ENZYME STUDIES IN THE TRICARBOXYLIC ACID CYCLE

The Purification and Properties of Aconitase

A THESIS

submitted for the degree of

DOCTOR of PHILOSOPHY

by

JOHN FRANCIS MORRISON

Trinity College

November, 1953.

A B S T R A C T

The purpose of this investigation was to obtain a highly purified preparation of aconitase and to study the properties of the purified enzyme. The thesis describes

(I) The method of purification

(II) The activation of aconitase by Fe^{2+} and reducing agents

(III) The effect of pH on the enzyme activity

(IV) A study of the kinetics of the enzyme

(V) The effect of fluorocitric acid on the enzyme activity

(VI) The effect of other inhibitors on the enzyme activity.

The results may be briefly summarised as follows:

A preparation of aconitase with a higher specific activity than previously reported has been obtained. The preparation was stable. Electrophoretic analysis showed that the aconitase protein formed 75-80% of the

total protein present. The results obtained during the fractionation were consistent with the idea that aconitase activity is associated with a single species of protein molecule. The final enzyme preparation possessed little activity in the absence of Fe^{2+} and a reducing agent. The activity could be increased 70-fold by the presence of these substances. The results indicate that the active form of the enzyme is an enzyme-Fe^{2+}-activator complex.

From a study of the effects of pH on the reactions catalysed by aconitase and of the reaction kinetics, it was concluded that the same catalytic centre of the aconitase protein is concerned in all the reactions.

Aconitase was inhibited in a competitive fashion by 'natural' fluorocitric acid, whilst synthetic fluorocitric acid inhibited it competitively and irreversibly. Whereas p-chloromercuribenzoate inhibited aconitase in the presence of substrate, o-phenanthroline, 2:2'-dipyridyl and versene inhibited only in the absence of substrate. Cyanide and azide did not inhibit the enzyme.

A C K N O W L E D G E M E N T S

It is a pleasure to express my sincere thanks to my supervisor, Professor Sir Rudolph Peters, F.R.S., for his constant interest and advice during the course of this work. I am also indebted to many members of the Department of Biochemistry for advice. In particular, I would like to thank Drs. R.B. Fisher, C.H. Elliott and R. Cecil who devoted much of their time to discussion of various aspects of enzyme action and made many helpful suggestions. Dr. Fisher also kindly carried out the statistical analyses.

The skilful technical assistance of Miss B. Read during the latter half of the work helped considerably. I would also like to thank Dr. E.O. Field for carrying out the electrophoretic analyses and Mr. K.W. Wakelin for practical advice. Finally, I would like to express my thanks to Pamela Morrison for her assistance in many ways, particularly for indexing and checking the references.

The work reported in this thesis was carried out during the tenure of an Australian National University scholarship.

TABLE of CONTENTS

P R E F A C E

Throughout this dissertation, the substrates of aconitase are referred to as acids. This is done not only because it is more convenient to write the structural formulae in the acid form, but also because at the pH values used, the substrates are not completely ionised. However, the substrates were added to the test systems as neutral sodium salts. DL-*iso*citric acid was used throughout, but the concentrations of this acid are expressed in terms of the *D*-stereoisomer.

The work is presented in the order which seemed most suitable and not in the order in which it was carried out. As a result, it will be found in some instances that the experiments designed to determine certain conditions are reported after those conditions have been used. For example, in Chapter II the relationship between the enzyme activity and the Fe^{2+} concentration was determined in phosphate buffer at pH 7.7, although the pH optimum of the enzyme in this buffer is reported in Chapter III.

The reaction rates were determined at 30° during the early part of the work when the formation of citric acid was estimated. As the formation of cis-aconitic acid was estimated in a Beckman spectrophotometer which was not fitted with a constant temperature device, the reactions were studied at a room temperature of 22°. This temperature was maintained for the later experiments.

A paper dealing with the purification of aconitase has been accepted by the Board of Editors for publication in the Biochemical Journal.

The tables and figures have been included on separate pages. A particular table or figure follows the page on which reference to it has been made.

GENERAL INTRODUCTION

(a) Historical

(b) Properties of aconitase

GENERAL INTRODUCTION

HISTORICAL

Oxidation of citric acid

Thunberg (1911) and Batelli & Stern (1910) discovered an enzyme in animal tissues which was capable of oxidising citric acid. Later work by Thunberg (1929) showed that this enzyme was widely distributed, occurring in moulds, yeasts, cucumber seeds and bacteria as well as in animal tissues. Bernheim (1928) prepared solutions of the enzyme from acetone powders of liver and showed that whereas the enzyme was capable of oxidising citric acid in the presence of methylene blue, as indicated by the reduction of this compound under anaerobic conditions, it could not directly oxidise citric acid in the presence of gaseous oxygen. He named the enzyme citric dehydrase or citric dehydrogenase. Bernheim did not identify the products of the oxidation of citric acid.

The early investigators suggested that the oxidation of citric acid may involve oxidation of the hydroxy

group to produce acetone dicarboxylic acid. Butterworth & Walker (1929) suggested that this reaction occurred in Aspergillus niger and Pseudomonas pyocyanea, but this idea has been questioned by Deffner (1938). Langecker (1934) and Müller (1935) showed that this reaction did not occur in animal tissues.

A significant advance in the elucidation of the mechanism of citric acid oxidation was made by Wagner-Jauregg & Rauen (1936). They found that 0.5 mole of oxygen was used and one mole of CO_2 was produced per mole of citric acid oxidised by the citric dehydrogenase of cucumber seeds. Furthermore, isocitric acid was oxidised at least as rapidly as citric acid. These workers failed to identify the products of the oxidation. It was Martius & Knoop (1937) and Martius (1937) who showed that the oxidation of citric acid gave rise to α-ketoglutaric acid which they identified by isolation. This result was obtained using a citric dehydrogenase preparation from liver. Krebs & Johnson (1937) found that the same reaction occurred in muscle.

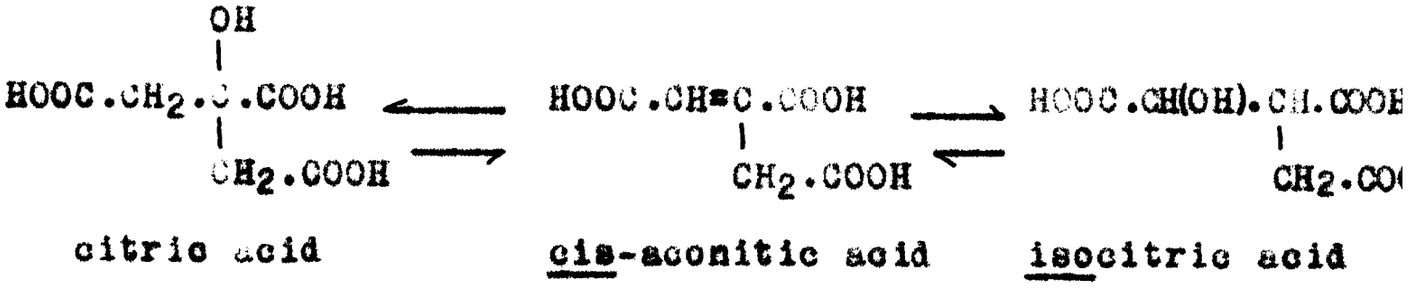
Discovery of aconitase

The means by which citric acid was converted to α-ketoglutaric acid was not immediately obvious. Whereas the ketonic oxygen of α-ketoglutaric acid was

in the α position, citric acid contained a β-oxygen atom, so that the direct oxidation of citric acid seemed improbable. The demonstration of Martius & Knoop (1937) of a new enzyme which catalysed the interconversion of citric, cis-aconitic and isocitric acids enabled the mechanism of the reaction to be made clear. Citric acid was firstly converted to isocitric acid which was then oxidised to α-ketoglutaric acid.

Breusch (1937) showed that, under anaerobic conditions, suspensions of pigeon breast muscle were able to convert both cis-aconitic acid and isocitric to citric acid and also . that added citric acid disappeared from the medium. He proposed the name aconitase for the enzyme(s) responsible for these reactions. Further evidence for the above reactions was provided by Martius (1938). He showed that enzyme preparations from a number of different sources were able to convert both cis-aconitic and isocitric acids to citric acid, while D-isocitric acid was formed from both cis-aconitic and citric acids. It was concluded that D-isocitric acid was concerned in the reaction as on the addition of synthetic DL-isocitric acid, about half of the isocitric acid remained unchanged as the L-stereoisomer.

This work established that citric dehydrogenase was, in fact, a mixture of two enzymes. Aconitase was responsible for the conversion of citric acid to isocitric acid, which was then oxidised to α-ketoglutaric acid by means of a second enzyme now known as isocitric dehydrogenase. Aconitase can be defined as the enzyme responsible for catalysing the equilibrium between citric, cis-aconitic and isocitric acids as shown below:



citric acid cis-aconitic acid isocitric acid

Characterisation of aconitase

In so far as the reaction involves a reversible hydration of a double bond, aconitase is analagous to fumarase. Martius (1937) originally thought that the two enzymes may be identical, despite the fact that the configuration of the double bond of the substrates differ in the two cases. In the case of cis-aconitic acid it is cis and in the case of fumaric acid it is trans. Experimental evidence soon came to light to show that the two enzymes were distinct. Jacobsohn & Tapadinhas (1940)

found that extracts of the leaves of the medlar hydrate cis-aconitic acid, but not fumaric acid and Jacobsohn et al. (1940) showed that whereas fluoride inhibited aconitase, it did not inhibit fumarase. On the other hand, iodoacetate had little effect on aconitase, but rapidly inhibited fumarase. Krebs & Eggleston (1944) found that the aconitase activity of aqueous muscle extracts fell rapidly on storage, whilst the fumarase activity remained unchanged. The most conclusive evidence that aconitase and fumarase are distinct enzymes comes from the work of Massey (1952). He showed that a crystalline, electrophoretically homogeneous preparation of fumarase did not attack cis-aconitic acid even when the enzyme was used in high concentrations.

The postulated "aconitases"

Jacobsohn & Soares (1940) and Jacobsohn et al. (1940) have proposed that two different aconitases are responsible for the conversion of cis-aconitic acid to citric and isocitric acids. They claimed that an α-aconitase converted cis-aconitic acid to isocitric acid (isocitric acid being an α-hydroxy acid) and a β-aconitase converted cis-aconitic acid to citric acid (citric acid being a β-hydroxy acid). The evidence

supporting this view was based on the findings that the relative rates of the two reactions varied in different animal and plant tissues. However, these authors were not able to separate the two activities, nor were they able to show any differential inhibition of the two reactions. Buchanan & Anfinsen (1949) found that the ratio of the two activities did not alter during the course of the purification of aconitase and Friedrich-Freska & Martius (1951) concluded, as a result of a mathematical treatment of the reaction kinetics, that only one enzyme was concerned in the establishment of the equilibrium. Ogston (1951) has also advanced theoretical arguments in favour of the idea that cis-aconitic acid can be converted to both isocitric acid and citric acid by a single enzyme. There is little evidence in favour of aconitase being two enzymes, but final proof that aconitase is a single enzyme must await isolation of the enzyme as an electrophoretically homogeneous protein. This question will be discussed further in those sections dealing with this aspect of aconitase activity.

Biological role of aconitase

Aconitase forms an essential link in the chain of enzymes catalysing the reactions of the well-established

Tricarboxylic acid cycle (Krebs, 1943). As this cycle is so well known, all the reactions will not be elaborated here. The discussion will be confined to those responsible for the formation and initial metabolism of citric acid, as it is these reactions which are concerned with the role played by aconitase in cellular metabolism.

It has now been established that the cycle commences with the condensation of oxalacetic acid and acetyl-coenzyme A to form citric acid, but during the years from 1937, modifications of the cycle have been postulated. Originally it was believed that pyruvic acid was the compound which condensed with oxalacetic acid, but later work indicated that an undefined derivative of pyruvic acid, known as "active acetate", was concerned. Lynen & Reichert (1951) and Lynen et al. (1951) showed that the "active acetate" was actually acetyl-coenzyme A. Krebs (1943) arbitrarily chose citric acid as the product of the condensation, although he made it clear that cis-aconitic acid could well be the product formed. In vitro experiments did not offer a solution of the problem. Krebs tended to the view that the reaction citric acid ⟶ cis-aconitic acid should occupy a place in the normal metabolism of muscle, rather than be confined to a side reaction as would be the case if

cis-aconitic acid were formed.

The isotopic experiments of Wood et al. (1941) and of Evans & Slotin (1941) suggested that cis-aconitic acid, rather than citric acid, was formed as a result of the condensation reaction. These workers found that oxalacetic acid, which contained isotopic carbon in the carboxyl group adjacent to the methylene group, gave rise to α-ketoglutaric acid which contained the isotopic carbon only in the carboxyl group adjacent to the carbonyl group. It was reasoned that if citric acid, which is a symmetrical molecule, were the direct product of the condensation, the isotopic carbon should have been found in both carboxyl groups. Ogston (1948) pointed out that it is possible for a symmetrical molecule to act in an asymmetric fashion if the linkage of the substrate to the enzyme involves a three point attachment. In fact, a three point attachment is necessary wherever a single optical antipode is formed enzymically from an inactive precursor. Under these conditions, the enzyme can distinguish identical groups of a symmetrical molecule so that the asymmetric occurrence of an isotope in a product could not be taken as conclusive evidence against its arising from a symmetrical precursor.

The hypothesis of Ogston (1948) was confirmed experimentally by Potter & Heidelberger (1949). These workers isolated labelled citric acid formed by a rat liver homogenate from oxalacetic acid, pyruvic acid and $^{14}CO_2$, and converted it enzymically to α-ketoglutaric acid. They found that all the ^{14}C was present in the carboxyl group of α-ketoglutaric acid adjacent to the carbonyl group. This meant that the citric acid was labelled in only one of the two 'identical' carboxyl groups and that the enzymes responsible for the further oxidation of citric acid attacked the molecule in an asymmetric fashion. The work of Stern & Ochoa (1949) and Ochoa et al. (1951) proved that citric acid was the product of the condensation reaction. These workers obtained a preparation of the enzyme responsible for the condensation reaction which was free from aconitase and showed that citric acid was the product formed. The enzyme was named the condensing enzyme.

Although the hypothesis of Krebs (1943) was accepted as the means whereby pyruvate was oxidised in vitro, until recently there was doubt as to whether the Tricarboxylic Acid Cycle functioned in vivo. Orten & Smith (1936) found that the intravenous injection

into dogs or rats of certain metabolites, chiefly malic, fumaric and succinic acids, greatly increased the urinary citric acid which they believed was formed in the kidney. These experiments were confirmed by Krebs, Salvin & Johnson (1938) and accepted by these authors as proof for the occurrence of the Tricarboxylic Acid Cycle in the living organism. However, Carteneson (1940) showed that the citric acid excreted as a result of the injection of malic acid was not due to the formation of citric acid from malic acid, but rather to the inhibition by malic acid of the normal breakdown of citric acid formed in the kidney. Peters (1952) found that fluoro-citric acid caused the accumulation of citric acid *in vivo*, whilst Peters & Wilson (1952) showed that fluoro-citric acid inhibited aconitase. These experiments provided positive evidence in favour of the idea that the Tricarboxylic Acid Cycle functions *in vivo*.

The significant function of aconitase in cellular metabolism is to convert citric acid to isocitric acid which is oxidised to α-ketoglutaric acid. α-Ketoglutaric acid is then oxidised still further by the other reactions of the Tricarboxylic Acid Cycle.

Distribution of aconitase

Aconitase is widely distributed in nature. The

enzyme has been found in all animal tissues examined, high concentrations being found in muscle, kidney and prostate. It has also been reported to occur in a number of plant tissues, yeasts and bacteria. For references see the review by Ochoa (1951).

PROPERTIES of ACONITASE

In order to complete the survey of the known facts concerning aconitase, a brief report of the properties of the enzyme is given at this stage. But these properties, with the exception of the work concerning the equilibrium data, will be discussed in more detail in the appropriate sections of this work.

Stability of aconitase

The instability of aconitase in aqueous solution has been reported by a number of investigators (Krebs & Eggleston, 1944; Ochoa, 1948; Buchanan & Anfinsen, 1949 and Peters & Wilson, 1952) and various attempts have been made to stabilize the enzyme. On the other hand, the enzyme is stable in frozen tissues (Buchanan & Anfinsen, 1949). It has also been found that dialysis of solutions of the enzyme against water and salt solutions causes rapid inactivation (Buchanan & Anfinsen, 1949). Dickman & Cloutier (1951) showed that Fe^{2+} and cysteine

both stabilised and activated crude and partially purified solutions of aconitase. These agents were also effective in re-activating crude preparations of aconitase which had been subjected to dialysis. The results suggested that the loss of enzyme activity was associated with the loss of Fe^{2+}, so the instability of the apoenzyme was more apparent than real.

Substrate specificity

Jacobsohn & Soares (1939) showed that aconitase did not hydrate citraconic (methyl-maleic) acid, while Bernheim (1928) found that aconitase did not oxidise aconitic acid (presumably the trans isomer). In fact, this acid caused inhibition of the oxidation of citric acid.

Equilibrium and rates of the aconitase reactions

Martius & Leonhardt (1943) found that the equilibrium of the aconitase system was 89.2% citric acid, 3.1% cis-aconitic acid and 7.7% isocitric acid at pH 7.4 and 37°. Krebs (1943) reported the equilibrium to be 89.5% citric acid, 4.3% cis-aconitic acid and 6.2% isocitric acid at pH 6.8 and 38°. Whilst Martius & Leonhardt estimated the cis-aconitic acid by difference, Krebs estimated it by the method of Johnson (1939). Krebs (1953) found that at 25° and pH 7.4, the equilibrium

mixture contained 90.9% citric acid, 2.90% cis-aconitic acid and 6.20% isocitric acid. The corresponding value for isocitric acid at 38° was found by Aggeston & Breus (1949) to be 6.6%. Friedrich-Freska & Martius (1951) assumed for their mathematical calculations that the equilibrium values were 88% citric acid, 5% cis-aconitic acid and 7% isocitric acid. Although the equilibrium values obtained by various workers differ to some extent, it is clear that the equilibrium lies predominantly in favour of citric acid.

Friedrich-Freska & Martius (1951) showed that the rates of the individual reactions responsible for the attainment of the equilibrium vary greatly. The rates at which cis-aconitic acid is converted to isocitric acid and citric acid are somewhat similar, but the rate of the reaction citric acid ⟶ cis-aconitic acid is much slower than that of the reaction isocitric acid ⟶ cis-aconitic acid. The result is that starting with cis-aconitic acid, isocitric acid reaches a maximum value of 50% and only then falls to the equilibrium value. When isocitric acid is used as the starting material, cis-aconitic acid reaches a maximum value of 15%.

Effect of pH on aconitase activity

Jacobsohn (1941) found that the optimum pH value of aconitase activity was in the neutral region. Johnson (1939) reported that the aconitase of crude tissue extracts showed a sharp maximum at pH 7.4, whereas with kidney slices, the pH optimum was 7.9.

Michaelis constants of aconitase

Racker (1950) found that the Michaelis constant of aconitase for citric acid was 1.1×10^{-3} M and for isocitric acid was 4×10^{-4} M. The Michaelis constant of aconitase for cis-aconitic acid was not determined.

Inhibition of aconitase

Krebs & Eggleston (1944) reported that aconitase was inhibited by Cu^{2+} ions, $HgCl_2$, HCN and sodium sulphide in relatively low concentrations. Higher concentrations of NaCl, pyrophosphate and alloxan also inhibited the enzyme. Dickman & Cloutier (1951) showed that aconitase was inhibited by dilute solutions of o-phenanthroline and 2:2'-dipyridyl and that the enzyme could be re-activated by the subsequent additions of Fe^{2+}.

Two competitive inhibitors of aconitase have been found. Bernheim (1928) and Saffran & Prado (1949) found that the inhibition of aconitase by trans-aconitic acid

was primarily competitive and the preliminary results of Peters & Wilson (1952) indicated that fluorocitric acid inhibited the enzyme in a competitive fashion.

C H A P T E R I

THE PURIFICATION of ACONITASE

CHAPTER I

THE PURIFICATION of ACONITASE

INTRODUCTION

Although aconitase has been known for some sixteen years, it has not been isolated in the pure state. Ochoa (1948) was able to concentrate the enzyme by ammonium sulphate fractionation, but there was no increase in the specific activity and no further attempts were made to purify it. Buchanan & Anfinsen (1949) obtained a 23-fold purification of aconitase by low temperature ethanol and ammonium sulphate fractionation. Electrophoretic analysis showed that the preparation consisted of "three non-homogeneous components" and the purity of the enzyme was estimated to be 30%.

The purification of aconitase has been greatly hampered by its apparent instability. Krebs & Eggleston (1944) found that glycerol stabilized crude enzyme extracts, but Buchanan & Anfinsen (1949) reported that

glycerol was without effect in stabilizing purified preparations. They also found that whilst cysteine stabilized the crude enzyme, it strongly inhibited the purified enzyme. Citric or cis-aconitic acid were found to be the most effective stabilizers of aconitase.

An important finding for the further study of the purification of aconitase was made by Dickman & Cloutier (1950). They found that crude solutions of aconitase could be both stabilized and activated by the addition of Fe^{2+} and cysteine. Further work (Dickman & Cloutier, 1951) showed that these agents were also effective in stabilizing and activating more highly purified solutions of the enzyme. Fe^{2+} was the only cation which gave consistent activation of aconitase. Ascorbic acid was as effective as cysteine as a reducing agent, whilst glutathione was only one half as effective.

An attempt was made to purify the enzyme further in the light of the findings of Dickman & Cloutier (1950, 1951).

EXPERIMENTAL

Citric acid estimation

The method of Lucher, Sherman & Vickery (1936) as modified by Buffa & Peters (1949) was used. Samples

ranging in volume from 1.0 to 3.0 ml. and containing up to 200 µg. of citric acid were used; within this range the colour given by the method was proportional to the amount of citric acid present.

Protein estimation

The protein concentration of aconitase solutions was determined according to Kalckar (1947) by measurement of the absorption in the Beckman spectrophotometer at a wavelength of 280 mµ after the appropriate dilution with 0.1 N NaOH. Under these conditions, a solution containing 1 mg. of protein/ml. was found to have an absorption of 1.5.

Activation of aconitase

A suitable sample of the enzyme solution was measured into a test tube and placed in an ice-bath. Non-neutralised ferrous ammonium sulphate and neutralised cysteine were added, so that on dilution of the solution with cold water to 10 ml., the final concentration of Fe^{2+} was 5×10^{-4} M and that of cysteine was 10^{-2} M. The mixture was then neutralised with N NaOH to pH 7.4 using a glass electrode and incubated in the ice-bath for 1 hr. before the aconitase activity was determined. Except where otherwise stated, the various enzyme

fractions were treated in this fashion before an activity determination was carried out.

Aconitase activity determination

To a series of test tubes 4.4 or 4.3 ml. of water were added. 0.5 ml. of a solution containing 20 µmoles of cis-aconitic acid at pH 7.4 was then added and the test tubes placed in a water-bath at 30°. After an equilibration period of 5 min., the reaction was started by the addition of 0.1 or 0.2 ml. of enzyme solution. The reaction was stopped after 15 min. by the addition of 0.5 ml. of 50% (w/v) trichloroacetic acid. If a visible precipitate formed it was filtered off through a No. 30 Whatman filter paper, otherwise the citric acid estimation was carried out on the acidified solution without further treatment. It was found that small amounts of protein did not interfere with the estimation. Under these conditions the amount of citric acid formed from cis-aconitic acid was proportional to the amount of enzyme added. The activity determinations were carried out at two enzyme levels to ensure that the substrate concentration was not limiting.

Definition of a unit of aconitase activity

One unit of aconitase activity was taken to be the

amount of enzyme which formed one μmole of citric acid from cis-aconitic acid in 15 min. at pH 7.4 and 30° in the absence of buffer.

Specific activity

Units of aconitase activity/mg. of protein was taken to be the specific activity.

Other enzyme activity determinations

isoCitric dehydrogenase activity was determined by the method of Ochoa (1951). One unit was defined as a change of Log. I_0/I of 0.01/min. Fumarase activity was determined by the method of Racker (1950). One unit was defined as a change of log. I_0/I of 0.001/min.

Reagents

cis-Aconitic anhydride (m.p. 77°) was prepared from trans-aconitic acid by the method of Kalachowski & Maslowski (1928). Sodium cis-aconitate was formed by neutralising the anhydride to pH 7.4 with N NaOH. Trichloromethyl paraconic acid was prepared by the method of Fittig & Miller (1889) and converted to DL-isocitric lactone by the method of Krebs & Eggleston (1944). Sodium isocitrate was formed by hydrolysis of the lactone with NaOH as described by Krebs & Eggleston (1944). I am grateful to Mr. R.W. Wakelin for preparing these two acids.

Absolute ethanol obtained commercially was used throughout. L-Cysteine hydrochloride used in the later work was a Roche product which was free from iron. The ammonium sulphate was B.D.H. Special Grade and the 2:2'-dipyridyl was a B.D.H. Spot Test Reagent. All other reagents were A.R.

RESULTS

Activation of aconitase

The work of Dickman & Cloutier (1951) on the activation of aconitase by Fe^{2+} and cysteine was repeated using their partially purified enzyme preparation. The results were in good agreement with those obtained by these authors. However, the activating effect of cysteine is open to criticism. It was found that the preparation of cysteine which gave similar results to those of Dickman & Cloutier contained appreciable amounts of iron. Nevertheless, the importance of Fe^{2+} and cysteine as activators of aconitase was confirmed. The cysteine used in the later experiments was completely free from iron.

Inactivation of aconitase

It was apparent that the presence of ions, such as phosphate, which are capable of forming insoluble complexes

with Fe^{2+}, would lead to inactivation of the system as a whole. In order to test this experimentally samples of the same enzyme preparation were activated and pre-incubated at 30° with 0.05 M phosphate buffer (pH 7.4) for various periods of time. Substrate was then added and the reaction allowed to proceed. In the control system, water replaced the buffer. Fig. 1 shows that phosphate causes rapid inactivation of the activated enzyme when substrate is absent. When phosphate and substrate are added simultaneously, the enzyme activity is greater than that obtained in the absence of phosphate. This increased enzyme activity was shown to be due to the influence of the ions present in solution on the pH optimum of the enzyme (see chapter III). The enzyme activity was not increased by the simultaneous addition of excess Fe^{2+} (5×10^{-3} M) and substrate, following pre-incubation for various periods of time in the presence of phosphate. The above finding is in contrast to the results of Dickman & Cloutier (1951) for they found that phosphate did not decrease the activity of an activated enzyme preparation. As similar results were obtained with veronal acetate, borate and glycerophosphate buffers at pH 7.4, buffers were not used during the course of enzyme

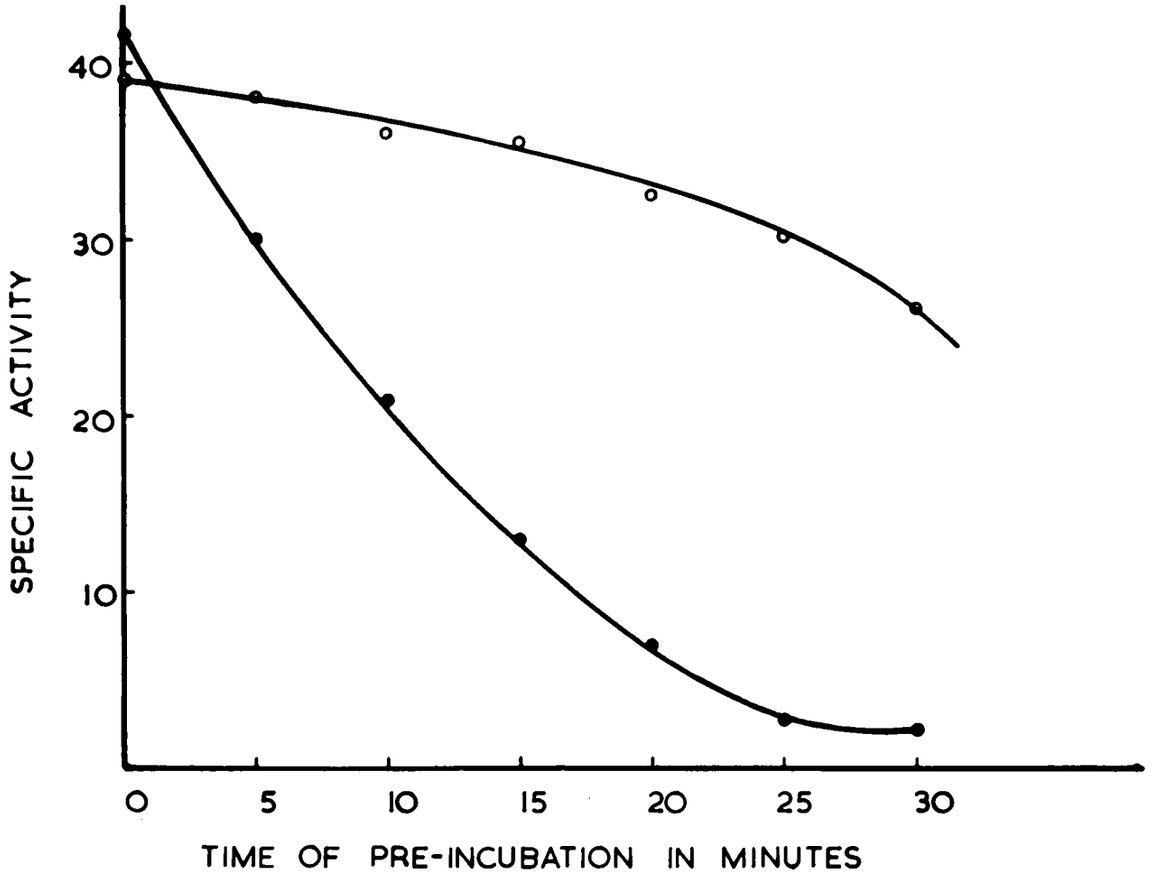


Fig. 1. The effect of incubating aconitase in the absence of substrate. The enzyme was activated with Fe^{2+} and cysteine and incubated at 30° for various periods of time. cis-Aconitic acid was added (final concentration 4 x 10^{-3} M) and the reaction was stopped after 15 min. by the addition of 0.5 ml. of 50% trichloroacetic acid. The solutions were analysed for citric acid.

○ No buffer present, reagents adjusted to pH 7.4.

● 0.05 M phosphate buffer (pH 7.4)

purification nor for the determination of enzyme activity. A check of the pH of the system before and after incubation showed that although there was a small alteration of the pH in the absence of buffer, the results were reproducible.

Preparation of aconitase by the method of Buchanan & Anfinsen

As Fe^{2+} and cysteine had not been used by Buchanan & Anfinsen (1949) for activation of their aconitase fractions, it was possible that their final preparation was, in fact, of higher specific activity than that reported. In order to determine whether or not this was the case, aconitase was prepared according to the method of these workers. A study was then made of the activity of various fractions in the presence and absence of Fe^{2+} and cysteine. Buchanan & Anfinsen carried out their tests at 38°; in the present work the tests were made at 30° and the activities were multiplied by 1.5, the temperature coefficient of the reaction found for 38°/30°.

The results of table 1 clearly show that in the absence of Fe^{2+} and cysteine, the specific activities of the intermediate fractions are much lower than those claimed by Buchanan & Anfinsen. It was only in the presence of these two agents that similar activities

TABLE 1. Comparison of Aconitase Activities in the Presence and Absence of Fe^{2+} and Cysteine

(Fractions were prepared according to the method of Buchanan & Anfinsen (1949). Activation by Fe^{2+} and cysteine was carried out as described in the text. Temp. 30°, pH 7.4. The results obtained were multiplied by 1.5, the temperature coefficient for 38°/30°, so that they are equivalent to activities at 38°).

Fraction	Buchanan & Anfinsen results		Present results					
	No additions		No additions		Fe^{2+} + Cysteine		Ratio of activities	
	Total Units	Specific activity	Total Units	Specific activity	Total units	Specific activity	$\frac{\text{activated}}{\text{non-activated}}$	enzyme
Extract	310,000	10.0	261,000	10.5	278,000	11.3	1.07	
1st pH 5.6 ethanol ppt.	133,000	18.9	144,000	26.9	220,500	41.1	1.53	
2nd pH 5.6 ethanol ppt.	102,000	68	100,500	29.3	175,000	51.5	1.74	
pH 6.8 ethanol ppt.	63,000	132	45,000	45	105,000	107	2.35	

could be obtained. A comparison of the activities of the activated and non-activated fractions shows that the degree of activation by Fe^{2+} and cysteine increases as purification proceeds, indicating that the co-factors of the enzyme are removed to some extent by the fractionation procedure. It will also be noted that the relative purification of the enzyme in each step differs markedly from that obtained by Buchanan & Anfinsen. Moreover, it was found that the addition of ammonium sulphate to 66% saturation did not increase the specific activity of the enzyme.

As the method of Buchanan & Anfinsen (1949) gave only a relatively small increase in the purity of aconitase, an effort was made to obtain a more highly purified preparation of the enzyme. The procedure utilised by these authors was developed further and some modifications of the initial fractionation steps were made. In view of the fact that maximum enzyme activity was obtained only after the addition of Fe^{2+} and cysteine, the various fractions were incubated with these agents before the activities were determined.

METHOD of ISOLATION of ACONITASE from PIG HEART

It will be appreciated that the attempts to purify aconitase further involved the trial of many purification procedures. The results of all these procedures are not described in detail as many brought about little or no increase in the purity of the enzyme, but brief mention of their use is made. The final method adopted for the enzyme purification was as follows:

Extraction of tissue

Immediately after the death of the animal, the hearts were removed, cut into strips of about ¼ inch thickness, freed from fat and connective tissue and placed in ice. At the laboratory, the hearts were minced through a coarse mincer and stored at -15°. Under these conditions, pig heart could be stored for some months without loss of enzyme activity. One kg. of frozen heart was allowed to thaw at room temperature and then homogenised in 200 g. lots for 2 min. in a pre-cooled Waring blendor with 600 ml. of 0.004 M citrate buffer (pH 4.7) and 130 ml. of chloroform. Chloroform reduced the haemoglobin content of the extract without denaturation of the enzyme and increased its specific activity. The homogenate was centrifuged at -1° for

15 min. at 1,000 g. The clear red-amber supernatant (pH 5.7) was poured off and filtered through a fluted filter paper (Whatman No. 531). Below pH 5.0 marked loss of enzyme activity occurred.

1st. ethanol fractionation

For convenience, the supernatant was divided into two equal amounts and fractionated simultaneously. From this stage onwards, all operations were carried out in the coldroom. The supernatant was brought to 0° in a dry ice-ethanol bath and 90% ethanol was added with mechanical stirring at the rate of 250 to 300 ml./hr., to a concentration of 15%. (90% ethanol was used throughout the fractionation). Freezing of the solution at this stage leads to loss of enzyme activity. Hyflo Super-Cel was added to the solution (5 g./l.) and filtration was carried out on a Buchner funnel under slight pressure (Whatman No. 30 filter paper). To the clear red-amber filtrate, saturated sodium chloride (1.25 ml./100 ml. of filtrate) was added. The ethanol concentration of the solution was brought to 45%, the temperature being lowered to -10°. The mixture was allowed to stand overnight as contact of the enzyme with ethanol for this period did not have any harmful effect.

2nd. ethanol fractionation

The precipitate was centrifuged off at -10° and stored at the same temperature whilst another kg. of pig heart was treated in the same fashion. The two precipitates were combined and dissolved in 800 ml. of ice-cold water. A small amount of insoluble matter was centrifuged off to yield a clear deep red-amber solution. The solution was brought to 0° and ethanol added to a concentration of 10% (the ethanol already present was neglected), the temperature being lowered to -4°. A small amount of a white precipitate was centrifuged off at -4°. The pH of the supernatant which was about 5.6, was adjusted to pH 6.8 with 5% sodium carbonate solution. Ethanol was added to a concentration of 23% whilst the temperature was lowered to -10°. The precipitate was centrifuged off at -10° and dissolved in 100 ml. of ice-cold water.

Ammonium sulphate fractionation

The aqueous solution was diluted 2.5 times with 0.125 M citrate buffer (pH 6.8). Ammonium sulphate (36.7 g./100 ml. of solution) was added slowly, the temperature being lowered to -6°. The yellow coloured precipitate was centrifuged off at -6° for 10 min. at 5,000 g. To the supernatant, ammonium sulphate

(9.2 g./100 ml. of supernatant) was added slowly and the temperature lowered to -10°. The precipitate was centrifuged off at -10° for 15 min. at 5,000 g. and dissolved in 90 ml. of 0.004 M citrate buffer (pH 5.7) to give a deep amber solution. This solution was dialysed overnight with stirring against the same buffer. There was no loss of enzyme activity, but dialysis against water and salt solutions caused large losses of enzyme activity.

Heat fractionation

The dialysed solution in 20 to 25 ml. lots was heated in a water-bath for 15 min. at 50° and cooled rapidly in an ice-bath. The white flocculent precipitate was centrifuged off at 0° and discarded.

Ethanol fractionation at pH 8.0.

The supernatant was cooled to 0° and ethanol was added to a concentration of 20%, the temperature being lowered to -5°. A small amount of precipitate was centrifuged off at -5° and discarded. The supernatant was adjusted to pH 8.0 with a saturated solution of sodium bicarbonate and ethanol was added to a concentration of 50%, the temperature being lowered to -12°. During the fractionation at alkaline pH values, care was taken to prevent CO_2 from distilling over from the dry

ice-ethanol bath into the protein solution. The reddish coloured precipitate was centrifuged off from a cloudy supernatant (due to the presence of sodium bicarbonate) at -12° and dissolved in 20 ml. of 0.004 M citrate buffer (pH 5.7).

Ammonium sulphate fractionation at pH 8.5

A saturated solution of ammonium sulphate was neutralised with strong ammonia, so that on dilution of 1 in 5, the pH was 8.5. After adjustment of the pH of the protein solution with ammonia, the ammonium sulphate solution was added dropwise until the saturation was 0.65. The temperature was gradually lowered to -10°. The precipitate was centrifuged off at -10°, dissolved in 10 ml. of 0.004 M citrate buffer (pH 5.7) and dialysed against the same buffer overnight at 0°. The final solution was amber in colour.

Attempts to purify the enzyme further were unsuccessful. Although the enzyme constituted the major portion of the final solution, it could not be induced to crystallize. The slow addition of ethanol always gave rise to an amorphous product. Attempts were also made to crystallize the enzyme from ammonium sulphate solutions by the addition of an amount just

insufficient to cause precipitation, followed by adjustment of the pH. Within the pH range 5.7 to 8.5. both in the presence and absence of Fe^{2+} and cysteine, the enzyme precipitated only in small amounts and in an amorphous form.

A brief account of the procedures which were tried without success during the course of the purification follows below. Large amounts of calcium phosphate gel were required to adsorb the enzyme from solution between pH 5.6 and 6.8 and no differential adsorption or elution could be obtained. Although the enzyme could be stirred with n-butanol for 30 min. at room temperature, this treatment did not alter the precipitation pattern after further ethanol fractionation. No protein was precipitated by the nucleic precipitants manganese sulphate and protamine sulphate. Fractionation with acetone precipitated the enzyme between 30 and 50% acetone concentration, but marked loss of activity occurred even though the fractionation was carried out between -5° and -10°. This finding is in contrast to that of Buchanan & Anfinsen (1949), but is consistent with the fact that acetone powders of pig heart are devoid of aconitase activity. The enzyme was not precipitated by sodium

chloride or ammonium acetate. Ethanol fractionation in the presence of zinc was ineffective, presumably because the zinc reacted with the citrate present.

In Table 2 is given a summary of the yields and degrees of purification of aconitase during the fractionation procedure. This procedure brings about a 24-fold increase in the purity of aconitase, with an overall recovery of about 12%. The final product has a specific activity of about 265 when the test is carried out in the absence of buffer. If the test is carried out in 0.05 M phosphate buffer (pH 7.4), the specific activity is increased to 336. As mentioned previously, this increase is due to the fact that the pH optimum of aconitase is dependent upon the ions present in solution (see Chapter III). It was calculated that at 38° the specific activity is about 500 which compares to the value of 235 claimed by Buchanan & Anfinsen (1949) for their final preparation.

General properties of aconitase

The enzyme in dilute citrate buffer at pH 5.7 retained its activity for many weeks when stored in the frozen state and was unaffected by repeated freezing and thawing. It was not found necessary to add Fe^{2+} and

TABLE 2. Summary of Yields and Specific Activities of Fractions obtained during the Purification of Aconitase from 2 Kg. Pig Heart. (see text for details)

Fraction	Volume (ml.)	Protein Concn. (mg./ml.)	Total Protein (g.)	Units per ml.	Total Units	Specific activity	Yield %
Extract	6,240	6.3	39.3	70	437,000	11.1	100
1st. ethanol ppt.	832	6.35	5.3	310	260,000	49	60
2nd ethanol ppt.	112	19.6	2.2	1880	212,000	96	49
Dialysed ammonium sulphate ppt.	116	8.4	0.98	1410	157,000	168	36
Supernatant after heating at 50°	102	7.0	0.72	1320	135,000	188	31
pH 8.0 ethanol ppt.	25	16.5	0.41	3840	96,000	232	22
pH 8.5 ammonium sulphate ppt.	15	12.8	0.19	3360	50,500	265	12

cysteine as stabilizing agents, although in the absence of both or either of these agents the enzyme activity was markedly reduced. Details of the activation of aconitase will be reported in Chapter II. The final enzyme preparation could also be freeze-dried without loss of activity, but it was found more convenient to keep it in the frozen state. Fig. 2 shows that aconitase is stable to heat until a temperature of 51° is attained, after which there is a sharp loss of activity. A characteristic feature of the enzyme was its wide range of precipitation. Aconitase activity was found in every fraction obtained with all solvents and salts used over the pH range from 5.7 to 8.5.

Electrophoretic analysis

The electrophoretic pattern of the final aconitase preparation is illustrated in fig. 3. It was not possible to carry out electrophoretic analyses under conditions where the enzyme was on the acid side of its isoelectric point. All the components migrated to the anode when the electrophoresis was carried out at pH 8.6, but at pH 5.7 at least two of the minor components still migrated to the anode whilst the main component migrated to the cathode. pH values below 5.0 could not be used because of the denaturation of the enzyme.

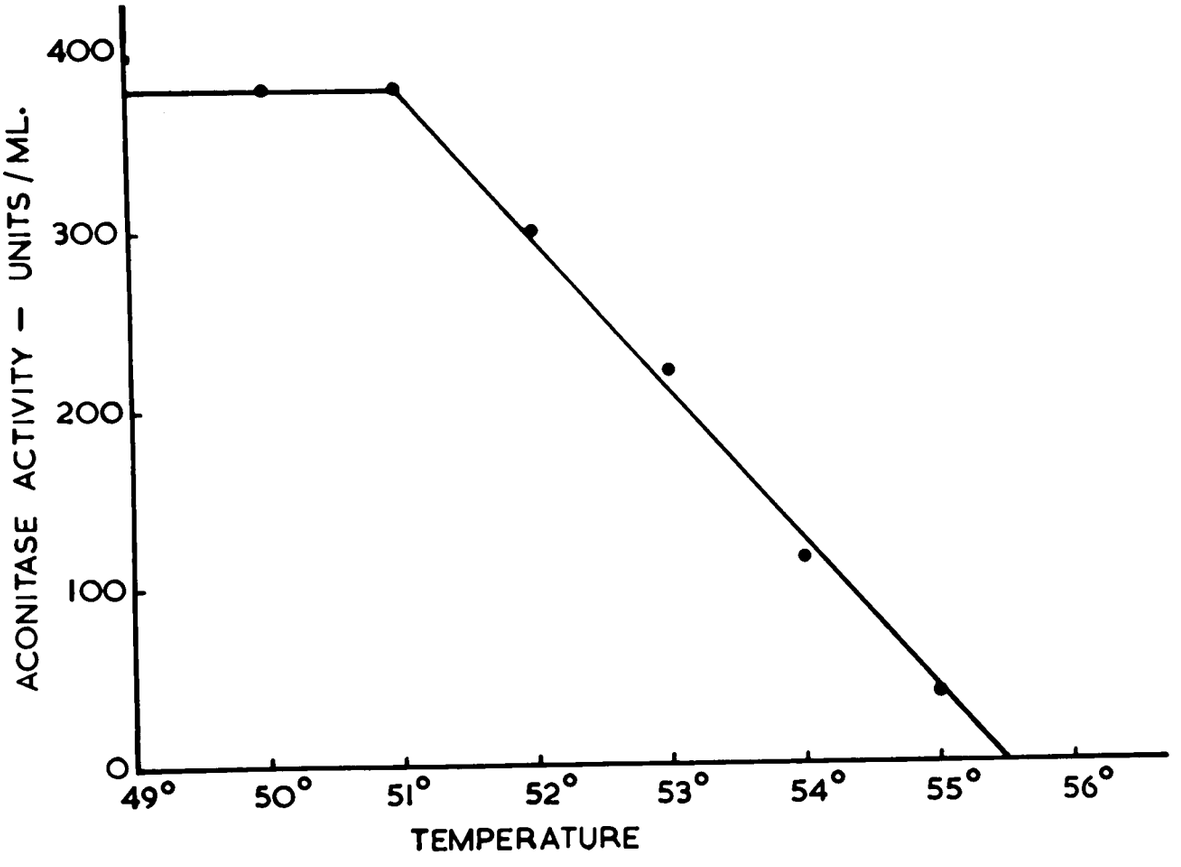


Fig. 2. The effect of temperature on aconitase activity. Heating was carried out in a water-bath for 15 min., using 2.0 ml. samples of the dialysed ammonium sulphate precipitate (protein concentration, 9 mg./ml.). Aconitase activity was determined after activation with Fe^{2+} and cysteine as described in the text. Temperature 30°; pH 7.4.

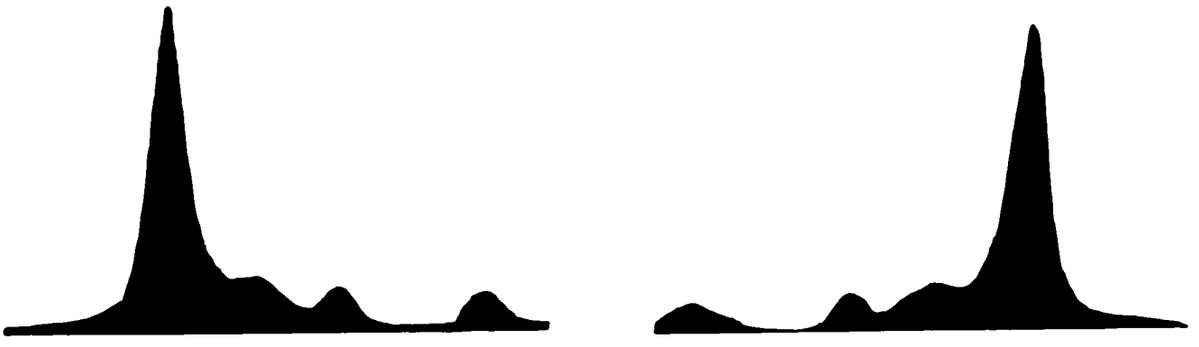


Fig. 3. Electrophoretic patterns of the aconitase preparation obtained after fractionation as described in the text. Veronal buffer, pH 8.6; ionic strength 0.1; temperature 1°; current 10 milliamperes; protein concentration 1%; time 108 min.

Identification of the main electrophoretic component

In order to determine whether or not the main component was responsible for aconitase activity, a second electrophoretic run was carried out after dialysis of a 1% solution of the protein against veronal buffer at pH 8.6 (μ = 0.075) which contained sodium citrate (μ = 0.025). A similar electrophoretic pattern to that shown in fig. 3 was obtained. Enzymic analysis of the main component after separation showed that this component was responsible for the aconitase activity, whilst the other components were devoid of activity. Both fractions were dialysed overnight against 0.004 M citrate buffer (pH 5.7) and activated with Fe^{2+} and cysteine before the aconitase activity was determined. Unfortunately, the enzyme lost activity during the dialysis and electrophoresis. The value obtained for the specific activity of the electrophoretically homogeneous protein was approximately half that of the original solution. The isolated main component was also capable of converting both cis-aconitic acid and isocitric acid to citric acid.

The main component was completely free from the amber coloured pigment which was associated with the enzyme during the stages of purification. The mobility

of the amber coloured protein was somewhat similar to that of aconitase, so that electrophoresis had to be continued for a long period to effect separation. It may be calculated that the purity of aconitase in this preparation is 75-80%. If the main component consists entirely of aconitase, the fact that a 24-fold purification yields an enzyme preparation with a purity of 75-80% appears to indicate that aconitase represents a large proportion of the total protein of heart muscle. It is, of course, possible that the isolated component is not homogeneous in the ultracentrifuge, but this point has not yet been investigated.

Rates of citric acid formation from cis-aconitic and isocitric acids by aconitase fractions in the presence and absence of Fe^{2+} and cysteine

Although the activation of aconitase is dealt with in Chapter II, the results of this aspect of the activation are given here as they are intimately concerned with the purification procedure.

During the purification, various fractions were tested for the ability to convert both isocitric acid and cis-aconitic acid to citric acid. Table 3 shows that the ratio of the two activities remains constant within

TABLE 3. Comparison of the Rates of Citric acid Formation from Isocitric acid and cis-Aconitic acid by Activated and Non-activated Fractions of Aconitase.

(Fractions were obtained and activated as described in the text)

Fraction	Specific Activities						Ratio of Activities of activated / non-activated } enzyme	
	Non-activated			Activated				
	(a)	(b)	Ratio	(c)	(d)	Ratio		
	Cis-aconitic acid	iso-citric acid	a:b	cis-aconitic acid	isocitric acid	c:d	cis-aconitic acid	isocitric acid
Extract	7.0	4.35	1.61	11.4	7.2	1.59	1.63	1.66
1st. ethanol ppt.	31	17.5	1.78	49	31	1.58	1.6	1.8
2nd. ethanol ppt.	50	32.5	1.54	96	56	1.71	1.9	1.7
Ammonium sulphate ppt.	72.5	45.5	1.59	151	97	1.56	2.1	2.1
Dialysed ammonium sulphate ppt.	7.0	4.7	1.49	142	85	1.67	20	18
Supernatant after heating at 50°	6.7	4.2	1.60	174	113	1.54	26	27

the limits of experimental error. The addition of Fe^{2+} and cysteine increased the two activities equally so that there was no alteration in the ratio. The activating effect of Fe^{2+} and cysteine during the early stages of the purification was small and remained reasonably constant. After dialysis the activation was greatly enhanced. Fe^{2+} and cysteine can, therefore, replace completely the dialysable co-factors. The addition of 2:2'-dipyridyl (10^{-4} M)to the dialysis medium caused the formation of ferrous tridipyridyl inside the dialysis sac and this complex slowly diffused out into the medium. It is an interesting fact that not only does Fe^{2+} activate aconitase, but that loss of enzyme activity coincides with loss of Fe^{2+}. It would thus appear likely that Fe^{2+} is the metal with which the enzyme is associated in vivo. The results also suggest that the Fe^{2+} is bound to the enzyme sufficiently strongly to resist splitting as a result of ethanol and ammonium sulphate fractionation, but the linkage is readily broken by dialysis.

Tests for the presence of other enzymes in the final aconitase preparation

The final aconitase preparation contained 196 units of isocitric dehydrogenase activity per mg. of protein

and 1100 units of fumarase activity per mg. of protein. Tests for the presence of lactic dehydrogenase were negative. It is possible that two of the three minor components of the electrophoretic pattern are due to the presence of isocitric dehydrogenase and fumarase.

DISCUSSION

The inactivating effect of phosphate on aconitase in the absence of substrate can at least partially explain the instability of the enzyme as previously reported, for the tissues were extracted with phosphate buffer. It would seem that the apparent instability of the enzyme was related to the loss of co-factors, rather than to the denaturation of the apoenzyme. The final aconitase preparation was stable, but both it and the fractions obtained during the purification required the addition of Fe^{2+} and cysteine for maximum activity.

The assay figures of Johnson (1939) for the aconitase activity of various animal organs are also open to criticism as the organs were extracted with phosphate buffer. From the results obtained here, it would appear that the values obtained would depend on the time that elapsed between the extraction and the test of enzyme activity as well as the amount of substrate present in

the extract.

The possible mechanism for the inactivation of aconitase by phosphate in the absence of substrate will be discussed in Chapter III.

The fact that the relative rates of the reactions cis-aconitic acid ⟶ citric acid and isocitric acid ⟶ citric acid are the same throughout the fractionation procedure and that each reaction is activated to the same extent by Fe^{2+} and cysteine provides good evidence in favour of the idea that aconitase is a single enzyme. More conclusive is the finding that an electrophoretically homogeneous protein is capable of carrying out those reactions associated with aconitase activity. These findings are in accord with the fact that the loss of one enzyme activity invariably leads to the loss of all activities.

SUMMARY

1. The activation of aconitase by Fe^{2+} and cysteine has been confirmed. Phosphate was shown to inactivate the activated enzyme rapidly in the absence of substrate.
2. Aconitase has been purified 24-fold by low temperature

ethanol and ammonium sulphate fractionation. The purity of the final preparation was estimated to be 75-80%.

3. The final aconitase preparation was contaminated to a small extent by isocitric dehydrogenase and furmarase.
4. Aconitase has been isolated from the final preparation as an electrophoretically homogeneous protein. This protein was free of pigment and was capable of converting both isocitric acid and cis-aconitic acid to citric acid.
5. During the purification, the degree of activation of aconitase by Fe^{2+} and cysteine increased, especially after dialysis.

C H A P T E R II

THE ACTIVATION of ACONITASE

CHAPTER II

THE ACTIVATION of ACONITASE

INTRODUCTION

Many enzymes exert their catalytic effects only in the presence of a metal or low molecular weight compounds. Some low molecular weight compounds, such as the phosphopyridine nucleotides and some metals, are considered to form complexes with the enzyme and are classified as coenzymes or prosthetic groups. Other low molecular weight compounds, such as cysteine and glutathione, activate certain enzymes by reaction, rather than by combination. One way in which this can happen is for these compounds to reduce disulphide linkages on the surface of those enzymes which require free -SH groups for activity. Thus, they differ from prosthetic groups and coenzymes in not taking part in the actual mechanism of catalysis and are better classified as activators. Glutathione is essential

for the activity of glyceraldehyde-3-phosphate dehydrogenase as shown by Krimsky & Racker (1952) and Racker & Krimsky (1952), but it is firmly bound to the enzyme and takes part in the reaction. Thus it must be classified in this instance as a prosthetic group rather than as an activator. As will be seen later, it would seem as though a number of prosthetic groups function by uniting the substrate with the enzyme through the active centre(s) and included in this group are some metal enzymes. Coenzymes appear to be linked to the enzyme at a site other than where the substrate is attached and act as acceptors of electrons or ions. Coenzymes are more highly dissociated than the prosthetic groups.

Green (1941) proposed the thesis that any substance which occurs in traces in the cell and which is necessary in traces in the diet of man or in the media for the growth of micro-organisms, must be an essential part of some enzyme system. This theory was based on the finding that a number of trace substances had been found to exert their effects either as prosthetic groups or coenzymes of enzymes. It, therefore, gave a rational basis for the explanation of how trace substances exert such profound biological effects. The small amounts of these substances required were

consistent with the fact that the concentration of particular enzymes in the cell is low. This hypothesis has been strengthened as a result of further research over the intervening years. It has also come to light that many enzymes have a specific requirement for a particular coenzyme or prosthetic groups, and this has led to a classification of enzymes according to the structure of these compounds. The fact that different enzymes require the same co-factor, although they have mutually exclusive specificities, indicates that the protein moiety is not only specific, but also determines the nature of the catalysis.

Although it is likely that the explanation of the requirement of an organism for trace metals is or will be found to be due to their role in enzyme systems, it is not only the trace metals that serve as prosthetic groups or activators. Of the so-called bulk metals (K, Na, Ca, Mg and Fe), all with the possible exception of Na are capable of activating enzymes. Other metals such as V and Rb have also been found in animal and plant tissues, but at the present time it is not known whether or not they participate in enzymic reactions.

The exact function of the metal in such enzyme

systems is not clear. In certain cases, the catalytic activity of the metalloenzyme is manifested to some extent in the metal itself, but the addition of the apoenzyme together with the metal greatly enhances the reaction rate. In such cases, it is considered that the function of the apoenzyme is to enhance the inherent properties of the metal. An example of this type of reaction is found with the oxidative decarboxylation of α-ketoglutaric acid. α-ketoglutaric carboxylase requires Mn^{2+} for activity, but Kalnitsky (1953) has shown that the same reaction occurs in the presence of Mn^{2+} and in the absence of the apoenzyme, although the reaction rate is slower in the absence of the apoenzyme. This cannot be the function of the apoenzyme in the case of all metalloenzymes. Although Zn^{2+} is essential for the activity of carbonic anhydrase, Neilin & Mann (1940) showed that inorganic zinc salts, simple and complex organic zinc compounds were devoid of catalytic activity. The function of the Zn^{2+} here might be to unite the enzyme and substrate in a co-ordination complex as suggested by Smith (1951), rather than to increase the ability of Zn^{2+} to catalyse the reaction. It is, of course, possible that Zn^{2+} does have some catalytic

activity, but it is so low that it cannot be measured.

Smith (1951) found that the addition of a peptidase capable of hydrolysing L-leucylglycine to Mn^{2+} caused striking changes in the absorption spectrum of the metal. When L-leucylglycine was added to Mn^{2+}, absorption changes were also observed. These findings led Smith to postulate the formation of an enzyme-substrate complex which involved co-ordination complexes of Mn^{2+}. He proposed that after the formation of the enzyme-substrate complex, the decrease in the activation energy of hydrolysis was produced by attraction of the electrons by Mn^{2+} while the protein centres oriented the substrate so that the net effect was an electronic deformation of the sensitive bond. The hydrolysis was then considered to be due to the catalytic effect of H^+ and OH^- ions.

In the absence of further information, it would seem reasonable to assume that a metal unites with the apoenzyme to form an active complex which is then in equilibrium with the free apoenzyme and free metal according to the equation:

$$\text{metal} + \text{enzyme} \rightleftharpoons \underset{\text{(active)}}{\text{metal-enzyme}}$$

(inactive)

From such an equation, it is possible to determine the hypothetical dissociation constant of the complex. This has been done for many enzymes with a result that it has been found that the affinity of apoenzymes for metals varies from system to system and within the same system for different metals. Dialysis studies have also shown this difference in affinity. Smith & Hanson (1949) found that the Mg^{2+} of carboxypeptidase is not removed by dialysis, whereas the Mg^{2+} of leucine aminopeptidase is readily removed by dialysis. Although enzymes vary as to their affinity for metal ions, after dialysis the metal concentration required for the enzyme to show its maximum activity is relatively low.

Not all enzyme systems show an absolute metal specificity. A number of phosphatases, phosphate transferring enzymes and α-keto-acid carboxylases can be activated by either Mn^{2+} or Mg^{2+}, although Mn^{2+} is more effective than Mg^{2+}, whilst other metals of the transition series are inactive. This indicates that the ability of a metal ion to activate enzyme systems is not simply related to the properties of the metal ion. The difference between Mn^{2+} and Mg^{2+} with respect to mass, ionic radius, ionic potential, mobility

and rate of diffusion is considerably greater than between Mn^{2+} and the other transition metals. It is probable that hydration and complex formation are more concerned with the ability of a metal to activate an enzyme system, than are the other physical properties. At the same time, it is possible that the properties of the 'naked' metallic ion may differ greatly from those which it possesses in a physiological medium where it is surrounded by other molecules of an inorganic and organic nature (Lehninger, 1950).

The activation of some metal enzymes has been found to be a very slow reaction. Mohamed & Greenberg (1945) found that the activation of arginase was dependent on time as well as other factors. Under certain conditions, days were required for the enzyme to be fully activated. Smith (1951) also found a similar slow rate of activation with peptidases. This slow rate of activation may be significant and find explanation in the time required for the formation of a co-ordination compound between the metal and the enzyme, but it is noteworthy that none of the enzymes known to show a very slow rate of activation has been obtained as an electrophoretically homogeneous protein.

Dickman & Cloutier (1951) showed that aconitase was a metalloenzyme which was dependent on the presence of Fe^{2+} and a reducing agent for maximum activity. Qualitative studies showed that Fe^{2+} was capable of forming complexes with the substrates of aconitase under the conditions of optimum enzyme activity and the results also suggested that the Fe^{2+} was capable of forming a complex with the apoenzyme. These authors suggested that the Fe^{2+} was responsible for the linkage between the apoenzyme and the substrate in the aconitase-tricarboxylic acid complex. It was assumed that the reducing agents played a double role in maintaining the iron as Fe^{2+} and in keeping the reducing groups of the protein in the reduced state, but no evidence was presented to indicate that this was the case.

It has been seen that some metal ions are capable of activating some enzymes; different theories to explain the function of the metal have been put forward, but the mode of activation has not yet been definitely established. Dickman & Cloutier (1951) explained their results concerning aconitase in terms of the hypothesis advanced by Smith (1951). Whilst their results were not inconsistent with this hypothesis, the authors

provided little or no direct evidence to show that the function of Fe^{2+} was to form a link between the enzyme and the substrate. No evidence was presented to indicate the role played by the reducing agents in the activation of the enzyme. The high residual activity of the Dickman & Cloutier aconitase preparation in the absence of Fe^{2+} and reducing agents did not permit of a more detailed investigation of the function of these compounds in the aconitase system. The aconitase preparation obtained by the method reported in chapter I provided a much better means of determining whether these compounds functioned as prosthetic groups, activators or both. The preparation showed little activity in the absence of Fe^{2+} and a reducing agent, whereas it showed considerable activity on the addition of these substances. This preparation was used for testing the effect of Fe^{2+} and reducing agents on aconitase activity. The results reported in this chapter indicate that both Fe^{2+} and reducing agents act as prosthetic groups of aconitase and so are concerned in the formation of the active enzyme complex. It is suggested that the active form of the enzyme is an enzyme-Fe^{2+}-reducing agent complex.

EXPERIMENTAL

Methods

Aconitase was activated by Fe^{2+} and all reducing agents in the same fashion as previously described (page 19). The enzyme solutions contained 60 µg. of the final aconitase preparation/ml. The enzyme activity was determined and citric acid estimated as previously described (page 20).

Reagents

Ascorbic acid was a Roche product, thioglycollate was a B.D.H. product which was re-distilled before use and glutathione was a gift from the Distillers Co. Ltd., Liverpool. As determined by thiol titration, the glutathione was 99% pure. All these compounds were free from iron. The other reagents were either A.R., or as previously mentioned.

RESULTS

Activation of aconitase by Fe^{2+} and cysteine

Fig. 4 shows the effect of Fe^{2+} and cysteine on the activity of aconitase. The final purified, dialysed preparation possesses little activity; cysteine alone

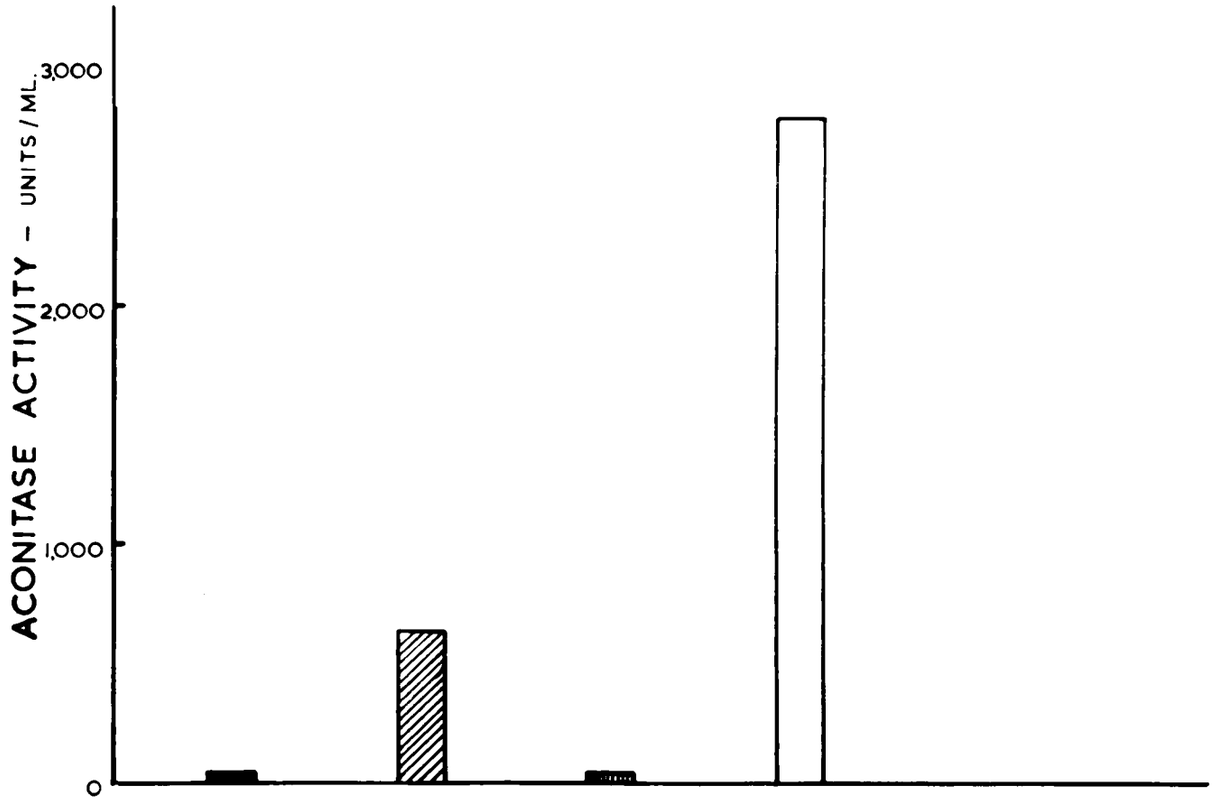


Fig. 4. Activation of aconitase by Fe^{2+} and cysteine. Aconitase was incubated for 1 hr. at 0° and pH 7.4 before the activity was determined as described in the text.

No additions

5×10^{-4} M Fe^{2+}

0.01 M cysteine

5×10^{-4} M Fe^{2+} + 0.01 M cysteine

does not increase the activity; Fe^{2+} alone brings about a 15-fold increase in the activity, whilst the addition of Fe^{2+} and cysteine increases the activity 70-fold. The experiments reported in Chapter I had shown that cysteine alone would activate aconitase preparations, but later this was shown to be due to the presence of Fe^{2+} in the cysteine preparation. The investigation of the activation of aconitase by Fe^{2+} alone involved some technical difficulties. On account of the small amount of protein present in solution, it showed little or no buffering capacity. The ferrous ammonium sulphate solution was acidic so that if it were not partly neutralised the pH of the Fe^{2+}-enzyme solution was below pH 5.0 with a result that there was denaturation of the enzyme. The adjustment of the pH of this solution to 7.4 was difficult and at this pH value at least some of the Fe^{2+} was in the colloidal state. Therefore, it is not possible to state that the above value represents an accurate estimation of the activation of aconitase by Fe^{2+}. Nevertheless, it is clear that Fe^{2+} and cysteine bring about a very marked activation of aconitase.

Time required for maximum activation of aconitase by

Fe^{2+} and cysteine

The conditions chosen in the previous experiments for the determination of aconitase activity were based on those of Dickman & Cloutier (1951). Although they were quite arbitrary, they provided a suitable means for following the purification of the enzyme. But having obtained a highly purified preparation, it was important to determine whether or not these conditions gave rise to maximum activation. The results of Dickman & Cloutier (1951) suggested that an interaction between the apoenzyme and the Fe^{2+} to form a Fe^{2+}-enzyme complex according to the equation:

$$Fe^{2+} + \text{enzyme} \rightleftharpoons Fe^{2+}\text{-enzyme}$$

was essential for enzyme activity. It was possible that the rate of the attainment of the equilibrium was slow and that the 1 hr. period of activation of the enzyme with Fe^{2+} and cysteine was not sufficiently long for the enzyme to be activated to the full extent. Therefore, an investigation was made of the relationship between the enzyme activity and the period of incubation.

It is clear from Table 4 that there is an initial rapid activation of aconitase by Fe^{2+} and cysteine, followed by a slower period of activation, so that about

TABLE 4

Effect of Pre-incubation Time on the Activation of Aconitase by Fe^{2+} and cysteine

(Aconitase was incubated at pH 7.4 and 0° with 5×10^{-4} M Fe^{2+} and 10^{-2} M cysteine. 0.2 ml. samples, equivalent to 12 µg. of the final aconitase preparation, were removed at intervals and added to test tubes containing 0.5 ml. of cis-aconitic acid (final concentration 4×10^{-3} M) at pH 7.4 and 4.3 ml. of water. Reaction was stopped after 15 min. by the addition of 0.5 ml. of 50% (w/v) trichloroacetic acid and the mixture analysed for citric acid. Temperature 30°)

Time of activation (hr.)	Aconitase activity (µg. citric acid formed/15 min.)
0	285
0.5	335
1.0	320
2.0	320
3.0	320
4.0	318
5.0	295
6.0	278
24.0	192

30 min. at 0° is required for maximum activation. This activity is retained for at least 4 hr., after which there is a slow decrease so that at 24 hr., the enzyme possesses only 60% of its maximum activity. These results indicate that the arbitrary conditions previously chosen for the incubation do bring about maximum activation of the enzyme.

There seemed to be three possible reasons by which the loss of enzyme activity after 4 hr. could be explained. They were:

(a) alteration of the pH of the mixture during the incubation period at 0°

(b) accumulation of the end-products of the oxidation of cysteine by Fe^{2+}

(c) denaturation of the enzyme in dilute solution.

A check of the pH of the mixture during the incubation showed that there was no alteration over the 24 hr. period. The accumulation of the end-products of cysteine oxidation could not explain the loss of activity, for when the enzyme was added to a mixture of Fe^{2+}, cysteine and water which had been incubated at 0° for 24 hr., the activity following a 30 min. incubation period at 0° was maximal. It is unlikely that there is any significant accumulation

of the end-products of the oxidation of cysteine, for although no special precautions were taken to exclude oxygen, the conditions were virtually anaerobic. It must be concluded that the loss of enzyme activity is due to the denaturation of the enzyme in dilute solution.

Activation of aconitase by the addition of Fe^{2+} and cysteine to the test medium

With the previous method of activating aconitase with Fe^{2+} and cysteine, it was assumed that there was no dissociation of the active complex when it was added to the test medium containing substrate. Whilst the concentrations of Fe^{2+} and cysteine in the enzyme solution during the period of activation were 5×10^{-4} M and 10^{-2} M respectively, when the enzyme solution was added to the test medium the concentrations were reduced to 10^{-5} M and 2×10^{-4} M. Dissociation of the enzyme complex was likely on account of the high dilution, so an investigation was made of the influence of the Fe^{2+} and cysteine concentration in the test medium on the aconitase activity. In these experiments, the activation was carried out at 30° for convenience.

It was found that when the non-activated aconitase was added to a medium containing the lower concentrations

of Fe^{2+} and cysteine and incubated for 5 min. at 30° before the addition of substrate, the enzyme showed no activity. When the concentrations of Fe^{2+} and cysteine in the medium were increased to 5×10^{-4} M and 10^{-2} M respectively, the enzyme showed the same activity over the initial period of the reaction as it did when the original method of activation was used. (The activation of aconitase in the presence of 5×10^{-4} M Fe^{2+} and 10^{-2} M cysteine in the test medium will be designated as Method II. The original method of activation, i.e. the activation of the enzyme in the presence of 5×10^{-4} M Fe^{2+} and 10^{-2} M cysteine so that a sample of this solution is added to the test medium, will be referred to as Method I). Fig. 5 shows the effect of adding the enzyme directly to a mixture of Fe^{2+}, cysteine and substrate. It will be noted that there is a distinct lag period of about 3 min. and that the reaction rate is slower than that of the control in which the enzyme was pre-incubated with Fe^{2+} and cysteine for 5 min. prior to the addition of substrate.

It may be concluded from these results that dissociation of the enzyme complex does not occur on dilution, at least in the presence of substrate. The

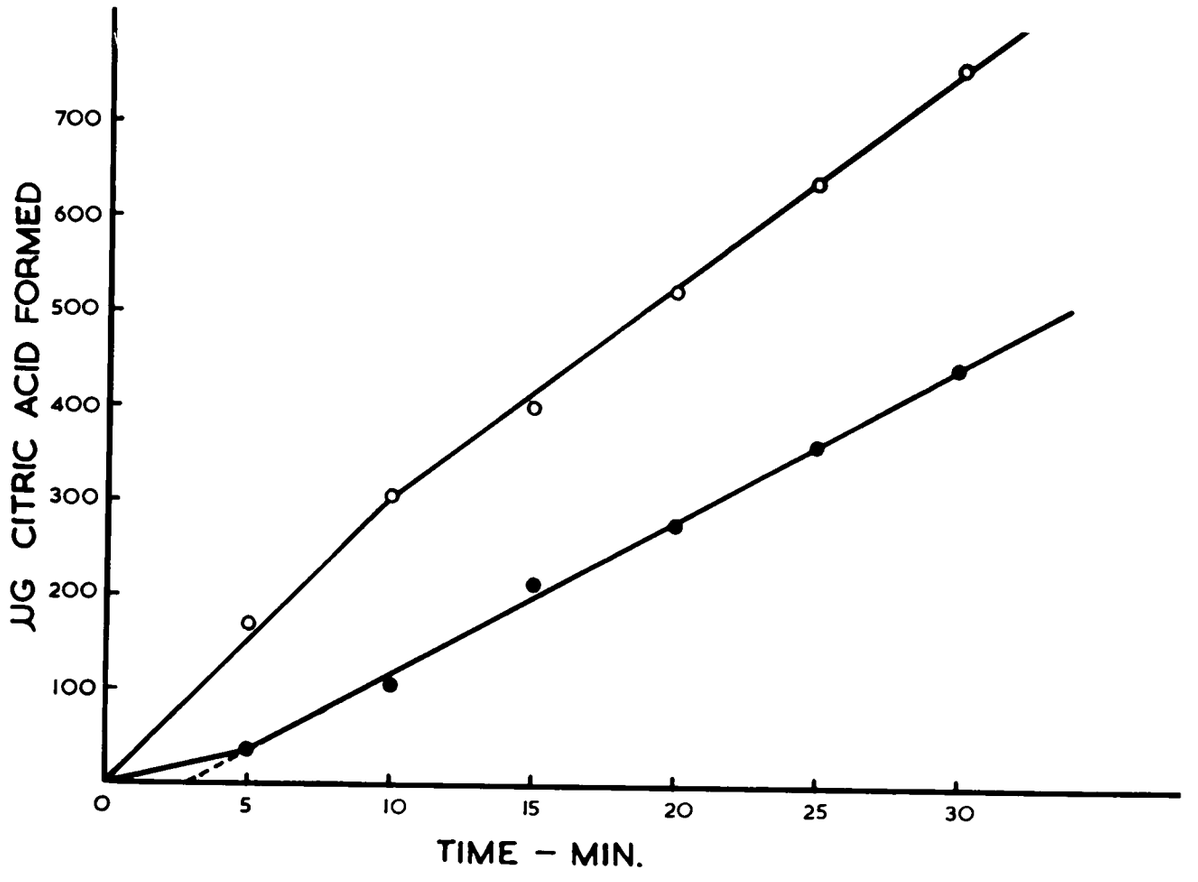


Fig. 5. The activation of aconitase by the addition of Fe^{2+} and cysteine to the test medium. 12 µg. of the non-activated aconitase preparation were added to the test medium which contained 5×10^{-4} M Fe^{2+}, 10^{-2} M cysteine and 4×10^{-3} M cis-aconitic acid. Total volume 5.0 ml., temperature 30°, no buffer, pH 7.4.

o aconitase incubated 5 min. with Fe^{2+} and cysteine before the addition of substrate

• aconitase added to a mixture of Fe^{2+}, cysteine and substrate without pre-incubation.

fact that a finite time is required for maximum activation of the enzyme is verified, but at 30° the period is greatly reduced. If it is assumed that the rate of activation of aconitase is doubled for each 10° rise in temperature, an activation period of 30 min. at 0° is equivalent to 3.7 min. at 30°, so the results are in reasonable agreement.

Activation of aconitase under aerobic and anaerobic conditions

The fact that a reducing agent was required to obtain maximum activation of aconitase suggested that higher enzyme activities might be obtained under strictly anaerobic conditions. Experiments showed that there was no increased enzyme activity when the activation and test were carried out under nitrogen. This was not altogether surprising in view of the fact that the usual test conditions were essentially anaerobic. Oxygen was admitted to the solution only during the time of mixing and apparently the relatively large amount of cysteine present was adequate to prevent oxidation of the Fe^{2+} or enzyme taking place.

If the Fe^{2+}-cysteine-aconitase mixture was rapidly shaken in air during the activation period, the enzyme

showed no activity on the addition of the substrate. Possibly this was due to the complete oxidation of cysteine in the presence of Fe^{2+}, with a result that the cysteine concentration fell to a level at which it was unable to participate in the activation of aconitase. It was also possible that the insoluble cystine produced as a result of the oxidation of cysteine could have adsorbed the enzyme thus preventing the activation taking place, or alternatively rendering the enzyme inactive as a result of the adsorption taking place through the active centre(s) of the enzyme.

Effect of shaking activated aconitase rapidly in air

It was of interest to determine whether or not activated aconitase could be inactivated by oxygen during the course of the reaction. The aconitase was activated by both methods I and II, substrate was added and the reaction rates determined with and without shaking. Fig. 6 shows that there is a slight loss of enzyme activity when the test medium is rapidly shaken in air, following activation by method I. On the other hand, there is a marked loss of activity under these conditions when the activation is carried out by method II. A comparison of the two controls, which were not

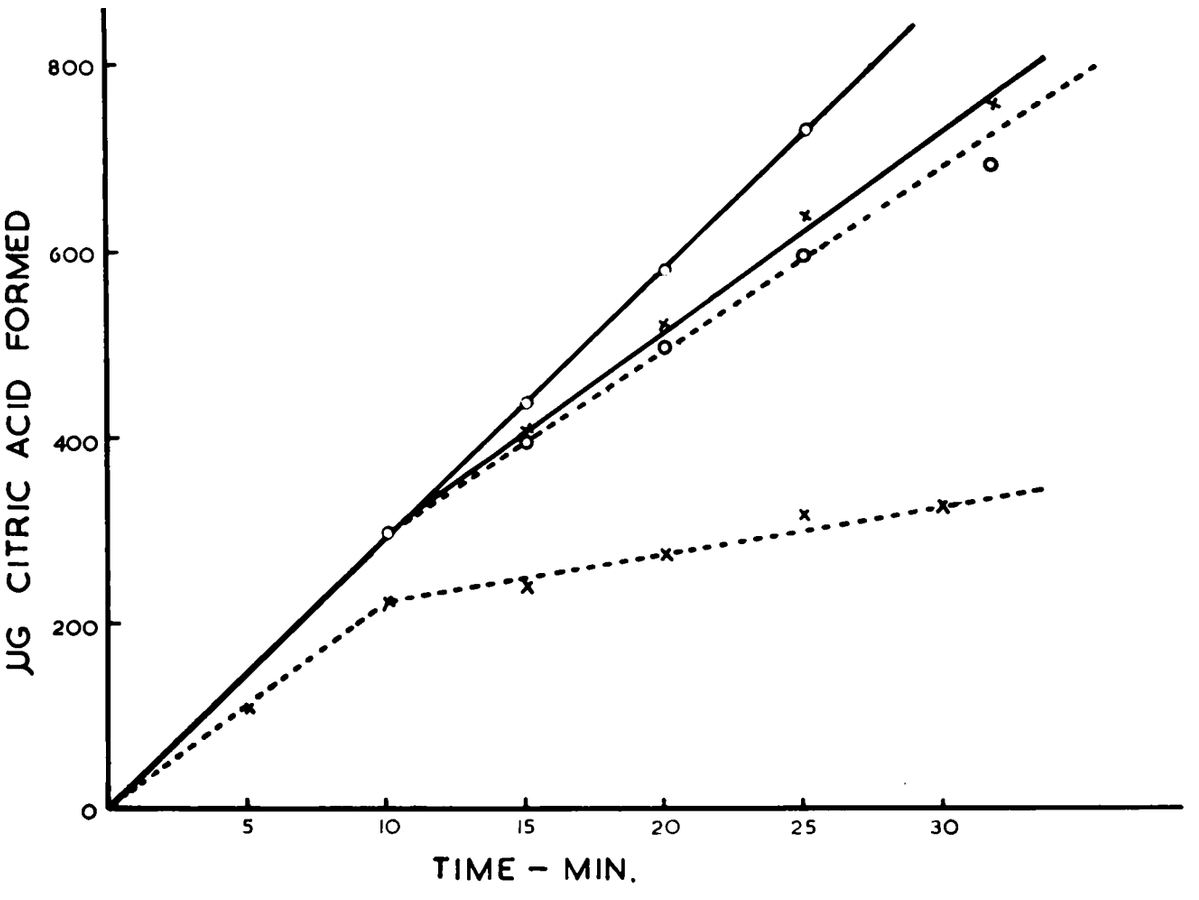


Fig. 6. The effect of rapid shaking in air on the activity of activated aconitase. The amount of aconitase added was equivalent to 12 µg. of the final aconitase preparation. Temperature 30°, no buffer pH 7.4.

o aconitase activated by method I as previously described (page 19)

x aconitase activated by method II as described in fig. 5.

——— non-shaken ..... shaken

shaken, shows that the initial rates are identical, but in the case of aconitase activated by method II, there is a small loss of activity after 10 min. This was a characteristic feature of this means of activation.

Three possible reasons to account for the inactivation of aconitase under these conditions are:

(a) Oxidation of essential -SH groups on the enzyme surface

(b) Formation of Fe^{3+} which is inhibitory

(c) Formation of cystine which is inhibitory.

As the inactivation of the enzyme is greater in the presence of larger amounts of cysteine, (a) and (b) cannot explain the inhibition. The cysteine concentration at any one time must be greater when the enzyme is activated by method II than it is when the enzyme is activated by method I, so that the degree of reduction of the iron and enzyme must be greater. The addition of cystine to the medium in an amount less than that required for a saturated solution did not cause any inhibition. Although cystine in solution did not inhibit aconitase, the adsorption of aconitase by suspended cystine could explain the inhibition. This idea is consistent with the finding that the presence of

high concentrations of cysteine which give rise to suspended cystine as a result of oxidation, caused more marked inhibition of aconitase. The loss of activity in the control of the enzyme activated by method II can also be explained by the formation of smaller amounts of cystine when the solution is not shaken.

These results are of importance as far as artificial systems for the oxidation of citric acid are concerned. Activation of aconitase by method I, so that the concentrations of Fe^{2+} and cysteine in the test medium are minimal, is clearly preferable if the aconitase is to act at a maximum rate for long periods of time under aerobic conditions.

Activation of aconitase by reducing agents

Dickman & Cloutier (1951) had shown that ascorbic acid was as effective as cysteine in activating aconitase, whereas glutathione was only about half as effective. As the preliminary results obtained with the more highly purified preparation of aconitase differed from those of these authors, the ability of reducing agents to activate aconitase was re-investigated.

Table 5 shows the relative effectiveness of four reducing agents in activating aconitase in the presence

TABLE 5

Effect of Reducing Agents on Aconitase Activity

(Aconitase was incubated with the reducing agent and 5×10^{-4} M Fe^{2+} for 1 hr. at 0°. 0.25 ml. samples, equivalent to 15 µg. of the final aconitase preparation, were added to media containing 1.5 ml. of 0.1 M phosphate buffer (pH 7.7), 0.5 ml. of cis-aconitic acid (final concentration 6.7×10^{-3} M) and 0.75 ml. of water. Reaction was stopped after 15 min. by the addition of 0.5 ml. of 50% (w/v) trichloroacetic acid and the mixture analysed for citric acid. Temperature 22°)

Reducing agent	Aconitase Activity (µg. citric acid formed)	
	0.01 M	0.025 M
Cysteine	178	265
Ascorbic acid	100	114
Glutathione	23	182
Thioglycollate	129	182

of a fixed amount of Fe^{2+}. At a level of 0.01 M. cysteine brings about the greatest activation of aconitase, whilst thioglycollate, ascorbic acid and glutathione are less effective in that order. It was surprising that glutathione activated the highly purified enzyme to such a small extent, but later experiments showed that the activation was increased when higher concentrations of glutathione were used. In view of this result, the experiment was repeated with the reducing agents at concentrations of 0.01 M and 0.025 M and both results are included here for comparative purposes. It is seen that when the level of the reducing agents is increased to 0.025 M, the degree of activation is increased in all cases. Cysteine is still the most effective, but the activation by glutathione is increased to such an extent that it is as effective as thioglycollate. The higher concentration of ascorbic acid caused only a small increase in the enzyme activity, so that at the higher level, it was least effective.

Specificity of Fe^{2+} in the activation of aconitase

As Fe^{2+} was markedly catalytic in the oxidation of cysteine, as first shown by Mathews & Walker (1909), it was of interest to determine whether or not those metals

which are capable of oxidising cysteine are also capable of activating aconitase. It was known that Mn^{2+} and Cu^{2+} are also capable of catalytically oxidising cysteine, so these metals were tested for ability to activate aconitase. No other metals are known to oxidise cysteine catalytically.

It was found that Cu^{2+} and Mn^{2+} at a concentration of 5×10^{-4} M did not bring about any activation of aconitase. Moreover, when this concentration of Cu^{2+} was added to the usual activation system containing Fe^{2+} and cysteine, the activity of the enzyme was inhibited 50%. These results confirm and extend those of Krebs & Eggleston (1944). On the other hand, Mn^{2+} was without effect. Thus it would seem as though there was no relationship between the ability of a metal to oxidise cysteine catalytically and its ability to activate aconitase. Results also indicated that there was a lack of any correlation between the other reducing agents and the metals capable of oxidising them catalytically, and the ability of these systems to activate aconitase. It is worthy of note that Cu^{2+} and Mn^{2+} which belong to the same transitions series as Fe^{2+} and which therefore have similar properties, do not activate aconitase.

This indicates the high specificity of aconitase for Fe^{2+}. No other metals were tested as activators of aconitase, but it is clear from the work of Dickman & Cloutier (1950) that Fe^{2+} is specific in this regard.

Role of Fe^{2+} in the action of aconitase

In order to elucidate the role played by Fe^{2+} in the action of aconitase, a quantitative study was made of the effect of the Fe^{2+} concentration on the activity of aconitase.

The observations on the relationship between the Fe^{2+} concentration and the reaction velocity in the presence of cysteine and ascorbic acid, as shown in fig. 7, conform to a simple Michaelis-Menten relation i.e. to the equation $v = \frac{Vx}{K_x + x}$ where v = initial velocity of the reaction at a standard concentration of substrate, in the presence of a reducing agent; x = concentration of Fe^{2+}, and K_x = hypothetical dissociation constant of the Fe^{2+}-enzyme complex formed according to the equation

$$Fe^{2+} + \text{enzyme} \rightleftharpoons Fe^{2+}\text{-enzyme}$$

These results are consistent with the hypothesis that Fe^{2+} combines with aconitase in the ratio of one Fe^{2+} ion to one active centre to form the Fe^{2+}-enzyme complex which

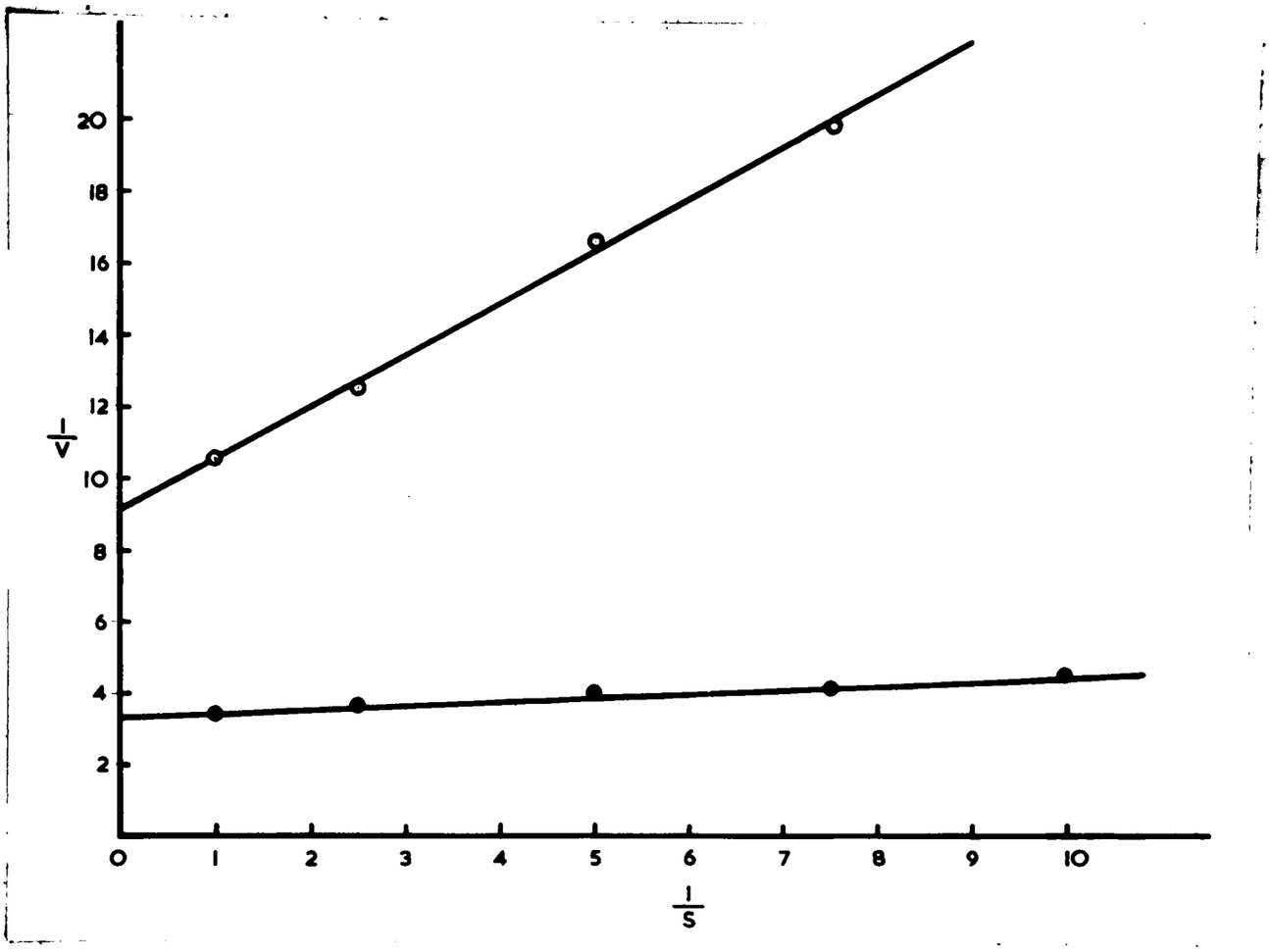


Fig. 7. The effect of Fe^{2+} concentration on aconitase activity. Aconitase was activated in the presence of 10^{-2} M cysteine or ascorbic acid and various concentrations of Fe^{2+} at pH 7.4 for 1 hr. at 0°. 0.25 ml. samples of the enzyme solutions, containing 15 µg. of the final aconitase preparation, were added to media containing 1.5 ml. of 0.1 M phosphate buffer (pH 7.7), 0.5 ml. of neutralised cis-aconitic acid (final concentration 6.7×10^{-3}M) and 0.75ml. of water. The reaction was stopped after 15 min. by the addition of 0.5 ml. of 50%(w/v) trichloroacetic acid and the solutions analysed for citric acid. Temperature 22°.

S = molar conc. of $Fe^{2+} \times 10^4$ o ascorbic acid

V = µg. of citric acid formed/15 min.$\times 10^{-3}$ e cysteine

is responsible for enzymic activity.

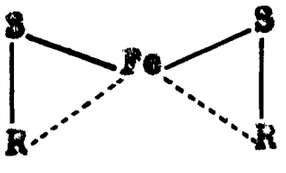
This interpretation is valid only because it was known from previous experiments that the above equilibrium was established during the pre-incubation of the enzyme with Fe^{2+} and the reducing agent for 1 hr. at 0°. As the substrate was not added and the reaction rate was not determined until after the equilibrium was established, it can be taken that the reaction rate was proportional to the amount of the Fe^{2+}-enzyme complex formed. As the final enzyme preparation was subjected to prolonged dialysis against citrate buffer (pH 5.7) before use, it was assumed that there was little or no Fe^{2+} remaining in the preparation. It was on this basis that the hypothetical dissociation constant was calculated to be 3.9×10^{-6} M in the presence of cysteine and 1.7×10^{-5} M in the presence of ascorbic acid. The above assumption may not be correct in view of the fact that the enzyme did possess slight residual activity in the absence of Fe^{2+} and a reducing agent. On this account the value obtained may not be correct and may also vary from one preparation to another. However, it is clear that aconitase has a very high affinity for Fe^{2+}.

It can also be seen from fig. 7 that the absolute

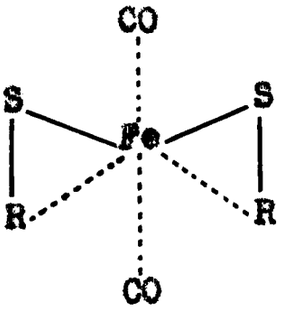
value of the maximum rate obtained in the presence of cysteine is over twice as great as that obtained in the presence of ascorbic acid. If the active complex is of the type Fe^{2+}-enzyme, then when the Fe^{2+} concentration rises sufficiently, the limiting concentration of the Fe^{2+}-enzyme complex obtained must be independent of the nature of the reducing agent. But the maximum velocity is dependent on the nature of the reducing agent which indicates that the reducing agent is concerned in the formation of the active complex. This point will be discussed further in a later section.

Reaction of Fe^{2+} with reducing agents

From the colour of the enzyme solutions during the activation period, it was clear that Fe^{2+} had reacted with cysteine, thioglycollate, ascorbic acid and glutathione to form coloured complexes. Schubert (1932) and Barron (1951) had shown that cysteine reacts with Fe^{2+} at alkaline pH values to form a complex of type (I)



I

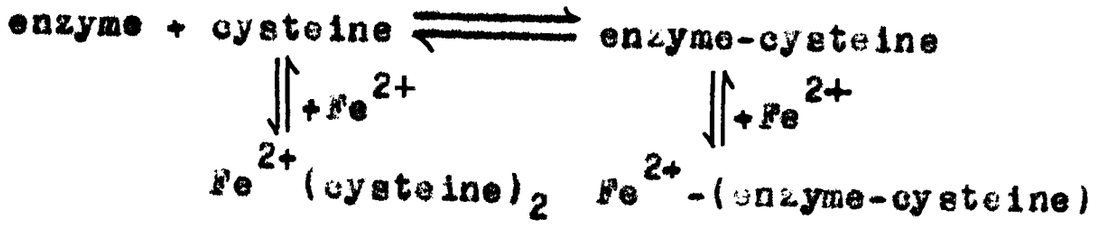


II

which can combine with CO to form a stable colourless complex, or with oxygen to form an unstable blue-violet complex of type (II). A blue-violet colour formed when the enzyme solution containing Fe^{2+} and cysteine was shaken, but faded on standing. A stable cherry-red complex formed as a result of the reaction between Fe^{2+} and thioglycollate. Schubert (1942) showed that this complex was also of type (I). A violet complex formed between Fe^{2+} and ascorbic acid, but no evidence for the structure of this complex could be found in the literature. However, the structure of ascorbic acid would seem to permit of the formation of a complex with Fe^{2+}. Glutathione and Fe^{2+} gave a stable golden yellow complex, but again no evidence could be found for the structure of this complex. Whereas the Fe^{2+}-cysteine complex was coloured only in the presence of oxygen, the other complexes were coloured in the absence of oxygen.

Equilibrium reactions of the aconitase system

The experiments to date had indicated that reducing agents as well as Fe^{2+} were concerned in the formation of the active aconitase complex. The equilibrium between the enzyme, Fe^{2+} and cysteine might be as follows:



so that the Fe^{2+}(enzyme-cysteine) complex was the active complex in the case of cysteine. If the affinity of the enzyme for cysteine were greater than the affinity of Fe^{2+} for cysteine, it might be expected that high concentrations of cysteine would give rise to maximum formation of the enzyme-cysteine complex. At the same time, the enzymic activity could be reduced as a result of the equilibrium between the Fe^{2+} and cysteine being displaced in favour of the formation of the $Fe^{2+}(cysteine)_2$ complex. The same argument might also be expected to apply in the case of ascorbic acid. Fig. 8 shows that this is the case with both reducing agents. It is clear also that the enzyme activity in the presence of cysteine is greater than in the presence of ascorbic acid. The similarity of the curves is more apparent when the enzyme activities are plotted as percentages of the activity in the presence of the reducing agent at a concentration of 10^{-2} M as shown in Fig. 9. These results are consistent with the idea that the activation processes are similar

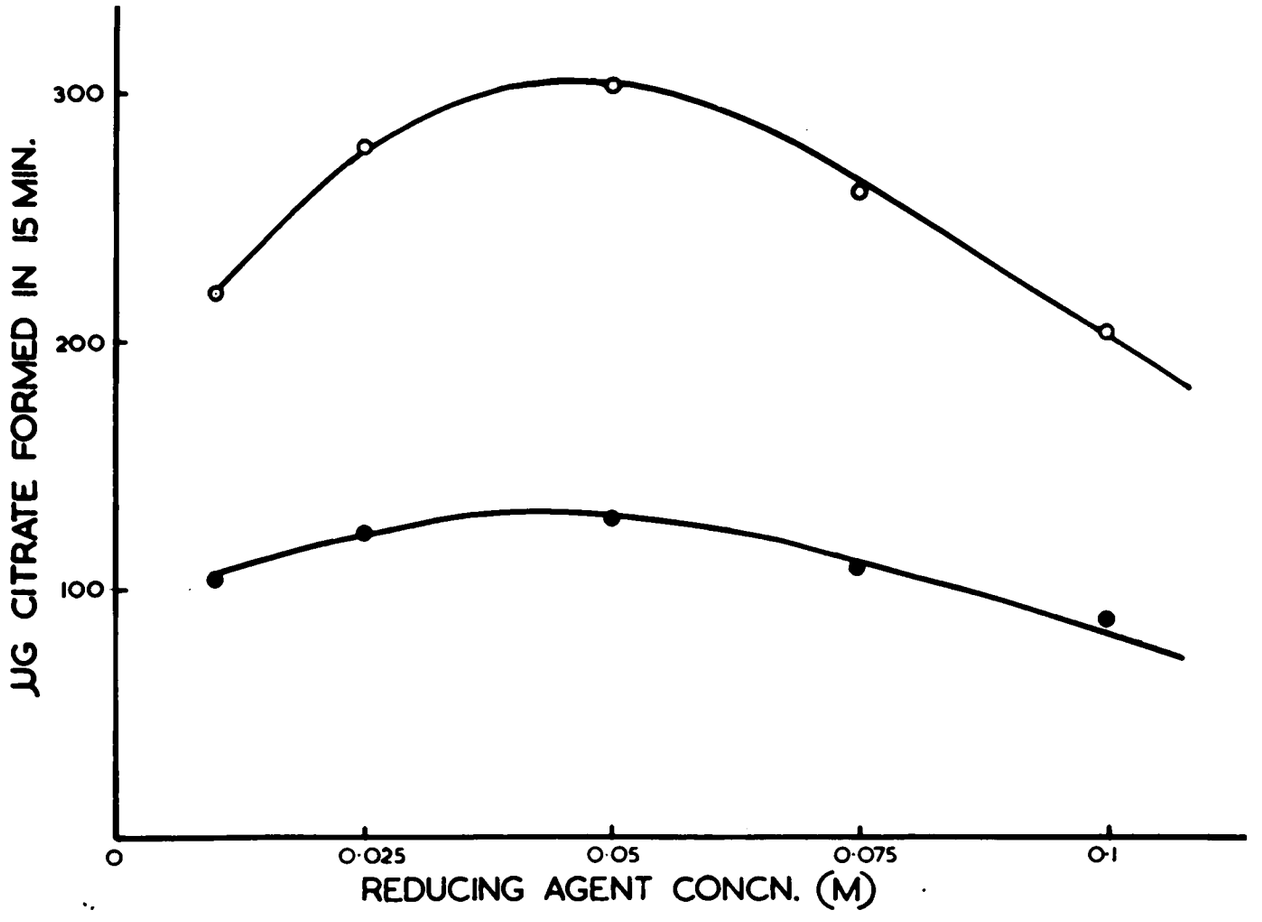


Fig. 8. The effect of increasing concentrations of cysteine and ascorbic acid on aconitase activity. Aconitase was incubated with various concentrations of cysteine or ascorbic acid and 5×10^{-4} M Fe^{2+} for 1 hr. at 0° and pH 7.4. Activity determinations were carried out as described in fig. 7.

o = cysteine • = ascorbic acid

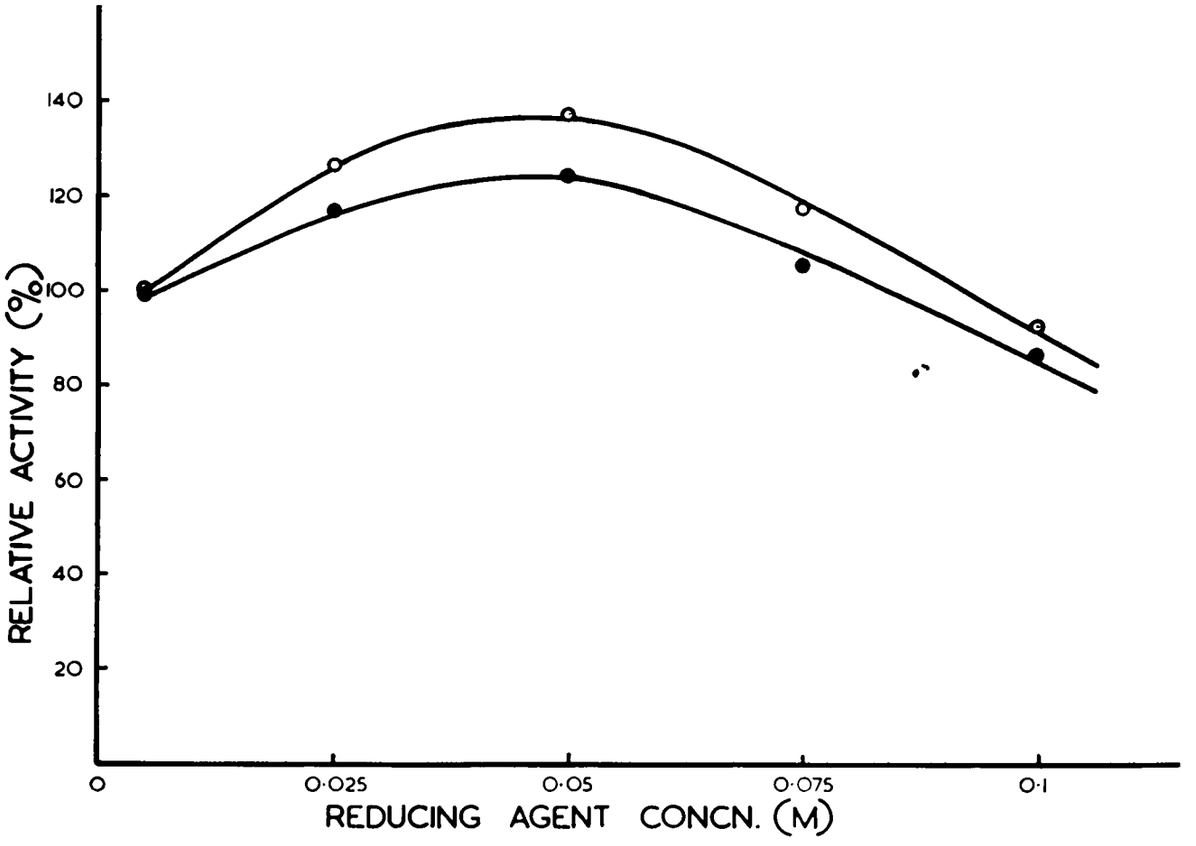


Fig. 9. The effect of increasing concentrations of cysteine and ascorbic acid on aconitase activity. Conditions as described in fig. 8.

o = cysteine ● = ascorbic acid

with both cysteine and ascorbic acid, the absolute activity of the active complex being dependent on the nature of the reducing agent.

Role of reducing agents in the action of aconitase

In the hope of establishing more securely the hypothesis that the active form of aconitase is a complex containing cysteine, ascorbic acid or other activators, an investigation was made of the effect of varying concentrations of the reducing agents on the aconitase activity in the presence of 5×10^{-4} M Fe^{2+}.

Cysteine, thioglycollate and ascorbic acid

The observations on the relationship between the concentration of cysteine, thioglycollate and ascorbic acid and the reaction velocity, as shown in Fig. 10, conform to a simple Michaelis-Menten relation, i.e. to the equation

$$v = \frac{Vc}{K_c + c} \qquad \text{where}$$

v = initial velocity of the reaction at a standard concentration of substrate, in the presence of 5×10^{-4} M Fe^{2+}.

c = concentration of the reducing agent

K_c = hypothetical dissociation constant of the reducing

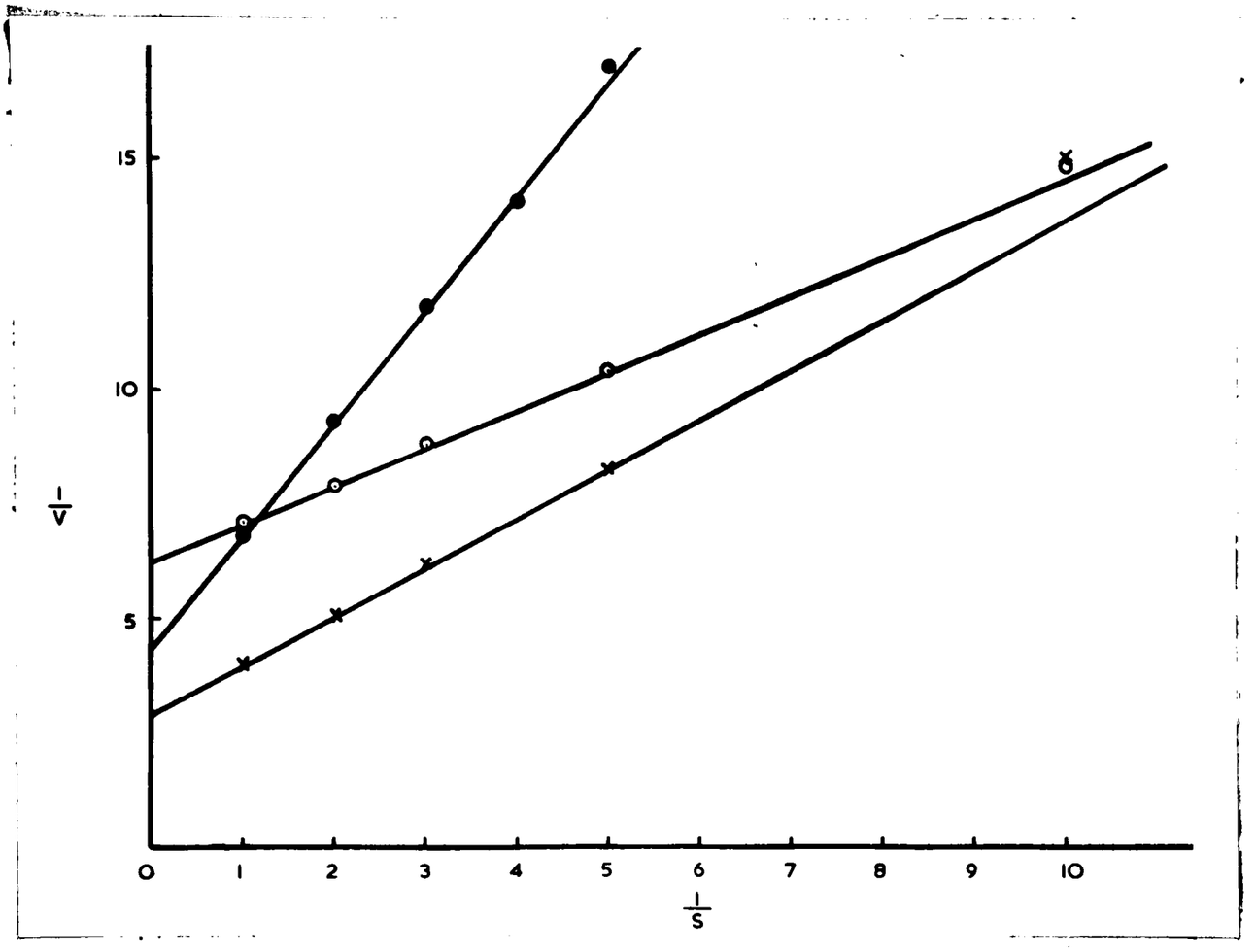


Fig. 10. The effect of cysteine, thioglycollate and ascorbic acid concentration on aconitase activity in the presence of Fe^{2+}. Aconitase (60 µg. of the final preparation/ml.) was incubated with 5×10^{-4} M Fe^{2+} and various concentrations of the reducing agents for 1 hr. at 0° and pH 7.4. The enzyme activity was determined as described in fig. 7.

δ = molar concentration of reducing agent x 10^2

V = µg. of citric acid formed/15 min. x 10^{-3}

x cysteine o ascorbic acid • thioglycollate

agent-enzyme complex, formed according to the equation

$$\text{enzyme} + \text{reducing agent} \rightleftharpoons \text{enzyme-reducing agent}$$

There was a tendency for the points obtained at low concentrations of the reducing agents to lie off a straight line; this fact may have some significance, but the experimental error was much greater with the low reaction rates. The results indicate that each of the reducing agents combines with aconitase in the ratio of one molecule of the reducing agent to one active centre to form an enzyme-reducing agent complex. However, this complex cannot be considered in itself as an active complex in view of the previous finding that the enzyme is inactive in the presence of a reducing agent alone. No activities were obtained unless Fe^{2+} was also present. Again this interpretation is valid only because the system had attained equilibrium before the reaction rates were determined.

The hypothetical dissociation constants of the enzyme-reducing agent complex were calculated to be 3.6×10^{-3} M for cysteine, 1.2×10^{-3} M for ascorbic acid and 6.0×10^{-3} M for thioglycollate. These values indicate that the affinity of the enzyme for ascorbic

acid > cysteine > thioglycollate. There is no relationship between the affinities of the enzyme for the reducing agents and the maximum activity of the enzyme in the presence of the same reducing agents. Cysteine gives the greatest maximum velocity, ascorbic acid gives the lowest, whilst thioglycollate gives an intermediate maximum velocity value. These findings are in keeping with the idea that the active form of the enzyme is the complex reducing agent-enzyme-Fe^{2+}, the properties of the complex being determined by the nature of the reducing agent.

The plot of the reciprocal of the velocity against the reciprocal of the square of the reducing agent concentration gave a curve rather than a straight line. If the reducing agents formed a $Fe^{2+}(\text{reducing agent})_2$ complex which combined with the enzyme, or if the active form of the enzyme was enzyme-$(\text{reducing agent})_2$, then this plot would give a straight line. Although Fe^{2+} forms complexes of the above type with the reducing agents, it is not these complexes which are responsible for the activation of aconitase.

Glutathione

When glutathione was tested for its ability to

activate aconitase, it was seen that very little activation was obtained at a concentration of 0.01 M. However, higher concentrations of glutathione gave an appreciable activation of the enzyme; maximum activation was obtained with glutathione at a concentration of 0.025 M. Having found that aconitase could be activated by glutathione, a study was made of the effect of glutathione concentration on the enzyme activity. Fig. 11 shows that there is no simple relationship between the concentration of glutathione and the enzyme activity, such as was found with cysteine, thioglycollate and ascorbic acid. The fact that a curve is obtained when the reciprocal of the velocity is plotted against the reciprocal of the glutathione concentration would seem to indicate that the mechanism of the activation is different from that obtained with the other reducing agents.

DISCUSSION

The marked activation of aconitase by Fe^{2+} in the presence of a reducing agent and the failure of other metal ions to activate, is indicative of Fe^{2+} being a specific integral component of the aconitase system.

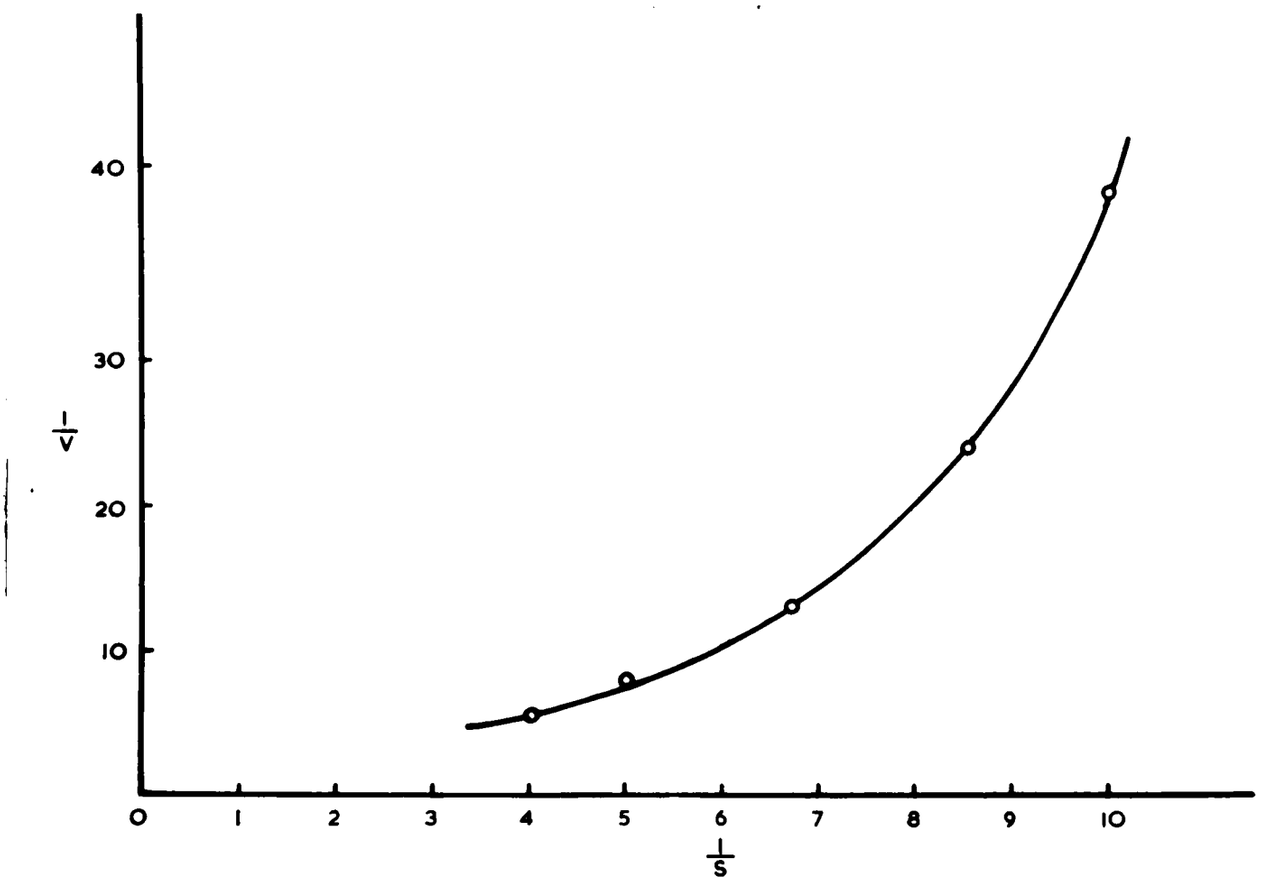


Fig. 11. The effect of glutathione concentration on aconitase activity. The conditions were the same as those described in fig. 10.

S = molar concentration of glutathione x 10

V = µg. of citric acid formed/15 min. x 10^{-3}

The observations on the relationship between the Fe^{2+} concentration and the enzyme activity are consistent with the idea that Fe^{2+} reacts with the enzyme in the ratio of one Fe^{2+} ion to one active centre of the enzyme according to the equation

$$Fe^{2+} + \text{enzyme} \rightleftharpoons Fe^{2+}\text{-enzyme}$$

Thus the Fe^{2+}-enzyme complex can be considered as an active component of the system. As the activation of the enzyme is increased by the presence of a reducing agent and influenced by the type of reducing agent, this cannot be the only reaction concerned in the activation.

The equilibrium between the enzyme and the activators is not established immediately; a definite time is required before the enzyme attains its maximum activity. This suggests that the free energy of the activation is significant. Thus the interaction cannot be entirely electrostatic as such a reaction is instantaneous and does not require activation energy.

The four reducing agents tested have the ability of increasing the enzyme above that obtained with Fe^{2+} alone, although they are not equally effective. The function of the reducing agents could be related to their

ability to maintain the iron as Fe^{2+}. Since the concentrations of the reducing agents were always in excess of that of Fe^{2+} and the oxidation-reduction potentials are such that they favour the reduction of the iron, then only a small amount of Fe^{3+} must be present under the conditions of these experiments. Therefore, it is unlikely that a Michaelis-Menten relationship between the concentration of the reducing agent and the enzyme activity would be found if this were the sole function of the reducing agents. Moreover, if this were the case, the maximum velocity of the enzyme should be independent of the nature of the reducing agent and this is not so.

If the reducing agents were concerned in reducing a group on the surface of the enzyme which was essential for activity and the reduction were an equilibrium reaction requiring relatively high concentrations of the reducing agent, it might be expected that there would be a Michaelis-Menten relationship between the concentration of the reducing agent and the enzyme activity. As the reducing agents are capable of reducing disulphide linkages, it would seem most likely that this was the type of group concerned. However, the

Michaelis-Menten relations indicate that the enzyme would have to be a monothiol enzyme and it is difficult to see to what this group could have been oxidised. However, if the enzyme molecules were monothiol complexes which can form dimers in solution as a result of the formation of disulphide linkages, then the activation of the enzyme by reducing agents could be concerned with the splitting of these linkages. Two molecules of the reducing agent would be required to reduce each dimer and two active centres, one on each molecule, would be exposed. The reducing agent would then be reacting with the enzyme in the ratio of one molecule of the reducing agent to one active centre of the enzyme. However, it would be expected that if the same type of disulphide bonds were concerned that when the concentration of each reducing agent, which was capable of activating the enzyme, was increased to a sufficiently high value, the maximum enzyme activity would be independent of the nature of the reducing agent. This is not the case, so the above hypothesis must be abandoned.

Before discussing another possible interpretation of the results obtained with the reducing agents, some

complicating features of the reaction must be mentioned. The four reducing agents which were capable of activating aconitase are also capable of forming complexes with Fe^{2+} and these complexes exist under the conditions used for the activation of the enzyme. It might seem as though it was the complexes which activated the enzyme, rather than the free components. As the results obtained are consistent with the idea that one molecule of the reducing agent reacts with each active centre of the enzyme, this cannot be the case. At least with cysteine and thioglycollate, the complex formed with Fe^{2+} is of the type $Fe^{2+}(RS)_2$, so the results would indicate that two molecules of the reducing agent were reacting with each active centre of the enzyme. The same argument applies if the Fe^{2+}-reducing agent complexes were concerned in reducing the enzyme. Moreover, if the function of these complexes were simply to reduce the enzyme, it would be expected that other metals of the same transition series e.g. Co^{2+}, Mn^{2+} etc. could replace Fe^{2+}.

The fact that the reducing agents, cysteine, thioglycollate and ascorbic acid, react with aconitase in the ratio of one molecule of the reducing agent to

one active centre can also be interpreted to mean that the reaction:

enzyme + reducing agent $\rightleftharpoons$ enzyme-reducing agent

occurs and that the enzyme-reducing agent complex is at least partially responsible for aconitase activity. It cannot be wholly responsible as the enzyme shows no activity in the presence of a reducing agent and absence of Fe^{2+}. Statistical analysis of the results obtained with cysteine and ascorbic acid as activators of aconitase showed that the maximum enzyme velocities and the hypothetical dissociation constants of the enzyme/ complexes were significantly different. The ability of cysteine to activate the enzyme was about twice as great as that of ascorbic acid, although the affinity of the enzyme for ascorbic acid was greater than it was for cysteine. With thioglycollate, the maximum enzyme velocity was found to be intermediate between the velocities obtained with cysteine and ascorbic acid, whilst the dissociation constant of the enzyme-thioglycollate complex was greater than either the enzyme-cysteine or the enzyme-ascorbic acid complexes. This suggests that different forms of aconitase, each

with distinct properties, are catalytically active.

The results with glutathione somewhat complicate the matter. There is no direct relationship between the enzyme activity and the concentration of glutathione. Below a certain concentration, its ability to activate the enzyme is sharply reduced. It is interesting to compare this result with those obtained by Kubowitz (1935) on the reaction between Fe^{2+} and glutathione, although the relationship between the two results is not clear. He found that it was possible to form a CO complex of ferrous glutathione only in the presence of high concentrations of glutathione and concluded that this was due to the high dissociability of the ferrous glutathione complex. With a Fe^{2+} concentration of 2×10^{-3} M, he found that the concentration of glutathione required to form the CO complex of ferrous glutathione was 10^{-1} M. On this basis, a Fe^{2+} concentration of 5×10^{-4} M would require a glutathione concentration of 2.5×10^{-2} M which is the concentration found to be required for the maximum activation of aconitase.

It has been established that Fe^{2+} is a specific activator of aconitase and forms a dissociable complex with the enzyme, also that this metal alone is not

sufficient to bring about maximum activation of the enzyme. Reducing agents are also required, in fact, the degree of activation of the enzyme by Fe^{2+} is dependent on the nature of the reducing agent present. It has also been established that cysteine, thioglycollate and ascorbic acid form dissociable complexes with the enzyme. The dissociation constant of each complex differs and bears no relationship to the maximum velocity of the enzyme. From these facts, it seems as though the activation of aconitase by Fe^{2+} and a reducing agent can best be explained by considering the active form of the enzyme to be a Fe^{2+}-enzyme-reducing agent complex. The results with glutathione do not completely fit in with this hypothesis. Glutathione does activate the enzyme, but if a Fe^{2+}-enzyme-glutathione complex is required for activity, the glutatnione does not dissociate in the same fashion as do the other reducing agents. Therefore, at present the mechanism of the activation of aconitase by glutathione must be regarded as being distinct from that with the other reducing agents.

It may be only fortuitous that all the organic compounds found to activate aconitase are reducing agents. Other types of organic compound have not been

tested for their ability to activate the enzyme, but they may well do so. In view of this uncertainty, it may be better to consider the active form of the aconitase complex as being Fe^{2+}-enzyme-A where A is a group of activators in which the reducing agents are included. It is possible that the essential feature of an activator is its ability to form a certain type of complex with Fe^{2+}.

The point arises as to whether a single or two different sites on the enzyme surface are concerned in the reactions between the enzyme and the Fe^{2+} and the activators. At this stage it is not possible to say which is the case, nor is it possible to state the exact function of the Fe^{2+} and the activator. They may be concerned in linking the substrates to aconitase, fixing the degrees of freedom of the enzyme molecule or both. However, as Fe^{2+} reacts with two molecules of the activators, at least in the case of cysteine and thioglycollate, and the enzyme reacts with one Fe^{2+} ion and one molecule of the activator, it does seem plausible that the form of the active enzyme complex is enzyme-Fe^{2+}-activator. Furthermore, since the substrates of aconitase are also capable of forming complexes with Fe^{2+}

it is tempting to suggest that the remaining two free valencies of the Fe^{2+} are concerned in substrate linkage. If this were the case, it is not so difficult to reconcile the fact that compounds of such different structure as cysteine and ascorbic acid can function as activators of aconitase. On the other hand, if Fe^{2+} alone were concerned with linking the substrate to the enzyme, there would be, in effect, competition between the activators of the enzyme and the substrates.

Although further information has been obtained concerning the relationship between Fe^{2+} and the activators and the aconitase activity, it is clear that the problem of the mode of action of aconitase is far from being solved. More light may be thrown on the problem by determination of the Michaelis constants and maximum velocities of aconitase for its three substrates in the presence of different activators, also by determination of whether or not Fe^{2+} can form a mixed complex with each of the activators and the substrates of the enzyme.

SUMMARY

1. It was shown that the final purified aconitase preparation possessed little activity. It was not

activated by cysteine alone; the addition of Fe^{2+} increased the activity 15-fold, whilst the addition of Fe^{2+} and cysteine gave a 70-fold increase in activity.

2. The optimum conditions required for maximum activation and maximum activity of aconitase were determined.

3. Aconitase was not activated by Mn^{2+} and Cu^{2+} was inhibitory.

4. A Michaelis-Menten relationship was shown to exist between the concentration of Fe^{2+} and the enzyme activity. It was concluded that a Fe^{2+}-enzyme complex was partly responsible for aconitase activity.

5. A Michaelis-Menten relationship was also found to exist between the enzyme activity and the concentration of the reducing agents cysteine, thioglycollate and ascorbic acid. It was concluded that these compounds form an enzyme-reducing agent complex which is partly reponsible for enzyme activity. Only higher concentrations of glutathione appreciably activated aconitase; there was no Michaelis-Menten relationship between the concentration of glutathione and the enzyme activity.

6. The conclusion was drawn that the active form of aconitase is either an enzyme-Fe^{2+}-reducing agent or an enzyme-Fe^{2+}-activator complex.

C H A P T E R III

THE INFLUENCE of BUFFERS on the pH OPTIMUM of ACONITASE

CHAPTER III

THE INFLUENCE of BUFFERS on the pH OPTIMUM of ACONITASE

INTRODUCTION

Jacobsohn (1941) found that the pH optimum of aconitase in the presence of phosphate buffer was in the neutral region. A more extensive study of the influence of pH on aconitase activity was made by Johnson (1939). He claimed that the pH-activity curve showed a sharp maximum at pH 7.4, but this value must be regarded as approximate. The pH values chosen for testing the aconitase activity were widely spaced and no mention was made of the buffers used to cover the extensive pH range.

As this was the only information concerning the effect of pH on aconitase activity and was gained from studies with crude aconitase preparations, a more detailed investigation was made of the effect of pH

and the type of buffer on the enzyme activity.

EXPERIMENTAL

Determination of aconitase activity

Aconitase was activated with Fe^{2+} and cysteine by the method previously described (page 19). The enzyme solution contained 60 µg. of the final aconitase preparation/ml. in the presence of 5×10^{-4} M Fe^{2+} and 10^{-2} M cysteine. The veronal acetate buffer was prepared according to Michaelis (1931), whilst the other buffers were 0.1 M. 0.15 ml. samples of the enzyme solution were added to media containing 2.5 ml. of the appropriate buffer, 0.5 ml. of cis-aconitic acid or isocitric acid (final concentration 4×10^{-3} M) and 1.85 ml. of water. The reaction was stopped after 15 min. at 30° by the addition of 0.5 ml. of 50% (w/v) trichloroacetic acid when citric acid was determined, or 0.5 ml. of 10% (w/v) metaphosphoric acid when cis-aconitic acid was estimated.

Determination of citric and cis-aconitic acids

Citric acid was estimated by the method previously described (page 18), whilst cis-aconitic acid was estimated by the method of Dickman (1952).

Reagents

N-Ethyl morpholine was kindly supplied by Prof. A. Albert. Veronal, glycerophosphoric acid, glycyl--glycine, boric and metaphosphoric acid were B.D.H. Laboratory Reagents. The other reagents were A.R.

RESULTS

During the purification of aconitase, the various fractions obtained were tested for aconitase activity in a system which did not include a buffer and the pH chosen for the test was an arbitrary value. As it is preferable to study enzymic reactions in a system in which the pH is stabilized by the presence of a buffer, the enzyme activity was determined firstly in the presence of different buffers at pH 7.4.

Aconitase activity in the presence of different buffers at pH 7.4

It was seen in Chapter I that the inactivating effect of various buffers could be eliminated if the enzyme were added to the buffer-substrate mixture. These activity tests were carried out in that fashion. Fig. 12 shows the effect of six different buffers at pH 7.4 on the activity of aconitase as compared to the activity when the reactions were carried out in the absence

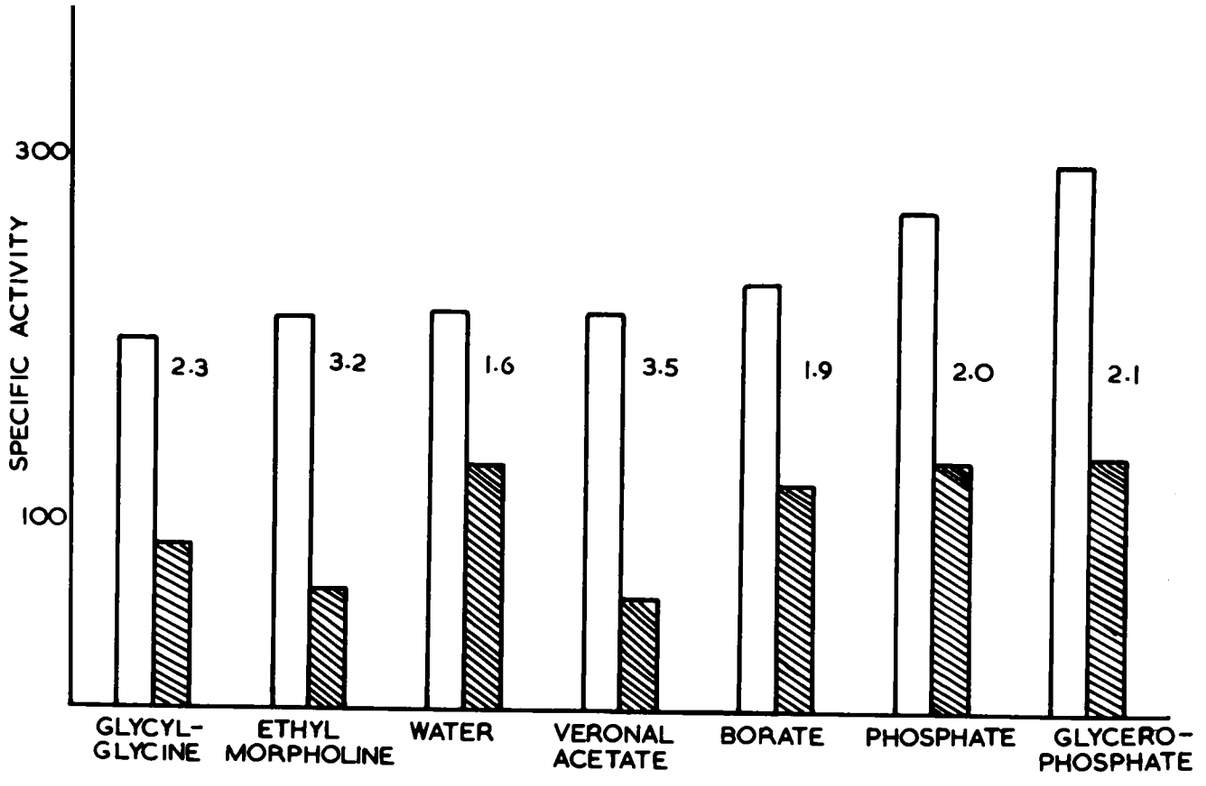


Fig 12. The activity of aconitase in the presence of different buffers at pH 7.4. Enzyme activity determined as described in the text. Temperature 30°. buffer 0.05 M.

unshaded bars = cis-aconitic acid ⟶ citric acid - (1)

shaded bars = iso-citric acid ⟶ citric acid - (2)

figures indicate the ratio of reaction (1) to reaction (2).

of buffer. For comparative purposes the reactions isocitric acid ⟶ citric acid and cis-aconitic acid ⟶ citric acid were studied. It is clear that the composition of the buffer has a profound influence on these reactions. In the reaction cis-aconitic acid ⟶ citric acid, the enzyme activity in glycyl-glycine is decreased as compared to the activity in the absence of buffer; veronal acetate and N-ethyl morpholine have no effect, whilst borate, phosphate and glycerophosphate increase the activity. In the reaction isocitric acid ⟶ citric acid, the activity is approximately the same in phosphate and glycerophosphate, and in the absence of buffer, whereas the activity is lower in borate, glycyl-glycine, N-ethyl morpholine and veronal acetate. The consequence is that the ratio of the rates of the two reactions varies according to the buffer used.

There seemed to be two possible explanations for the above results; that the various buffers have different abilities to activate and inactivate the two reactions, or that the pH optimum or optima of the reactions are influenced by the ions present in solution. As the latter explanation seemed more likely, the pH optimum of the enzyme in catalysing both reactions was determined in the presence of different buffers.

Influence of buffers on the pH optimum of aconitase

It can be seen from figs. 13 & 14 that the pH optimum of aconitase is influenced by the buffer in which the reaction is carried out. The following pH optima were found for both the reactions: glycerophosphate, 7.5; phosphate, 7.7; N-ethyl morpholine, 8.1 and veronal acetate 8.6. The absolute rates of each reaction were approximately the same at the pH optimum for each buffer, so that the ratio of the rates of the reactions cis-aconitic acid ⟶ citric acid to iso-citric acid ⟶ citric acid was about 1.8 in each case. This compares with values ranging from 1.6 to 3.5 which were obtained previously with these buffers at pH 7.4.

It was not possible to determine the activity of aconitase over a wide range of pH in the absence of buffer on account of the poor buffering capacity of the above aconitase substrates at higher pH values. (The approximate pK values of these acids are reported in Table 6. It was of interest to determine them as to the author's knowledge no values have been reported in the literature). However, the activity of the enzyme was determined for the reaction cis-aconitic acid ⟶ citric acid at the lower pH values. In figs. 13 & 14 it is seen that over the pH range which could be studied, the

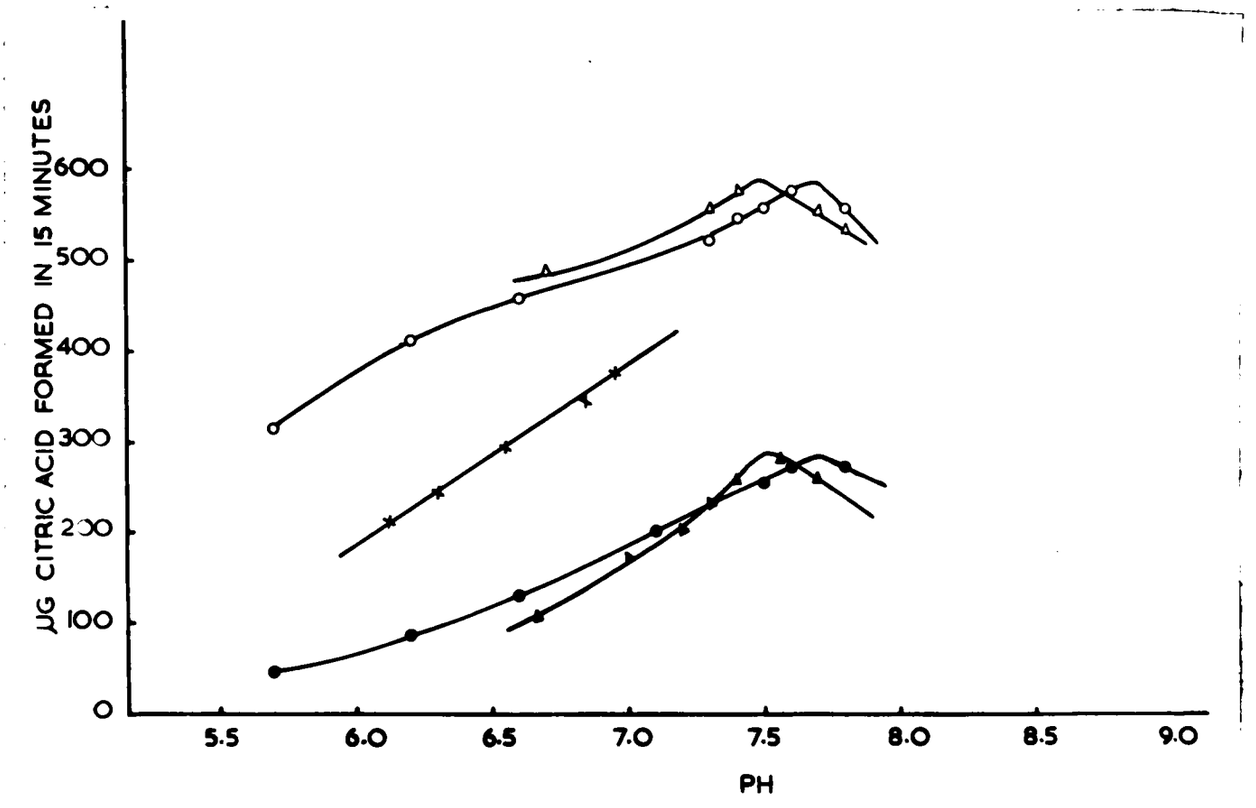


Fig. 13. Effect of buffers on the pH optimum of aconitase. Enzyme activities determined as described in the text. Temperature 30°, buffer 0.05 M.

x = no buffer
O = phosphate
Δ = glycerophosphate

} cis-aconitic acid ⟶ citric acid

● = phosphate
▲ = glycerophosphate

} isocitric acid ⟶ citric acid

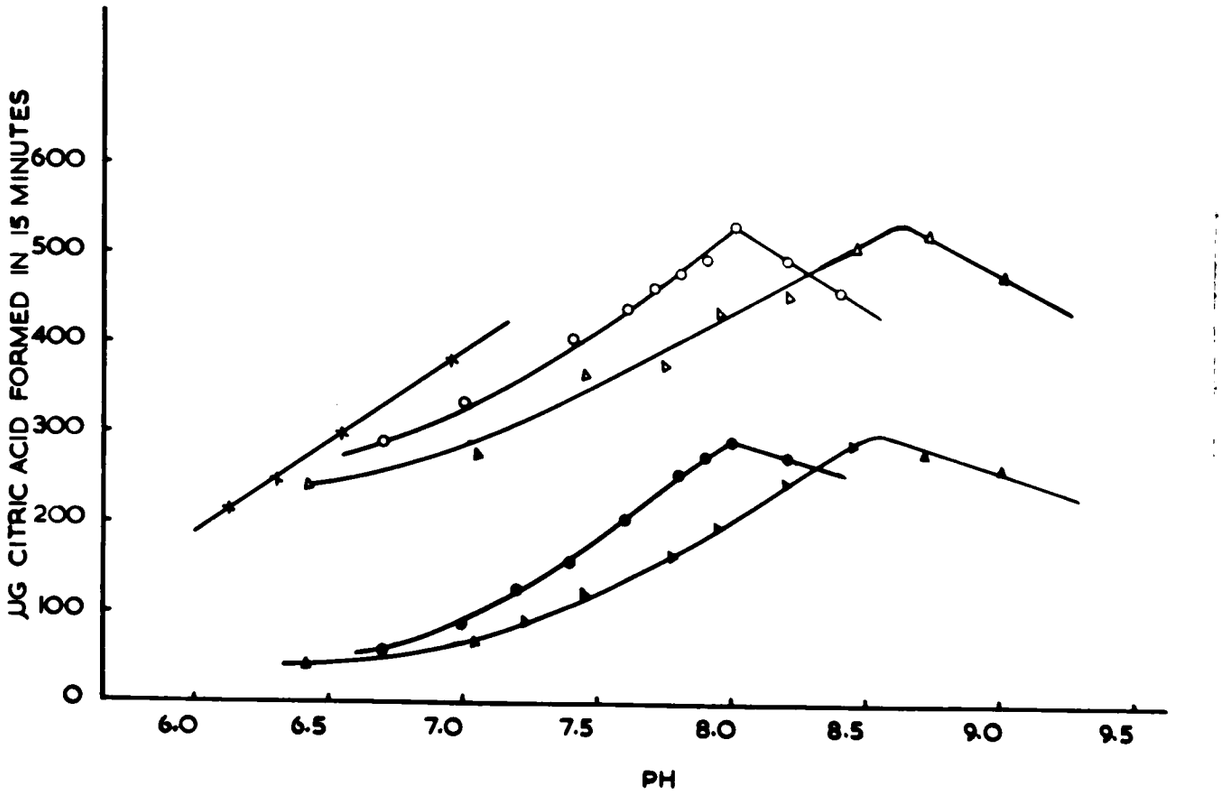


Fig. 14. Effect of buffers on the pH optimum of aconitase. Conditions the same as those of fig. 13.

x = no buffer)
) <u>cis</u>-aconitic acid ⟶ citric acid
o = N-ethyl morpholine)
Δ = veronal acetate)

• = N-ethyl morpholine)
) <u>iso</u>citric acid ⟶ citric acid
▲ = veronal acetate)

TABLE 6

pK Values of Citric, cis-Aconitic and isoCitric Acids

(0.04 M solutions of the acids were titrated with 0.873 M NaOH at 20°. Stirring was by means of a rapid stream of N_2. isoCitric acid was the DL- isomer. The values in brackets are those obtained for citric acid by Hastings & Van Slyke (1922) at 20°).

Acid	pK_1	pK_2	pK_3
Citric	3.2	4.7	5.6
	(3.1)	(4.75)	(5.4)
cis-Aconitic	2.1	4.1	6.2
isoCitric	3.1	4.4	5.2

enzyme had not reached its maximum activity. If the line obtained is produced to the level of the optimal enzyme activity in the other buffers, it can be estimated that the pH optimum in the absence of buffer is about pH 8.0.

As the pH optimum of the reactions cis-aconitic acid ⟶ citric acid and isocitric acid ⟶ citric acid was the same in the presence of a particular buffer and altered to the same extent in different buffers, it was of interest to determine if this were also the case for the reaction isocitric acid ⟶ cis-aconitic acid. The pH-activity curves obtained with phosphate and veronal acetate buffers, as shown in fig. 15, indicate that this is indeed the case.

Inactivation of aconitase by buffers

Fig. 16 illustrates the rapid inactivation of aconitase that occurs when the enzyme is incubated in the absence of substrate for various periods of time with different buffers. The pH of each buffer was such that the aconitase activity was maximal. The rate of inactivation differs for each buffer and appears to be a function of the pH value.

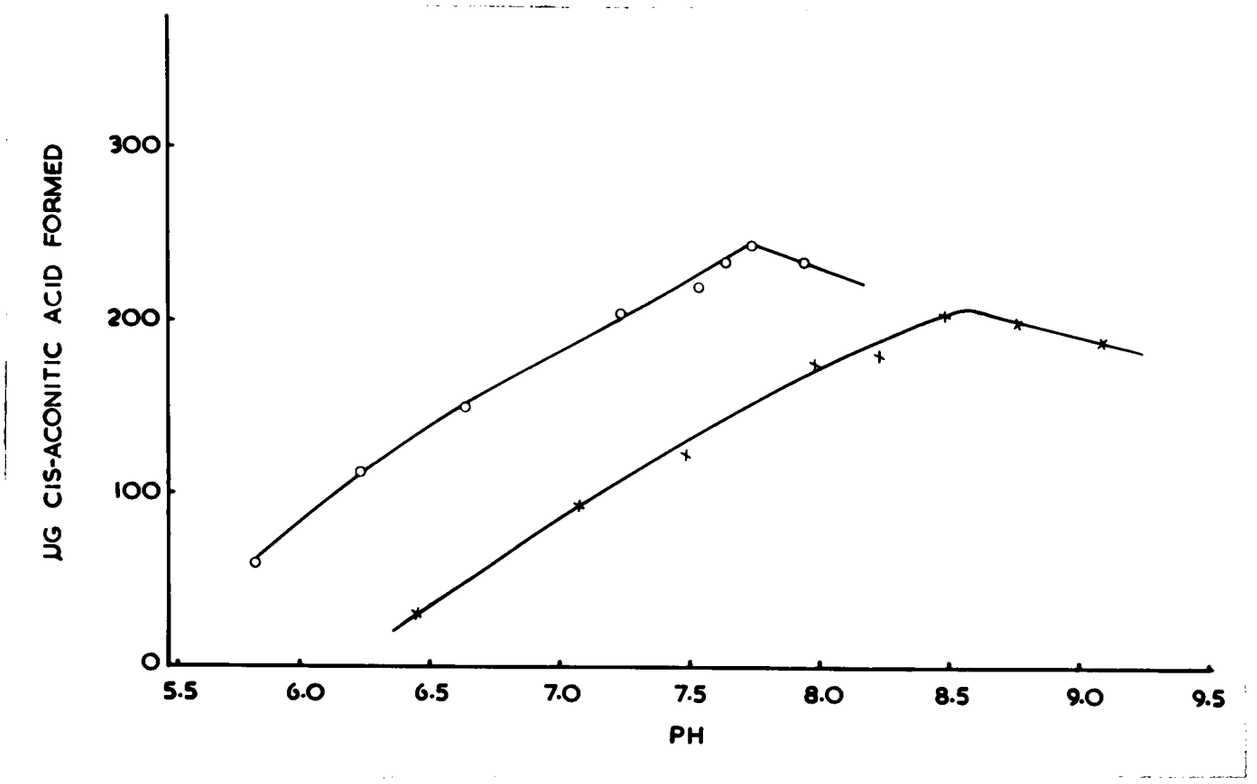


Fig. 15. Effect of buffers on the pH optimum of aconitase. Conditions the same as those described in fig. 13 except that the reaction was stopped by the addition of 0.5 ml. of 10% (w/v) metaphosphoric acid.

o = phosphate
x = veronal acetate

} <u>iso</u>citric acid ⟶ <u>cis</u>-aconitic acid

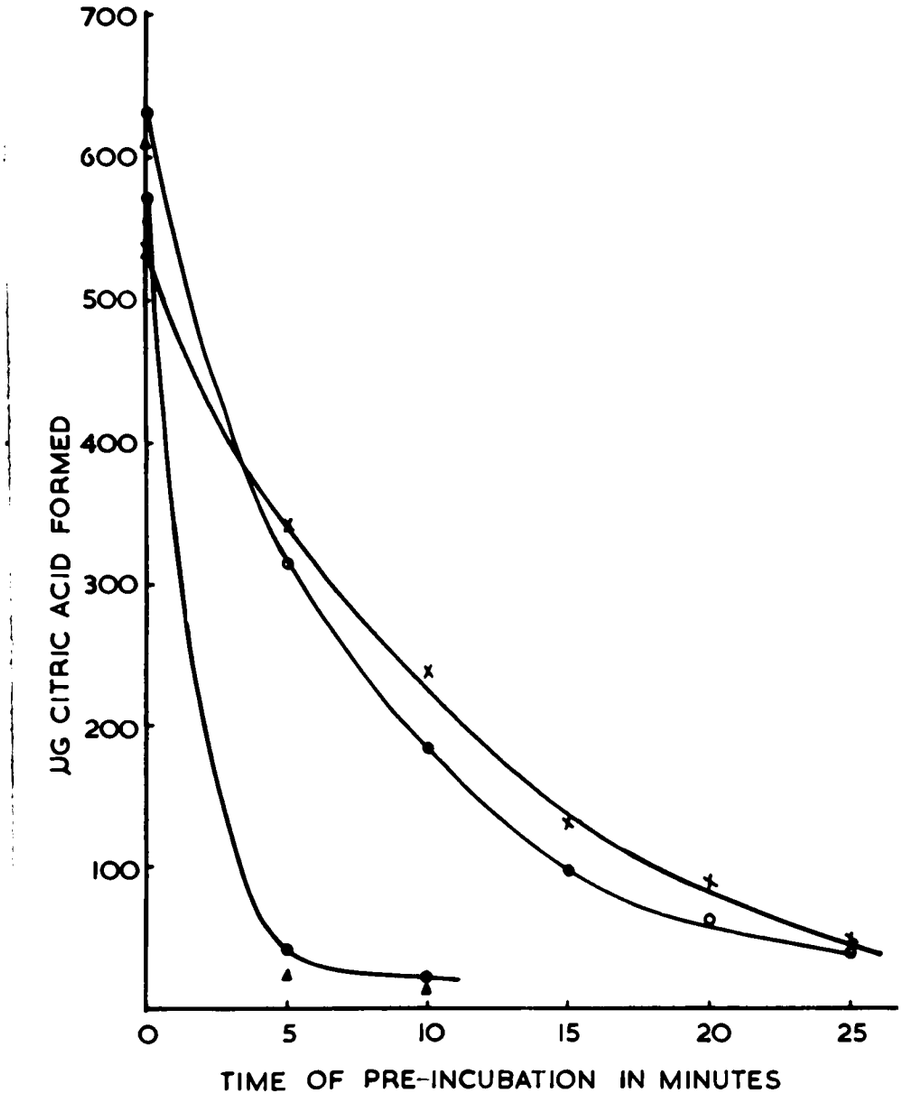


Fig. 16. Inactivation of aconitase by buffers in the absence of substrate. Aconitase activated with Fe^{2+} and cysteine was incubated at 30° for various periods of time in the presence of 0.05 M buffer. cis-Aconitic acid was added and the reaction stopped after 15 min. Solutions were analysed for citric acid.

x = glycerophosphate (pH 7.5)
o = phosphate (pH 7.7)
● = N-ethyl morpholine (pH 8.1)
▲ = veronal acetate (pH 8.6)

DISCUSSION

These results explain not only the different activities which were found in various buffers at pH 7.4, but also the apparent activation of aconitase by phosphate buffer at pH 7.4, as reported in Chapter I. The increases in enzyme activity are not due to the activation of the enzyme, but to the fact that the pH value chosen was closer to the optimum value in some buffers than it was in others. The alteration of the optimum pH of an enzyme is not a new phenomenon, but it is unusual to find that it is shifted to such an extent. Massey (1953) has reported similar effects of ions on the pH optimum of fumarase.

Michaelis & Davidsohn (1911) pointed out that the pH-activity curve of saccharase could be explained by assuming that the enzyme was an ampholyte so that the two halves of the pH-activity curve corresponded to the dissociation of the acidic and basic groupings on the surface of the enzyme. They suggested that the enzyme showed maximum activity when it existed in the isoionic form so that when the pH was altered in either direction, the amount of enzyme available for combination with the substrate was reduced. Thus, the enzymic activity was

decreased. Kuhn (1923) showed that the fact underlying the pH-activity curve of saccharase was the variation with pH of the rate of breakdown of the enzyme-substrate complex into enzyme and products. The theories of Michaelis were therefore rejected.

There is no reason to suppose that the influence of pH on all enzymes is due to the same factors. Before any attempt is made to explain the reasons for a particular pH-activity curve, it is necessary to determine both the pH-Michaelis constant and pH-maximum velocity curves (see Dixon, 1953). In the final analysis, it is also necessary to consider the effects of pH on the stability of the enzyme, the ionisation of the substrate and the state of aggregation of the enzyme molecules. As these factors have not been investigated, no attempt will be made to explain the pH-activity curves obtained with aconitase, or the effect of ions on the position of the pH optimum. However, some points, as far as the present results are concerned, might be mentioned.

It is interesting that the buffers which depend on anions for their buffering capacity shift the pH optimum to the acid side of the pH optimum obtained in the absence

of buffer. Cationic buffers, on the other hand, shift the pH optimum to the alkaline side. It is also noteworthy that the pH optimum in each buffer lies at the end of the alkaline range of the buffer, so that it was not possible to follow the alkaline branch of the pH-activity curve to an appreciable extent.

It is significant that the pH optimum of aconitase in catalysing the three reactions isocitric acid ⟶ citric acid, isocitric acid ⟶ cis-aconitic acid and cis-aconitic acid ⟶ citric acid is the same. This would seem to indicate that not only was the same enzyme responsible for catalysing these reactions, but also that the properties of the active centre(s) were similar. It might be supposed from these results that the same catalytic centre(s) was concerned in carrying out all the reactions associated with aconitase activity.

The inactivation of aconitase by buffers in the absence of substrate is likely to be a non-ionic reaction on account of the relatively slow rate of the inactivation. In the case of the inactivation by phosphate, it could be due to the interaction of the free Fe^{2+} with the phosphate so as to upset the equilibrium between the free enzyme, free Fe^{2+} and the Fe^{2+}-enzyme complex. The attainment of the new equilibrium

would be a slow process. The ionic reaction between phosphate and the free Fe^{2+} would conceivably be rapid, but the rate at which the Fe^{2+} was removed from the enzyme would be slow, as this reaction is the reverse of the activation process. However, this theory would not hold in the case of N-ethyl morpholine as this buffer does not form co-ordination compounds with Fe^{2+}. As the rate of inactivation increases with a rise in the pH, it seems more likely that the inactivation of the enzyme is not concerned specifically with Fe^{2+}, but with a number of groups on the enzyme surface.

The ability of the substrates of the enzyme to prevent the inactivation which occurs in these buffers must be due to the fact that the enzyme has a greater affinity for the substrates than it has for the buffer ions. Nevertheless, according to the theory of enzyme action postulated by Michaelis & Menten (1913), there is always a small amount of enzyme which is not combined with substrate. Therefore, it is likely that the substrate protects the enzyme to a large extent, but not completely.

SUMMARY

1. It has been shown that the pH optimum of aconitase

is influenced by the ions present in solution.

2. The pH optimum of aconitase in catalysing the reactions isocitric acid ⟶ citric acid, isocitric acid ⟶ cis-aconitic acid and cis-aconitic acid ⟶ citric acid is the same in the presence of a particular buffer and altered to the same extent in different buffers.
3. The inactivation of aconitase by buffers in the absence of substrate has been shown.
4. The significance of these findings has been discussed.

C H A P T E R IV

THE KINETICS of ACONITASE

CHAPTER IV

THE KINETICS of ACONITASE

INTRODUCTION

Since the discovery of aconitase by Martius & Knoop (1937), it has been generally accepted that citric acid is transformed into isocitric acid via cis-aconitic acid. However, Martius & Lynen (1950) and Friedrich-Freska & Martius (1951) claimed that there was a direct conversion of citric acid to isocitric acid as they did not observe a latent period at the start of the reaction. Further, as a result of the mathematical treatment of the reaction velocities, Friedrich-Freska & Martius (1951) supposed that after combination of the substrate with the enzyme, there is formed an intermediate complex which can decompose into enzyme-cis-aconitic acid, enzyme-isocitric acid or enzyme-citric acid. They regarded cis-aconitic acid as being only a by-product of the aconitase

reaction. These authors also studied the six initial reaction velocities associated with aconitase activity and claimed that the difference in the rates could account for the transient accumulation of isocitric acid above the equilibrium value that occurs when the reaction is started with cis-aconitic acid. On account of the agreement between their experimental findings and mathematical predictions, they concluded that a single enzyme could catalyse the conversion of each of the three acids to the other two.

The mathematical treatment which Friedrich-Freska & Martius (1951) applied to their results has been strongly criticised by Laeser (1952). Krebs & Holzach (1952) re-investigated the problem of the lag period in the conversion of citric acid to isocitric acid and showed that it did occur.

As all the previous studies on the kinetics of aconitase had been made with crude or partially purified preparations of the enzyme, the previous work was repeated with a more highly purified enzyme preparation. The question of the lag period was investigated, also the relative rates of the reactions catalysed by aconitase. The Michaelis constants of aconitase for citric,

cis-aconitic and isocitric acids were determined.

EXPERIMENTAL

Estimation of cis-aconitic acid

cis-Aconitic acid was estimated in the first place by the method of Dickman (1952) which was based on the reduction of potassium permanganate by a compound containing a double bond, in the presence of metaphosphoric acid. The results indicated that there was an initial rapid formation of cis-aconitic acid from isocitric acid which was followed by a slower production of cis-aconitic acid. Moreover, there was no relationship between the amount of enzyme added and the formation of cis-aconitic acid during the initial stages of the reaction. Investigation showed that the addition of the enzyme to a mixture of buffer, isocitric acid and metaphosphoric acid (which was used to stop the reaction) gave rise to a compound which was capable of reducing the potassium permanganate and so estimated as cis-aconitic acid. The amount of this compound which formed rose with increasing amounts of enzyme, although the increase was not linear. If this blank value were subtracted from the subsequent estimations, a linear rate was obtained

which was proportional to the amount of enzyme added.

A similar phenomenon was encountered when the formation of cis-aconitic acid was determined in a Beckman spectrophotometer at a wavelength of 240 mμ by the method of Racker (1950). Although none of the components of the system gave any appreciable absorption alone, on adding the enzyme there was a rapid increase in the extinction coefficient. With isocitric acid as the substrate, this rapid increase in the extinction coefficient continued over a period of 1 min. after which the reaction rate became linear. Fig. 17 shows what happens on adding the enzyme to a mixture of buffer and citric acid. The concentration of citric acid used for curves 1, 2 and 3 affects both the point where the rapid increase in the absorption ceases and the time for that point to be reached, but the subsequent linear rates of the reactions are the same. With lower concentrations of citric acid, a smaller increase in the extinction coefficient is obtained before the reaction rates become linear.

The enzyme used in these experiments had been activated with Fe^{2+} and cysteine. As the concentration of the enzyme was small, it seemed more likely that

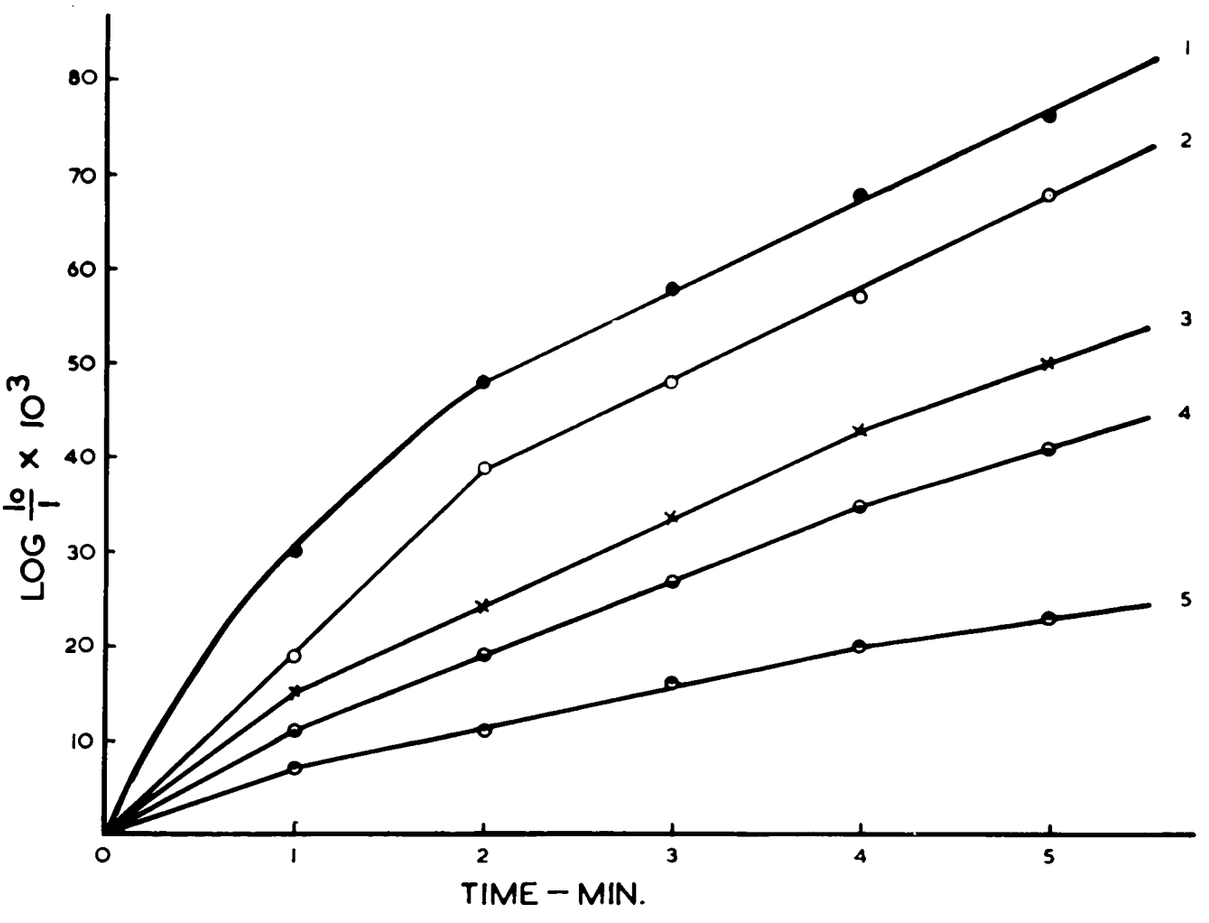


Fig. 17. The enzymic and non-enzymic reaction rates on adding aconitase activated with Fe^{2+} and cysteine to various concentrations of citric acid. The system contained 0.05 M phosphate buffer (pH 7.7), 8 µg. of the aconitase preparation and the following amounts of citric acid:

1. 5×10^{-2} M 2. 2×10^{-2} M 3. 1×10^{-2} M
4. 5×10^{-3} M 5. 1×10^{-3} M.

the rapid increase in the absorption was due to the interaction of the Fe^{2+} with the substrates rather than to the interaction of the substrates with the enzyme. On adding Fe^{2+} and cysteine to both citric and isocitric acids in the same concentration in which they were present in the enzyme solution, identical rapid increases in the extinction coefficients were obtained. At the point where the reaction became linear in the presence of the enzyme, the non-enzymic reaction ceased. If these values were subtracted from those obtained in the presence of the enzyme, the rates were linear from the start of the reaction. In view of this finding, it was important to distinguish the non-enzymic from the enzymic reaction rate. The method of Racker (1950) was used for following the production of cis-aconitic acid in the experiments reported in this chapter and only the enzymic reaction rates have been plotted in the graphs.

Estimation of citric and isocitric acids

Citric acid was estimated by the method previously described (page 18). Isocitric acid was estimated by the method of Ochoa (1951). As the final aconitase preparation contained isocitric dehydrogenase, a

non-activated solution was used as the source of this enzyme. The aconitase activity of this solution was so small that there was no detectable difference in the reaction rate when it was added to a medium containing the activated enzyme.

Activation of aconitase

Aconitase was activated with Fe^{2+} and cysteine before use by the method previously described (page19). The enzyme solutions contained 32 µg. of the final aconitase preparation/ml. in the presence of 5×10^{-4} M Fe^{2+} and 10^{-2} M cysteine.

Reagents

Coenzyme II was prepared by the method of Lepage & Mueller (1949) and had a purity of 48%. The other reagents were the same as previously stated.

RESULTS

Relative reaction rates

The relative rates of the six reactions catalysed by aconitase are shown in fig. 18 and a diagrammatic representation of these rates is shown in fig. 19. These results agree reasonably well with those obtained by Friedrich-Freska & Martius (1951), with the exception

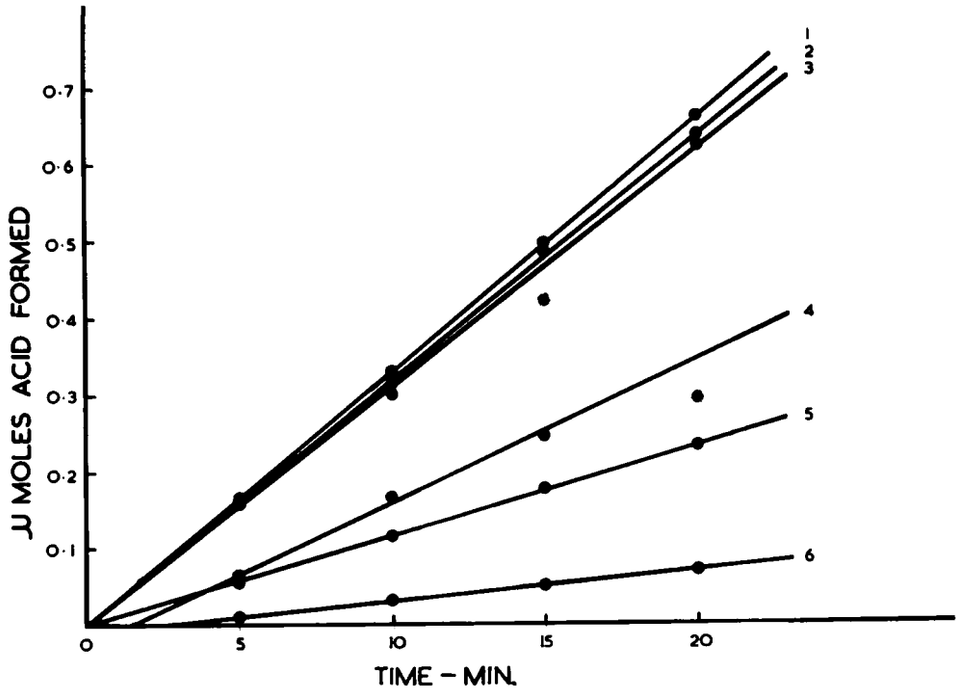


Fig. 18. The relative rates of the reactions catalysed by aconitase. The reaction mixture contained 0.05 M phosphate buffer (pH 7.7), together with either cis-aconitic acid (5×10^{-3} M), isocitric acid (5×10^{-3} M) or citric acid (5×10^{-2} M). Total volume 3.0 ml., temperature 22°. Reactions 2 & 6 were stopped at 5 min. intervals by the addition of 0.3 ml. of N. HCl. The solutions were neutralised by the addition of 0.3 ml. of N NaOH before aliquots were taken for the estimation of isocitric acid. Reactions 3 & 4 were stopped at 5 min. intervals by the addition of 0.5 ml. of 50% trichloroacetic acid and samples were analysed for citric acid. The formation of cis-aconitic acid was determined directly in the Beckman spectrophotometer, readings being taken at 1 min. intervals. A solution containing 0.1 µmole of cis-aconitic acid/ml. gave an extinction coefficient of 0.400. The amount of enzyme solution used in each test is given in brackets.

1. isocitric acid ⟶ cis-aconitic acid (0.1 ml.)
2. cis-aconitic acid ⟶ isocitric acid (0.1 ml.)
3. cis-aconitic acid ⟶ citric acid (0.4 ml.)
4. isocitric acid ⟶ citric acid (0.4 ml.)
5. citric acid ⟶ cis-aconitic acid (0.2 ml.)
6. citric acid ⟶ isocitric acid (0.5 ml.)

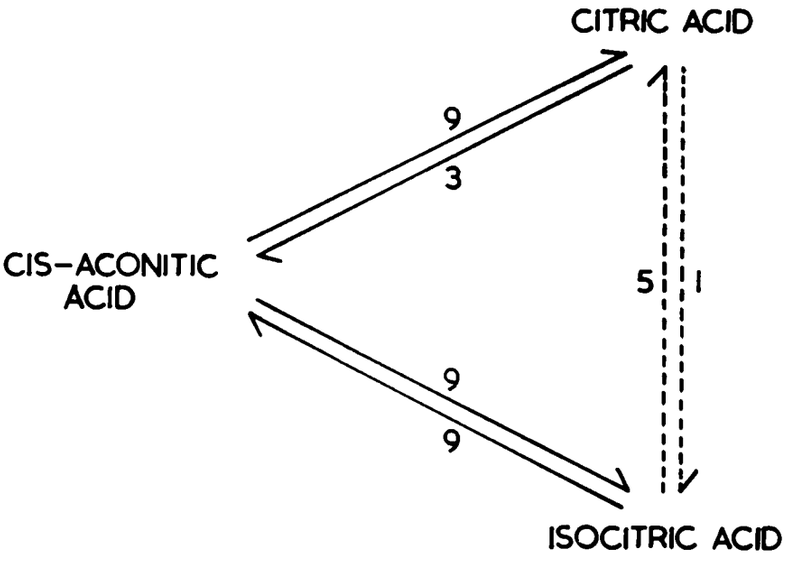


Fig. 19. The relative rates of the reactions catalysed by aconitase. A diagrammatic representation. The experimental conditions were as described in fig. 18.

of the reaction citric acid ⟶ isocitric acid. The rate of this reaction was found to be one-third of that reported by these authors. It will be noted that the rates in the case of the reactions isocitric acid ⟶ citric acid and citric acid ⟶ isocitric acid are linear only after 5 min. It was these rates which were taken to be the initial rates of the reactions as quoted in Fig. 19, although strictly speaking they are not initial rates. When the lines representing the rates of these reactions are produced backwards they cut the time axis which indicates that there is an initial latent period. Thus, the conversions must occur via an intermediate substance which is most likely cis-aconitic acid, as no other intermediate substance is known.

Determination of the lag phase

Fig. 20 illustrates the lag period of the reaction citric acid ⟶ isocitric acid in more detail. The formation of isocitric acid was studied by measuring the reduction of coenzyme II in the presence of isocitric dehydrogenase. The pH in this instance was 7.2 rather than 7.7. The reason for this alteration was that it was not possible to study the reduction of coenzyme

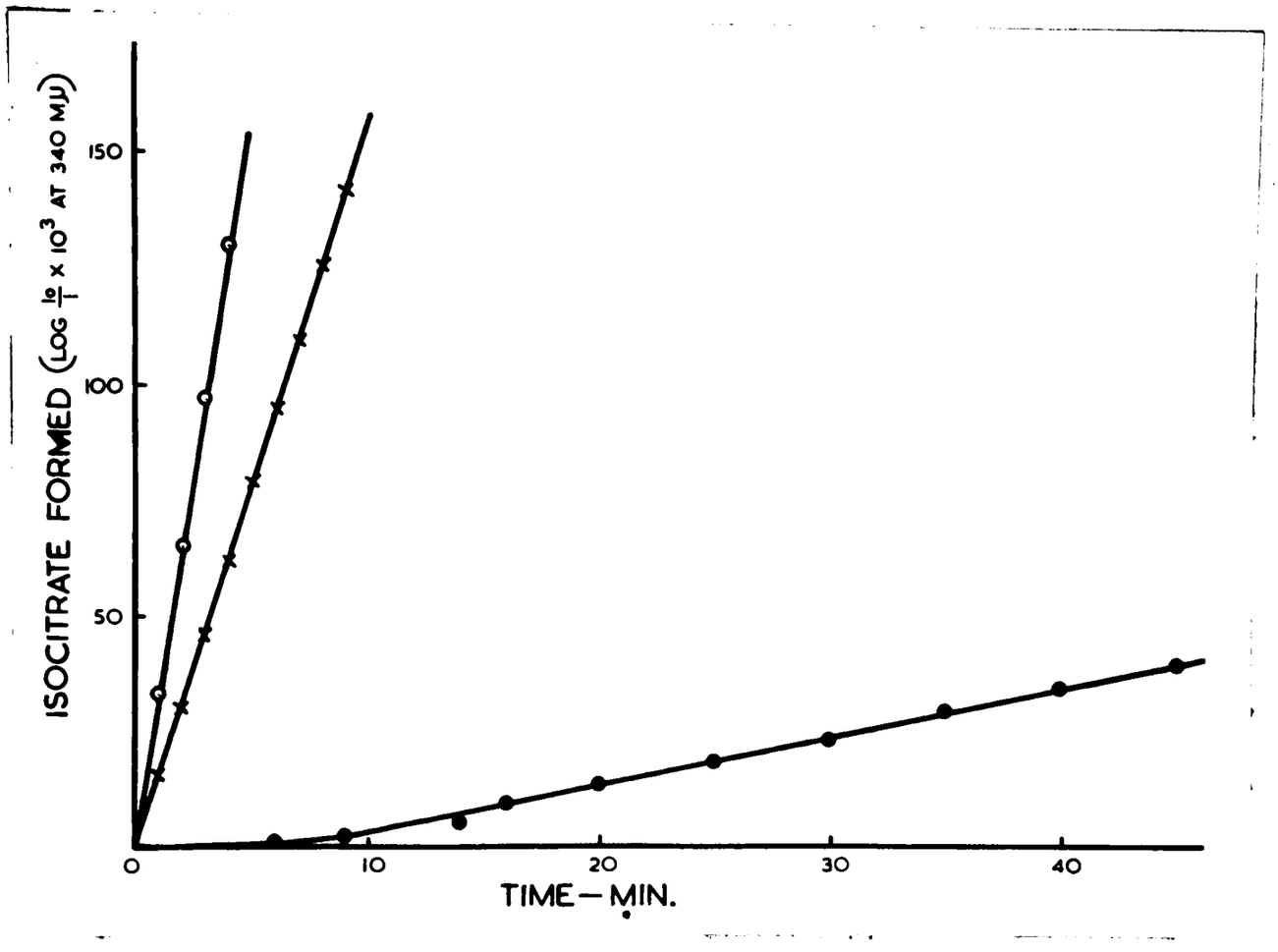


Fig. 20. Time curves for the formation of isocitric acid from cis-aconitic and citric acids. The system contained 0.05 M phosphate buffer (pH 7.2), 1.8 μmoles of $MnCl_2$, 0.135 μmoles of Co II, 3 μg. of the activated aconitase preparation, 15 μg. of the non-activated aconitase preparation (isocitric dehydrogenase), together with isocitric acid (5×10^{-3} M), cis-aconitic acid (5×10^{-3} M) or citric acid (5×10^{-2} M). Total volume 3.0 ml., temperature 22°.
o = isocitric acid; • = citric acid; x = cis-aconitic acid

II by isocitric dehydrogenase in the presence of phosphate buffer at pH 7.7 on account of the precipitation of manganese. The oxidation of isocitric acid was the fastest reaction, so this was a valid means of estimating the rate at which isocitric acid was formed from citric and cis-aconitic acids. It can be seen that there is no lag period in the conversion of cis-aconitic acid to isocitric acid whereas the lag period is marked with citric acid as the substrate, in fact, there was no measurable amount of isocitric acid formed until 6 min. after the start of the reaction. These findings are in agreement with those of Krebs & Holzach (1952).

Effect of the simultaneous addition of isocitric acid and citric acid on the rate of formation of cis-aconitic acid

In an endeavour to determine whether or not the same enzyme centre(s) was concerned in the formation of cis-aconitic acid from both citric and isocitric acids, the rate of cis-aconitic acid formation was determined in the presence of each acid separately and in the presence of both acids. So that the results should not be complicated by the different affinities of the enzyme for the two acids, they were added in a

concentration ten times greater than their Michaelis constants. (The actual Michaelis constant values are reported in the following section).

The results of Table 7 show that the rate of the conversion of isocitric acid to cis-aconitic acid is influenced by the presence of citric acid, in fact, the rate in the presence of both acids is equal to half the combined rates obtained with each acid separately. This result would seem to indicate that the same centre(s) is concerned in the two reactions.

Determination of Michaelis constants

The Michaelis constants of aconitase for citric acid was found to be 5.6×10^{-3} M; for cis-aconitic acid, 1.2×10^{-4} M and for D-isocitric acid, 4.8×10^{-4} M at pH 7.7 and 22° in the presence of 0.05 M phosphate buffer. Racker (1950) found that the Michaelis constants of aconitase for citric acid was 1.1×10^{-3} M and for D-isocitric acid, 4×10^{-4} M at pH 7.4 and 25°. Racker (1950) did not measure the Michaelis constant of aconitase for cis-aconitic acid. Thus, the affinity of aconitase for cis-aconitic acid is four times greater than for isocitric acid and thirty times greater than for citric acid.

TABLE 7

Influence of Citric Acid on the Rate of the Reaction isoCitric Acid to cis-Aconitic Acid

(The reactions were carried out in the presence of 0.05 M phosphate buffer (pH 7.7), 5 µg. of the aconitase preparation, 3.6×10^{-2} M citric acid and/or 4.8×10^{-3} M isocitric acid. Total volume, 3.0 ml., temperature 22°.)

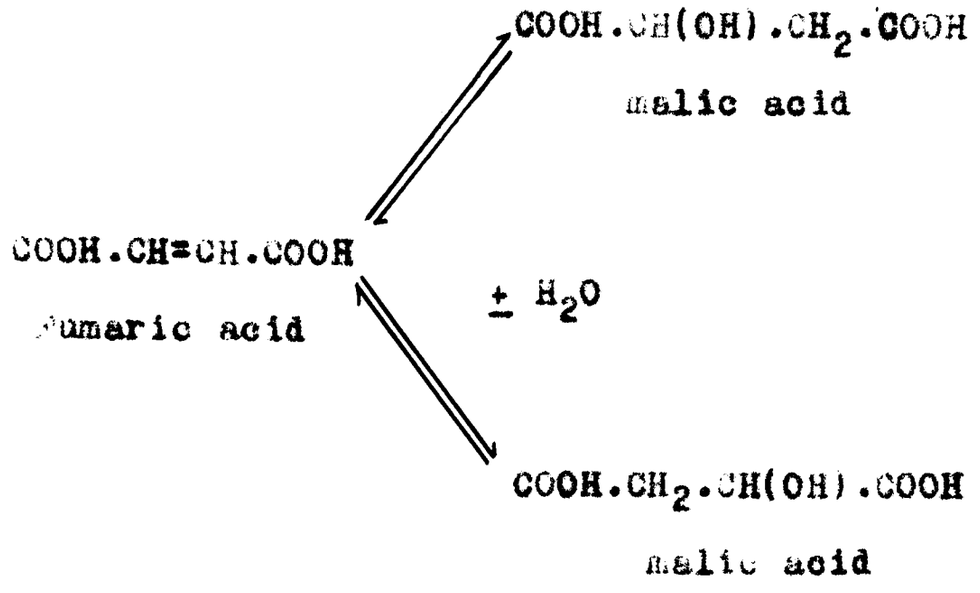
Substrate	Reaction rate (ΔE/min. x 10^3)
citric acid	15
isocitric acid	45
citric acid + isocitric acid	29

The Michaelis constant of aconitase for cis-aconitic acid was determined from the reaction cis-aconitic acid ⟶ citric acid. It would have been of interest to determine the value also from the reaction cis-aconitic acid ⟶ isocitric acid, for if they were the same it would have been strong evidence in favour of the idea that both reactions are catalysed at the same centre(s) of the same enzyme. Unfortunately, the rate of isocitric acid formation could not be studied at pH 7.7 with isocitric dehydrogenase and coenzyme II in the presence of phosphate buffer for reasons previously mentioned. Attempts were made to determine the amount of isocitric acid present in samples of the reaction mixture after various periods of time, but reliable results could not be obtained on account of the small amounts of isocitric acid formed from low concentrations of cis-aconitic acid.

DISCUSSION

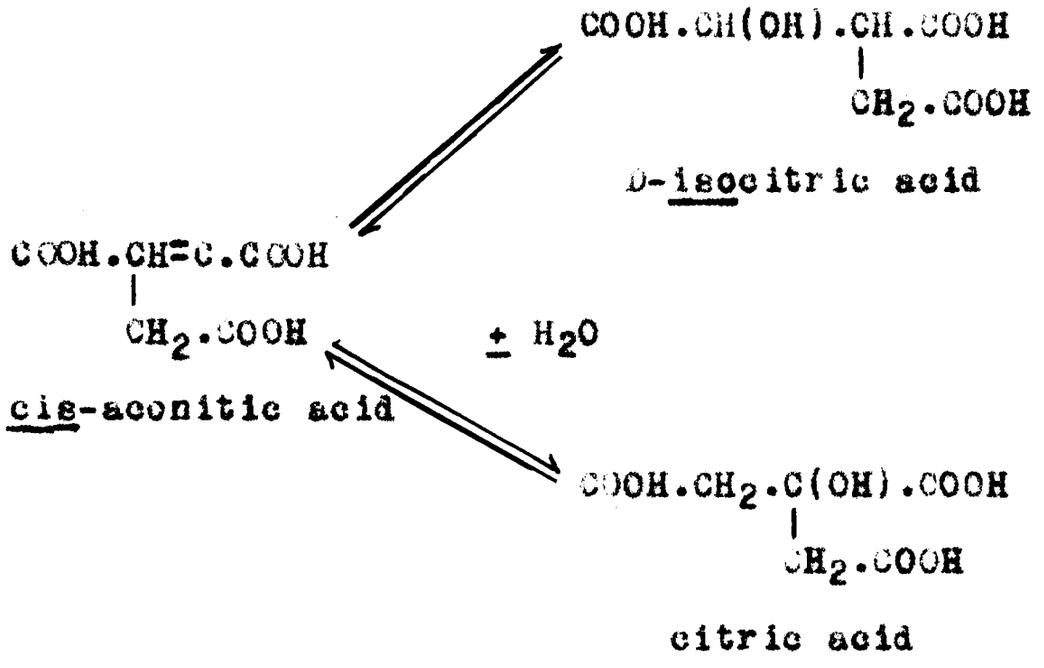
Aconitase has been considered as an unusual enzyme in so far as it has three substrates. However, as pointed out by Ogston (1951), there is no reason why it should not be considered in a broad sense as being

similar to fumarase. The reaction catalysed by fumarase can be written as follows:



Theoretically, water can be added in two ways. (As L-malic acid is formed, the enzyme must be linked to the substrate so that the water molecules can approach from one direction only, otherwise, D-malic acid would also be formed. The same reasoning applies in the case of aconitase as only D-<u>iso</u>citric acid is formed. Ogston (1951) has proposed that the linkage of <u>cis</u>-aconitic acid to the enzyme involves a three point attachment in order to explain this fact). On account of fumaric acid being a symmetrical molecule, the same compound is formed irrespective of the way in which the water

Molecules are added across the double bond of fumaric acid. Aconitase can be considered as catalysing essentially the same reaction which can be written in a similar fashion:



As cis-aconitic acid is an asymmetrical molecule, the products formed as a result of the addition of a water molecule across the double bond can be distinguished.

Citric acid formed as a result of the condensation reaction between acetyl-coenzyme A and oxalacetic acid is further metabolised by conversion to cis-aconitic acid and isocitric acid as shown by Krebs & Holzach (1952) and confirmed here. As the reaction is usually written

citric acid → *cis*-aconitic acid → *iso*citric acid

there has been a tendency to think of the conversion of citric acid to *iso*citric acid as two separate reactions possibly catalysed by two distinct enzymes. However, if the reaction is thought of, not as written above, but as written previously, there is no difficulty in thinking of aconitase as a single enzyme which does not have an absolute specificity. That is, whilst it can distinguish between citric and *iso*citric acids as indicated by the marked difference in the Michaelis constants, it can convert both acids to *cis*-aconitic acid which in a sense can be considered as an end-product. As aconitase is a catalyst, the reverse reactions must also occur and so citric acid can be converted to *iso*-citric acid. This reaction must be then considered as being due to the establishment of an equilibrium, rather than a direct conversion of one acid to the other.

If the above concept is correct, two conditions must hold; citric acid must be converted to *iso*citric acid via *cis*-aconitic acid and the same catalytic centre(s) must be concerned in all the reactions catalysed by aconitase. With regard to the first point, the results of Krebs & Holzach (1952) and those

reported here indicate that citric acid is converted to isocitric acid via cis-aconitic acid. Thus the intermediate complex of Friedrich-Freska & Martius (1951) can be considered as being nothing more than the complex formed between aconitase and cis-aconitic acid. No experimental evidence has been previously presented to indicate that only one catalytic centre of the enzyme is concerned with all the reactions. The results obtained here can be interpreted to show that this is the case. It was found that citric acid was converted to cis-aconitic acid at a rate which can be arbitrarily called 1, whilst isocitric acid was converted to cis-aconitic acid at a rate of 3. If two different centres were concerned in these reactions, it would be expected that the simultaneous addition of both isocitric and citric acids would give a rate greater than 3, theoretically 4, as a result of summation. If only one centre were concerned, the rate should be equal to half the combined rate of each reaction. That is, there would be an equal chance of either acid reacting with the enzyme. The results showed that the latter is actually what happens for the rate in the presence of both substrates is 2. Another possible explanation must also be borne in mind. If two centres were

concerned and the reaction taking place at each was inhibited 50% by the other substrate, the reaction rate in the presence of both substrates would be 2. However, other evidence, particularly the pH-activity curves obtained for three of the reactions in different buffers favours the idea of one centre.

Friedrich-Freska & Martius (1951) explained the transient accumulation of isocitric acid above the equilibrium value when the reaction was started with cis-aconitic acid as being due to the difference in the initial reaction rates. The same reasoning was applied to explain the transient accumulation of cis-aconitic acid when the reaction was started with isocitric acid. As the rates found here are essentially the same as those of Friedrich-Freska & Martius, this explanation seems tenable. Although these authors claimed that there was no lag period with the reactions citric acid ⟶ isocitric acid and isocitric acid ⟶ citric acid, the validity of the argument is not affected as only the four true initial reaction rates are concerned. The rates of the reactions citric acid ⟶ isocitric acid and isocitric acid ⟶ citric acid are consistent with the idea that they are the sum of two reactions

i.e. the sum of the reactions citric acid ⟶ *cis*-aconitic acid and *cis*-aconitic acid ⟶ *iso*citric acid and *vice versa*.

Martius & Leonhardt (1943) found that *cis*-aconitic acid was converted to both *iso*citric acid and citric acid at equal rates, whereas Friedrich-Freska & Martius (1951) found that *cis*-aconitic acid was converted to *iso*citric acid at almost 1.5 times the rate at which it was converted to citric acid. The results obtained here agree with those of Martius & Leonhardt (1943). The fact that *cis*-aconitic acid is converted to both *iso*citric and citric acids at an equal rate raises an interesting point. Ogston (1951) analysed the aconitase reactions theoretically and concluded that when *cis*-aconitic acid was linked to the enzyme, the water molecules of the medium have an equal chance of reacting to form either citric or *iso*citric acid. He quotes the results of Martius & Leonhardt (1943) in support of this idea. Kacser (1952) has also concluded from a mathematical treatment of the reactions catalysed by aconitase that the water molecules approach from solution.

At present there is no experimental evidence that the water molecules concerned in the reaction approach

the enzyme from the medium. It is also possible that they could react with cis-aconitic acid by prior adsorption on the enzyme surface. Under these circumstances, it might be expected that the enzyme would have an orientating influence on the water molecules so that citric acid would be formed in preference to isocitric acid or vice versa. But the finding that the rates are equal is inconsistent with this idea. On the otherhand, if the water molecules approached cis-aconitic acid linked to the enzyme from the medium, it might also be expected that the acid itself being an asymmetric molecule would influence the way in which the water moleculeswould be added across the double bond. The only way in which the finding that cis-aconitic acid is converted to both citric and isocitric acid at an equal rate can be explained, is that the effect of the enzyme on cis-aconitic acid is to eliminate the normal directional influence of cis-aconitic acid on the water molecules approaching from solution.

SUMMARY

1. It was shown that Fe^{2+} and cysteine react with citric and isocitric acids in such a way as to

estimate as *cis*-aconitic acid.

2. The relative rates of the reactions catalysed by aconitase have been determined.
3. A definite lag period was shown to occur in the conversion of citric acid to *iso*citric acid.
4. The rate of formation of *cis*-aconitic acid from citric acid plus *iso*citric acid was shown to be equal to half the sum of the rates of *cis*-aconitic acid formation from each acid separately.
5. The Michaelis constants of aconitase for citric, *cis*-aconitic and *iso*citric acids were determined.
6. Some aspects of aconitase action have been discussed.

C H A P T E R V

THE INHIBITION of ACONITASE by FLUOROCITRIC ACID

CHAPTER V

THE INHIBITION of ACONITASE by FLUOROCITRIC ACID

INTRODUCTION

Before the metabolic effects of fluorocitric acid are discussed, it is necessary to make brief mention of fluoroacetic acid. It is from the latter acid that fluorocitric acid is synthesised in the animal body by a mechanism classified by Peters (1952) as lethal synthesis. That is, whilst fluoroacetic acid is not itself toxic, it is converted into fluorocitric acid which is extremely toxic.

The toxicity of fluoroacetic acid and the accompanying physiological effects are now well known. For a comprehensive review of this aspect, see Peters (1952). The first indications of the biochemical lesions which could account for the physiological effects came from the work of Bartlett & Barron (1947). These workers

found that fluoroacetic acid strongly inhibited the oxidation of acetic acid *in vitro* and that the oxidation of pyruvic acid was also inhibited with the accumulation of acetic acid. They advanced the hypothesis that fluoroacetic acid competitively inhibits the oxidation of acetic acid and it was to this failure of acetic acid oxidation that the toxic effects were due.

Peters (1948) and Liébecq & Peters (1949) found that fluoroacetic acid also inhibited the *in vitro* oxidation of fumaric acid without the accumulation of acetic acid. They concluded that the hypothesis of Bartlett & Barron (1947) could not completely account for the action of this inhibitor. It was also shown that, as a result of the inhibition of the oxidation of fumaric acid, citric acid accumulated, whereas non-poisoned tissues readily oxidised citric acid. The authors tested the effect of fluoroacetic acid on a number of isolated enzymes of the Tricarboxylic Acid Cycle and found that none was inhibited. From these findings, it was concluded that fluoroacetic acid was not the inhibitor, but was transformed into another substance which was inhibitory. They suggested that fluoroacetic acid could undergo activation in a similar

manner to acetic acid and after activation condense with oxalacetic acid to form a fluorotricarboxylic acid; the fluorotricarboxylic acid could then inhibit or "jam" the tricarboxylic acid Cycle at the citric acid stage. The same conclusion was reached by Martius (1949). This hypothesis was consistent with that of Bartlett & Barron (1947) for it might be expected that there would be an initial competition between fluoroacetic acid and acetic acid for the enzyme responsible for the activation, but it was not to this that the toxic effects were due. In the absence of oxalacetic acid, acetic acid rather than citric acid would accumulate.

Isolation of a fluorotricarboxylic acid

Elliott & Kalnitsky (1950) presented evidence which they interpreted as being support for the formation of a fluorocitric acid, but their methods have been strongly criticised by Peters et al. (1953a). More conclusive evidence was presented by Buffa et al. (1951). They succeeded in isolating tricarboxylic acid fractions from kidney tissue poisoned with fluoroacetic acid which contained fluorine and which were potent inhibitors of citric acid oxidation. Later Peters et al. (1951) and Peters et al. (1953a) isolated the active principle in

a crystalline form and showed that it was a monofluoro-tricarboxylic acid. At the time, it was not certain whether the inhibitor was fluorocitric or fluoroisocitric acid. The possibility that it was fluoro-cis-aconitic acid was eliminated as the molecule did not contain a double bond. Further work by Peters et al. (1953b) showed that synthetic fluorocitric acid had the same infra red absorption spectrum as did the enzymically synthesised compound. Therefore, the inhibitory substance was actually fluorocitric acid.

Site of action of fluorocitric acid

The hypothesis of Liébecq & Peters (1949) that fluoroacetic acid was activated and condensed with oxalacetic acid to form a fluorotricarboxylic acid which inhibited the Tricarboxylic Acid Cycle at the citric acid stage was strengthened by the work of Buffa & Peters (1949). They found that in vivo fluoroacetic acid gave rise to large accumulations of citric acid in many animal tissues. These results were confirmed by Potter & Busch (1950), Lindenbaum et al. (1951) and Mandel et al. (1951). At the same time, there was no accumulation of α-ketoglutaric acid so it was concluded that either isocitric dehydrogenase or aconitase must be inhibited. The

experiments of Lotspeich et al. (1952) showed that the oxidation of isocitric acid was not inhibited and therefore that the accumulation of citric acid was not due to the inhibition of isocitric dehydrogenase. Thus, aconitase must be the enzyme which is inhibited.

Originally, Buffa & Peters (1949) concluded that aconitase was not affected for they found that the tricarboxylic acid fractions which contained fluorine did not inhibit soluble aconitase. Moreover, the aconitase activity of poisoned heart tissue was as high as that of normal heart tissue. However, the later work of Lotspeich et al. (1952) showed that the reactions citric acid ⟶ isocitric acid, isocitric acid ⟶ citric acid, cis-aconitic acid ⟶ citric acid and cis-aconitic acid ⟶ isocitric acid were inhibited. The reaction citric acid ⟶ isocitric acid was inhibited to the same degree as the reverse reaction, whilst the two reactions starting with cis-aconitic acid were less sensitive to the inhibitor. The previous failures to demonstrate the inhibition were due to the use of too high a concentration of substrate and too low a concentration of inhibitor so that the formation of the enzyme-inhibitor complex was prevented. Peters & Wilson (1952)

found that the reactions citric acid ⟶ cis-aconitic acid and isocitric acid ⟶ cis-aconitic acid were also inhibited. In this preliminary work, the inhibition appeared to be competitive, the affinity of the fluorocitric acid for aconitase being twenty times greater than the affinity of citric acid for the enzyme.

The above studies of the inhibition of aconitase by fluorocitric acid were carried out with crude aconitase extracts and impure solutions of the inhibitor and the interpretation of the results was rendered somewhat difficult by the instability of the enzyme. As the inhibitor had been crystallized at the time this work was begun, a supply of synthetic fluorocitric acid was available and a stable highly purified solution of aconitase had been obtained, the work of Peters & Wilson (1952) was re-examined and extended.

EXPERIMENTAL

Formation of cis-aconitic acid

The formation of cis-aconitic acid from citric and isocitric acids was followed by the method of Racker (1950). In plotting the graphs, the point at which the non-enzymic reaction ceased was taken as the zero point

(see page 117).

Activation of aconitase

In all instances, aconitase was activated with Fe^{2+} and cysteine as previously described (page 19). Solutions contained between 30 and 50 µg. of the final aconitase preparation/ml. in the presence of 5×10^{-4} M Fe^{2+} and 10^{-2} M cysteine.

Reagents

Fluorocitric acid synthesised enzymically will be referred to as natural fluorocitric acid. Samples were prepared by the method of Peters et al. (1953a). Specimens of synthetic fluorocitric acid had been prepared by Dr. D.E.A.Rivett (1953). Both were supplied by Professor Peters and had been standardised on kidney particles by Peters & Wakelin.

RESULTS

Stabilization of aconitase during pre-incubation in the presence of phosphate

The use of phosphate buffer in the previous experiments for the determination of aconitase activity had not caused complications as none involved pre-incubation of the enzyme with buffer. However, as it

was desired to repeat some of the experiments of Peters & Wilson (1952) in which aconitase had been pre-incubated with fluorocitric acid for 15 min. in the presence of phosphate prior to the addition of substrate, it was necessary to find a means of preventing or reducing the phosphate inactivation of the enzyme in the absence of substrate. The experiments could have been carried out in the absence of buffer, but it seemed preferable to use a buffer to maintain a constant pH.

It was found that when the aconitase activity of a $CHCl_3$-citric acid buffer extract of heart muscle was destroyed by the addition of HCl to pH 4.0, the addition of this extract to the aconitase system during the pre-incubation with phosphate buffer prevented the inactivation of the enzyme. It was not clear from the initial experiments whether the function of the crude extract was to stabilize the enzyme during the pre-incubation with phosphate or to activate the enzyme directly. Further experiments showed that the addition of the extract after the pre-incubation did not affect the enzyme activity. On the other hand, when the extract was added to the system prior to the pre-incubation with phosphate, there was little or no loss of enzyme activity.

Thus the extract was capable of preventing the inactivation of aconitase by phosphate in the absence of substrate, rather than being capable of activating the enzyme to a greater extent than that obtained with Fe^{2+} and cysteine.

Aconitase could also be stabilized to an appreciable extent by the addition of small amounts of substrate to the system during the pre-incubation of the enzyme with phosphate. Fig. 21 shows the marked loss of enzyme activity that occurs during the pre-incubation and that small increasing amounts of isocitric acid reduce the loss of activity. In the presence of 0.5 µmole of isocitric acid/3ml., about 70% of the enzyme activity remained after 15 min. pre-incubation as compared with the non-incubated enzyme. It will be seen later (Table 10) that as the incubation period is increased, greater losses of enzymic activity occur. These results provide further evidence in favor of the idea that phosphate inhibits aconitase irreversibly.

isocitric acid (0.5 µmole/3ml.) was chosen for stabilizing aconitase during the pre-incubation period in the presence of phosphate. Although the loss of enzyme activity was greater than when the inactivated crude aconitase extract was used, this method had the

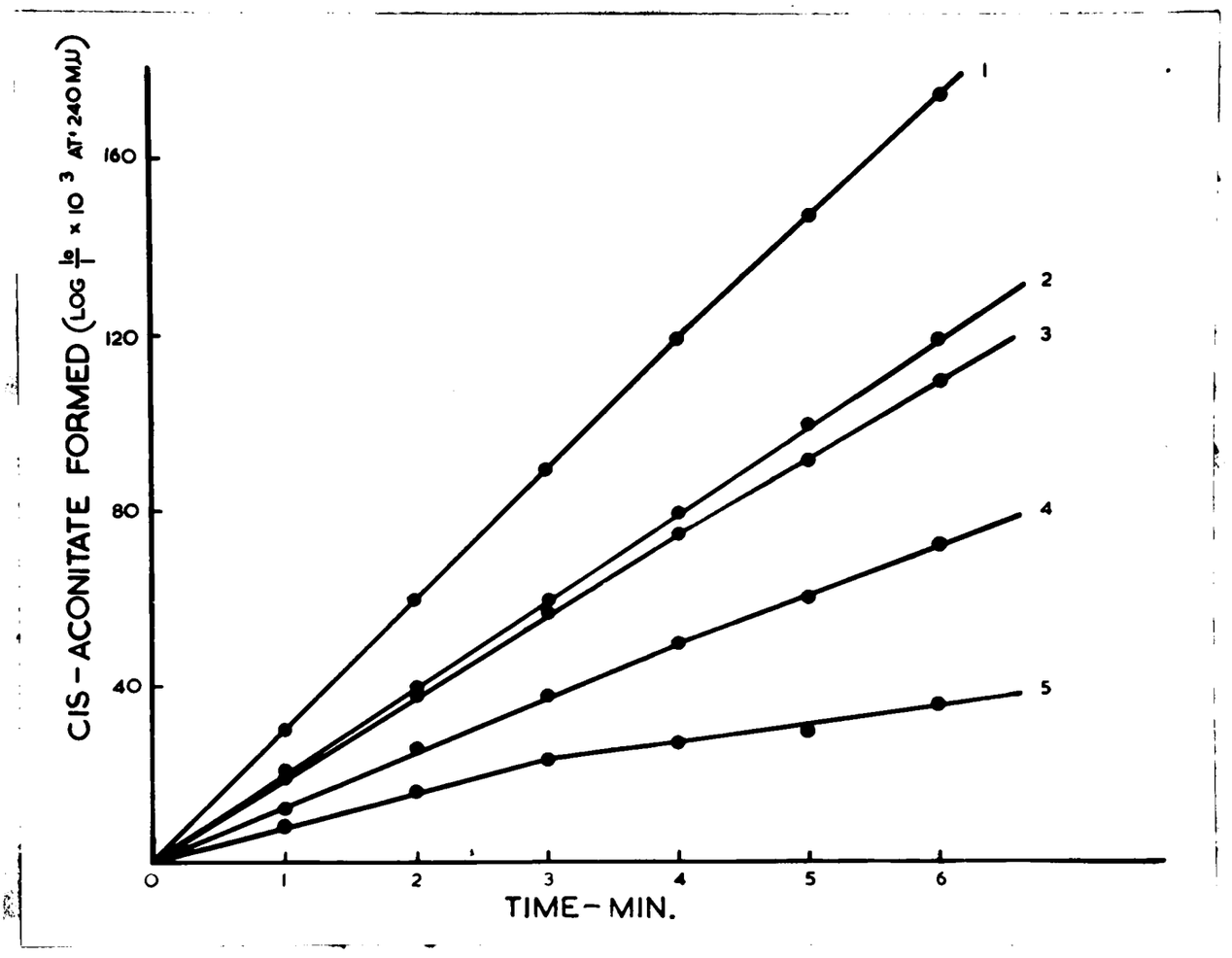


Fig. 21. The stabilization of aconitase by isocitric acid during incubation in the presence of phosphate. 5 µg. of the final aconitase preparation, activated by Fe^{2+} and cysteine, were incubated with 0.05 M phosphate buffer (pH 7.7) for 15 min. in the presence of various amounts of isocitric acid. Following the incubation period, 16 µmoles of isocitric acid were added and the reaction rate determined. Total volume 3.0 ml., temperature 22°.

1. No pre-incubation
2. pre-incubation with 0.5 & 1.0 µmole of isocitric acid
3. pre-incubation with 0.25 µmole of isocitric acid
4. pre-incubation with 0.1 µmole of isocitric acid
5. pre-incubation without isocitric acid

advantage of not introducing unknown components into the system.

Studies with Synthetic Fluorocitric Acid

An investigation of the inhibition of aconitase by synthetic fluorocitric acid was made firstly as relatively larger amounts of this compound were available. It was hoped that the knowledge gained would permit of a full investigation of the effect of natural fluorocitric acid on aconitase with the smaller amounts available. The reaction isocitric acid ⟶ cis-aconitic acid was studied in most instances because of the ease with which it could be followed.

Inhibition of aconitase by fluorocitric acid with pre-incubation

Table 8 shows that when the enzyme is pre-incubated with low concentrations of synthetic fluorocitric acid for 15 min. prior to the addition of substrate, marked inhibitions are obtained; a 5-fold increase in the concentration of fluorocitric acid increases the percentage inhibition to only a small extent. In order to determine the type of inhibition, two different concentrations of substrate were added following pre-incubation

TABLE 8

Inhibition of Aconitase by Synthetic Fluorocitric acid

(Fluorocitric acid was pre-incubated with 5 µg. of the final aconitase preparation, activated by Fe^{2+} and cysteine, in the presence of 0.05 M phosphate buffer (pH 7.7) and 0.5 µmole of isocitric acid for 15 min. 16 µmoles of isocitric acid were added and the reaction rate was determined in a Beckman spectrophotometer at a wavelength of 240 mµ. Total volume 3.0 ml., temperature 22°)

Fluorocitric acid concentration (x 10^{-5} M)	Inhibition (%)
1.2	61
2.4	68
3.0	75
6.0	79

of the enzyme with the inhibitor. The results of fig. 22 indicate that the inhibition appears to be irreversible for the degree of inhibition was independent of the substrate concentration. At 2 min. the inhibition was 83% when 2 μmoles of isocitric acid were added and 79% when 16 μmoles of isocitric acid were added.

Because of the small amount of enzyme used in these tests (3-5 μg. of protein/3 ml.), the molar ratio of fluorocitric acid to enzyme must have been high. It was, therefore, possible that the fluorocitric acid could be acting as a non-specific denaturing agent. As a check on this point, the above experiments were carried out in the presence of crystalline bovine serum albumin at a concentration one thousand times that of the enzyme. As the results were not altered by the presence of a large excess of inactive protein the possibility that the inhibition of aconitase by fluorocitric acid was non-specific was rendered much less likely. The possibility that the inhibition was due to an impurity in the fluorocitric acid is also unlikely. Identical results were obtained with a preparation which had been fractionated by precipitation as a barium salt.

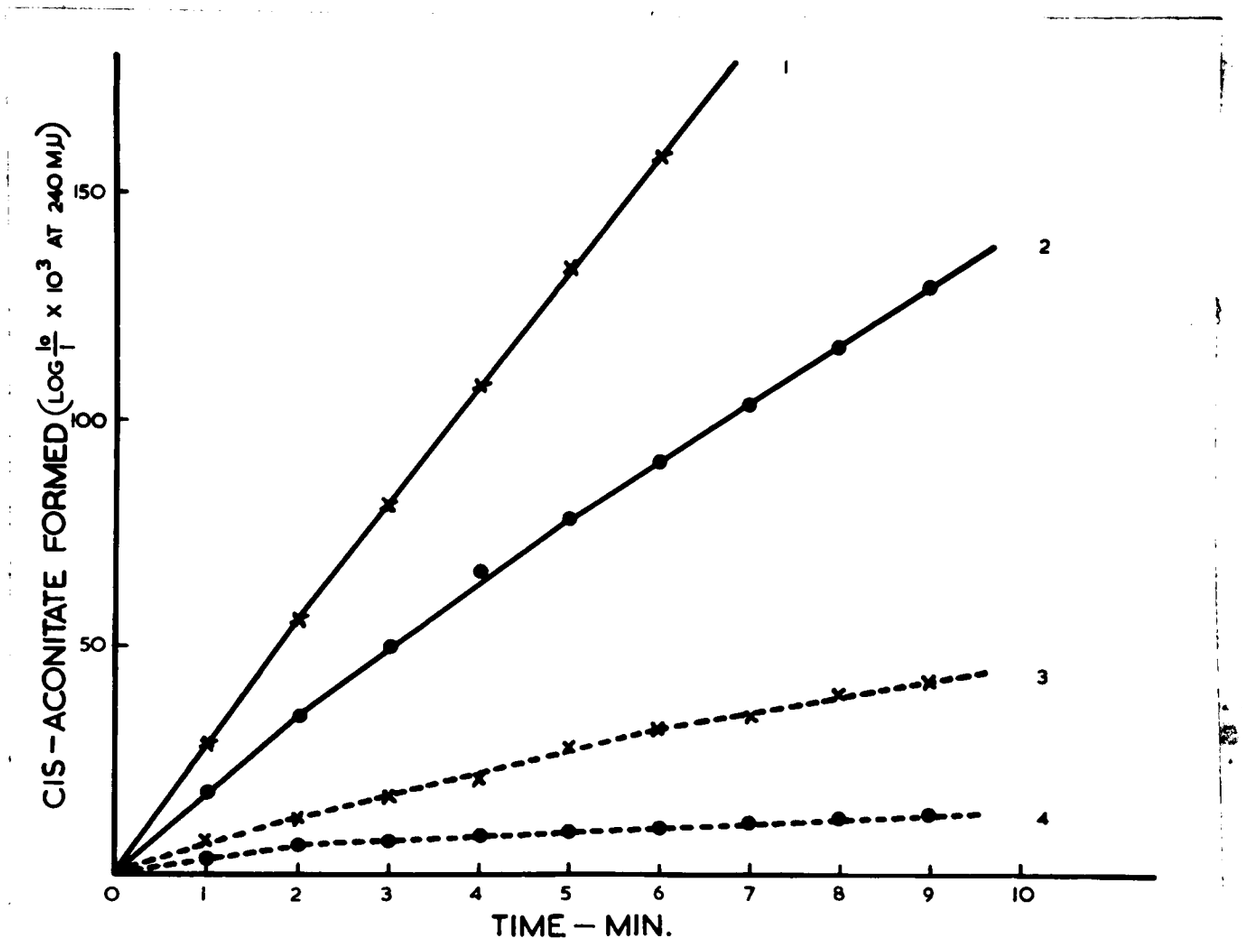


Fig. 22. Inhibition of aconitase by synthetic fluorocitric acid. 5 µg. of the final aconitase preparation, activated by Fe^{2+} and cysteine were incubated with fluorocitric acid (2.4×10^{-5} M) for 15 min. in the presence of 0.05 M phosphate buffer (pH 7.7) and 0.5 µmole of isocitric acid. Substrate was then added and the reaction rates determined as described in the text. Total volume 3.0 ml., temperature 22°.

x = reaction rate in the presence of 16 µmoles of isocitric acid

● = reaction rate in the presence of 2 µmoles of isocitric acid

——— control ..... fluorocitric acid

Inhibition of aconitase by fluorocitric acid without pre-incubation

It was found that the inhibition was very much dependent on the substrate concentration when the enzyme was not pre-incubated with fluorocitric acid. Table 9 shows a comparison of the inhibitions obtained with and without pre-incubation. It can be seen that pre-incubation makes little difference to the inhibition when 2 μmoles of isocitric acid are added, but when 16 μmoles of isocitric acid are added, there is an appreciable difference. These findings were not consistent with the idea that fluorocitric acid was an irreversible inhibitor of aconitase for if that were the case, the inhibition would be independent of the substrate concentration. On the other hand, if the inhibition were competitive pre-incubation of the enzyme and inhibitor should not increase the inhibition. According to the theory of Michaelis & Menten (1913), the equilibria between the enzyme and substrate and between the enzyme and inhibitor should be set up immediately.

Competitive inhibition of aconitase by fluorocitric acid

A more detailed investigation was made of the effect

TABLE 9

The Effect of Pre-incubation of Aconitase with Synthetic Fluorocitric Acid on the Inhibition of the Reaction Isocitric Acid to cis-Aconitic Acid

(5 µg. of the final aconitase preparation, activated with Fe^{2+} and cysteine, was pre-incubated with 2.4×10^{-5} M fluorocitric acid for 15 min. in the presence of 0.05 M phosphate buffer (pH 7.7) and 0.5 µmole of isocitric acid. The indicated amounts of isocitric acid were then added and the initial reaction rates determined as described in Table 8. When pre-incubation was not carried out, 3 µg. of the final enzyme preparation were added directly to media containing buffer and substrate. Total volume 3.0 ml., temperature 22°)

Substrate concentration (µmoles/3 ml.)	Rate (ΔE/min. x 10^3) control	fluorocit.	Inhibition (%)
Pre-incubation			
2	23	8	65
	24	8	66
16	32	15	53
	36	15	58
No pre-incubation			
2	20	10	50
	25	11	56
16	32	27	16
	34	30	12

of the substrate concentration on the inhibition of aconitase by fluorocitric acid without pre-incubation. Fig. 23 shows that when the results are plotted according to the method of Lineweaver & Burk (1934), a graph characteristic of competitive inhibition is obtained. It was noted that after a time the reaction rates in the presence of fluorocitric acid fell off. The time at which they fell off was shorter in the presence of lower substrate concentrations. This could not be explained as being due to the conversion of cis-aconitic acid to citric acid for the control rates, which were faster than those with the inhibitor present, were linear at this time. The rates used in the plot were those obtained before the change.

From the above results it must be concluded that there is at least an initial competition between iso-citric acid and fluorocitric acid for the active centre(s) of the enzyme. Nevertheless, it was difficult to reconcile this finding with that of the effect of pre-incubation. An investigation was therefore made of the conditions affecting the inhibition and the reversal of the inhibition.

Table 10 shows that the time of pre-incubation of

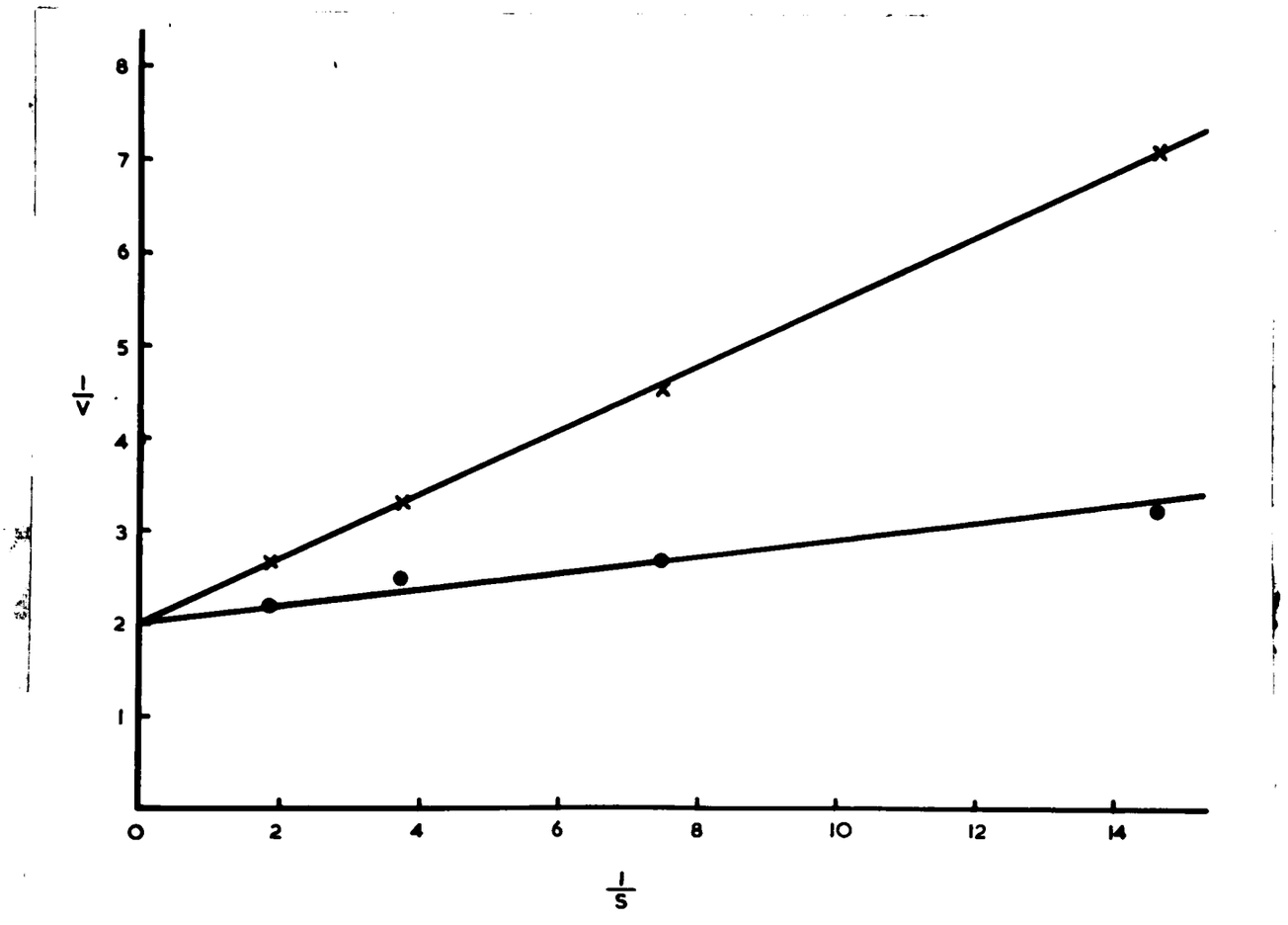


Fig. 23. Competitive inhibition of aconitase by synthetic fluorocitric acid. The graph is plotted according to the method of Lineweaver & Burk (1934). Aconitase activity was determined in the presence of 0.05 M phosphate buffer (pH 7.7) using 5 µg. of the final aconitase preparation activated by Fe^{2+} and cysteine. Total volume 3.0 ml., temperature 22°.

S = molar concentration of isocitric acid x 10^2

V = change of $\log. Io/I$ per min. x 10

o = control x = fluorocitric acid (2.4×10^{-5} M)

TABLE 10

The Effect of Pre-incubation Time of Aconitase with Synthetic Fluorocitric Acid on the Inhibition of the Reaction isoCitric Acid to cis-Aconitic Acid

(Conditions of the pre-incubation were the same as those of Table 9. Following the pre-incubation period, 2 μmoles of isocitric acid were added and the reaction rate determined for 2 min. 16 μmoles of isocitric acid were then added and the second reaction rate determined. Total volume 3.0 ml., temperature 22°).

Pre-incubation time (min.)	Rate (ΔE/min. x 10^3) 2 μmoles			16 μmoles		
	Control	F.cit.	Inhibition(%)	Control	F. cit.	Inhibition(%)
0	50	22	56	79	54	32
10	42	17	59	57	37	35
20	31	12	61	42	28	33
30	24	8	67	30	23	23

the enzyme and fluorocitric acid does not significantly affect the inhibition when 2 µmoles of isocitric acid are added. Moreover, when the isocitric acid concentration is increased, the degree of reversal is independent of the pre-incubation time. These results also show that the inhibition can be reversed to some extent by higher substrate concentrations. Fig. 24 shows the effect of adding 15 µmoles of isocitric acid immediately as compared to adding the same amount after the reaction has been started with 2 µmoles. A large inhibition is obtained with 2 µmoles and on the addition of 15 µmoles, the inhibition is reversed, but not to the extent that the reaction rate is the same as that obtained on adding 15 µmoles at the start.

The above results suggest that during a pre-incubation period or during the reaction with low substrate concentrations, a secondary reaction occurs between the enzyme and fluorocitric acid so that the reversal of the inhibition is rendered more difficult. The same reaction may occur with higher concentrations of substrate, but the time required is greater. Because of the time factor, further investigation of this point was not possible. When the reaction is allowed to

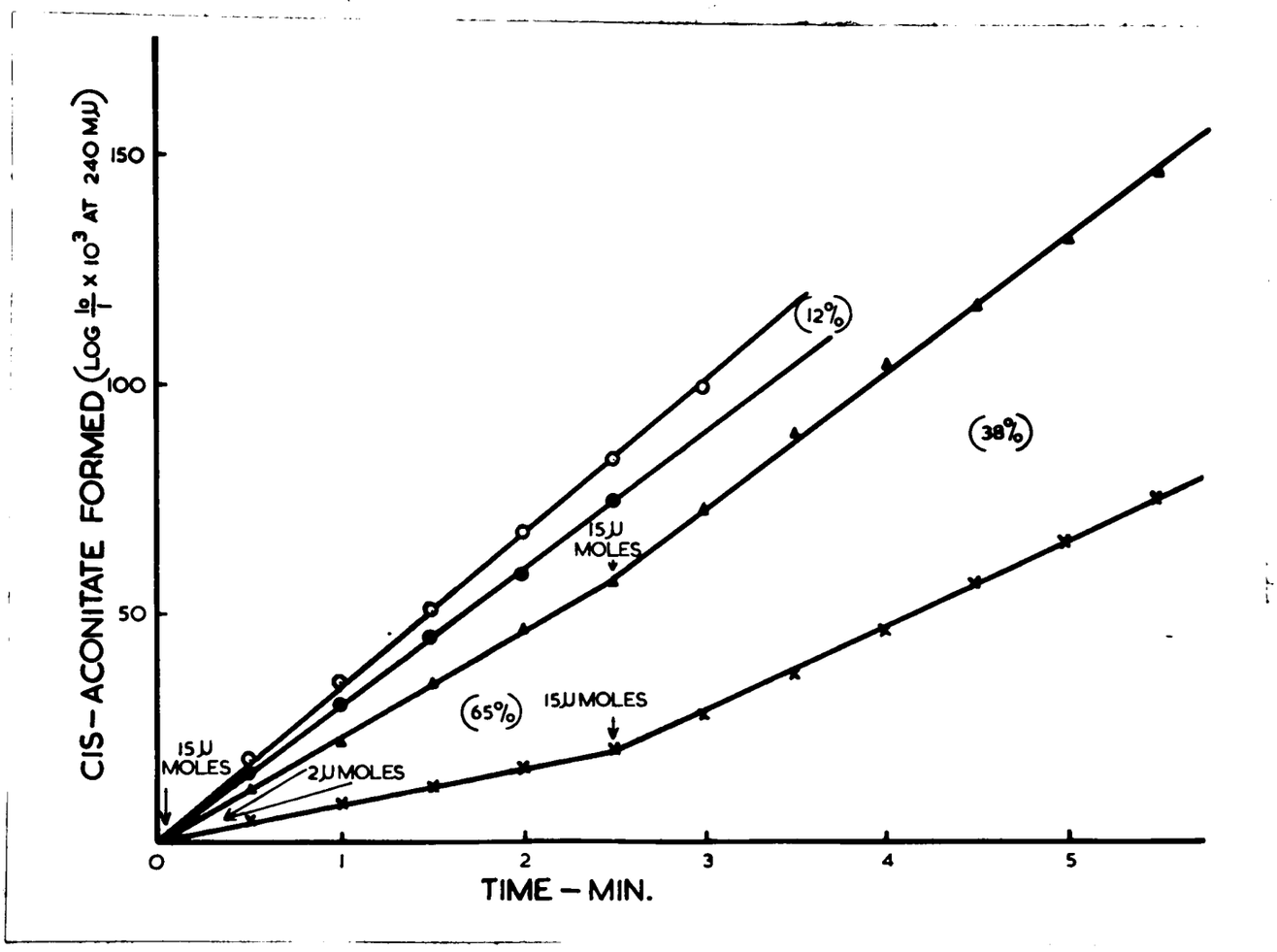


Fig. 24. Increased inhibition of aconitase by synthetic fluorocitric acid as a result of starting the reaction with a low concentration of isocitric acid. Conditions were as described in fig. 23. The concentration of fluorocitric acid was 2.4×10^{-5} M.

o = control) 15 μmoles of isocitric acid added
● = fluorocitric acid) at the start of the reaction

▲ = control) 2 μmoles of isocitric acid added
× = fluorocitric acid) at the start of the reaction, followed by 15 μmoles of isocitric acid.

Arrows indicate the time at which the substrate was added. Figures in brackets show the degree of inhibition.

proceed for a longer period of time, it is complicated by the conversion of cis-aconitic acid to citric acid.

Reversal of the inhibition of aconitase by fluorocitric acid

As seen in fig. 24 the inhibition of aconitase by fluorocitric acid could be reversed to some extent on the addition of higher concentrations of substrate. It was of interest to determine whether or not the inhibition could be completely reversed. Fig. 25 shows that a further increase in the substrate concentration reduces the inhibition still further, but at this level of inhibitor it was not practical to add larger amounts of substrate. It can be seen that in the control tube the final rate is decreased because of the dilution of the solution. When the concentration of fluorocitric acid was reduced to one quarter of the concentration, that is to 0.6×10^{-5} M, the inhibition could be completely reversed as shown in fig. 26. The initial inhibition in this case was small, being only 20% as compared with 60% with 2.4×10^{-5} M fluorocitric acid. It cannot be concluded from this result that the inhibition of aconitase by higher concentrations of fluorocitric acid would be reversed completely if the substrate

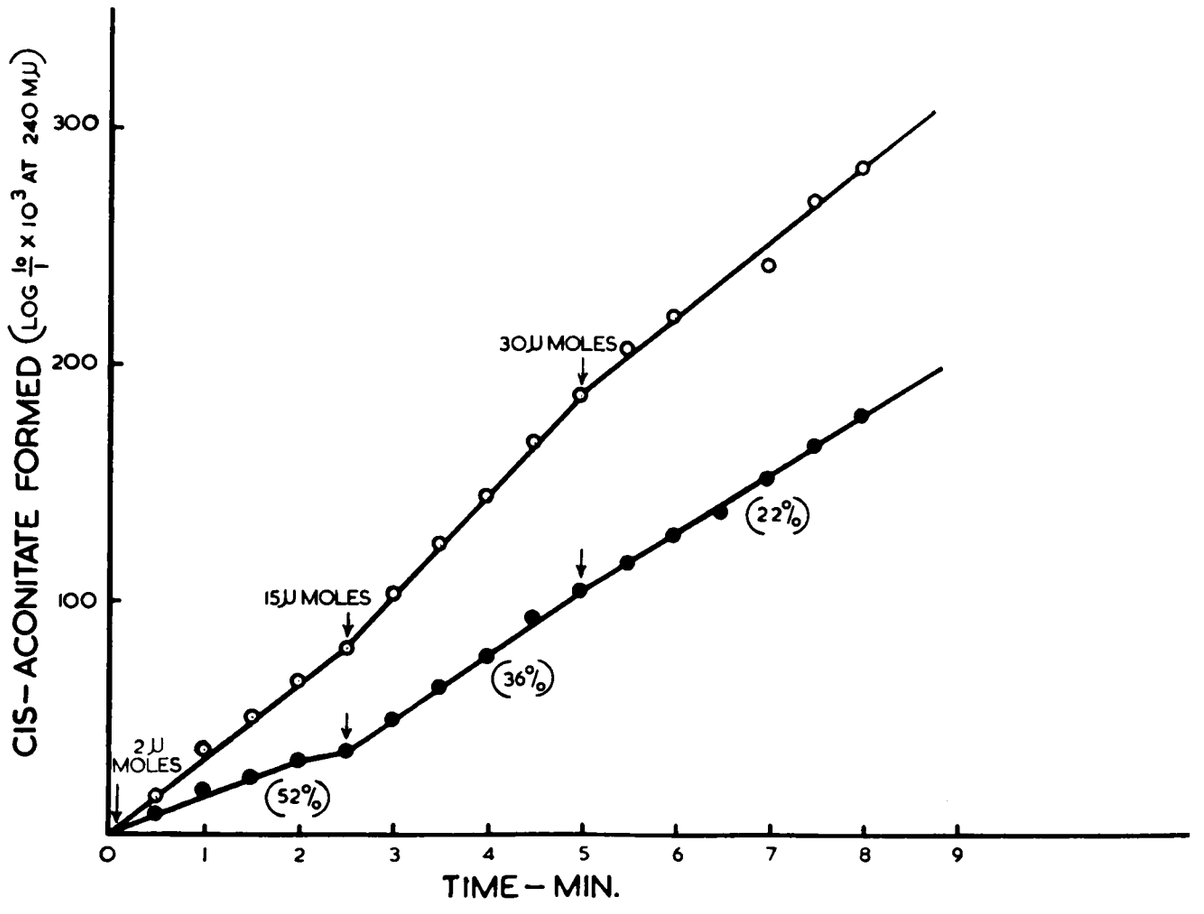


Fig. 25. Inhibition of aconitase by synthetic fluorocitric acid and reversal of the inhibition by increasing concentrations of isocitric acid. Conditions were the same as those of fig. 23.

o = control • = fluorocitric acid (2.4×10^{-5} M)

Arrows indicate the time at which substrate was added. Figures in brackets show the degree of inhibition.

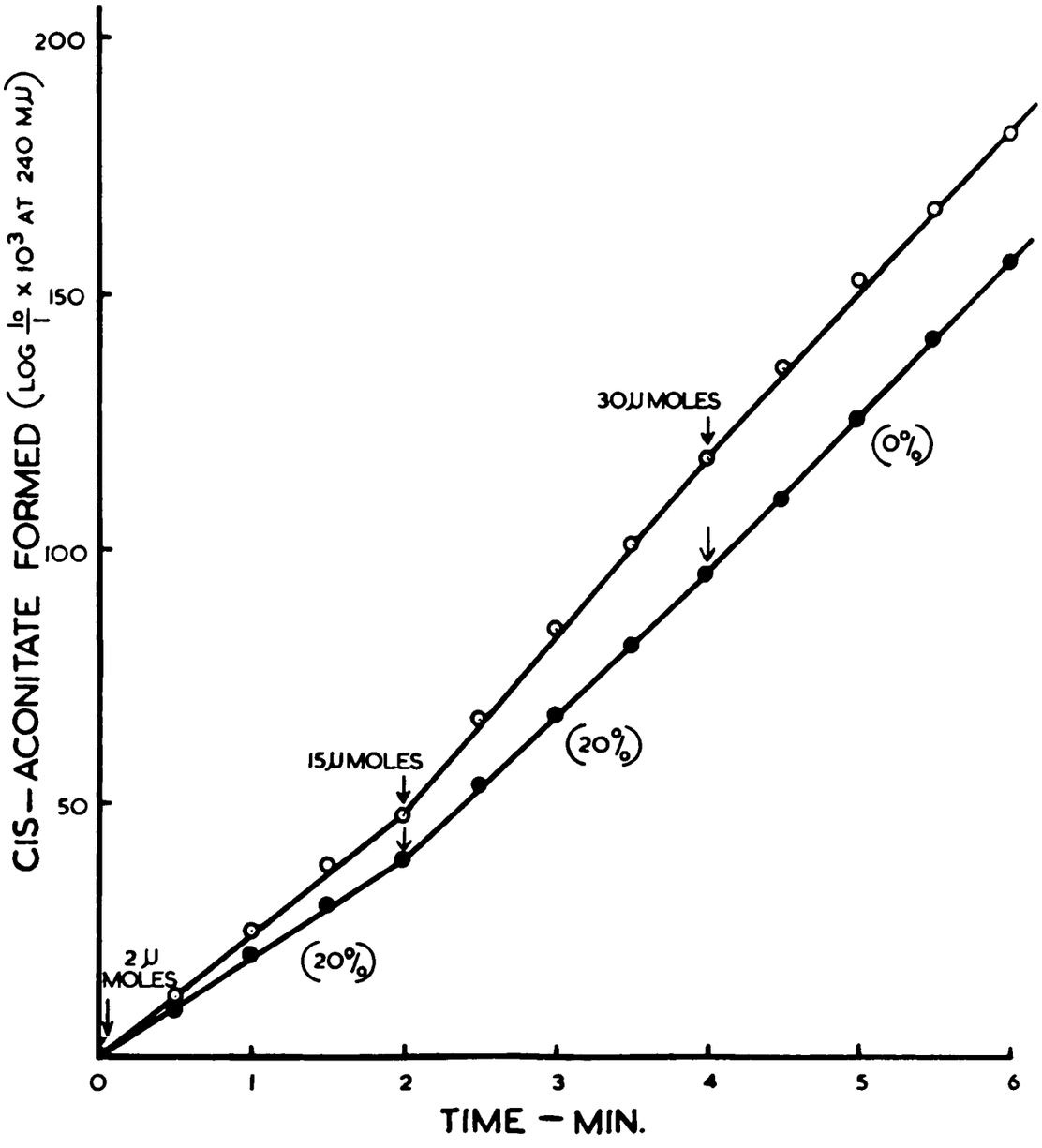


Fig. 26. Inhibition of aconitase by synthetic fluorocitric acid and reversal of the inhibition by increasing concentrations of isocitric acid. Conditions were the same as those of fig. 23.

o = control • = fluorocitric acid (0.6×10^{-5} M)

Arrows indicate the time at which substrate was added. Figures in brackets show the degree of inhibition.

concentration could be increased to a sufficiently high level. It is possible that lower concentrations of the inhibitor do not bring about the same irreversible effects.

Dissociation constant of the aconitase-fluorocitric acid complex

It was seen in fig. 23 that when aconitase was not pre-incubated with fluorocitric acid, there was a true competitive inhibition between the inhibitor and the substrate for the active centre(s) of the enzyme. From this graph it was calculated that the Michaelis constant (K_m) for isocitric acid was 4.4×10^{-4} M and the apparent Michaelis constant (K_p) in the presence of fluorocitric acid was 16×10^{-4} M. From the equation

$$K_i = \frac{[I] \times K_m}{K_p - K_m}$$

, the dissociation constant of the aconitase-fluorocitric acid complex (K_i) was calculated to be 8.4×10^{-6} M. Thus the enzyme has a very high affinity for fluorocitric acid.

The same competitive inhibition was obtained with citric acid as substrate. In this case the dissociation constant was found to be 12×10^{-6} M, but the difference could easily be accounted for by experimental error.

The ratio of isocitric acid to fluorocitric acid required for 50% inhibition was approximately 40 to 1, whereas the ratio of citric acid to fluorocitric acid was approximately 400 to 1. Thus the inhibition in the presence of a fixed amount of citric acid is greater than in the presence of the same amount of isocitric acid. It also follows that the inhibition could be more readily reversed by isocitric acid than it could be by citric acid. The ability of these two acids to reverse the inhibition is approximately proportional to their Michaelis constants.

Studies with Natural Fluorocitric Acid

The natural fluorocitric acid was tested under the same conditions as those used in the initial experiments with the synthetic material, whereupon it was found that this compound was very much less toxic. When used at a concentration of 3.2×10^{-5} M, only a small inhibition was obtained in the presence of 2 µmoles of isocitric acid. This was surprising in view of the marked toxicity of this compound *in vivo* and the marked inhibitions obtained with the synthetic, though consistent with the earlier findings of Peters

et al. (1953b). However, it was possible that the natural fluorocitric acid could inhibit aconitase in a competitive fashion if the substrate to inhibitor ratio were reduced. Peters & Wilson (1952) had concluded that this was so using relatively impure preparations of fluorocitric acid, but it could not be assumed that this was the case with the crystalline fluorocitric acid. As the amounts of fluorocitric acid available were limited, it was not possible to use high concentrations, so the substrate concentration was reduced. This was not ideal as it meant that the reaction rates were considerably reduced with a corresponding reduction in accuracy. But, nevertheless, under these conditions the inhibition of aconitase could be shown.

Inhibition and reversal of the inhibition of aconitase by fluorocitric acid

Fig. 27 illustrates the initial inhibition of aconitase by 1.6×10^{-4} M fluorocitric acid and the subsequent overcoming of this inhibition by increased amounts of substrate. Similar results were obtained using the inhibitor at a concentration of 3.2×10^{-5} M. Pre-incubation of the enzyme with fluorocitric acid prior to the addition of substrate may increase the initial

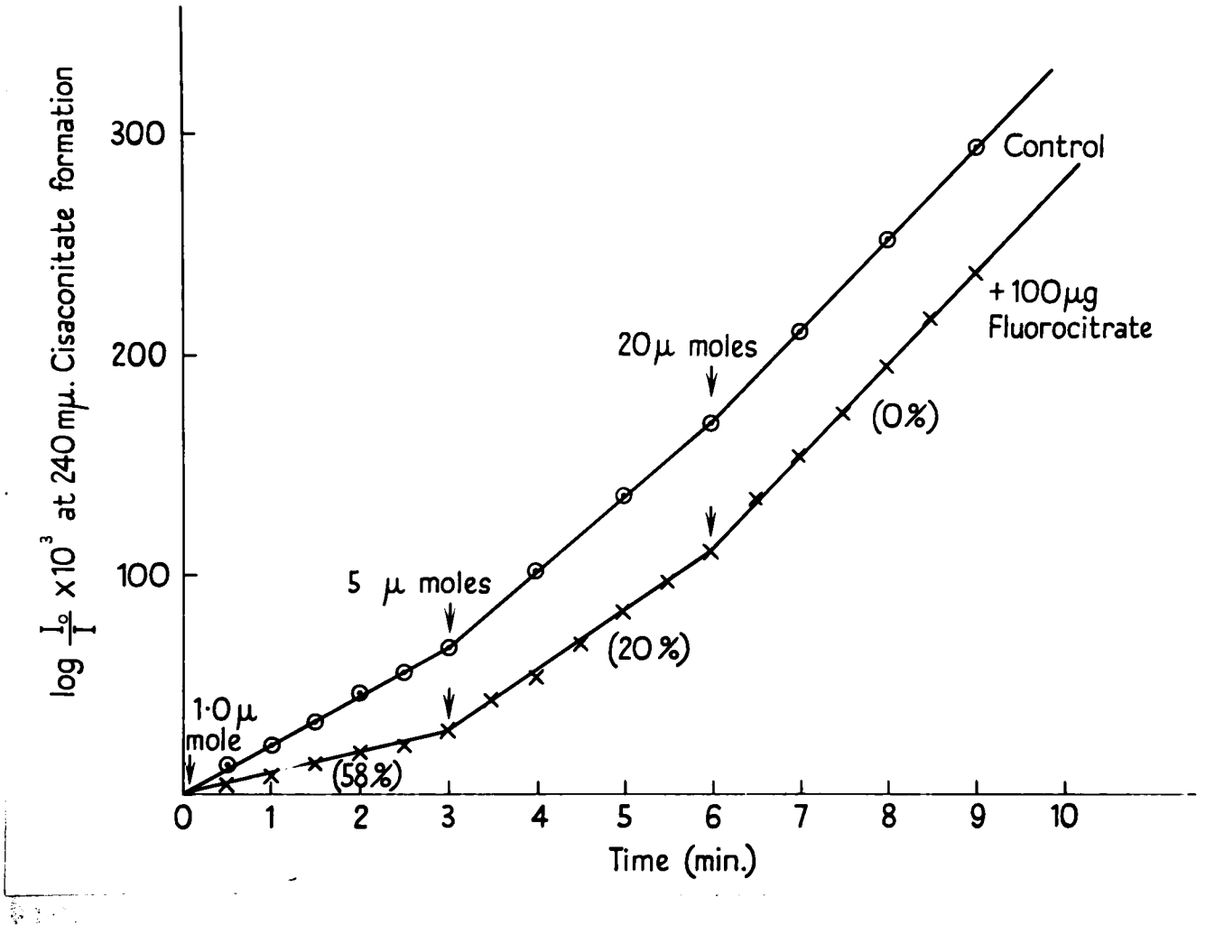


Fig. 27. Inhibition of aconitase by natural fluorocitric acid and reversal of the inhibition by increasing concentrations of isocitric acid. Conditions were the same as those of fig. 23.

o = control x = fluorocitric acid (1.6×10^{-4} M)

Arrows indicate the time at which substrate was added. Figures in brackets show the degree of inhibition.

inhibition. Only one comparison was made and this did show that pre-incubation increased the initial inhibition. However, definite conclusions cannot be drawn from this single experiment, besides which the comparison was made with low concentrations of the inhibitor where the degree of inhibition was small.

Competitive inhibition of aconitase by fluorocitric acid

Although it was clear from the above results that fluorocitric acid was a competitive inhibitor of aconitase, it was of interest to determine the dissociation constant of the aconitase-fluorocitric acid complex for comparison with the value obtained using the synthetic material. Fig. 28 confirms the fact that the inhibition is competitive. By means of the equation used previously (page 161), the dissociation constant of the enzyme-inhibitor complex was calculated to be 8.7×10^{-5} M. This value is ten times greater than that obtained with the synthetic fluorocitric acid and is consistent with the lower order of inhibition obtained with natural fluorocitric acid.

Apparent irreversible inhibition of aconitase

Peters (1952) showed that the sensitivity of the isolated aconitase to natural fluorocitric acid was

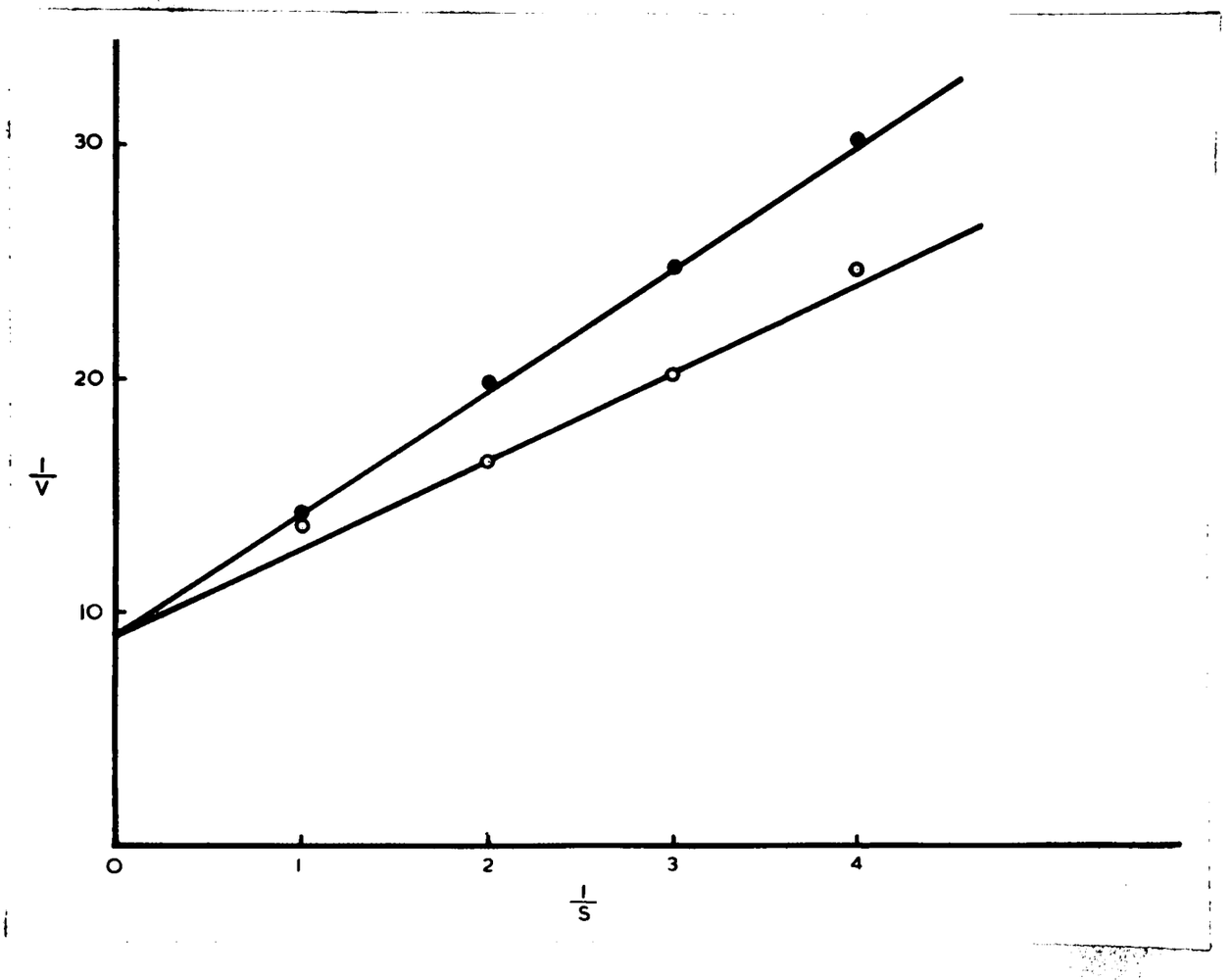


Fig. 28. Competitive inhibition of aconitase by natural fluorocitric acid. The graph is plotted according to the method of Lineweaver & Burk (1934). Aconitase activity was determined in the presence of 0.05 M phosphate buffer (pH 7.7) using 5 µg. of the final aconitase preparation activated by Fe^{2+} and cysteine. Total volume 3.0 ml., temperature 22°.

S = molar concentration of isocitric acid x 1500

V = change of log. I_o/I per 15 sec. x 10

o = control • = fluorocitric acid ($3.\overline{3}$ x 10^{-5} M)

very much less than that of the aconitase present in the kidney particles. Moreover, whereas the inhibition of aconitase of the kidney particles was apparently irreversible, that with the isolated aconitase was apparently reversible. The results as far as the isolated aconitase is concerned have been confirmed here. Peters (1952) has proposed that this difference is due to two factors, firstly to the concentration of the fluorocitric acid within the kidney particles and secondly to an exaggeration of the inhibition due to the intervention of structural factors. The result is that the concentration of fluorocitric within the particles is relatively high whereas the concentration of citric acid is relatively low. Thus the inhibitor behaves as though it were irreversible.

It was also possible that the fluorocitric acid isolated from the kidney particles was not the inhibitory substance, but was converted to the inhibitory substance when incubated with the kidney particle test system. This idea was consistent with a finding of Peters (personal communication) that the disappearance of citric acid was not inhibited immediately by fluorocitric acid and that the inhibition was apparently

irreversible. It was realized that this result could also be explained as being due to permeability factors. There was no doubt, however, that there was an initial competition between citric acid and fluorocitric acid because the inhibition of citric acid disappearance was dependent on the initial concentration of citric acid.

In order to determine whether or not this was the case, fluorocitric acid was incubated by Peters & Wakelin with the kidney particles under the usual test conditions. After acid inactivation of the aconitase, the supernatant was tested for the presence of an irreversible inhibitor in the system cis-aconitic acid ⟶ citric acid. This system was used on account of the high absorption of the extract at 240 mμ which did not permit of the study of the reaction isocitric acid ⟶ cis-aconitic acid. No evidence was found for the presence of an irreversible inhibitor, but it must be pointed out that the technical difficulties were great on account of the relatively large amounts of citric acid which were present in the extract containing fluorocitric acid. Citric acid had to be added during the incubation so that the kidney particles did not become inactivated.

Effect of fluorocitric acid on a crude preparation of aconitase

The effect of natural fluorocitric acid on the aconitase of a crude heart extract and of an extract of kidney particles was tested. In both cases only small inhibitions were obtained with 3.2×10^{-5} M fluorocitric acid when the enzyme and inhibitor were incubated for 15 min. before the addition of 16 μmoles of isocitric acid. Thus no irreversible inhibition could be shown with crude enzyme preparations. On the other hand, large inhibitions were obtained with synthetic fluorocitric acid under the same conditions.

DISCUSSION

At the outset, it is clear that the inhibitory action of the synthetic fluorocitric acid is not only greater, but also differs in some respects from that obtained with the natural fluorocitric acid. A discussion of the possible reasons for this difference will be deferred until the mode of action of each is discussed separately.

Synthetic fluorocitric acid

The results obtained with synthetic fluorocitric acid can best be interpreted by considering that two

types of inhibition occur, one being competitive or reversible and the other being irreversible.

According to the enzyme-substrate theory of Michaelis & Menten (1913), with competitive inhibition the equilibria between the enzyme, substrate and inhibitor is set up rapidly. Consequently, pre-incubation of the inhibitor with the enzyme should have no effect. However, with this inhibitor pre-incubation does have an appreciable effect on the degree of inhibition. On the other hand, when the enzyme is not pre-incubated with the inhibitor and the reciprocals of the initial velocities obtained in the presence and absence of the inhibitor are plotted against the reciprocals of the substrate concentrations according to the method of Lineweaver & Burk (1934), a graph characteristic of competitive inhibition is obtained. This result is consistent with the idea that there is a true competitive inhibition between the substrate and the inhibitor for the active centre(s) of the enzyme, at least during the initial stages of the reaction. The failure of high substrate concentrations to completely reverse the inhibition with the fluorocitric acid at a concentration of 2.4×10^{-5} M may mean that a proportion of the

inhibition is not reversible; it may also have been due to the technical limitations of increasing the substrate concentration to a sufficiently high level. Although the results indicated that the inhibition of aconitase by 0.6×10^{-5} M fluorocitric acid could be completely reversed, it is possible that as the inhibition was small, any irreversible inhibition would not have been detected. It cannot be concluded from these results whether or not synthetic fluorocitric acid also causes an irreversible inhibition of aconitase.

The concept of irreversible inhibition comes from two findings. In the first place, the marked effect of pre-incubation on the inhibition; in the second place, the fact that the reaction rates in the presence of the inhibitor fall after a time, even though the control rates which are faster, are still linear. These results suggest that a slow irreversible inhibition is taking place. If the reaction rates could have been studied for a longer period, it is likely that the change of rate would have been emphasised. However, the reaction isocitric acid ⟶ cis-aconitic acid could not be studied for long periods on account of the further conversion of cis-aconitic acid to citric acid

which complicates the measurement of cis-aconitic acid production. The results suggest that the time at which the irreversible inhibition manifests itself is a function of the substrate concentration. It would also seem as though the addition of low substrate concentrations was equivalent to pre-incubation. Prolonged pre-incubation followed by a low substrate concentration is no more effective than the addition of a low substrate concentration alone. But pre-incubation does increase the inhibition when higher concentrations of substrate are used.

It would seem as though the results can be best explained according to the following equations:

$$E + S \rightleftharpoons ES \longrightarrow E + P$$

$$E + I \rightleftharpoons EI' \longrightarrow EI'$$

where E = enzyme, S = substrate, I = inhibitor

ES = enzyme-substrate complex,

P = reaction products

EI = enzyme-inhibitor complex,

EI' = secondary complex formed between the enzyme and inhibitor.

Firstly, there is a competitive inhibition between the

inhibitor and the substrate for the active centre(s) of the enzyme; this equilibrium is set up immediately. This is followed by a slower reaction on the enzyme surface such that the inhibitor acts either in an irreversible fashion, or in a way that makes displacement of it from the enzyme surface more difficult. It is this latter reaction which occurs during the period of pre-incubation of the enzyme with the inhibitor, or during the course of the reaction.

If the second reaction between the enzyme and the inhibitor were irreversible, it might be expected that pre-incubation of the enzyme with fluorocitric acid for prolonged periods would lead to complete inactivation of the enzyme. Unfortunately, it was not possible to determine this because of the loss of enzyme activity that occurs when the enzyme is kept in dilute solution.

Although there is no conclusive proof of the irreversible inhibition of aconitase by synthetic fluorocitric acid, it does seem as though the above hypothesis offers the best explanation of the results.

Natural fluorocitric acid

There is no evidence of complicating factors in the inhibition of aconitase by natural fluorocitric acid.

The inhibition is competitive and can be readily reversed by higher substrate concentrations. One outstanding fact with this compound which is confirmed here, is the difference in the toxicity of it *in vivo* and in the kidney particle test system of Buffa *et al.* (1951) as compared with its toxicity in the isolated aconitase system. Perhaps the best explanation of this is the suggestion of Peters (1952) that the inhibitor is concentrated within the kidney particles or the mitochondria of cells so that the molar ratio of the inhibitor to enzyme is very much higher than that obtained in the isolated system with the amounts of fluorocitric acid used.

From the results obtained with the isolated aconitase it would appear as though the inhibition should be readily reversed, but the attempts made by Peters & Wakelin (1953) with the kidney particle system and by Hastings, Peters & Wakelin (1953) and others with the intact animal have been so far unsuccessful. No evidence was obtained for an irreversible inhibition, so it seems as though the inhibition *in vivo* is competitive. It is significant that smaller doses of fluoroacetic acid, which give rise to smaller amounts of fluorocitric acid *in vivo*, do cause an accumulation of citric acid in

animal tissues, presumably by the inhibition of aconitase, but the animals recover. This could be due to the fact that higher concentrations of citric acid within the mitochondria overcome the inhibition. When larger amounts of fluorocitric acid are formed and concentrated within the mitochondria, larger amounts of citric acid accumulate and the animals die. This could be due to the fact that very high concentrations of citric acid would be required to overcome the inhibition, so that before the citric acid to inhibitor ratio reaches a sufficiently high value, the secondary effects of high concentrations of citric acid bring about the death of the animal. The secondary effects could include changes within the mitochondria such as alterations in the ionic strength and binding of metallic ions (see Peters, 1952).

Citric acid would be the least effective substrate of aconitase in overcoming the inhibition as it has the lowest affinity for the enzyme. If cis-aconitic acid could be concentrated within the mitochondria, smaller amounts would be required for the reversal of the inhibition, as the enzyme has the highest affinity for this substrate. In these circumstances, the inhibition might be overcome without the development of the secondary

effects. The difficulty is in getting the tricarboxylic acids through the cell membrane; it is also possible that they pass through the mitochondrial membrane only at a slow rate. These permeability troubles might be eliminated if cis-aconitic acid were added to the kidney particle preparation or injected into the intact animal as an ester or amide. The success of this therapy would depend not only on the ability of the tissue to hydrolyse the ester or amide, but also on the rate of the hydrolysis. If high concentrations of cis-aconitic acid were to give rise to inhibitory trans-aconitic acid, the use of the ester or amide of isocitric acid might be better.

Comparison of the action of natural and synthetic fluorocitric acids

The difference in the inhibitory action of the natural and synthetic fluorocitric acids on the isolated aconitase can be most readily explained as being due to the presence of different stereoisomers in each preparation. Although no direct evidence is available to support this conclusion, it is clear that the mode of synthesis of the synthetic compound would give rise to the formation of the four stereoisomers. On the other hand, it is unlikely that more than two stereoisomers would be formed

enzymically. Peters et al. (1953) found that there was a difference in the ability of natural and synthetic fluorocitric acids to inhibit the disappearance of citric acid in kidney particles, but in this instance the synthetic fluorocitric acid was only one half as active as the natural fluorocitric acid. These authors concluded on the basis that the four stereoisomers of the synthetic preparation are present in equal amounts, that only one optically active centre is concerned in the inhibition. In the experiments with the isolated aconitase, the synthetic material is far more active than the natural. Not only is there a difference in degree as far as the competitive inhibition is concerned, but the synthetic fluorocitric acid also exhibits an irreversible inhibition. It would seem as though the isomers present in the synthetic material, but not present in the natural fluorocitric acid, are responsible for the different effects.

The reasons for the difference in the action of the two fluorocitric acid preparations on the aconitase of the kidney particles and on the isolated aconitase have been discussed with Professor Peters. From the results obtained with the isolated aconitase, it would be

expected that the synthetic fluorocitric acid would inhibit the aconitase of the kidney particles to a greater extent than the natural fluorocitric acid, but this is not the case. The results of Peters et al. (1953b) suggest that the 'unnatural' stereoisomers of the synthetic material do not enter the kidney particles, or if they do, they do not come into contact with the aconitase. If on the other hand, all four stereoisomers of the synthetic material are free to react with the aconitase, but no reaction takes place, it must be postulated that there is some structural difference between the enzyme within the kidney particles and in the isolated state. Perhaps with the isolated enzyme, the 'unnatural' stereoisomers of the synthetic fluorocitric acid react with groups on the enzyme surface which are not exposed when the enzyme is fixed and surrounded by other enzyme molecules in the kidney particles. As the inhibition of the isolated aconitase by synthetic fluorocitric acid is at least partially competitive, this reaction probably involves one or more of the active centres as well as other groups on the enzyme surface.

A more conclusive explanation of these differences must await the separation of the stereoisomers of

synthetic fluorocitric acid and the determination of which stereoisomer(s) is formed enzymically.

SUMMARY

1. The competitive inhibition of aconitase by natural fluorocitric acid has been confirmed.
2. Synthetic fluorocitric acid has been shown to inhibit aconitase in both a competitive and irreversible fashion.
3. The dissociation constants of the enzyme-inhibitor complexes for both the natural and synthetic fluorocitric acids have been measured. The affinity of aconitase for the synthetic compound is much greater than for the natural compound.
4. The possible reasons for the difference between the two preparations of fluorocitric acid in inhibiting the isolated aconitase have been discussed. The difference between the action of these compounds on the isolated aconitase system as compared to their action *in vivo* and on the kidney particle system of Buffa *et al.* (1951) has also been discussed.

CHAPTER VI

THE EFFECT of OTHER INHIBITORS on AZOTASE ACTIVITY

CHAPTER VI

THE EFFECT OF OTHER INHIBITORS on ACONITASE ACTIVITY

INTRODUCTION

Dickman & Cloutier (1951) found that aconitase was inhibited when the activation of the enzyme by Fe^{2+} and ascorbic acid was carried out in the presence of 10^{-4} M p-chloromercuribenzoate. This suggested that aconitase was dependent on the presence of a free -SH group for activity. The results of Krebs & Eggleston (1944) that aconitase is inhibited by Hg^{2+}, Cu^{2+} and alloxan were also consistent with this idea.

The inhibition of aconitase by o-phenanthroline (Dickman & Cloutier, 1951), sodium sulphide, HCN and pyrophosphate (Krebs & Eggleston, 1944) was in keeping with the fact that aconitase is a Fe^{2+} enzyme.

As the preliminary results of the effect of p-chloromercuribenzoate, o-phenanthroline, 2:2'-dipyridyl, versene, (ethylenediamine tetra-acetic acid), cyanide and azide on the activity of aconitase seemed of interest,

they are reported in this Chapter.

EXPERIMENTAL

Methods

Aconitase was activated by Fe^{2+} and either cysteine or ascorbic acid and the enzyme activity was determined by the methods previously described (page 19/20).

Reagents

p-Chloromercuribenzoate was kindly supplied by Dr. L.A. Stocken. o-Phenanthroline, 2:2'-dipyridyl, and versene were B.D.H. products. The other reagents were either A.R. or as previously described.

RESULTS

p-Chloromercuribenzoate

Table 11 shows the inhibition of aconitase by increasing concentrations of p-chloromercuribenzoate and the effect of cysteine in reversing the inhibition when added immediately after the p-chloromercuribenzoate. The concentration of p-chloromercuribenzoate required to inhibit the enzyme was relatively high, but as the enzyme had been activated by cysteine (the concentration in the medium was 0.3×10^{-4} M), the effective

TABLE 11

Inhibition of Aconitase by p-Chloromercuribenzoate. Reversal of the Inhibition by Cysteine.

(The system contained 0.05 M phosphate buffer (pH 7.7), cis-aconitic acid (5.7×10^{-3} M) and various concentrations of p-chloromercuribenzoate. Aconitase (equivalent to 10 µg. of the final preparation) activated by Fe^{2+} and cysteine was added. Excess cysteine (10^{-2} M) was added after the addition of the enzyme. Total volume 3.0 ml. Reaction was stopped after 15 min. by the addition of 0.5 ml. of 50% (w/v) trichloroacetic acid and the solutions analysed for citric acid. Temperature 22°)

p-Chloromercuribenzoate concentration ($\times 10^{-4}$ M)	Inhibition (%)	
	No excess cysteine	excess cysteine
10	83	33
7	76	19
4	58	12

concentration of the inhibitor would be considerably reduced. The enzyme was not pre-incubated with the inhibitor on account of the inactivation of aconitase by phosphate in the absence of substrate.

Table 12 shows that the inhibition is increased when p-chloromercuribenzoate is added to the enzyme before the substrate, whereas there is no difference between adding the enzyme to a mixture of substrate and inhibitor and adding the inhibitor after the addition of substrate. These results indicate that the substrate does protect the enzyme from the inhibitor during the initial stages of the reaction and suggest that the inhibition is due to p-chloromercuribenzoate reacting with the active centre(s) of the enzyme. According to the Michaelis-Menten theory of enzyme action, the inhibition should increase with time as there is always a proportion of the enzyme not in combination with substrate which can react with the inhibitor. Fig. 29 shows that this is so. The inhibition calculated at 15 min. is 59% and agrees well with the previous results, but it is clear that this is only a qualitative result. The inhibition increases during the initial stages of the reaction so that when the reaction is carried out for a fixed period of time the inhibition found is not a quantitative result, but merely

TABLE 12

The Reduction of the Inhibition of Aconitase by p-Chloromercuribenzoate by Substrate

(Conditions were as described in Table 11. The concentration of p-chloromercuribenzoate was 4×10^{-4} M. Total volume 3.0 ml., temperature 22°.

1. enzyme added to substrate
2. enzyme added to substrate plus inhibitor
3. enzyme added to inhibitor, followed by substrate
4. enzyme added to substrate, followed by inhibitor)

No.	Rate (µg. citric acid formed/15 min.)	Inhibition (%)
1.	128	-
2.	54	58
3.	19	86
4.	55	58

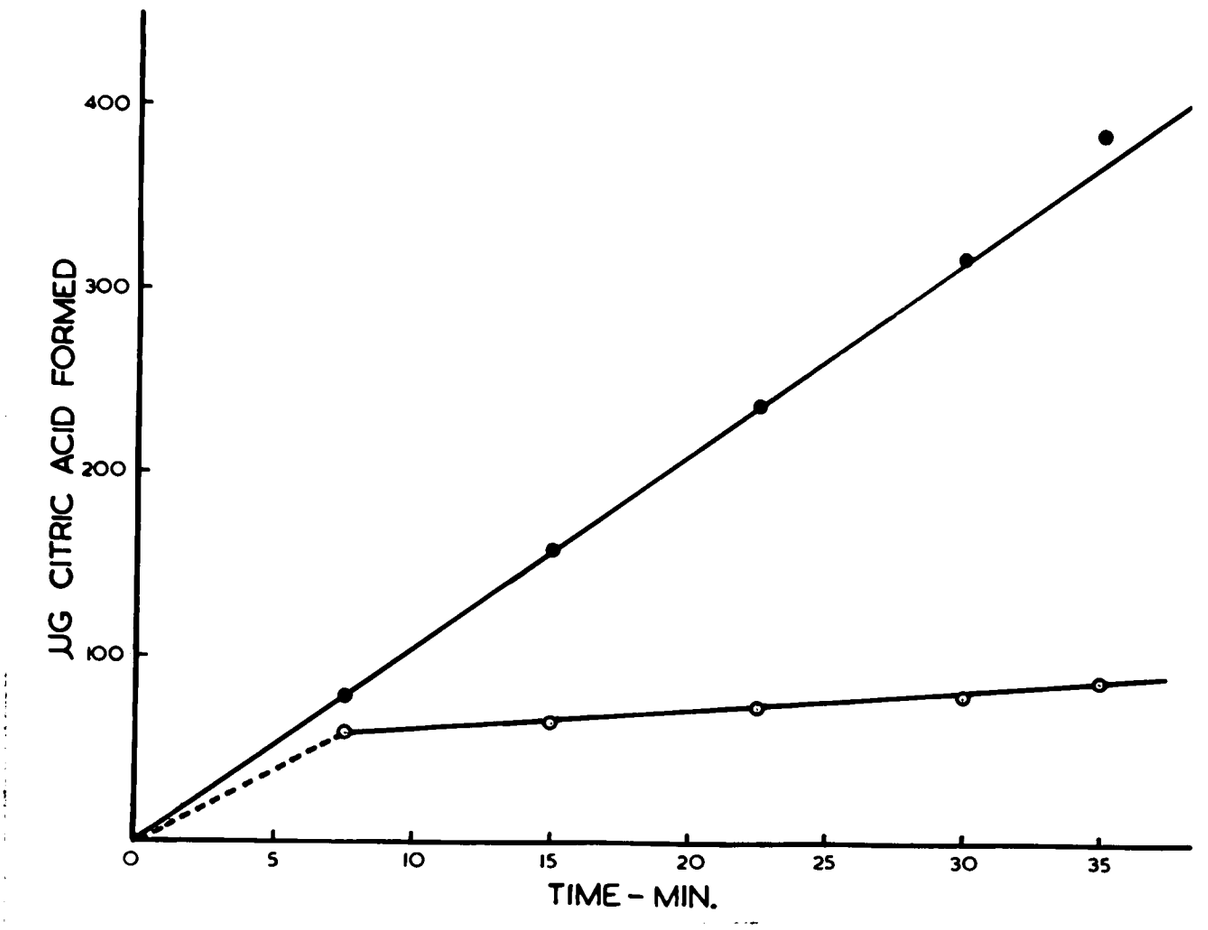


Fig. 29. Time curve for the inhibition of aconitase by p-chloromercuribenzoate. Conditions were as described for table 11. The concentration of p-chloromercuribenzoate was 4×10^{-4} M.

indicates that inhibition does occur.

It cannot be concluded from these results that an -SH group on the surface of the enzyme is concerned with aconitase activity for the p-chloromercuribenzoate could also bring about inhibition by upsetting the equilibrium that exists between the enzyme and cysteine. The addition of excess cysteine could then overcome the inhibition by restoring the equilibrium. The problem was resolved by activating the enzyme with ascorbic acid rather than with cysteine for ascorbic acid does not react with p-chloromercuribenzoate.

The time course of the inhibition of aconitase, activated with ascorbic acid, by p-chloromercuribenzoate was similar to that obtained when the enzyme was activated with cysteine (fig. 29), but the enzyme activated with ascorbic acid was more sensitive to the inhibitor. The inhibition calculated at 15 min. with 10^{-4} M p-chloromercuribenzoate was 73% whereas with the cysteine activated enzyme, there was no inhibition with twice this concentration of inhibitor.

As the maximum inhibition of the enzyme by p-chloromercuribenzoate did not develop until between 5 and 10 min. after the start of the reaction, it was

possible that the supposed reversal of the inhibition of the cysteine activated enzyme was, in fact, a prevention of the development of the inhibition. Therefore, attempts were made to reverse the inhibition of the ascorbic acid activated enzyme by adding excess cysteine 5 min. after the start of the reaction. Fig. 30 shows the marked inhibition that develops and the partial reversal of the inhibition by cysteine, but not by ascorbic acid.

The above evidence suggests that aconitase requires a free -SH group for activity.

o-Phenanthroline, 2:2'-dipyridyl and versene

o-Phenanthroline, 2:2'-dipyridyl and versene are known to form complexes with Fe^{2+}. However, concentrations of these substances up to 10^{-2} M did not inhibit the reaction cis-aconitic acid ⟶ citric acid when added simultaneously with substrate. From the colour of the solutions to which o-phenanthroline and 2:2'-dipyridyl had been added, it was clear that at least some of the Fe^{2+} present had reacted to form a complex. On the other hand, when these substances were added to the enzyme solution at the start of the 1 hr. period of activation or at the end of the activation period followed by a

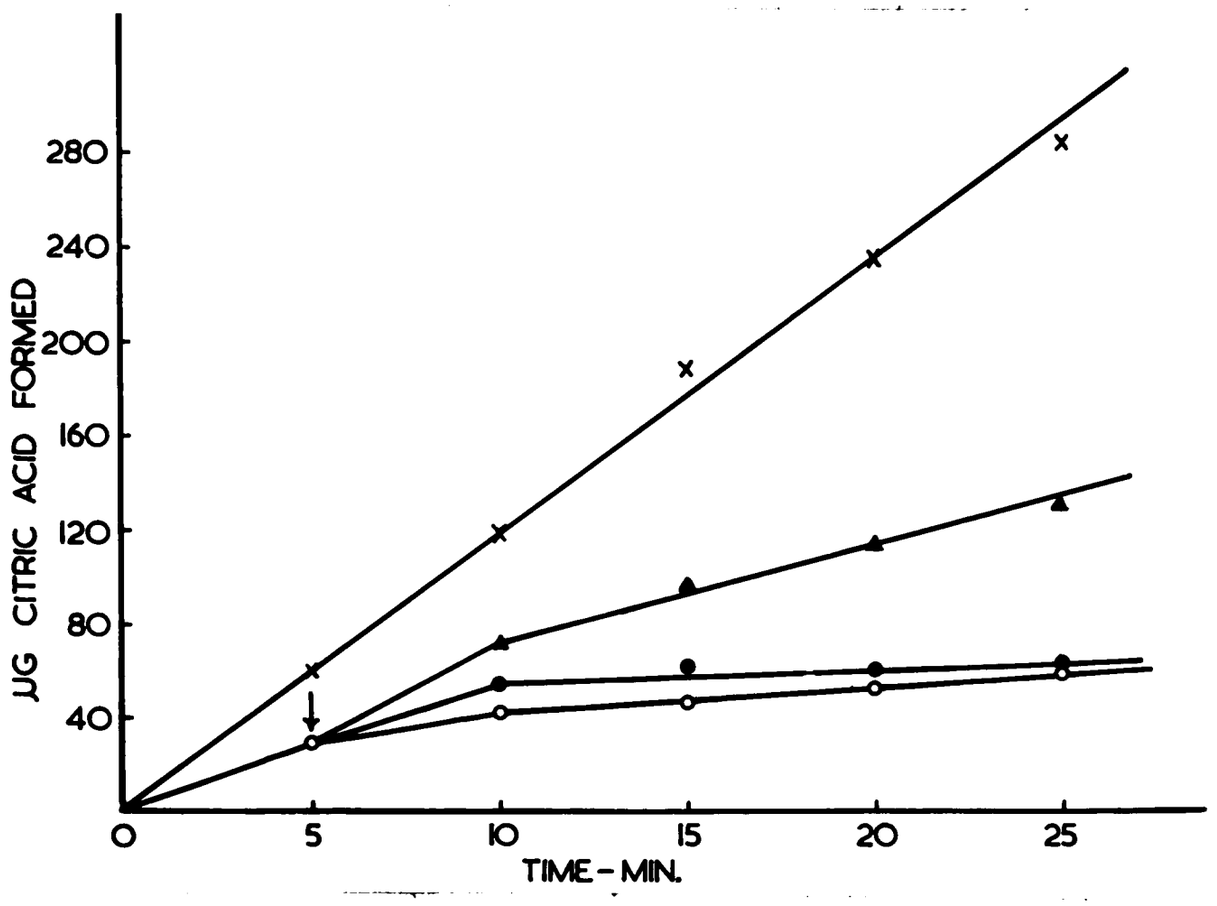


Fig. 30. Time curve for the inhibition of aconitase by p-chloromercuribenzoate; part reversal of the inhibition by cysteine and non-reversal of the inhibition by ascorbic acid. Conditions were as described in Table 11, except that the enzyme was activated with Fe^{2+} and ascorbic acid and 15 µg. of the final aconitase preparation was used. Cysteine and ascorbic acid were added 5 min. after the addition of the enzyme, final concentration 10^{-2} M. The concentration of p-chloromercuribenzoate was 10^{-4} M.

x = control o = p-chloromercuribenzoate

● = p-chloromercuribenzoate + ascorbic acid

▲ = p-chloromercuribenzoate + cysteine

further incubation period of 1 hr., or pre-incubated with the enzyme in the presence of low concentrations of substrate, marked inhibitions of the enzyme were obtained. Table 13 shows the effect of adding versene and o-phenanthroline to the enzyme solution during the period of activation.

Cyanide and azide

The reactions cis-aconitic acid ⟶ citric acid and isocitric acid ⟶ cis-aconitic acid were not inhibited by concentrations of cyanide and azide up to 10^{-2} M. Moreover, when the activation of aconitase was carried out in the presence of 10^{-2} M cyanide, there was little or no inhibition of either reaction, although the colour of the enzyme solution indicated that ferrocyanide had been formed. Further tests showed that cyanide was not capable of replacing the other reducing agents in activating aconitase. Krebs & Eggleston (1944) claimed that aconitase was inhibited by cyanide, whereas Jacobsohn (1940) concluded that the prosthetic group of aconitase did not contain a heavy metal as the enzyme activity was not inhibited by even high concentrations of cyanide. The above results are in agreement with those of Jacobsohn (1940).

TABLE 13

Inhibition of Aconitase by Versene and o-Phenanthroline

(Aconitase was incubated in the presence of 5×10^{-4} M Fe^{2+} and 10^{-2} M cysteine for 1 hr. at 0° after the addition of the inhibitor. The enzyme activity was then determined as described in Table 11. 15 µg. of the final aconitase preparation, temperature 22°)

Inhibitor	Concentration of inhibitor (M)	Time of addition[o] (min.)	Inhibition (%)
Versene	10^{-2}	0	72
		60	57
o-Phenanthroline	5×10^{-3} [x]	0	64
		60	60

[x] Higher concentrations precipitated from solution at 0°

[o] The time of addition indicates the period for which the enzyme had been incubated with Fe^{2+} and cysteine prior to the addition of the inhibitor.

DISCUSSION

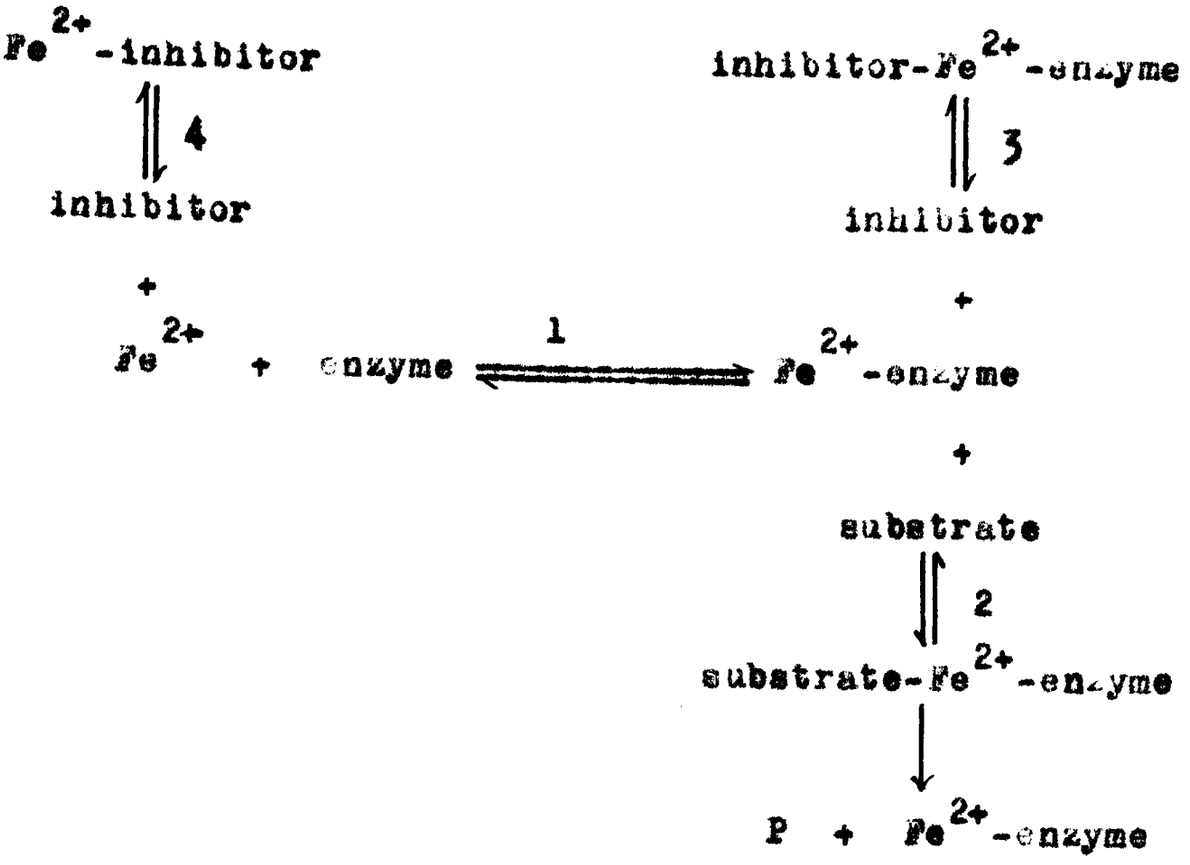
As it is known that versene and o-phenanthroline have high affinities for Fe^{2+}, it seems reasonable to conclude that the inhibition of aconitase is due to their interaction with Fe^{2+}, so that Fe^{2+} ceases to play its normal role in the mechanism of aconitase action. When these compounds are added at the start of the activation period, the inhibition could be due to the prevention of the formation of the Fe^{2+}-enzyme complex; when added after the formation of the Fe^{2+}-enzyme complex i.e. following a 1 hr. period of activation, the inhibition could be due to

(a) reaction of the inhibitor with the free Fe^{2+} in equilibrium with the Fe^{2+}-enzyme complex

(b) direct reaction of the inhibitor with the Fe^{2+} on the surface of the enzyme

(c) a combination of both.

(a) and (b) are illustrated by reactions (3) and (4) of the scheme below:



If Fe^{2+} functions as a means of linking the substrate to the enzyme, the above scheme illustrates the reactions that could occur when substrate and inhibitor are added simultaneously to the activated enzyme. It might be expected that the inhibitors could compete with the substrate for the enzyme (reactions 2 & 3) as they both react with Fe^{2+} and so cause inhibition. The fact that inhibition was not found even with high concentrations of the inhibitors is not surprising in view of the high affinity of the enzyme for cis-aconitic acid (K_m = 1.2 x 10^{-4} M) and the high substrate concentration

used (6.7×10^{-3} M). It is possible that competitive inhibition may be obtained in the presence of lower substrate concentrations, but this point has not yet been investigated.

Inhibition could also conceivably occur as a result of the reaction between the inhibitor and the free Fe^{2+} in equilibrium with the Fe^{2+}-enzyme (reaction 4). (The colour of the solutions on the addition of o-phenanthroline or 2:2'-dipyridyl indicated that this reaction had occurred to at least some extent). The result of this would be a shift of the equilibrium of reaction (1) to the left. But due to the high affinity of the Fe^{2+}-enzyme complex for substrate, the amount of free Fe^{2+}-enzyme present would be small. Thus only a small proportion of the total enzyme would be inactivated. Of course, if the theory of Michaelis & Menten (1913) holds, inhibition would occur in time. There is also another factor which would tend to delay the onset of the inhibition. The affinity of the enzyme for Fe^{2+} is high (dissociation constant of the Fe^{2+}-enzyme complex is 3.9×10^{-6} M); a finite time is required for the establishment of the equilibrium of reaction (1) starting with Fe^{2+} and enzyme, so the rate of establishment of the new equilibrium

would be slow even in the absence of substrate. The rate in the presence of substrate would be slower still.

No allowance has been made in the above scheme for the fact that cysteine which was used to activate the enzyme, and the substrates of aconitase are also capable of reacting with Fe^{2+} and may influence the reactions taking place. However, it does seem as though the failure of compounds capable of forming complexes with Fe^{2+} to inhibit aconitase can be reasonably explained if the Fe^{2+} is concerned in the linkage of the substrate to the enzyme.

The Fe^{2+} could also function by fixing the degrees of freedom of the enzyme molecule so that substrate could combine at another site on the enzyme surface. In this case, the reason for the protection of the enzyme from inhibition by substrate is not so apparent, but there do seem to be two plausible explanations. It is possible that the inhibitors cannot approach the Fe^{2+} whilst substrate is linked to the enzyme. However, reaction with the free Fe^{2+} would still be possible, but the rate of dissociation of the Fe^{2+} from the surface of the enzyme may be slowed down even further whilst the enzyme is in combination with substrate. Therefore, inhibition may

not be observed over the period for which the reaction was carried out.

The failure of cyanide to inhibit a heavy metal enzyme is an interesting phenomenon in view of the general acceptance of the idea that metalloenzymes are sensitive to cyanide. The failure of cyanide to inhibit the activation of aconitase could be due to the fact that the affinity of the enzyme for Fe^{2+} is greater than that of cyanide, or that ferrocyanide is capable of activating the enzyme. It is also possible that if the substrate is linked to the enzyme by way of the Fe^{2+}, that the cyanide molecules in combination with the Fe^{2+} can be rapidly displaced by the substrate.

The compounds which react with Fe^{2+} can be divided into three classes as far as their effect on aconitase is concerned. In the first class are those compounds which activate, namely cysteine, thioglycollate, ascorbic acid and glutathione. In the second are cyanide and azide which do not affect the activity of the enzyme, whilst in the third are those compounds which do inhibit the enzyme, namely versene, o-phenanthroline and 2:2'-dipyridyl. The activators doubtlessly possess special properties as distinct from the other two classes, but it

does seem as though the ability of a compound, which is capable of forming a Fe^{2+} complex, to inhibit aconitase might be related to the dissociation constant of the complex.

SUMMARY

1. Aconitase was inhibited by p-chloromercuribenzoate and the inhibition was partly reversed by excess cysteine.
2. The inhibition of aconitase by p-chloromercuribenzoate developed with time when substrate was present. It was greater when p-chloromercuribenzoate was added prior to the addition of substrate.
3. Versene, o-phenanthroline and 2:2'-dipyridyl inhibited aconitase only in the absence of substrate. Cyanide did not inhibit the enzyme.
4. The inhibition of aconitase has been discussed.

R E F E R E N C E S

Barron, E.S.G. (1951). Adv. Enzymol. 11, 201.

Bartlett, G.R. & Barron, E.S.G. (1947). J. biol. Chem. 170, 67.

Batelli, F. & Stern, L. (1910). Comp.rend.soc.biol. 69, 552.

Bernheim, F. (1928). Biochem. J. 22, 1178.

Breusch, F.L. (1937). Z. physiol. Chem. 250, 262.

Buchanan, J.M. & Anfinsen, C.B. (1949). J. biol. Chem. 180, 47.

Buffa, P. & Peters, R.A. (1949). J.Physiol. 110, 488.

Buffa, P., Peters, R.A. & Wakelin, R.W. (1951). Biochem. J. 48, 467.

Butterworth, J. & Walker, P.K. (1929). Biochem. J. 23, 926

Deffner, M. (1938). Liebigs Ann. 536, 44.

Dickman, S.R. (1952). Anal. Chem. 24, 1064.

Dickman, S.R. & Cloutier, A.A. (1950). Arch. Biochem. 25 229.

Dickman, S.R. & Cloutier, A.A. (1951). J.biol.Chem. 188, 379.

Dixon, M. (1953). Biochem. J. 55, 161.

Eggleston, L.V. & Krebs, H.A. (1949). Biochem.J. 45, 578.

Elliott, W.B. & Kalnitsky, G. (1950). J.biol.Chem. 186,487.

Evans, E.A. & Slotin, L. (1941). J.biol.Chem. 141, 439.

Fittig, R. & Miller, H.E. (1889). Liebigs.Ann. 255, 43.

Friedrich-Freska, H. & Martius, C. (1951). Z.Naturforschung 6b, 296.

Green, D.E. (1941). Adv. Enzymol. 1, 177.

Hastings, A.B. & Van Slyke, D.D. (1922). J. biol.Chem. 53, 269.

Hastings, A.B., Peters, R.A. & Wakelin, R.W. (1953). Abstr. XIX Internat. Physiol. Congress - page 674.

Jacobsohn, K.P. (1940). Bull.soc.Port.sci. 13, 75.

Jacobsohn, K.P. (1941). Arch.Port.sci.biol. 6, 13.

Jacobsohn, K.P. & Soares, M. (1939). Compt.rend.soc.biol. 131, 652.

Jacobsohn, K.P. & Soares, M. (1940). Compt.rend.soc.biol. 133, 112.

Jacobsohn, K.P. & Tapadinhas, J. (1940). Compt.rend.soc. biol. 133, 109.

Jacobsohn, K.P., Soares, M. & Tapadinhas, J. (1940) Bull.soc.chim.biol. 22, 48.

Johnson, M.J. (1939). Biochem.J. 33, 1046.

Kacser, H. (1952). Biochim.Biophys.Acta. 9, 406.

Kalckar, H.M. (1947). J.biol.Chem. 167, 461.

Kalnitsky, G. (1953). J.biol.Chem. 201, 817.

Kandel, A., Johnson, L.B. & Chenoweth, M.B. (1951). Fed. Proc. 10, 312.

Keilin, D. & Mann, T. (1940). Biochem. J. 34, 1163.

Krebs, H.A. (1943). Adv. Enzymol. 3, 191.

Krebs, H.A. (1953). Biochem. J. 54, 78.

Krebs, H.A. & Eggleston, L.V. (1943). Biochem. J. 37, 334.

Krebs, H.A. & Eggleston, L.V. (1944). Biochem. J. 38, 426.

Krebs, H.A. & Holzach, O. (1952). Biochem. J. 52, 527.

Krebs, H.A. & Johnson, W.A. (1937). Enzymologia. 4, 148.

Krebs, H.A., Salvin, S. & Johnson, W.A. (1938). Biochem. J. 32, 113.

Krimsky, I. & Racker, E. (1952). J. biol. Chem. 198, 721.

Kubowitz, F. (1935). Biochem. Z. 282, 277.

Kuhn, R. (1943). Naturw. 11, 732.

Langecker, H. (1934). Biochem. Z. 273, 43.

Lehninger, A.L. (1950). Physiol. Rev. 30, 393.

LePage, G.A. & Mueller, G.C. (1949). J. biol. Chem. 180, 975.

Liébecq, C. & Peters, R.A. (1949). Biochim. Biophys. Acta. 3, 215.

Lindenbaum, A., White, M.R. & Schubert, J. (1951). J. biol. Chem. 190, 585.

Lineweaver, H. & Burk, D. (1934). J.Am.Chem.Soc. 56, 658.

Lotspeich, W.D., Peters, R.A. & Wilson, T.H. (1952). Biochem. J. 51, 20.

Lynen, F. & Reichert, E. (1951). Angew.Chem. 63, 47.

Lynen, F., Reichert, E. & Rueff, L. (1951). Liebigs Ann. 574, 1.

Malachowski, R. & Maslowski, M. (1928). Ber.dtsch.chem. Ges. 61, 2521.

Mårtensson, J. (1940). Acta physiol.Scand. 1. Suppl.2.

Martius, C. (1937). Z.physiol.Chem. 247, 104.

Martius, C. (1938). Z.physiol.Chem. 257, 29.

Martius, C. (1949). Liebigs.Ann. 561, 227.

Martius, C. & Knoop, F. (1937). Z.physiol.Chem. 246, I.

Martius, C. & Leonhardt, H. (1943). Z.physiol.Chem. 278, 208.

Martius, C. & Lynen, F. (1951). Adv. Enzymol. 10, 167.

Massey, V. (1952). Biochem. J. 51, 490.

Massey, V. (1953). Biochem. J. 53, 67.

Mathews, A.P. & Walker, S. (1909). J.biol.Chem. 6, 299.

Michaelis, L. (1931). Biochem.Z. 234, 139.

Michaelis, L. & Davidsohn, H. (1911). Biochem.Z. 35, 386.

Michaelis, L. & Menten, M.L. (1913). Biochem. Z. 49, 333.

Mohamed, M.S. & Greenberg, D.M. (1945). Arch.Biochem. 8, 349

Müller, D. (1935). Biochem. Z. 275, 347.

Ochoa, S. (1948). J. biol. Chem. 174, 133.

Ochoa, S. (1951). The Enzymes (Academic Press Inc. New York) 1, 1217.

Ochoa, S., Stern, J.R. & Schneider, M.C. (1951). J.biol. Chem. 193, 691.

Ogston, A.G. (1948). Nature, Lond. 162, 963.

Ogston, A.G. (1951). Nature, Lond. 167, 693.

Orten, J.M. & Smith, A.H. (1938). J.biol.Chem. 124, 43.

Peters, R.A. (1948). Proc.Roy.Soc.Med. 41, 781.

Peters, R.A. (1952). Proc.Roy.Soc. B 139, 143.

Peters, R.A. & Wakelin, R.W. (1953). Personal Communication

Peters, R.A. & Wilson, T.H. (1952). Biochim.Biophys.Acta. 9, 310.

Peters, R.A., Wakelin, R.W. & Buffa, P. (1951). Proc. Biochem.Soc. 50, xiii.

Peters, R.A., Wakelin, R.W., Buffa, P. & Thomas, L.C. (1953a). Proc.Roy.Soc.B 140, 497.

Peters, R.A., Wakelin, R.W., Rivett, D.E.A. & Thomas, L.C. (1953b). Nature, Lond. 171, 1111.

Potter, V.R. & Busch, H. (1950). Cancer Res. 10, 353.

Potter, V.R. & Heidelberger, C. (1949). Nature, Lond. 164, 180.

Pucher, G.W., Sherman, C.C. & Vickery, H.B. (1936). J. biol.Chem. 113, 235.

Racker, E. (1950). Biochim.Biophys.Acta. 4, 211.

Racker, E. & Krimsky, I. (1952). J.biol.Chem. 198, 731.

Rivett, D.E.A. (1953). J.Chem.Soc. (In the press)

Saffran, M. & Prado, J.L. (1949). J. biol.Chem. 180, 1301.

Schubert, M. (1932). J.Am.Chem.Soc. 54, 4077.

Smith, E.L. (1951). Adv. Enzymol. 12, 191.

Smith, E.L. & Hanson, H.P. (1949). J.biol.Chem. 179, 803.

Stern, J.R. & Ochoa, S. (1949). J.biol.Chem. 179, 491.

Thunberg, T. (1911). Scand.Arch.Physiol. 24, 23 & 72.

Thunberg, T. (1929). Biochem.... 206, 109.

Wagner-Jauregg, T. & Rauen, H. (1936). Z.physiol.Chem. 237, 227.

Wood, H.G., Werkman, C.H., Hemingway, A. & Nier, A.O. (1941). J.biol.Chem. 139, 483.