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 Kacser (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

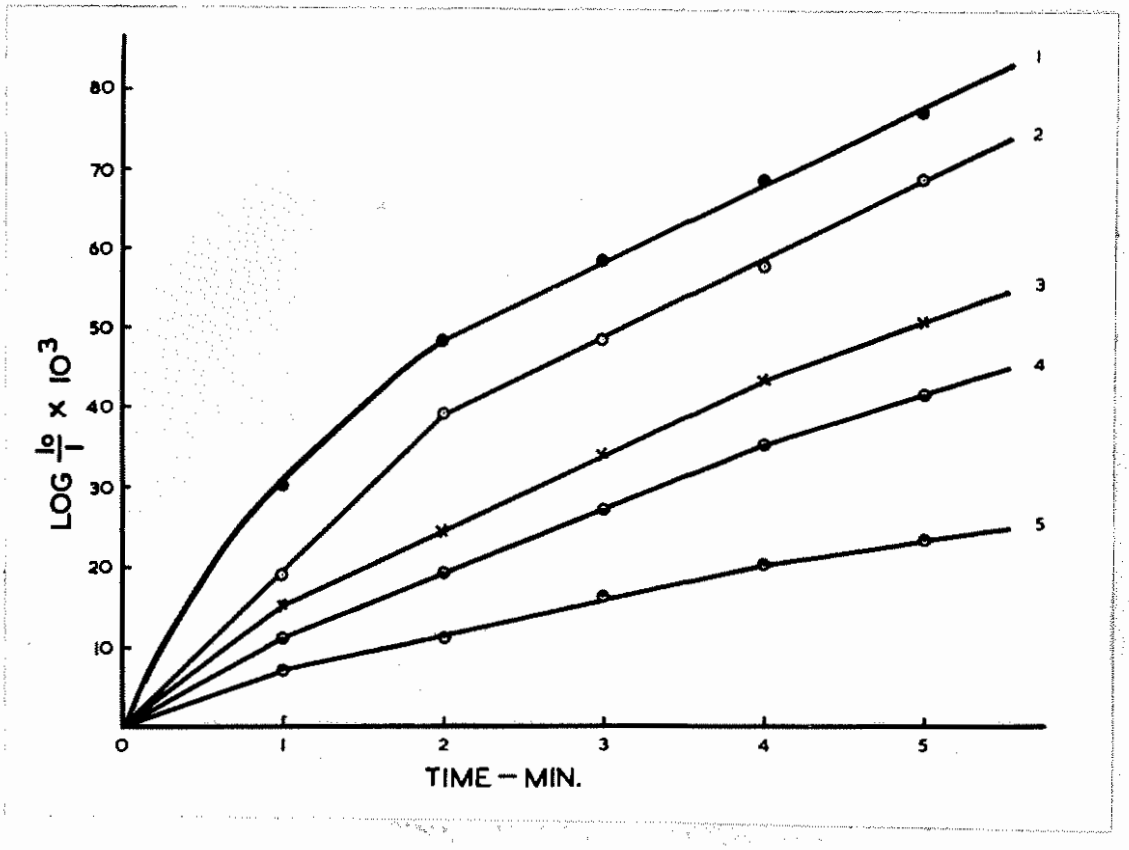


Fig. 17. The enzymic and non-enzymic reaction rates on adding aconitase activated with Fe^{2+} and cysteine to various concentrations of citric acid. The system contained 0.05 M phosphate buffer (pH 7.7). 8 µg. of the aconitase preparation and the following amounts of citric acid:

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 (page 19). 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

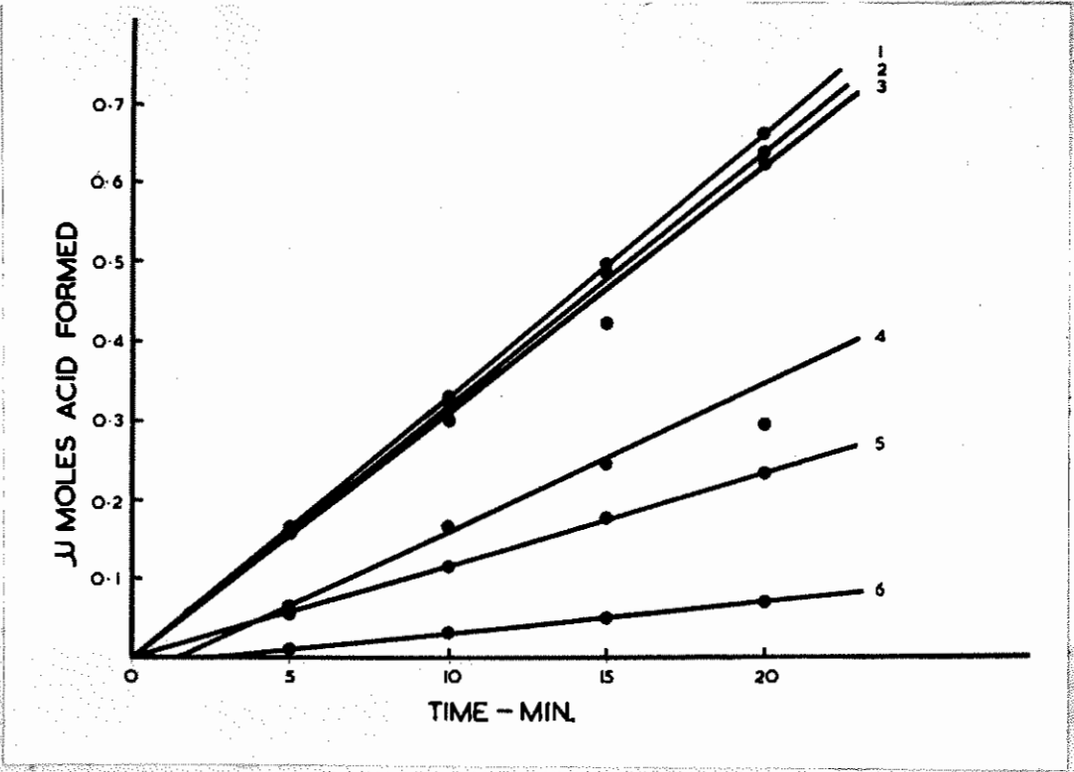


Fig. 18. The relative rates of the reactions catalysed by aconitase. The reaction mixture contained 0.05 M phosphate buffer (pH 7.7), together with either *cis*-aconitic acid (5×10^{-3} M), *iso*citric acid (5×10^{-3} M) or citric acid (5×10^{-2} M). Total volume 3.0 ml., temperature 22°. Reactions 2 & 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 *iso*citric 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. *iso*citric acid ⟶ *cis*-aconitic acid (0.1 ml.)
2. *cis*-aconitic acid ⟶ *iso*citric acid (0.1 ml.)
3. *cis*-aconitic acid ⟶ citric acid (0.4 ml.)
4. isocitric acid ⟶ citric acid (0.4 ml.)

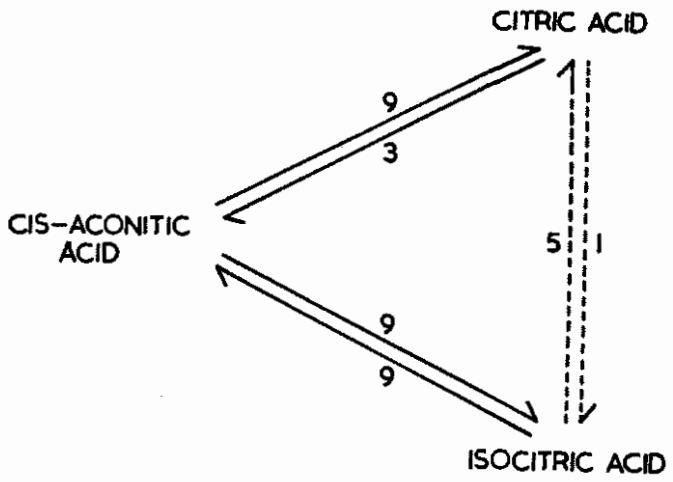


Fig. 19. The relative rates of the reactions catalysed by aconitase. A diagrammatic representation. The experimental conditions were as described in fig. 18.

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 iso-citric 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

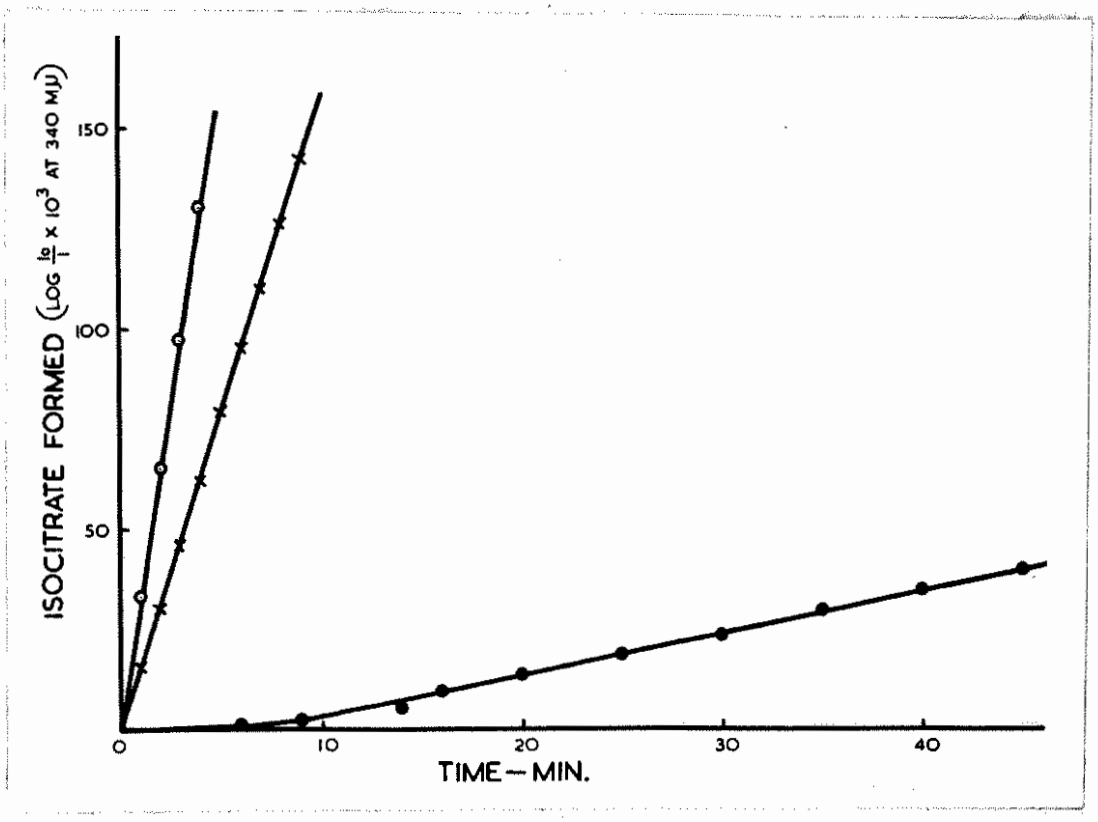


Fig. 20. Time curves for the formation of isocitric acid from cis-aconitic and citric acids. The system contained 0.05 M phosphate buffer (pH 7.2), 1.8 μmoles of $MnCl_2$, 0.135 umoles of Co II, 3 μg. of the activated aconitase preparation, 16 μg. of the non-activated aconitase preparation (isocitric 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 22^0. 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 3.6×10^{-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°.)

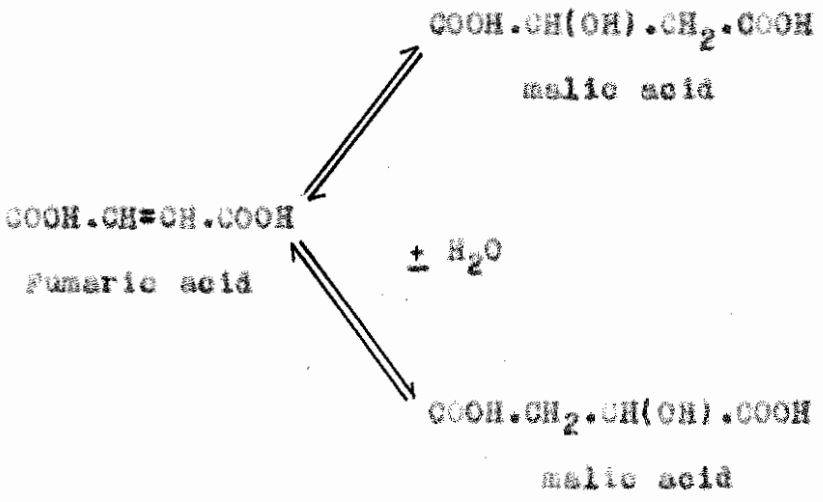
Substrate	Reaction rate (ΔE/min. x 10^3)
citric acid	15
isocitric acid	45
citric acid + isocitric acid	29

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

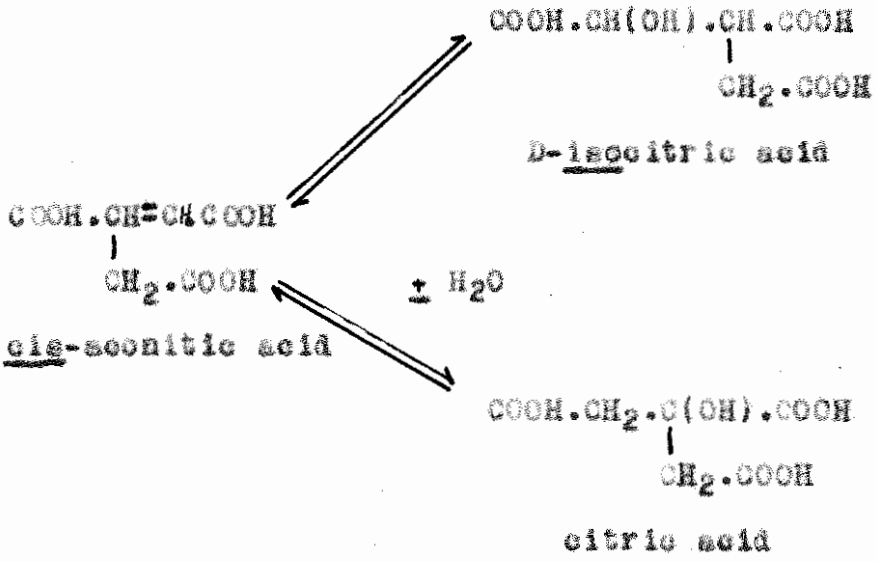
Aconitase has been considered as an unusual enzyme in so far as it has three substrates. However, as pointed out by Ogston (1951), there is no reason why it should not be considered in a broad sense as being

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-isocitric acid is formed. Ogston (1951) has proposed that the linkage of cis-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 → isocitric acid

there has been a tendency to think of the conversion of citric acid to isocitric 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 isocitric 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 isocitric 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 ½. 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 ½, 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 fluoro*iso*citric 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 Kandel *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 favour 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

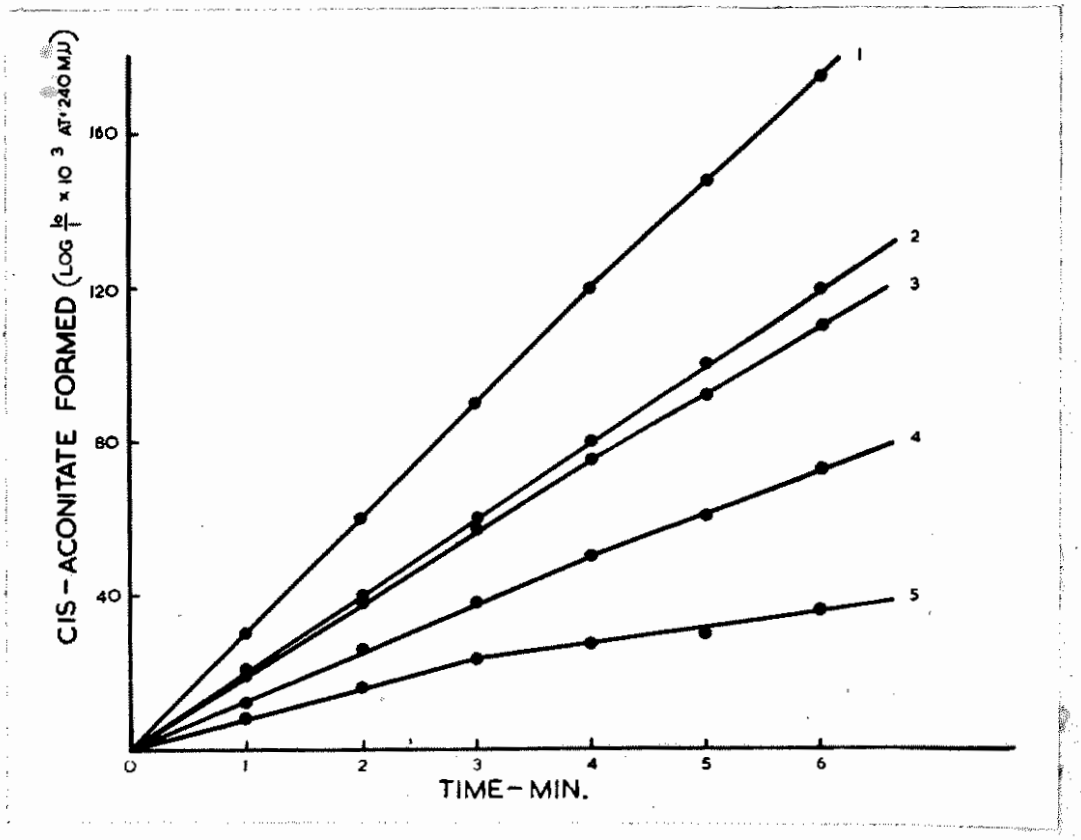


Fig. 21. The stabilization of aconitase by isocitric acid during incubation in the presence of phosphate. 5 µg. of the final aconitase preparation, activated by Fe^{2+} and cysteine, were incubated with 0.05 M phosphate buffer (pH 7.7) for 15 min. in the presence of various amounts of isocitric acid. 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 6 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.

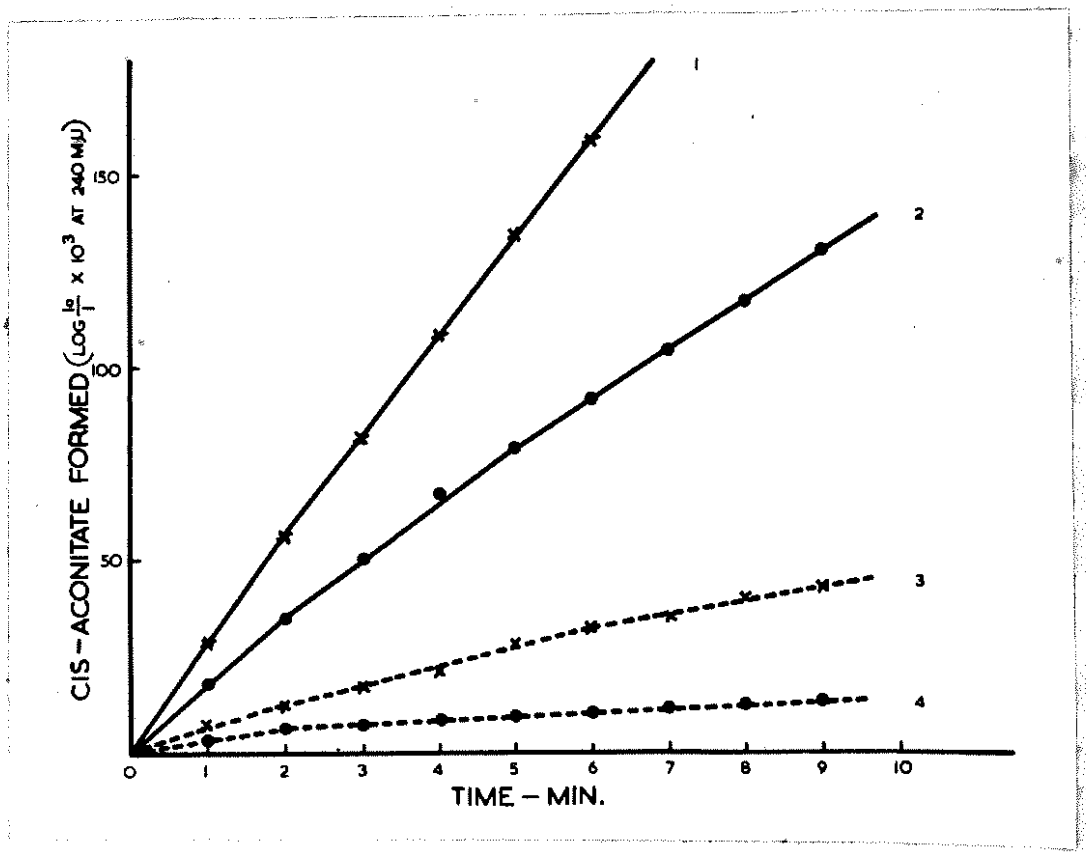


Fig. 22. Inhibition of aconitase by synthetic fluorocitric acid. 5 µg. of the final aconitase preparation, activated by Fe^{2+} and cysteine were incubated with fluorocitric acid (2.4×10^{-5} M) for 15 min. in the presence of 0.05 M phosphate buffer (pH 7.7) and 0.5 µmole of isocitric acid. Substrate 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 15 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

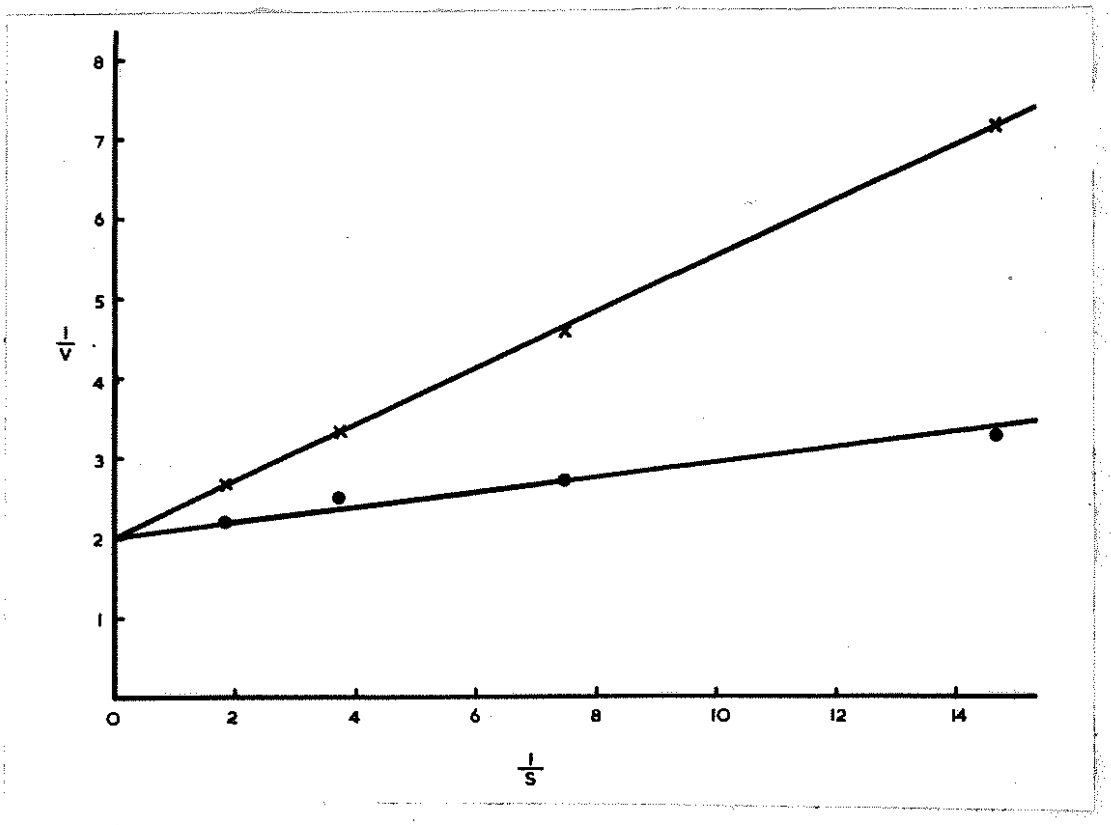


Fig. 23. Competitive inhibition of aconitase by synthetic fluorocitric acid. The graph is plotted according to the method of Lineweaver & Burk (1934). Aconitase activity was determined in the presence of 0.05 M phosphate buffer (pH 7.7) using 5 µg. of the final aconitase preparation activated by Fe^{2+} and cysteine. Total volume 3.0 ml., temperature 22^{0}.

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 ($\Delta\varepsilon$/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

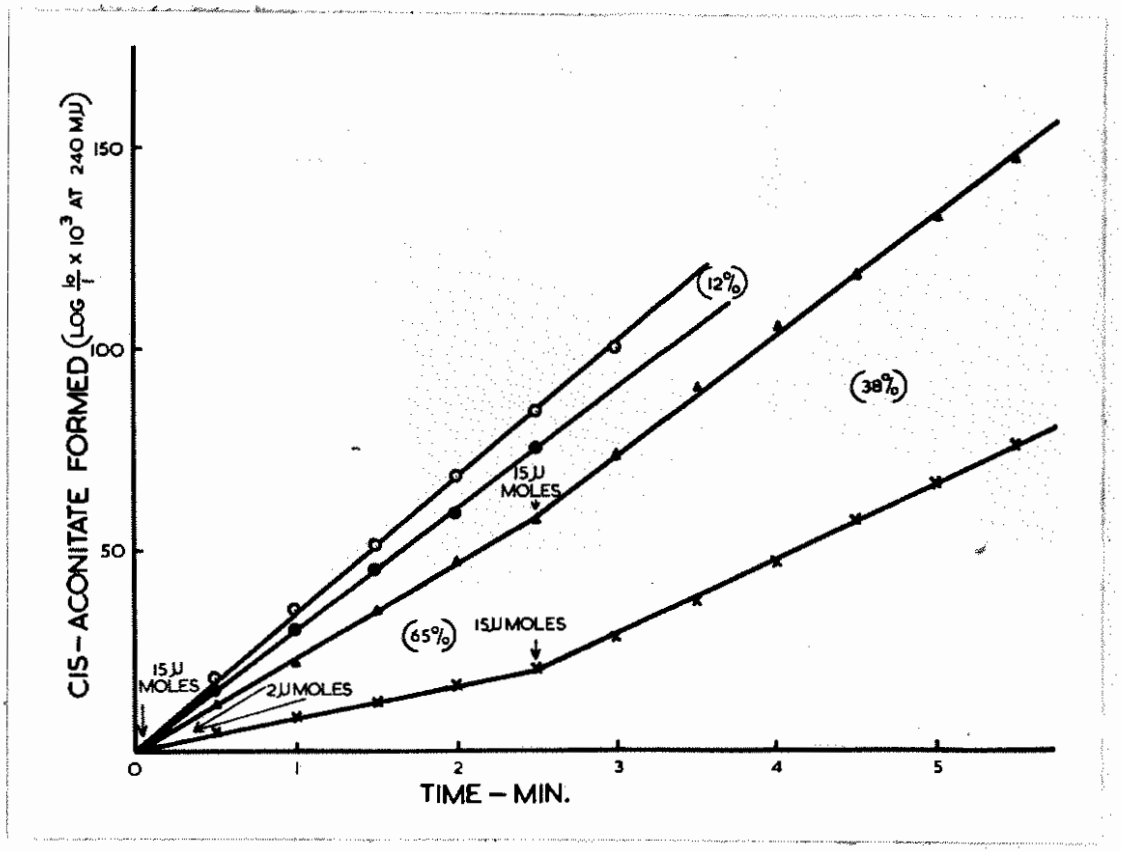


Fig. 24. Increased inhibition of aconitase by synthetic fluorocitric acid as a result of starting the reaction with a low concentration of isocitric acid. Conditions were as described in fig. 23. The concentration of fluorocitric acid was 2.4×10^{-5} M.

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

▲ = control) 2 μmoles of isocitric acid added
x = 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

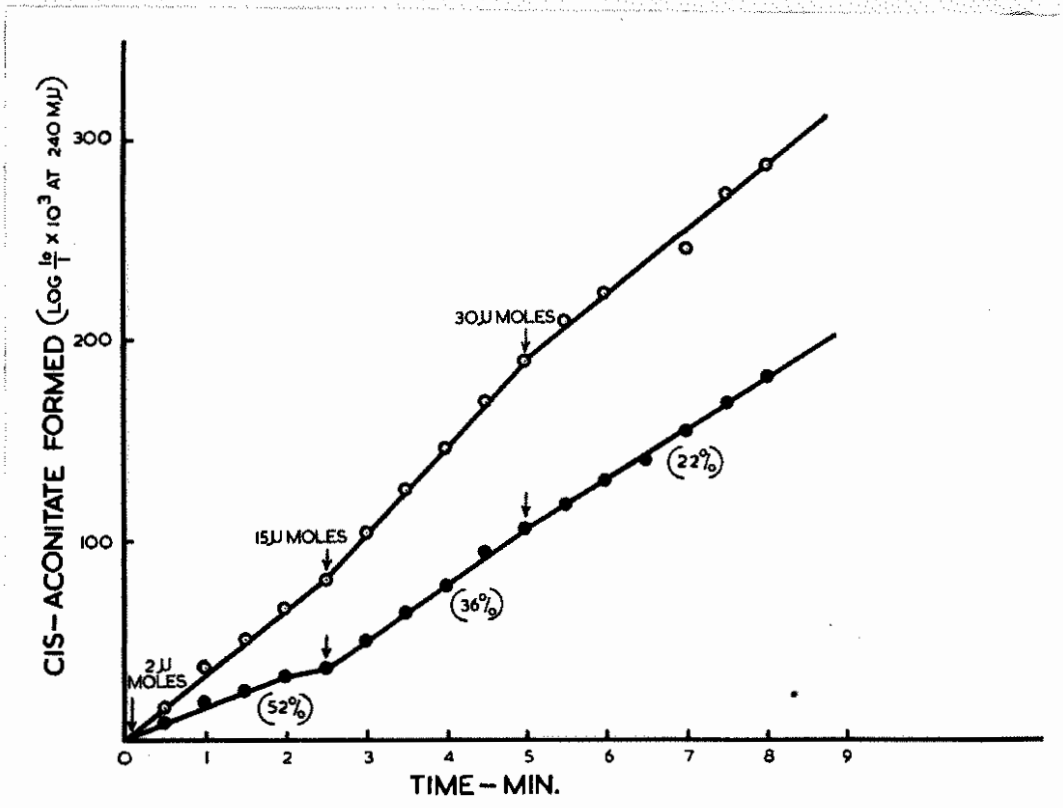


Fig. 25. Inhibition of aconitase by synthetic fluorocitric acid and reversal of the inhibition by increasing concentrations of <u>iso</u>citric acid. Conditions were the same as those of fig. 23.

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.

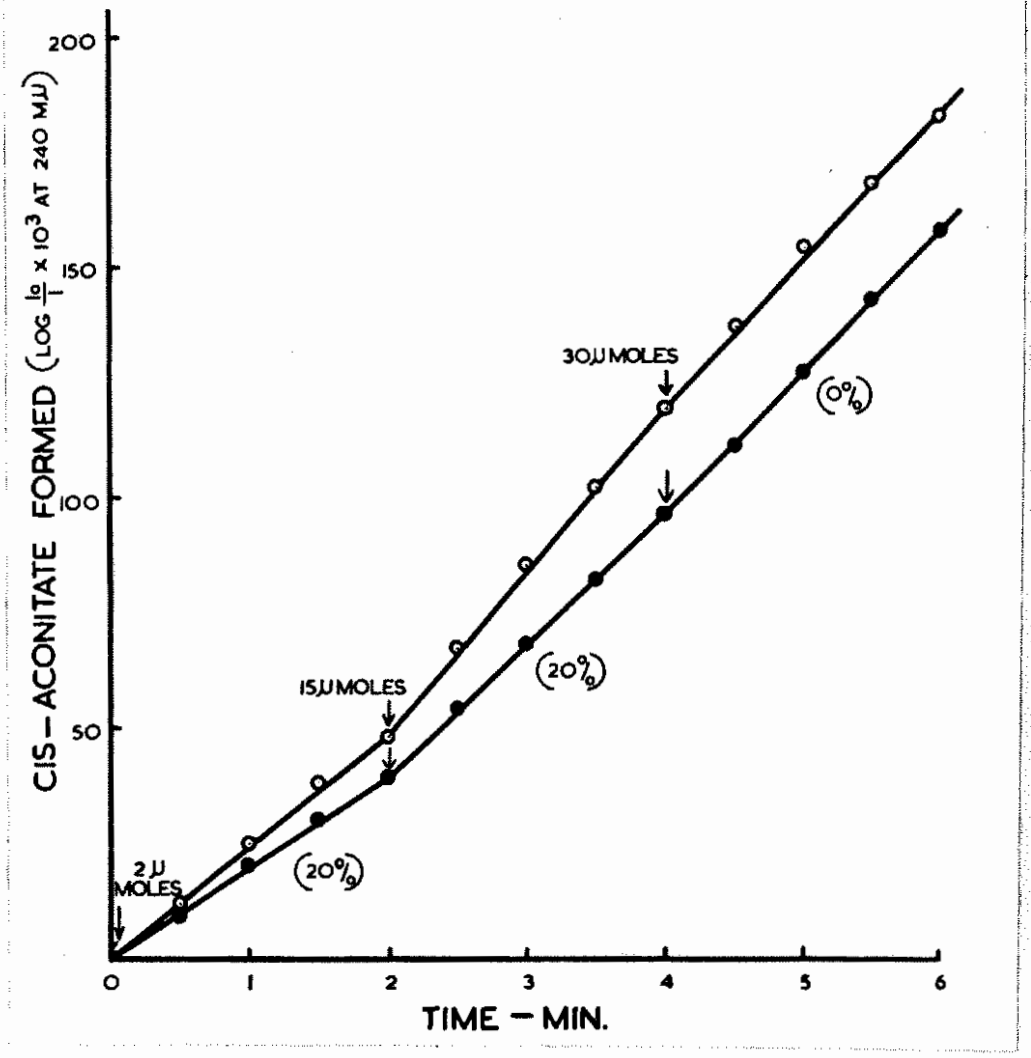


Fig. 26. Inhibition of aconitase by synthetic fluoro-citric acid and reversal of the inhibition by increasing concentrations of <u>iso</u>citric acid. Conditions were the same as those of fig. 25.

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

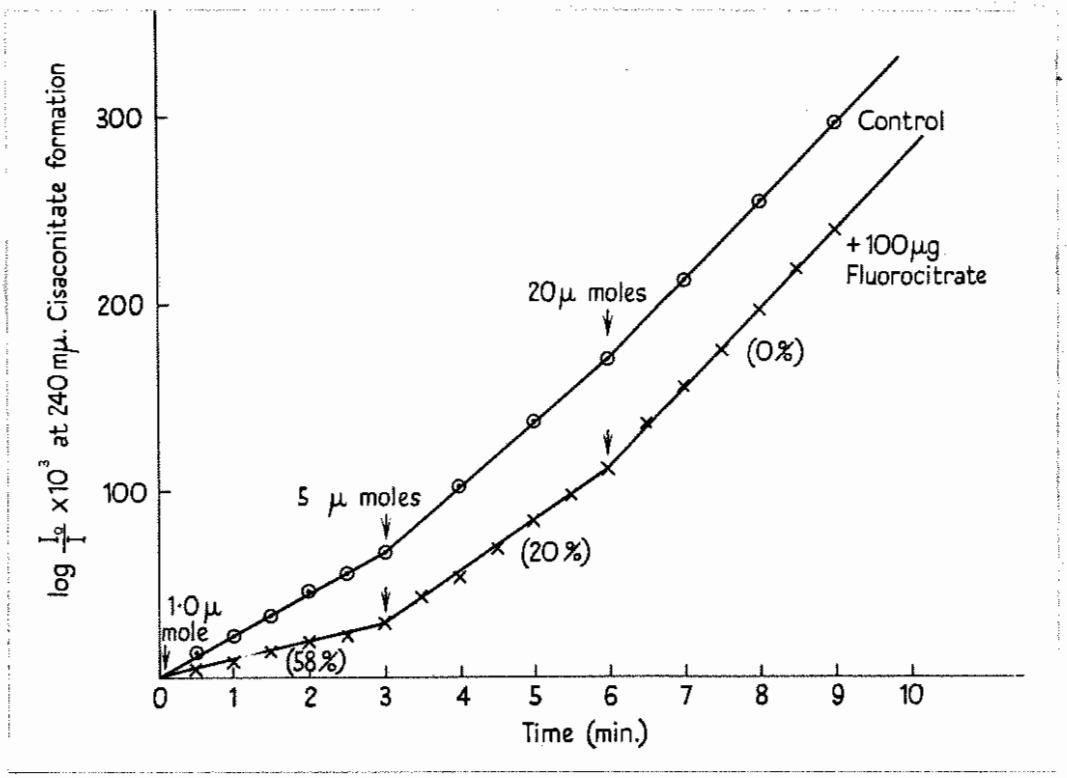


Fig. 27. Inhibition of aconitase by natural fluorocitric acid and reversal of the inhibition by increasing concentrations of isocitric acid. Conditions were the same as those of fig. 23.

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

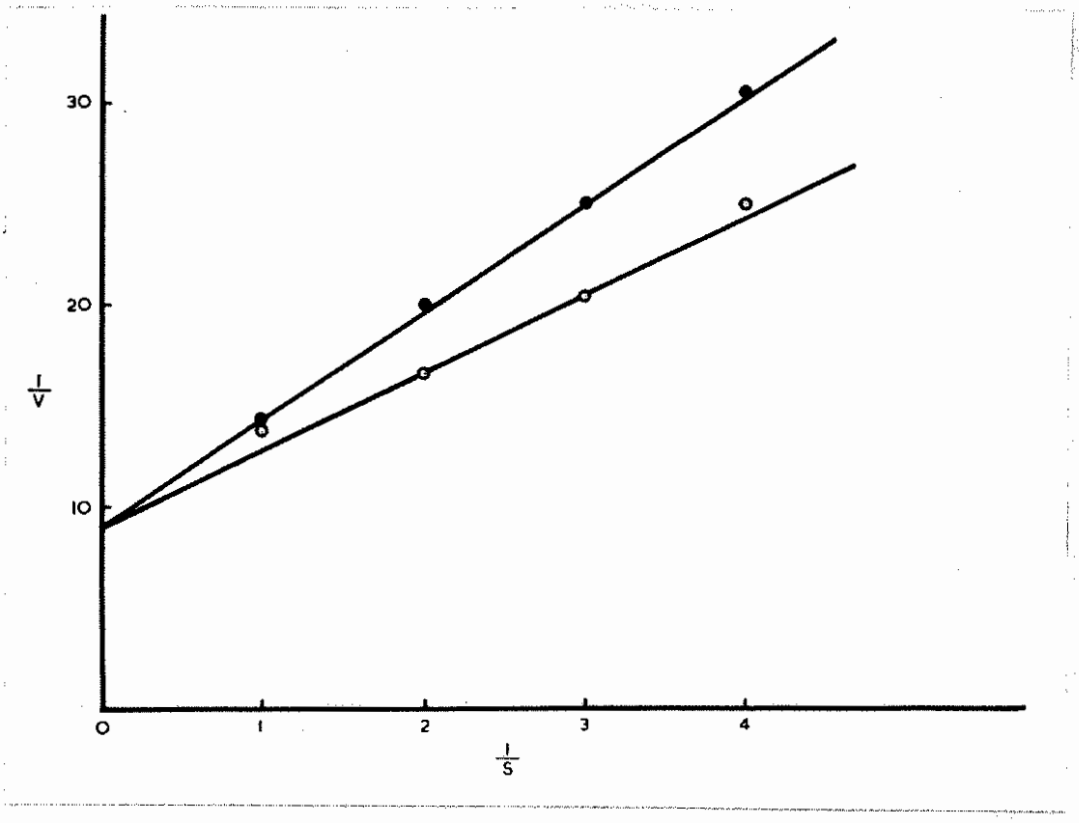


Fig. 28. Competitive inhibition of aconitase by natural fluorocitric acid. The graph is plotted according to the method of Lineweaver & Burk (1934). Aconitase activity was determined in the presence of 0.05 M phosphate buffer (pH 7.7) using 5 µg. of the final aconitase preparation activated by Fe^{2+} 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 (3.2×10^{-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 fluoro-citric acid and that the inhibition was apparently

irreversible. It was realised 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 <u>iso</u>citric acid ⟶ <u>cis</u>-aconitic acid could not be studied for long periods on account of the further conversion of <u>cis</u>-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 ghigh 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 ACONITASE ACTIVITY

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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 (6.7×10^{-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

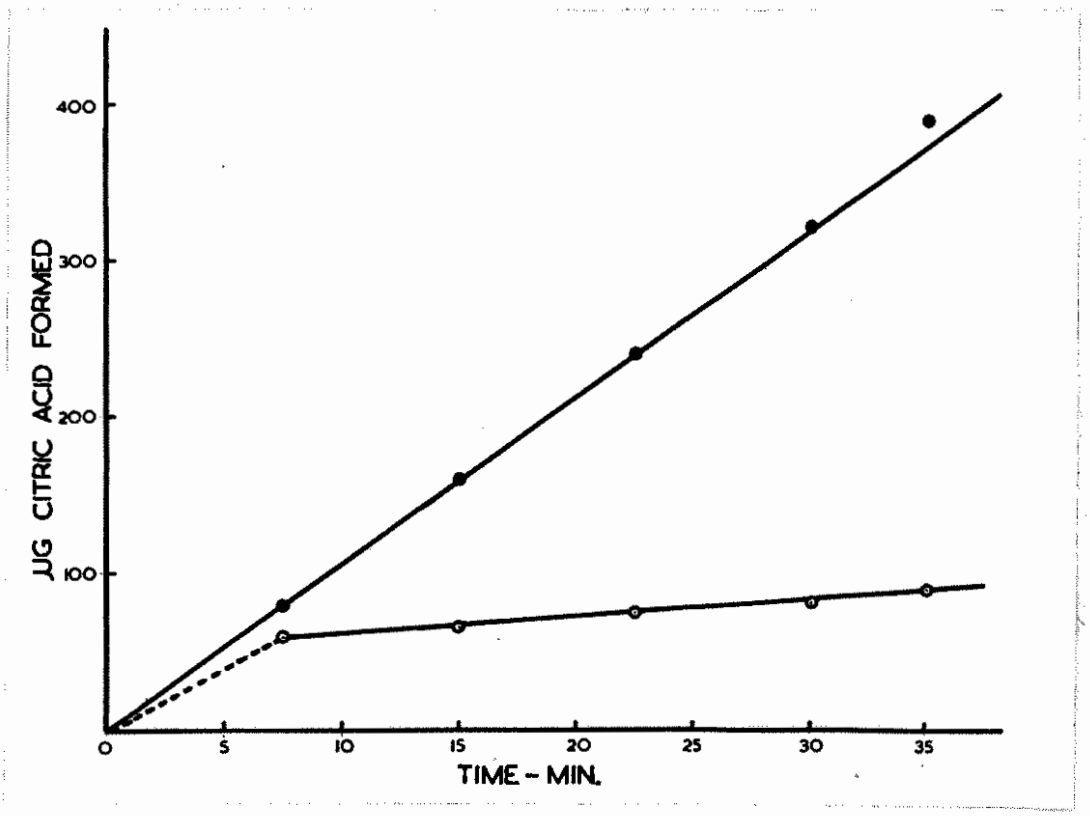


Fig. 29. Time curve for the inhibition of aconitase by p-chloromercuribenzoate. Conditions were as described for table 11. The concentration of p-chloromercuribenzoate was 4×10^{-4} M.

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

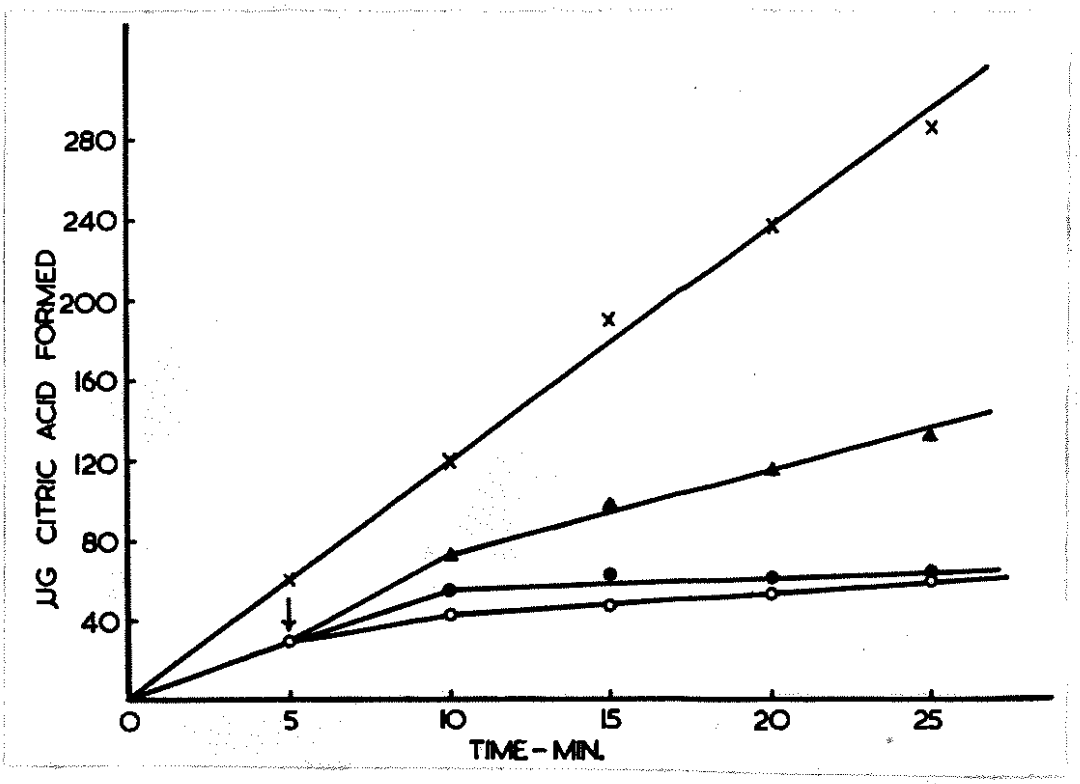


Fig. 30. Time curve for the inhibition of aconitase by p-chloromercuribenzoate; part reversal of the inhibition by cysteine and non-reversal of the inhibition by ascorbic acid. Conditions were as described in Table 11, except that the enzyme was activated with Fe^{2+} and ascorbic acid and 15 µg. of 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

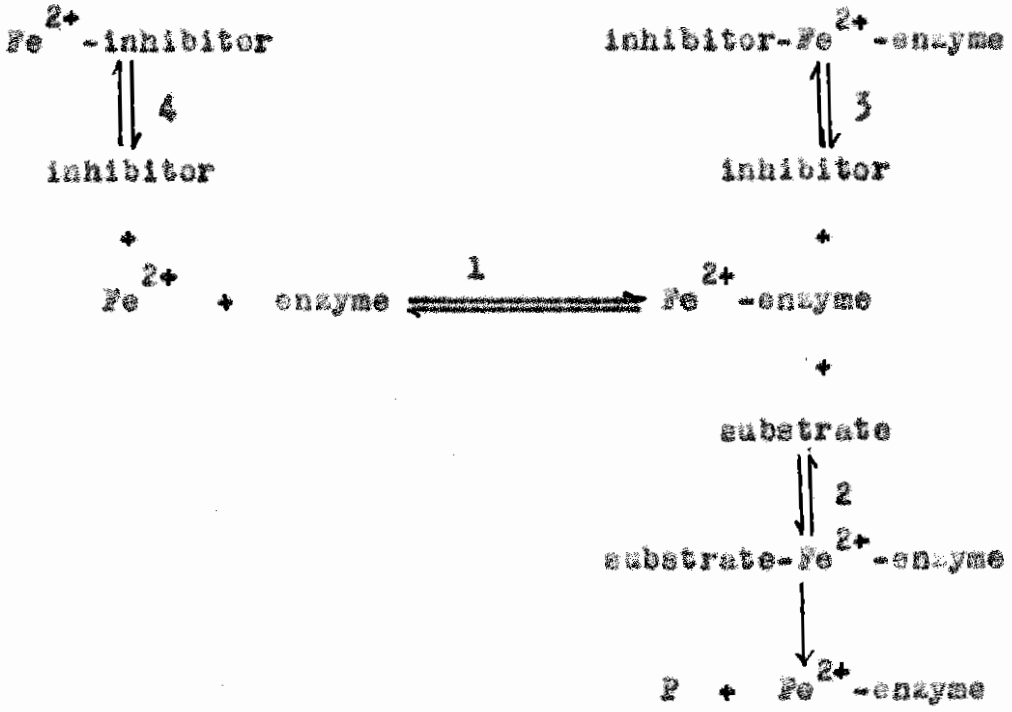
As it is known that versene and o-phenanthroline have high affinities for Fe^{2+}, it seems reasonable to conclude that the inhibition of aconitase is due to their interaction with Fe^{2+}, so that Fe^{2+} ceases to play its normal role in the mechanism of aconitase action. When these compounds are added 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 ($K_m = 1.2 \times 10^{-4}$ 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, T.K. (1929). Biochem. J. 23, 926

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

Dickman, S.R. (1952). Annl. 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, W.A. (1939). Biochem.J. 33, 1046.

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

Lindenbaum, A., White, M.H. & 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, 1.

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

Martius, C. & Lynen, F. (1950). 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.T. (1949). J.biol.Chem. 179, 803.

Stern, J.R. & Gehon, E. (1949). J.biol.Chem. 179, 491.

Thunberg, T. (1911). Scand.Arch.Physiol. 24, 25 & 72.

Thunberg, T. (1929). Biochem.Z. 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.