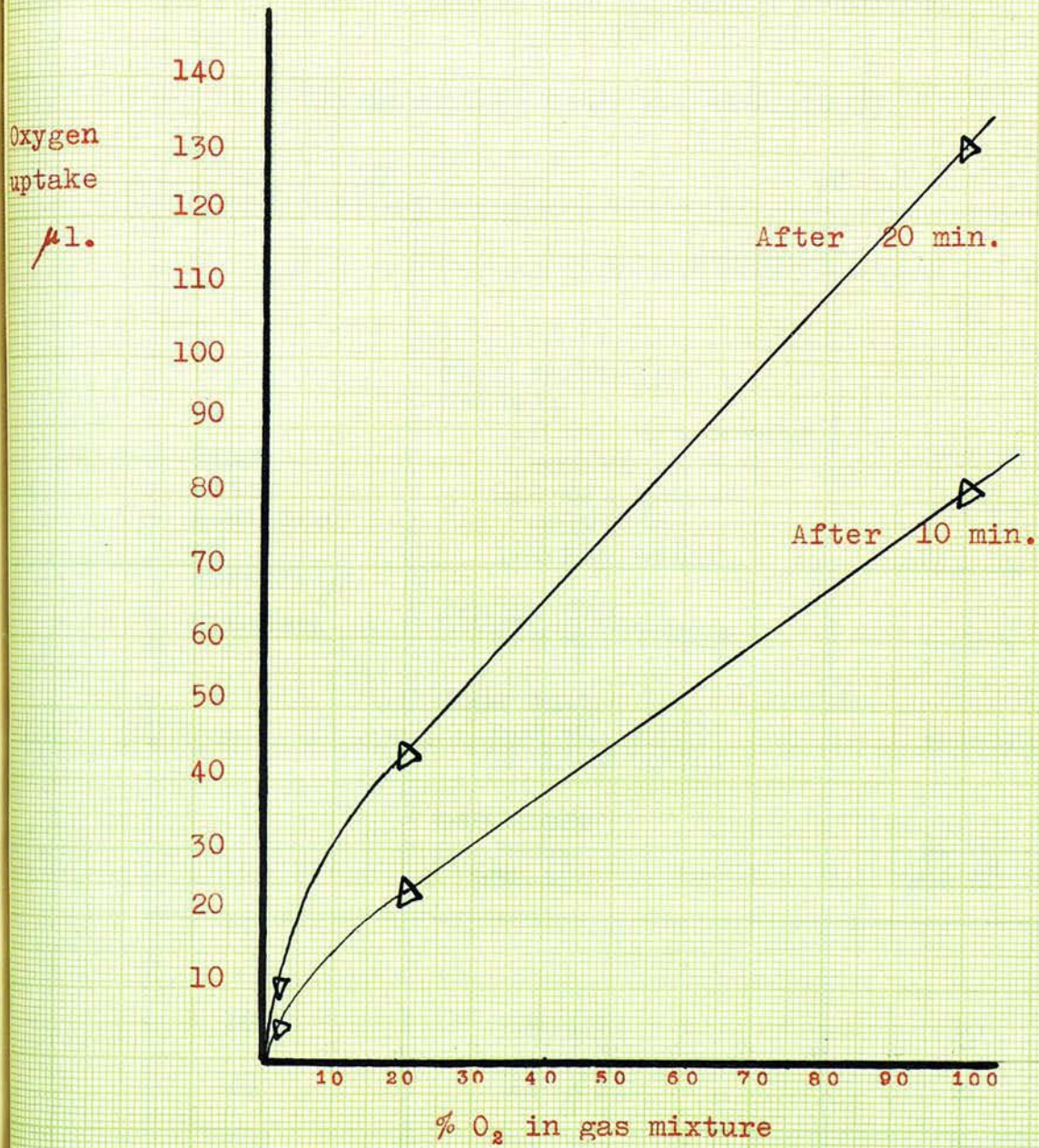


Table 5.

The effect of carbon monoxide.

Oxygen uptake with and without CO.

38°

pH 9.0

	Manometers.	
	1.	2.
Time after addition of substrate.	Main manometer vessel. 1.0 ml. M/5 borate buffer pH 9.0 0.3 ml. enzyme solution 1.2 ml. water.	→ → →
Minutes.	Centre cup 0.2 ml. 10%KOH	→
	Side bulb 0.4 ml. M/30 lithium urate	→
	Gas space 2% O <sub>2</sub> 98% Ar	2% O <sub>2</sub> 98% CO
10	9 μl.	10
20	17 μl.	20
30	24 μl.	29

curious action. At a concentration of M/200 no effect was produced on the enzyme action, but at higher concentrations, e.g. M/100, inhibition was produced but only after the reaction had been proceeding for about five minutes. Even when the concentration of thiocyanate was increased to M/10 the reaction still proceeded normally for the first five minutes and was then inhibited to a very great extent. Inhibition was, however, never absolutely complete. The action of KCNS is shown in ~~Table~~ Fig. VII.

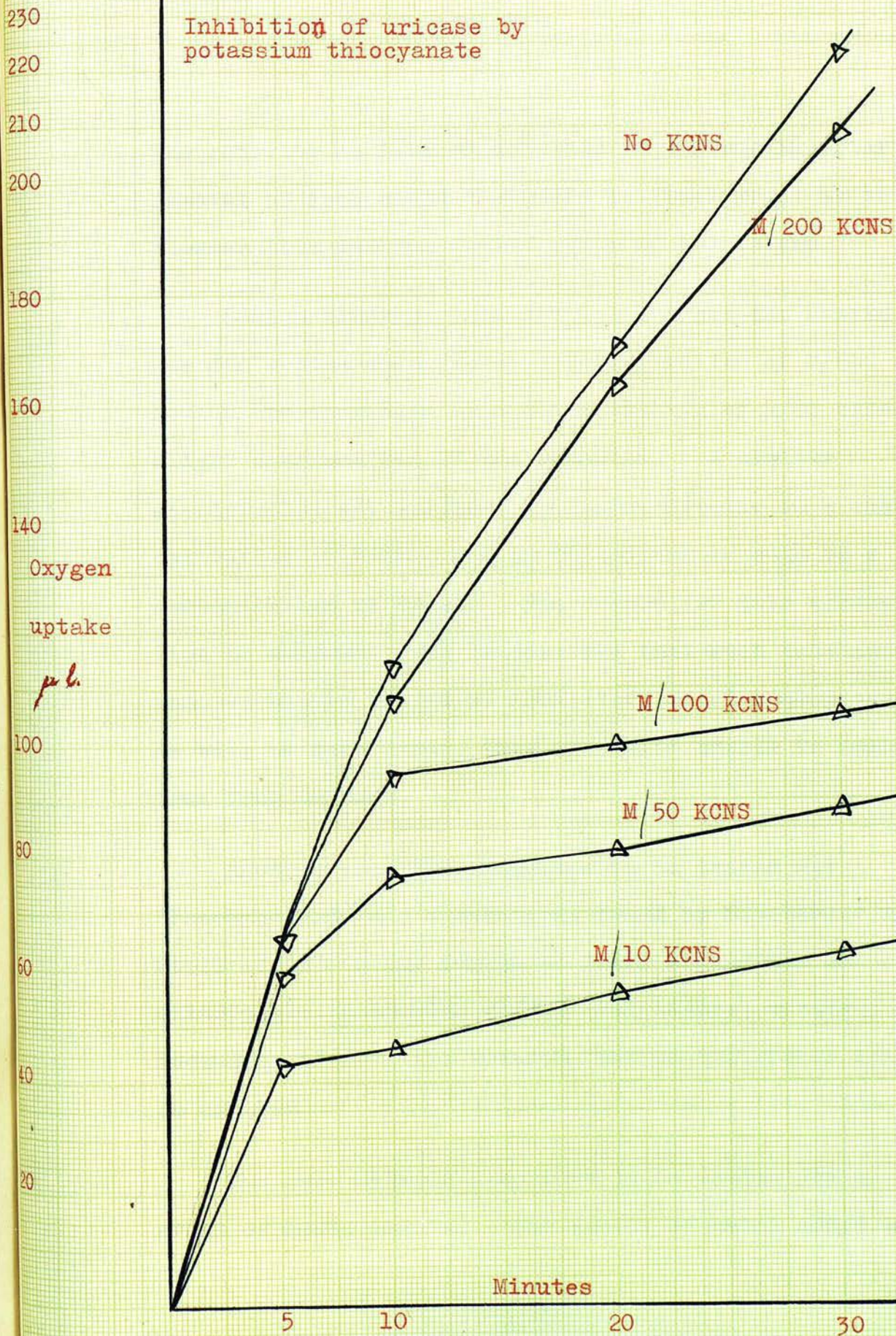
The addition of small amounts of iron, e.g.  $2 \mu\text{g.}$ , made no difference to the effect nor was it altered when the thiocyanate, instead of being present in the flask from the beginning of the experiment, was added from a second side bulb after the reaction had been proceeding for some time.

#### 4. Other Inhibitors.

The majority of inhibitors, or possible inhibitors, tested were substances which were known to react with iron. The reason for this was that if a substance known to react with iron was found to inhibit the enzyme, it might be found possible to use such an inhibitor in such a way as to remove iron from the enzyme in bulk.



Fig. VII

Inhibition of uricase by  
potassium thiocyanate

The activity or lack of activity of the enzyme after removal of iron would obviously be final proof as to whether iron was the active group of the enzyme.

Sodium fluoride M/60 did not inhibit the enzyme nor did sodium sulphide at a concentration of M/50. Sodium pyrophosphate at a concentration of M/30 caused slight acceleration of the reaction. A similar acceleration was produced by phenanthroline in a concentration of M/250, and by thioglycollic acid in a concentration of M/240. The reason for this acceleration is unknown. It is possible that such substances removed minute traces of inhibitory metal contaminant. As will be seen later, they are quite unable to remove iron from uricase.

Another possible inhibitor to be tested was "Phaeophorbide a", a substance found by Willstätter and Stoll (1913) in the course of their researches on chlorophyll to combine avidly with several metals, including iron. It, however, had no inhibitory action on uricase.

The last type of inhibitor to be tested was a group of heavy metals. The amount of metal added was

~~such that~~ to each manometer flask was fifty times greater than the amount of iron in the uricase in the flask. Such amounts were of course very minute.

Under such conditions copper caused almost complete inhibition of the enzyme; cobalt and manganese inhibited to about 50%, while nickel caused no inhibition whatever.

Added iron neither inhibited nor accelerated the action of uricase.

#### ATTEMPTS TO REMOVE IRON.

If uricase is an iron proteid in which iron acts as the active group of the enzyme, it might be expected that after the removal of iron from the protein, activity would disappear and would return on the addition of small amounts of iron.

Such a removal of a heavy metal active group from an enzyme was achieved by Kubowitz (1938) in the case of the copper proteid, polyphenol oxidase. Kubowitz treated his copper proteid with cyanide and then dialysed it for five days against running distilled water. By this means the copper combined with the cyanide to form a diffusible copper complex which was

removed, leaving an inactive protein containing no copper. When copper was added to the protein, the copper proteid was reformed and full activity was restored.

By this method Kubowitz was also able to remove copper from haemocyanin.

A similar method was therefore investigated in the case of uricase. To a sample of uricase, potassium cyanide was added and the solution dialysed against a fixed volume of M/10 borate buffer containing cyanide for 60 hours at 0° and then against running, ice-cold, distilled water for a further 24 hours. At the end of this time, however, the enzyme still retained its full activity and the iron content remained unaltered. The method of Kubowitz therefore does not remove iron from uricase. Modifications of the procedure were tried and the use of other inhibitors such as potassium thiocyanate instead of cyanide was investigated without any iron being removed. Dialysis with thioglycollic acid and sodium fluoride was equally ineffective. All these procedures were repeated, using uricase reduced by the addition of the substrate lithium urate, care being taken to exclude

air, but no removal of iron was accomplished.

Finally, dialysis in the presence of solutions of other heavy metals such as copper, cobalt, manganese and nickel, resulted in no alteration of the iron content of the enzyme.

Although these experiments gave negative results, yet they serve as confirmation of the view that the iron content of uricase is more than a mere impurity. It would be expected that traces of iron present as impurity would have been removed by these procedures, but yet neither the iron content nor the activity was altered in the dialysis experiments. The fact that both these properties were unaltered is evidence, admittedly of a negative kind, that uricase might be an iron proteid with iron as the active group, but at the same time it indicates that the iron in uricase, whatever its function, is bound tightly to protein.

Another line of approach was also tried in an attempt to remove iron. A solution of uricase was treated with sodium sulphide and left overnight in an atmosphere of argon. A duplicate experiment in which lithium urate was added was also carried out. Next

morning the enzyme was precipitated by the addition of ammonium sulphate to half saturation, the precipitate was washed several times with half saturated ammonium sulphate, and the activity and iron content of the enzyme were determined. Both remained unaltered.

Similar experiments, using other substances, e.g. cyanide, in place of sulphide were likewise without effect.

#### STOICHIOMETRIC EXPERIMENT WITH CO.

In the course of his experiments with polyphenol oxidase, Kubowitz (1938) proved conclusively that copper was the active group of the enzyme and that the copper was oxidised and reduced in the course of enzyme action. A large amount of enzyme was placed in a manometer flask in an atmosphere of 100% carbon monoxide. No gas was absorbed or evolved. Substrate was now added from a side bulb and a small uptake of CO immediately occurred, one molecule of CO being absorbed for every two atoms of copper in the enzyme. The carbon monoxide reacted with the reduced form of the enzyme, in which the copper has been reduced by the substrate, to form a copper carbonyl compound. On

the addition of cyanide from a second side bulb, an amount of CO equal to that originally taken up was evolved.

A similar experiment was therefore carried out with uricase. Three manometers were employed. The first vessel contained 30 mg. pure uricase in 4.4 ml. borate buffer pH 10. The side bulb contained 0.4 ml. lithium urate and the gas space 100% CO. The second and third manometers were controls, the second containing 100% argon instead of CO and the third containing 4.4 ml. borate buffer instead of enzyme. The manometers were shaken in a thermostat at 20° in a dark room for 24 hours, the substrate being tipped into the main vessels from the side bulbs after the first five minutes. The results of the experiment are shown in Table 6. It is seen that the CO uptake is negligible. This type of experiment therefore gives no information as to the part played by iron uricase.

It is to be noted that the amount of the enzyme in each manometer (30 mg.) in this experiment is relatively enormous when compared with the few  $\mu$ g. required for an ordinary manometric test.

Table 6.

## Stoichiometric experiment with CO.

The enzyme solution contained 30 mg. enzyme dissolved in 4.4 ml. borate buffer pH 10.0

20°

pH 10.0

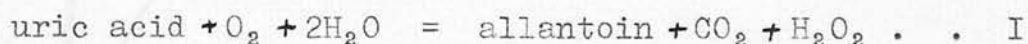
Light excluded.

	Manometers.		
	1.	2.	3.
Time after addition of lithium urate.	Main manometer vessel. 4.4 ml. enzyme solution 30 mg. enzyme.	→	4.4 ml. borate buffer pH 10.0
Minutes	Side bulb 0.4 ml. M/30 lithium urate	→	→
	Gas space 100% CO	100% Ar	100% CO
	$\mu\text{l. CO}$	$\mu\text{l. CO}$	$\mu\text{l. CO}$
10	$\pm 0.0$	+ 0.5	$\pm 0.0$
25	-1.0	$\pm 0.0$	$\pm 0.0$
50	-0.5	+ 0.5	$\pm 0.0$
170	-2.0	+ 0.5	$\pm 0.0$
260	-3.0	+ 0.5	-1.0
830	-8.0	+ 2.0	-6.5

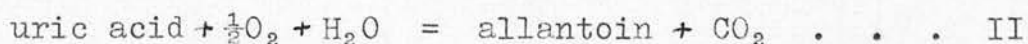


THE COURSE OF THE REACTION.

In the case of crude uricase, the oxygen uptake curve is a straight line. On the other hand, <sup>the</sup> oxygen uptake curve for purified uricase tends to fall off with time, even though the substrate concentration is kept up (Fig. VIII). It is probable that this is due to the poisoning of the now more labile and sensitive enzyme by products of the reaction, such, for example, as hydrogen peroxide. The production of hydrogen peroxide in the reaction can be readily demonstrated by using a suitable amount of substrate and allowing the reaction to proceed to completion. Using 1.12 mg. uric acid as substrate, and allowing the reaction to proceed till oxygen uptake has ceased, a matter of 60 minutes, then, if the equation for the reaction, is



the theoretical oxygen uptake for the reaction will be 149  $\mu$ l.  $\text{O}_2$  but, on the other hand, if the reaction is



the theoretical oxygen uptake will be only half this value, i.e. 74.5  $\mu$ l.  $\text{O}_2$  for the completed reaction.

The results of such an experiment are shown in

Fig. VIII

Course of the reaction with a purified preparation of uricase.

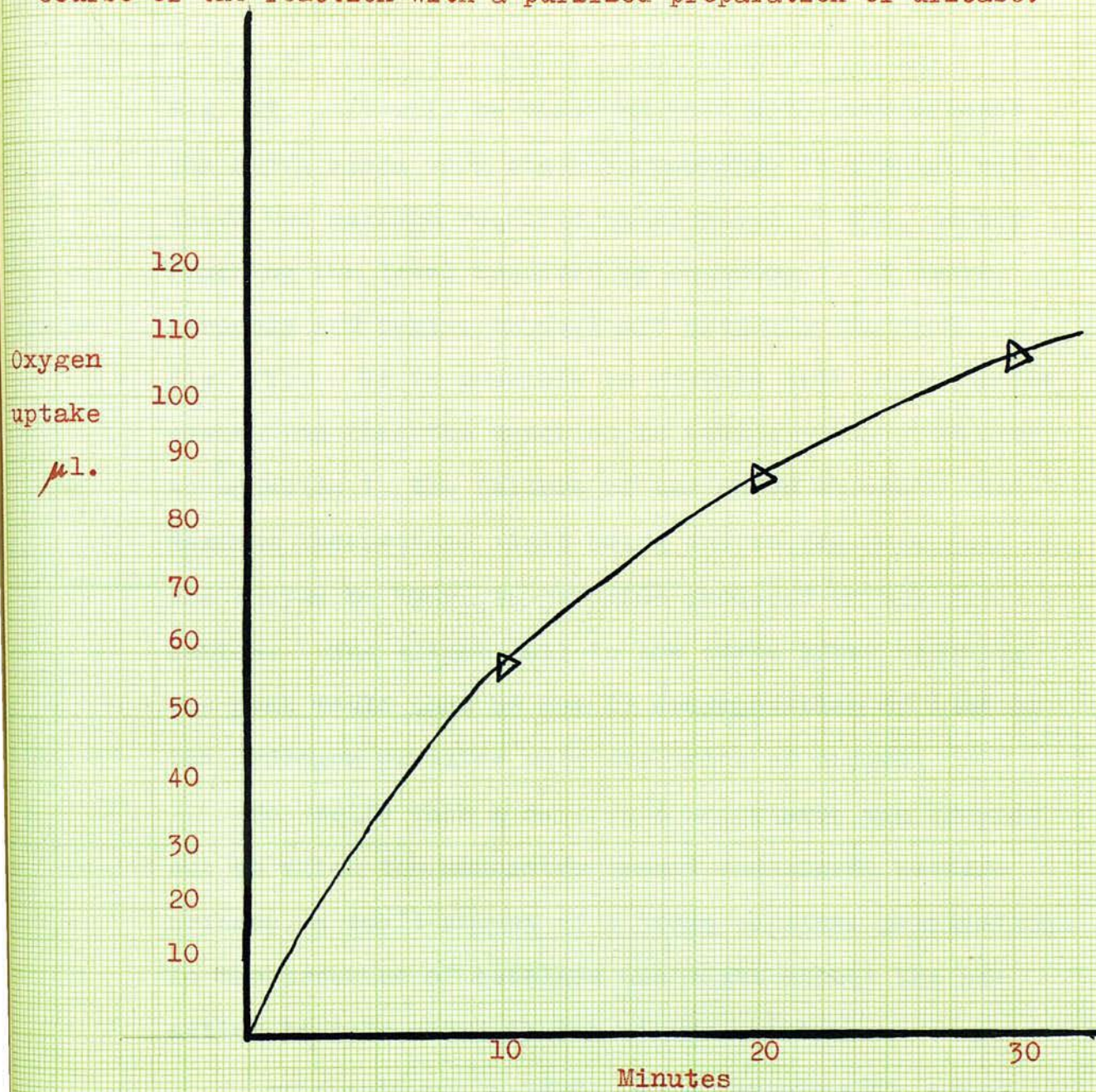
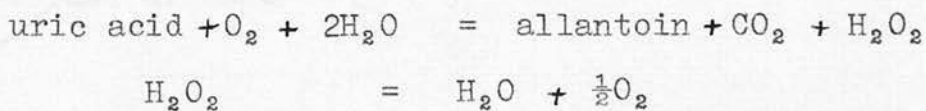


Fig. IX. It will be observed that with pure uricase the curve rises very steeply, ~~rapidly~~, to a point well above the maximum required by equation II but, before reaching the level required by equation I, it stops abruptly and begins to fall off slightly (Curve A, Fig. IX). It is possible to explain this curve on the assumption that the reaction is taking place according to equation I, the failure of the curve to reach the maximum theoretical limit being due to the poisoning of the enzyme by hydrogen peroxide produced in the reaction. The slight fall in the curve after reaching the peak would then be due to decomposition of some of the hydrogen peroxide with evolution of oxygen.

To verify this a duplicate experiment was carried out in which the manometer vessel contained, besides uricase, a small amount of a crude catalase preparation. In the presence of catalase one might expect the reaction to take place in two stages, thus:-



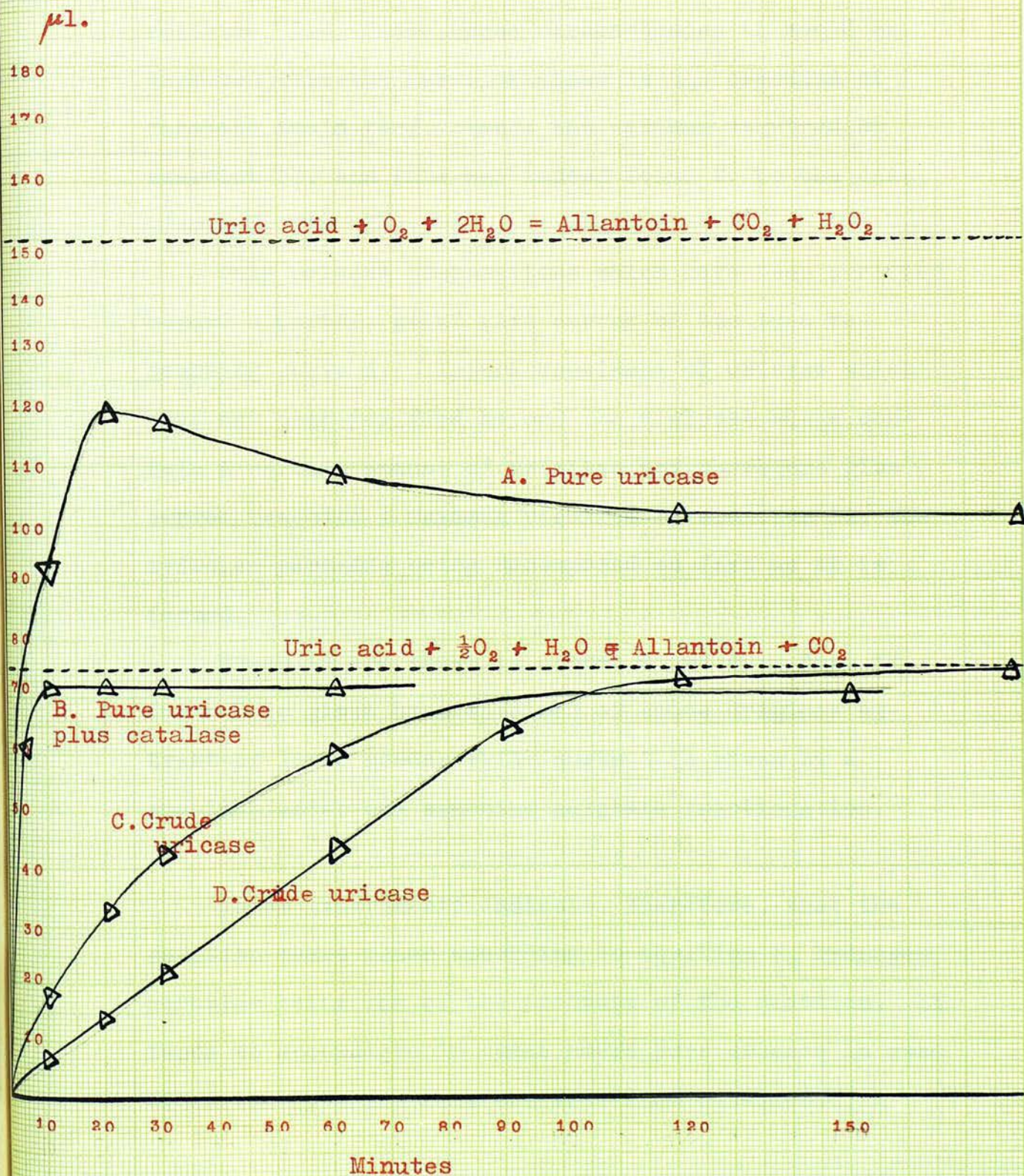
the hydrogen peroxide being decomposed by the catalase as quickly as it was produced.

The sum of these two reactions is, of course,

Fig. IX

Oxygen uptake  
 $\mu\text{l.}$ 

Course of the reaction.



reaction II. Under these circumstances it would be expected that the curve obtained in the presence of catalase would tend towards the maximum required by equation II, and this was indeed found to be the case, (Curve B, Fig. IX).

Only uricase preparations which have been purified beyond a certain point give curves of the type resembling curve A. Impure specimens of uricase which have not been purified beyond the dialysis stage contain a certain amount of catalase and therefore give curves reaching a maximum required by equation II, the hydrogen peroxide being decomposed as fast as it is formed.

Examples are shown in curves C and D in Fig. IX. Curve D is given by a specimen of crude original extract of liver powder while curve C is given by a specimen after one ammonium sulphate fractionation.

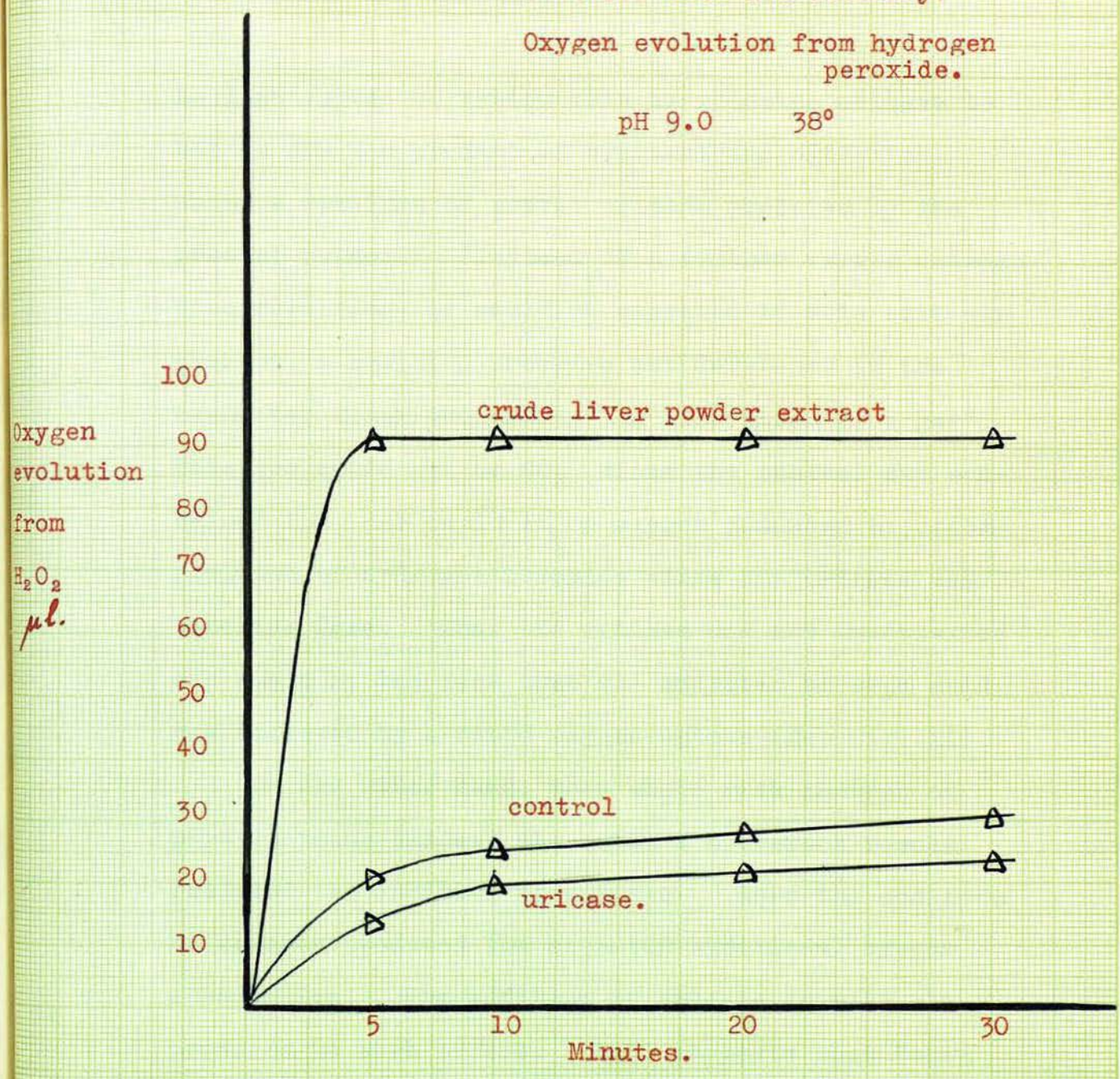
These experiments indicate, therefore, that the reaction takes place according to equation I, hydrogen peroxide being one of the products of the reaction. It tends to accumulate in cases where pure uricase is used since such preparations are free from catalase.

The absence of catalase activity in pure uricase preparations has also been demonstrated directly. Three manometers were used, each containing 1.0 ml. borate buffer pH 9 in the main chamber and 0.4 ml. 1/8% hydrogen peroxide in the side bulb. In addition, the first manometer contained some pure uricase, the second some catalase, while the third contained no enzyme. The manometers were shaken at 38° and the hydrogen peroxide tipped in from the side bulb after five minutes. The oxygen evolved was measured over a period of 30 min. The results are shown in Fig. X. It will be seen that in the vessel containing uricase no more oxygen is evolved than in the control vessel without enzyme. In the vessel containing catalase, oxygen evolution is very rapid. Uricase therefore in the pure state is free from catalase activity.

Arising out of these experiments, a point raised by E. G. Ball (1939) in connection with purine oxidation is worthy of mention. Ball, working in Warburg's laboratory, succeeded in isolating the enzyme xanthine oxidase in a highly purified state from milk. Xanthine oxidase is an enzyme which is responsible for the oxidation of xanthine and hypoxanthine to uric acid.

Fig. X

Test for catalase activity.  
Oxygen evolution from hydrogen peroxide.  
pH 9.0 38°



Ball attempted to verify this by allowing uricase to act on the end product of hypoxanthine oxidation, using a specimen of purest uricase prepared by the present author. "Instead of a further oxygen consumption resulting, as would be expected if uric acid was present, a positive pressure was developed in the manometer flask, indicating the liberation of a gas". This gas could not be  $\text{CO}_2$  since the central cup contained  $\text{KOH}$ , nor could it be oxygen produced by decomposition of hydrogen peroxide since the uricase was catalase free. That the uricase was not inactivated was shown by the fact that the addition of uric acid was followed by a rapid oxygen uptake (See Fig. XI).

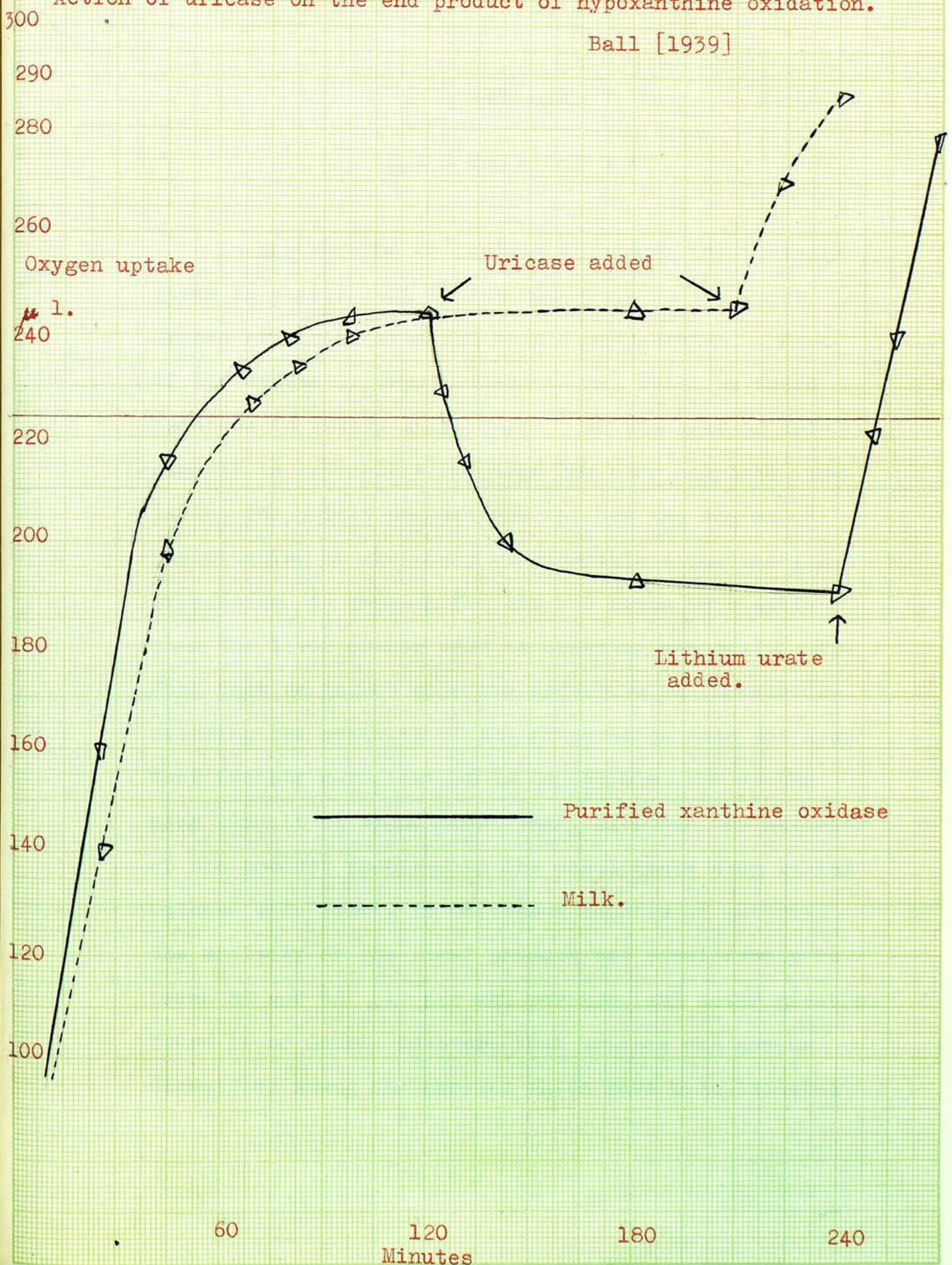
On the other hand, when whole milk instead of pure xanthine oxidase was used, a different result followed the addition of uricase. Here the addition of uricase produced the expected additional oxygen consumption. The addition of uricase to milk alone produced no such effect. Ball suggests that "when purified preparations of both xanthine oxidase and uricase are used, some factor is missing that is essential for the conversion of the end product of hypoxanthine oxidation into a substrate for uricase". Ball is investigating this point further.



Fig. XI

Action of uricase on the end product of hypoxanthine oxidation.

Ball [1939]



PART IV.D I S C U S S I O N.

In any attempt to isolate an enzyme the investigator is always left in some doubt as to the exact degree of purity attained by his preparation. Even if his enzyme is crystallised it may still be found possible to purify it further. In the case of uricase it is impossible to judge how near complete isolation the purest preparation is, but the degree of purification is obviously considerable since the final preparation is between 500 and 700 times more active than the liver powder used as starting material. The fact that no method used was effective in further increasing the activity of the preparation does not mean that further purification is impossible, but at the same time it is significant that, although the activity of the product produced in the penultimate step varied between wide limits in different batches of material, the activity of the final product was consistently between 85 and 90  $\mu$ l. per mg. per min. and never greater. There is no doubt, however, that uricase can now be classified among the enzymes which have been isolated

in a very pure state although not perhaps in absolute purity.

A clue to the degree of purity may be found in the iron content. If it is assumed that the iron is an integral part of the molecule then it is doubtful if much further purification would be possible since proteins containing a greater amount of heavy metal than 0.2% are seldom found. Crystalline catalase, for example, contains 0.11 - 0.15% of iron, while polyphenol oxidase contains 0.2% of copper.

Cytochrome, however, contains 0.34% of iron and hepatocuprein 0.34% of copper. Taking these figures as the upper limits for the metal content of a protein, then uricase might be considered to be at least 50% pure.

It must be emphasised that although uricase contains iron and appears to be an iron proteid, it has not been definitely proved that the iron functions as the active group of the enzyme. The final proof that this is so, would be the removal of iron with the production of an inactive protein and the restoration of activity on the addition of iron. This has not been found possible, nor has a stoichiometric experiment

with carbon monoxide given any clue as to the part played by iron. At the same time, when it is considered that uricase, owing to its inhibition by cyanide, is apparently a metal complex, it is very tempting to assume that such a large amount of iron as 0.15 - 0.20% is not without significance and that it is indeed the active group.

There are objections to the theory that iron is present simply as an impurity. In the first place it is bound so firmly to the protein that its presence as a casual contaminant is extremely improbable, while if the impurity in uricase were an iron protein compound then the iron content of such a compound must obviously be much higher than 0.2% - a very unlikely eventuality.

On the other hand there is evidence that iron may not be the active group. Although uricase is inhibited by cyanide and is therefore presumably a heavy metal compound, nevertheless it is not inhibited by other substances which combine with heavy metals, and in particular it is not inhibited by those compounds which are known to combine with iron.

Until iron has actually been reversibly removed

from uricase, the question cannot be regarded as being definitely settled.

In a short note some months ago, Holmberg (1939) claims to have purified uricase 1000 - 1400 times (i.e. further than the present author) and to have obtained a product containing much less iron but no experimental details have been given nor has the note been followed up by a more detailed publication. In the absence of experimental data it is impossible to judge Holmberg's claims fully.

There are certain points of resemblance between uricase and catalase. Catalase, as shown by Sumner and Dounce (1939) also contains iron, part of the iron being present in the form of haemin and part in the form of an unknown complex. Catalase, like uricase, contains a mere trace of copper.

All attempts by Sumner and Dounce to remove iron reversibly from catalase have failed.

If it is assumed that the activity of uricase is due to the iron, then for a specific activity of  $85 \mu\text{l. per mg. per min.}$  1 mg. enzyme containing  $15 \mu\text{g. Fe}$  will bring about the reaction of  $85 \mu\text{l.}$  of oxygen per minute, or 1 mg. Fe will bring about the reaction

of 57,000  $\mu\text{l.}$  oxygen per minute.

Again for a specific activity of 85  $\mu\text{l.}$  per mg. per minute 1 mg. enzyme catalyses the transfer of 85  $\mu\text{l.}$   $\text{O}_2$  per minute or  $3.8 \times 10^{-6}$  moles oxygen per minute. But this 1 mg. enzyme contains 0.0015 mg. Fe or  $2.7 \times 10^{-8}$  moles Fe. Therefore 1 atom iron catalyses the utilisation of 140 molecules of oxygen per minute.

If we assume that the enzyme is 100% pure and that its molecular weight is 70,000 (the value for the yellow enzyme) then for a specific activity of 85  $\mu\text{l.}$  per mg. per min.,

1 mg. enzyme, i.e.  $\frac{1}{70,000} \times 10^{-3}$  moles, catalyses the utilisation of 85  $\mu\text{l.}$   $\text{O}_2$  i.e.  $\frac{85}{22400 \times 10^{-3}}$  moles oxygen per minute. Therefore one molecule of enzyme catalyses the utilisation of 260 molecules oxygen per minute.

These figures are all quite reasonable when compared with those for other enzymes.

S U M M A R Y.

A method has been evolved whereby the enzyme uricase can be isolated from pig liver, and a preparation 500 - 700 times more active than the starting material has been obtained. The purest preparation has a specific activity of 85 - 90  $\mu$ l. per mg. per min. compared with 0.12 - 0.15  $\mu$ l. per mg. per min. for the dry liver powder used as starting material.

The pure enzyme is a white protein, insoluble in water, almost insoluble in phosphate buffer pH 7.4 but soluble in alkaline solutions such as borate buffer pH 10. Solutions of the enzyme are almost colourless. The enzyme contains 0.15 - 0.20% iron, a mere trace of copper, no cobalt or manganese, and 14.4% of nitrogen.

The activity of the enzyme is retained for several weeks when it is preserved in the form of a solution in borate buffer at 0° but the free protein loses its activity more rapidly. The enzyme cannot be dried without great loss of activity.

The velocity of the enzyme action is proportional to the oxygen pressure being only 7% as great in a

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mixture of 2% oxygen and 98% argon as in 100% oxygen. When argon is replaced by carbon monoxide no inhibition occurs.

On the other hand the enzyme is completely inhibited by cyanides in a concentration as low as  $M/20,000$ .

Inhibition by cyanide suggests that the enzyme is a heavy metal compound and the possibility exists that iron is the active group of the enzyme although this has not been conclusively proved.

The iron appears to be tightly bound to the protein and all attempts to remove it have failed.

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## CLXXXIV. THE PURIFICATION OF URICASE

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In the course of an attempt to isolate the enzyme uricase, which oxidizes uric acid to allantoin, it has been found possible to obtain a preparation 550 times purer than the liver powder which is used as starting material [Davidson, 1938]. The purest preparation contains 0.15–0.20 % Fe, a trace of Cu, no Co or Mn and 14.4 % N. Small quantities are colourless, and even very large quantities have only a very pale brown colour. It is therefore not a haemin derivative. It is quite insoluble in water, almost insoluble in phosphate buffer *pH* 7.4 but soluble in alkaline solution, e.g. borate buffer *pH* 10. Truszkowski [1934] and Ro [1931] have already shown that uricase is soluble in alkaline solution.

In order to determine the enzymic activity a manometric test has been developed. The main chamber of the manometer vessel contains 1.0 ml. *M*/5 borate buffer *pH* 9 and 1.5 ml. enzyme preparation plus water. The potash tube contains 0.2 ml. 10 % KOH, and the side bulb 2.24 mg. uric acid as *M*/30 lithium urate. (0.56 g. uric acid is dissolved in 35 ml. boiling *N*/10 lithium hydroxide and the solution is made up to 100 ml. with water. This solution must be freshly prepared each day.) The gas space contains pure oxygen and the oxygen uptake is measured at 38° over a period of 30 min.

By the specific activity of the enzyme is meant the number of  $\mu\text{l. O}_2$  which are taken up per min. in the first 30 min. by 1 mg. of dry enzyme preparation. The specific activity of the best preparation is 85–90  $\mu\text{l.}$  per mg. per min. as compared with a value of 0.12–0.15  $\mu\text{l.}$  per mg. per min. for the dry liver powder from which it is obtained.

The most convenient starting material has been found to be pig's liver, which has already been used by Kleinmann & Bork [1933] and by Keilin & Hartree [1936] for uricase studies. The pig's liver is finely minced and ground in a mortar with five parts of acetone. The solid material is centrifuged off and shaken with three parts of acetone. After centrifuging, the liver powder is dried at first rapidly in a current of air and then overnight in vacuum desiccators, and is then finely powdered and passed through a sieve. The resulting light brown powder has a specific activity of 0.12–0.15  $\mu\text{l.}$  per mg. per min. and retains its activity for several months when stored in desiccators at room temperature. Samples showing a specific activity of less than 0.10  $\mu\text{l.}$  per mg. per min. are discarded.

250 g. liver powder are stirred with 2.5 l. ice-cold *M*/10 phosphate buffer *pH* 7.4. After standing for 20 min. at 0° the mixture is centrifuged, and the extract, which contains much protein but only little uricase is discarded. The solid residue is then stirred with 5 l. *M*/10 borate buffer *pH* 10 at 38°. After 20 min. the mixture is rapidly cooled to 0° and centrifuged. The clear extract contains the bulk of the enzyme and has a specific activity of 0.5  $\mu\text{l.}$  per mg. per min.

An equal volume of saturated ice-cold ammonium sulphate solution is added to this solution, and the resulting precipitate, which contains the enzyme, is centrifuged off at 0° and dissolved with shaking in 5 l. ice-cold distilled water.

1/10 vol. saturated ice-cold ammonium sulphate is then added (no precipitate being formed), and the solution, which has a  $pH$  of 7.2-7.4, is heated to  $55^{\circ}$  for 5 min. with vigorous stirring, whereby a heavy flocculent precipitate of denatured protein is formed. The solution is rapidly cooled to  $0^{\circ}$  and the precipitate is centrifuged off and discarded. The enzyme solution, which now has a specific activity of 1.0-1.5  $\mu$ l. per mg. per min., is treated with enough saturated ammonium sulphate solution at  $0^{\circ}$  to bring the degree of saturation to 0.5. The resulting precipitate, which contains the enzyme, is centrifuged off at  $0^{\circ}$  and dissolved in 1 l. of ice-cold distilled water. This solution is dialysed for 15 hr. in cellophane sacs against running distilled water at  $0^{\circ}$ . A brown precipitate which appears during the dialysis and which contains the enzyme is centrifuged off and washed with ice-cold water on the centrifuge. It is then rubbed in a cooled mortar with 400 ml. *M/10* phosphate buffer  $pH$  7.4 at  $0^{\circ}$  and centrifuged on the high speed centrifuge (15,000 r.p.m.). The extract, which contains much protein but little enzyme, is discarded. The residue is rubbed in a mortar with 250 ml. *M/10* borate buffer  $pH$  10 at room temperature and is centrifuged on the high speed centrifuge. The extraction with 250 ml. borate buffer is repeated, the extracts, which contain the bulk of the enzyme, are combined, and the residue, which consists of brown insoluble protein, is discarded.

The clear, pale yellow uricase solution which now has a specific activity of 10-25  $\mu$ l. per mg. per min. is cooled to  $0^{\circ}$ , treated with an equal volume of saturated ice-cold ammonium sulphate solution, and the resulting precipitate, which contains the enzyme, is centrifuged off at  $0^{\circ}$  and dissolved in 60 ml. ice-cold water.

To the solution just enough (0.5-1.0 vol.) ice-cold 80% acetone is added so that a flocculent precipitate appears after 20 min. The precipitate is centrifuged off and discarded. Acetone is removed from the supernatant fluid by vacuum distillation at  $20^{\circ}$  with two drops octyl alcohol and the resulting slightly turbid solution, after standing overnight at  $0^{\circ}$ , is centrifuged on the high speed centrifuge. A small precipitate of very high activity is thus obtained, and is washed three times on the centrifuge with ice-cold distilled water. It is then rubbed up with 5 ml. *M/10* borate buffer  $pH$  10 at room temperature, in which the enzyme dissolves, leaving a small inactive brownish residue which is centrifuged off at high speed and discarded.

The uricase solution is almost colourless. When it is dialysed against running distilled water at  $0^{\circ}$  the free enzyme is obtained. Specific activity 85-90  $\mu$ l. per mg. per min. The yield of the purest preparation is about 5 mg. per 100 g. liver powder.

On standing the activity of the free enzyme gradually diminishes but in solution at  $pH$  10 it is stable for several weeks. Some of the enzyme frequently precipitates out in an insoluble form. The enzyme cannot be dried without great loss of activity.

The velocity of the test reaction is dependent on the oxygen pressure and in a mixture of 2% oxygen and 98% argon it is only 7% as great as 100% oxygen. If the argon is replaced by carbon monoxide no inhibition by the carbon monoxide is found.

Very small quantities of cyanide, however, inhibit the reaction reversibly, as has been already pointed out by Keilin & Hartree [1936] and by Truszkowski [1930]. The enzyme therefore would appear to be a heavy metal compound, but it has nevertheless not yet been proved that the catalytic activity of the enzyme is due to the iron. Attempts to remove iron by dialysis with cyanide after the manner in which Kubowitz [1938] removed copper from phenoloxidase, and by

other methods, have not so far been successful. The colourless nature of the enzyme suggests that the iron is not present in the form of a haemin derivative. On the other hand the comparatively large amount of iron (of the same order as that found by Sumner & Dounce [1937] in crystalline catalase) and the consistency with which the value 0.15–0.20 % is found in repeated preparations, suggests that the iron is more than a mere impurity.

If it is assumed that the catalytic activity of the enzyme is due to the iron, then 1 mg. iron brings about the reaction of 57,000  $\mu$ l. oxygen per min.

#### SUMMARY

A method for the purification of the enzyme uricase is described.

The purest preparation has a specific activity, under stated conditions, of 85–90  $\mu$ l. per mg. per min. and contains 0.15–0.20 % Fe.

Although uricase seems to be a heavy metal compound it has not yet been proved that the iron acts as the active group of the enzyme.

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