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Biochemistry of Fluoroacetate Poisoning: the Effect of Fluorocitrate on Purified Aconitase

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The toxic effect of fluoroacetate has been proved to be due to the enzymic synthesis of fluorocitrate (Peters, 1952; Peters, Wakelin, Buffa & Thomas, 1953). The hypothesis was advanced that fluorocitrate leads to the accumulation of citrate in animal tissues as a result of the inhibition of aconitase (Lotspeich, Peters & Wilson, 1952). Peters & Wilson (1952), working with impure fluorocitrate and crude aconitase preparations, produced tentative evidence that the inhibition was competitive. In order to establish this point the experiments of Peters & Wilson (1952) were repeated and extended using the highly purified, stable aconitase preparation of Morrison (1954). A comparison was also made of the effects of the enzymically prepared and synthetic fluorocitrate upon the enzyme.

The conclusion that the inhibition of aconitase by enzymically prepared fluorocitrate is competitive has been confirmed; at the same time interesting differences in the action of the two fluorocitrate preparations on aconitase have appeared. For a preliminary communication, see Morrison & Peters (1954).

EXPERIMENTAL

Preparation and activation of aconitase. Aconitase was prepared and activated in the presence of 5×10^{-4} M-Fe^{2+} and 10^{-2} M cysteine as described by Morrison (1954). The

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enzyme solutions contained between 30 and 50 μg. of the final aconitase preparation/ml.

Formation of cis-aconitate. Aconitase activity was determined by following the formation of cis-aconitate from isocitrate at 240 mμ. by the method of Racker (1950). It was found that when aconitase, activated with Fe^{2+} and cysteine, was added to both citrate and isocitrate there was a rapid initial increase in the extinction coefficient. With isocitrate as substrate, this rapid increase continued over a period of about 1 min., after which the reaction rate became linear. With citrate as substrate, the reaction rate became linear after about 2 min. This rapid increase in the extinction coefficient was a non-enzymic reaction, for when Fe^{3+} and cysteine were added to citrate or isocitrate in the same concentrations in which they were present in the enzyme solution, identical rapid increases in the extinction coefficient were obtained. At the point where the reaction rate in the presence of the enzyme became linear, the non-enzymic reaction ceased. In view of this finding, it was important to distinguish the non-enzymic from the enzymic reaction rate. Only the enzymic reaction rates have been plotted in the graphs.

Reagents. Fluorocitrate synthesized enzymically will be referred to as 'natural' fluorocitrate. Samples were prepared by the method of Peters, Wakelin, Buffa & Thomas (1953). Specimens of synthetic fluorocitrate (Rivett, 1953) were given by Dr D. E. A. Rivett. Both preparations had been standardized on kidney particles by the method of Buffa, Peters & Wakelin (1951).

Trichloromethyl paraconic acid was prepared by the method of Fittig & Miller (1889) and converted into 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). L-Cysteine hydrochloride was obtained from Roche Products Ltd. All other reagents were A.R.

RESULTS

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

The rapid inactivation of aconitase by phosphate buffer in the absence of substrate has previously been reported by Morrison (1954). As it was desired to repeat some of the experiments of Peters & Wilson (1952), in which aconitase had been pre-incubated with fluorocitrate for 15 min. in the presence of phosphate, it was necessary to find a means of preventing or reducing the inactivation of the enzyme during a pre-incubation period. It was found that the addition of a crude heart-muscle extract, in which the aconitase activity had been destroyed by the addition of hydrochloric acid to pH 4, prevented the phosphate inactivation of the enzyme. However, this method had the disadvantage of introducing unknown components into the system. Aconitase could also be stabilized to an appreciable extent by the addition of small amounts of *iso*citrate during the pre-incubation of the enzyme in the presence of phosphate. Fig. 1 shows the marked loss of enzyme activity that occurs during 15 min. pre-incubation with 0·05 M phosphate buffer, pH 7·7, and that small increasing amounts of *iso*citrate reduce the loss of activity. In the presence of 0·5 μmole of *iso*citrate/3 ml., about 70 % of the enzyme activity remained as compared with the activity of the non-incubated enzyme. This concentration of *iso*citrate was chosen for stabilizing aconitase when pre-incubation of the enzyme in the presence of phosphate buffer was carried out.

Studies with natural fluorocitrate

Several experiments were made with varying quantities of fluorocitrate. Fig. 2 illustrates the results of an experiment in which natural fluorocitrate ($1·6 \times 10^{-4}$ M) was pre-incubated with aconitase in the presence of phosphate buffer and 0·5 μmole of *iso*citrate; at the end of the incubation period, varying quantities of *iso*citrate were added at intervals and the rate of *cis*-aconitate formation determined.

Test for the presence of an irreversible inhibitor. The greater toxicity of natural fluorocitrate to the kidney-particle aconitase than to the isolated aconitase (Peters, 1952; Peters & Wilson, 1952) suggested the possibility that the fluorocitrate isolated from the kidney particles may not be the substance which is markedly toxic, but that it is converted into this substance when incubated with the kidney particles. Moreover, whereas the inhibition of the isolated aconitase by natural fluorocitrate is readily reversed, it is not yet known whether the inhibition *in vivo* is reversible; the attempts to reverse the *in vivo* inhibition have been unsuccessful (Hastings, Peters & Wakelin, 1953).
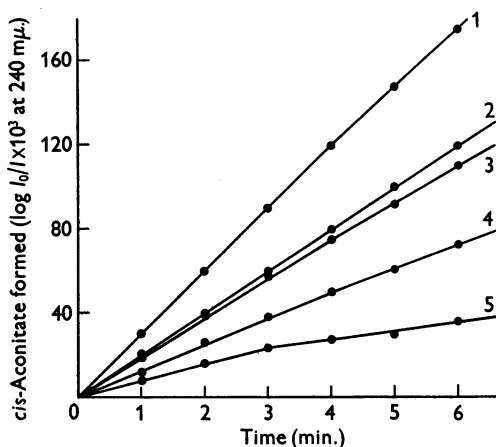

Fig. 1. The stabilization of aconitase by *iso*citrate during incubation in the presence of phosphate. The final aconitase preparation (5 μg.), activated by Fe^{2+} and cysteine, was incubated with 0·05 M phosphate buffer, pH 7·7, for 15 min. in the presence of various amounts of *iso*citrate. Following the incubation period, 16 μmoles of *iso*citrate were added and the reaction rates determined as described in the text. Total volume 3·0 ml., temp. 22°. Curve 1, no pre-incubation; 2, pre-incubation with 0·5 and 1·0 μmole of *iso*citrate; 3, pre-incubation with 0·25 μmole of *iso*citrate; 4, pre-incubation with 0·1 μmole of *iso*citrate; 5, pre-incubation without *iso*citrate.
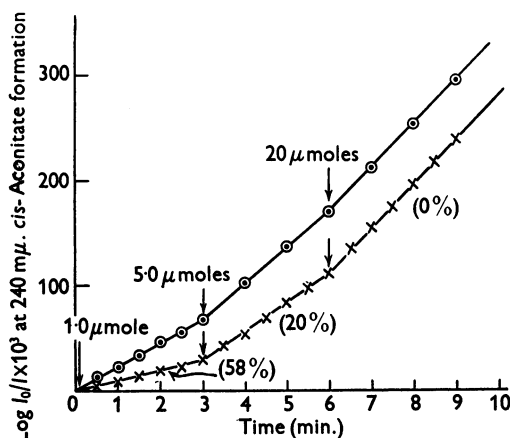

Fig. 2. Inhibition of aconitase by natural fluorocitrate and reversal of the inhibition by increasing concentrations of *iso*citrate. The reaction rates were determined in the presence of 0·05 M phosphate buffer, pH 7·7, as described in the text, using 5 μg. of the final aconitase preparation activated by Fe^{2+} and cysteine. Before the first addition of substrate the enzyme was incubated in the presence of 0·5 μmole of *iso*citrate ± fluorocitrate for 15 min. Total volume 3·0 ml., temp. 22°. ⊙, control; ×, fluorocitrate ($1·6 \times 10^{-4}$ M). Arrows indicate the time at which substrate was added. Figures in brackets show the degree of inhibition.

In order to determine whether or not natural fluorocitrate was converted into an irreversible inhibitor, it was incubated with the kidney particles under the usual test conditions described by Buffa *et al.* (1951). After acid-inactivation of the aconitase, the supernatant was tested for the presence of an irreversible inhibitor in the system, *cis*-aconitate → citrate. 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, *iso*citrate → *cis*-aconitate. No evidence was found for the presence of an irreversible inhibitor. This appears to exclude the possibility that fluorocitrate is converted into some other 'active' compound, but it must be pointed out that the technical difficulties were great because of the relatively large amounts of citrate which were present in the extract containing fluorocitrate. Citrate had to be added during the incubation so that the kidney particles did not become inactivated. After the addition of 20 μmoles of *iso*citrate, the plot of the reaction rate of the sample containing the inhibitor became parallel with the plot of the control rate, thereby showing complete reversal of the inhibition and proving the competitive nature of the inhibitor. Fig. 3 shows that when the initial reaction rates in the presence and absence of fluorocitrate are plotted according to the method of Lineweaver & Burk (1934), a graph characteristic of competitive inhibition is obtained. From this graph, the dissociation constant of the aconitase–fluorocitrate complex was calculated to be 8.7×10^{-5} M.

Studies with synthetic fluorocitrate

Synthetic fluorocitrate proved to be not only more toxic to the isolated aconitase than the natural fluorocitrate, but it also exhibited both a reversible and an irreversible type of inhibition.

Inhibition of aconitase by fluorocitrate with pre-incubation. Fig. 4 shows that when aconitase is pre-incubated with fluorocitrate (2.4×10^{-5} M) in the presence of phosphate buffer and a small amount of *iso*citrate, marked inhibitions are obtained after the reaction is started by the addition of larger amounts of *iso*citrate. The inhibition is nearly 90 % when the reaction is started with 2 μmoles of *iso*citrate and about 80 % when 16 μmoles *iso*citrate are used. These results appeared to indicate that the inhibition was largely irreversible as it was nearly independent of the substrate concentration.

Because of the small amount of enzyme used in these tests (3–5 μg. protein/3 ml.), the molar ratio of fluorocitrate to enzyme must have been high. Therefore, it was possible that the fluorocitrate could be acting as a non-specific denaturing agent. As a check on this point, the above experiments were repeated in the presence of crystalline bovine serum albumin at a concentration 1000 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 was non-specific was rendered much less likely.
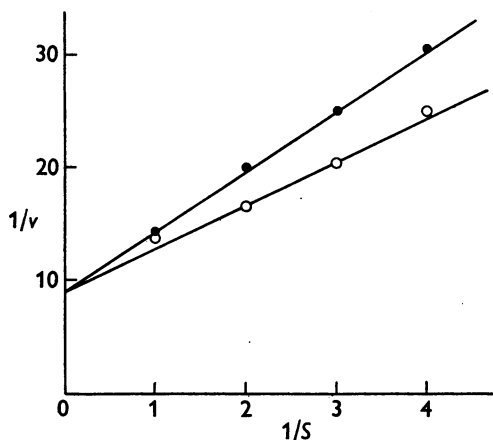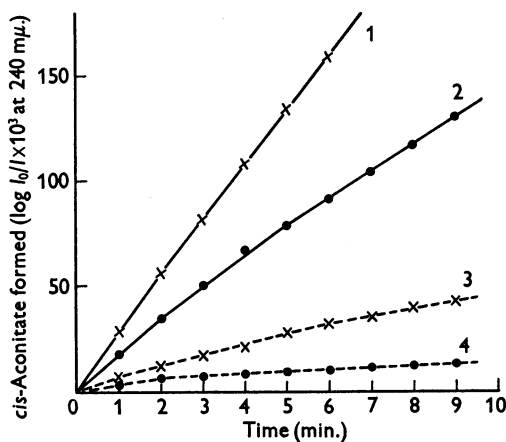
Fig. 3. Competitive inhibition of aconitase by natural fluorocitrate. The graph is plotted according to the method of Lineweaver & Burk (1934). The reaction rates were determined as described in the text, under the same conditions as those of Fig..2. Total volume 3·0 ml., temp. 22°. S, molar concentration of *iso*citrate × 1500; v = change of $\log I_0/I/15$ sec. × 10.   ○, control;   ●, fluorocitrate (3.2×10^{-5} M).

Fig. 4. Inhibition of aconitase by synthetic fluorocitrate. The final aconitase preparation (5 μg.), activated by Fe^{2+} and cysteine, was incubated with fluorocitrate (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 *iso*citrate. Substrate was then added and the reaction rates were determined as described in the text. Total volume 3·0 ml., temp. 22°. ×, reaction rate in the presence of 16 μmoles of *iso*citrate alone (curve 1); with fluorocitrate (curve 3); ●, reaction rate in the presence of 2 μmoles of *iso*citrate alone (curve 2) and with fluorocitrate (curve 4).

Inhibition of aconitase by fluorocitrate without pre-incubation. It was found that the inhibition was dependent on the substrate concentration when the enzyme was not pre-incubated with fluorocitrate. Table 1 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 $2\,\mu$moles of *iso*citrate are added, but when $16\,\mu$moles of *iso*citrate are added, there is an appreciable difference. These findings are not consistent with the idea that synthetic fluorocitrate is simply an irreversible inhibitor of aconitase.

A more detailed investigation was made of the effect of the substrate concentration on the inhibition of aconitase by fluorocitrate without pre-incubation. Fig. 5 shows that when the reciprocals of the initial reaction velocities in the presence and absence of fluorocitrate are plotted against the reciprocals of the *iso*citrate concentration according to the method of Lineweaver & Burk (1934), a graph characteristic of competitive inhibition is obtained. Similar results were obtained with citrate as substrate. From these results, it must be concluded that there is at least initial competition between the aconitase substrates and fluorocitrate for the active centre(s) of aconitase. It was noted that after a time the reaction rates in the presence of fluorocitrate decreased. This could not be explained as being due to the further conversion of *cis*-aconitate into citrate because the control rates, which were faster, were linear at this time. The rates used in the Lineweaver–Burk plot were those obtained before the change.

It was calculated that the dissociation constant of the aconitase–fluorocitrate complex was $8\cdot4 \times 10^{-6}\,\mathrm{M}$

Table 1. *The effect of pre-incubation of aconitase with synthetic fluorocitrate on the inhibition of the reaction,* isocitrate → cis-aconitate

The final aconitase preparation ($5\,\mu$g.), activated with Fe^{2+} and cysteine, was pre-incubated with $2\cdot4 \times 10^{-5}\,\mathrm{M}$ fluorocitrate for 15 min. in the presence of $0\cdot05\,\mathrm{M}$ phosphate buffer, pH $7\cdot7$, and $0\cdot5\,\mu$moles of *iso*citrate. The indicated amounts of *iso*citrate were then added and the initial reaction rates determined as described in the text. When pre-incubation was not carried out, $3\,\mu$g. of the final activated enzyme preparation were added directly to media containing buffer and substrate. Total volume $3\cdot0$ ml., temp. $22°$.

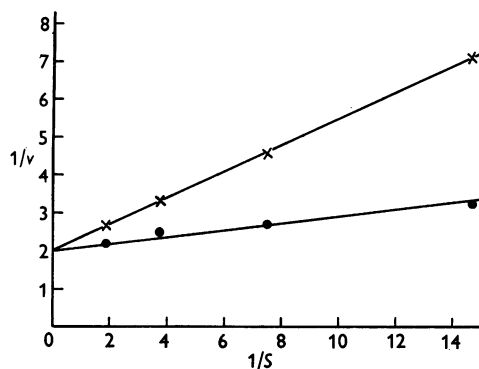| Substrate concentration (μmoles/3 ml.)	Rate ($\Delta\epsilon$/min. $\times 10^3$)		
	Control	Fluoro-citrate present	Inhibition (%)
Pre-incubation			
2	23	8	65
	24	8	66
16	32	15	53
	36	15	58
No pre-incubation			
2	20	10	50
	25	11	56
16	32	27	16
	34	30	12

Fig. 5. Competitive inhibition of aconitase by synthetic fluorocitrate. The graph is plotted according to the method of Lineweaver & Burk (1934). The reaction rates were determined as described in the text, under the same conditions as those of Fig. 2. Total volume $3\cdot0$ ml., temp. $22°$. *S*, molar concentration of *iso*citrate $\times 10^2$; *v*, change of log I_0/I/min. $\times 10$. ●, control; ×, fluorocitrate ($2\cdot4 \times 10^{-5}\,\mathrm{M}$).

Table 2. *The effect of pre-incubation time of aconitase with synthetic fluorocitrate on the inhibition of the reaction,* isocitrate → cis-aconitate

Conditions of the pre-incubation were the same as those of Table 1. Following the pre-incubation period, $2\,\mu$moles of *iso*citrate were added and the reaction rate was determined for 2 min. as described in the text. *iso*Citrate ($16\,\mu$moles) was then added and the second reaction rate determined. Total volume $3\cdot0$ ml., temp. $22°$.

Pre-incubation time (min.)	Rate ($\Delta\epsilon$/min. $\times 10^3$)					
	$2\,\mu$moles			$16\,\mu$moles		
	Control	Fluorocitrate present	Inhibition (%)	Control	Fluorocitrate present	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

with isocitrate and 12×10^{-6} M with citrate as sub-
strates. This difference could be accounted for by
experimental error. It was also estimated that for
50 % inhibition the ratio of isocitrate to fluorocitrate
was approximately 40:1, whereas the ratio of citrate
to fluorocitrate was of the order of 400:1. Thus the
inhibition in the presence of a fixed amount of
citrate is greater than in the presence of the same
amount of isocitrate. It also follows that the
inhibition should be more readily reversed by iso-
citrate than by citrate.

Factors affecting the degree of inhibition and the
reversal of the inhibition. Table 2 shows that the time
of pre-incubation of the enzyme with fluorocitrate
does not significantly affect the degree of inhibition
when the reaction is started with 2 μmoles of iso-
citrate. Moreover, the reversal of the inhibition
which takes place after the addition of 16 μmoles of
isocitrate is independent of the pre-incubation time.
It should be noted that the extent of the reversal of
the inhibition in this experiment was appreciably
greater than would have been expected from the
data of Table 1 and Fig. 4. The results of Fig. 6
confirm the marked inhibition of aconitase by
fluorocitrate that is obtained in the presence of
2 μmoles of isocitrate. They also show that on the
addition of 15 μmoles of isocitrate, the inhibition is
reversed, but not to the extent that the reaction rate
is the same as that obtained on adding 15 μmoles of
isocitrate at the start.

The above results suggest that during a pre-
incubation period or during the reaction with a low
substrate concentration, a secondary reaction
occurs between the enzyme and fluorocitrate so that
the reversal of the inhibition is rendered more
difficult.

As seen in Fig. 6 and Table 2, the inhibition of
aconitase by fluorocitrate could be reversed to some
extent on the addition of higher concentrations of
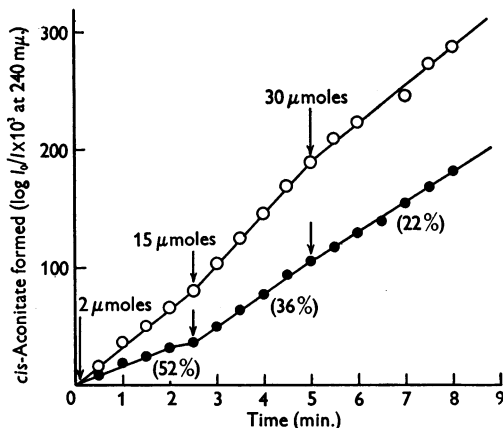substrate. It was of interest to determine whether

Fig. 7. Inhibition of aconitase by synthetic fluorocitrate
and part-reversal of the inhibition by increasing concen-
trations of isocitrate. Conditions were the same as those
of Fig. 2. ○, control; ●, fluorocitrate (2.4×10^{-5}M).
Arrows indicate the time at which substrate was added.
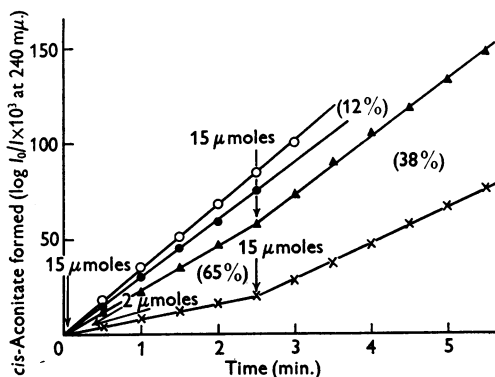Figures in brackets show the degree of inhibition.

Fig. 6. Increased inhibition of aconitase by synthetic
fluorocitrate as a result of starting the reaction with
a low concentration of isocitrate. Conditions were as
described in Fig. 2. The concentration of fluorocitrate was
2.4×10^{-5}M. With 15 μmoles of isocitrate added at the
start of the reaction; ○, control; ●, fluorocitrate. With
2 μmoles of isocitrate added at the start of the reaction,
followed by 15 μmoles of isocitrate; ▲, control; ×,
fluorocitrate. Arrows indicate the time at which the sub-
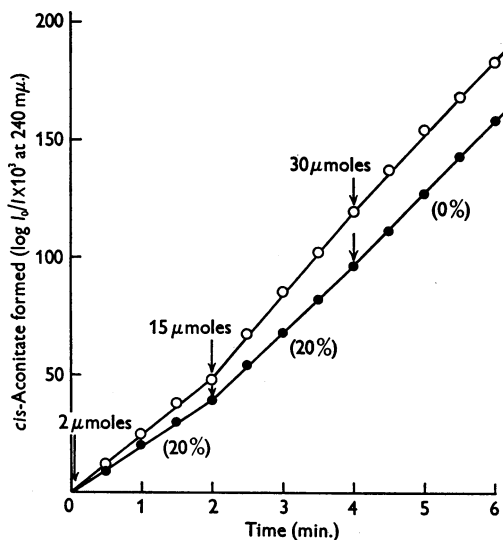strate was added. Figures in brackets show the degree of
inhibition.

Fig. 8. Inhibition of aconitase by synthetic fluorocitrate
and reversal of the inhibition by increasing concentrations
of isocitrate. Conditions were the same as those of Fig. 2.
○, control; ●, fluorocitrate (0.6×10^{-5}M). Arrows
indicate the time at which substrate was added. Figures
in brackets show the degree of inhibition.

or not the inhibition could be completely reversed. Fig. 7 shows that a further increase in the *isocitrate* concentration reduces the inhibition still further, but at this level of the inhibitor it was not practical to add larger amounts of substrate. It can be seen that the final reaction rate in the control tube decreased because of the dilution of the solution. When the concentration of fluorocitrate was reduced to one-quarter the concentration, that is to 0.6×10^{-5} M, the inhibition could be completely reversed as shown in Fig. 8. The initial inhibition in this case was small, being only 20 % as compared with 60 % with 2.4×10^{-5} M fluorocitrate.

Inhibition of isocitric *dehydrogenase.* It was found that there was no significant inhibition of *isocitric* dehydrogenase by synthetic fluorocitrate (2.4×10^{-5} M) following pre-incubation of the enzyme with the inhibitor for 15 min.

DISCUSSION

It appears to be settled that the effect of fluorocitrate in causing the accumulation of citrate in animal tissues is due to its inhibitory effect on aconitase. It is also clear that the inhibitory action of the synthetic fluorocitrate is not only greater, but also differs in some respects from that obtained with the natural fluorocitrate. However, it must be pointed out that the relatively small amount of natural fluorocitrate which was available did not permit of extensive investigations at the higher levels of inhibition. A discussion of the possible reasons for this difference in toxicity will be deferred until the mode of action of each compound is discussed separately.

Natural fluorocitrate

There was no evidence of complicating factors in the inhibition of aconitase by natural fluorocitrate. The inhibition is competitive and can be readily reversed by higher concentrations of substrate. One outstanding fact with this compound which is confirmed here, is its greater toxicity *in vivo* and in the kidney-particle test system of Buffa *et al.* (1951) as compared with its toxicity in the isolated aconitase system (Peters, 1952; Peters & Wilson, 1952). The only explanation for this at present advanced 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 with the soluble enzyme with the amounts of fluorocitrate used.

Synthetic fluorocitrate

It is clear from the Lineweaver–Burk plot (Fig. 5) that synthetic fluorocitrate can act as a competitive inhibitor of aconitase, at least during the initial stages of the reaction. However, if the fluorocitrate were simply a competitive inhibitor of aconitase, pre-incubation of the enzyme and inhibitor should not increase the degree of inhibition. This, together with the fact that the reaction rates in the presence of the inhibitor fall after a time, even though the control rates are still linear, suggest that a slow irreversible type of inhibition is also taking place. If the reaction rates could have been studied for longer periods, it is likely that this change of rate would have been emphasized. However, the reaction *isocitrate* → *cis*-aconitate could not be studied for long periods on account of the further conversion of *cis*-aconitate into citrate, which complicates the measurement of *cis*-aconitate production. The latter results also seem to indicate that the time at which the irreversible type of inhibition manifests itself is a function of the substrate concentration. It appears as though the addition of substrate at low concentrations is equivalent to pre-incubation; prolonged pre-incubation of enzyme and inhibitor followed by the addition of substrate in low concentration is no more effective in increasing the degree of inhibition than the addition of a low substrate concentration alone. But pre-incubation does increase the inhibition when higher substrate concentrations are used.

It would seem as though the results can best be explained according to a combination of the following equations along usual lines:

$$E + S \rightleftharpoons ES \rightarrow E + P, \qquad (1)$$

$$E + I \rightleftharpoons EI \rightarrow EI', \qquad (2)$$
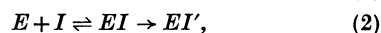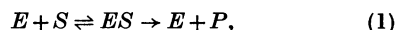
where E = enzyme, S = substrate, I = inhibitor, ES = enzyme–substrate complex, P = reaction products, EI = enzyme–inhibitor complex, and EI' = secondary complex formed between the enzyme and inhibitor. First, there is competitive inhibition between the inhibitor and substrate for the active centre(s) of the enzyme; this equilibrium is set up rapidly. 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 that would occur during the period of pre-incubation of the enzyme with the inhibitor, or during the course of the reaction.

The failure to reverse completely the inhibition of aconitase by higher concentrations of fluorocitrate and the fact that the inhibition with lower concentrations of fluorocitrate can be completely reversed cannot be quoted as evidence either for or against the above scheme. The failure to reverse the inhibition completely with higher concentrations of fluorocitrate may have been due only to the technical limitations of increasing the substrate concentration to a sufficiently high level. On the

other hand, the small degree of inhibition obtained with the lower concentrations of fluorocitrate may not have permitted the detection of a small amount of an irreversible type of inhibition.

Comparison of the action of natural and synthetic fluorocitrate

Since the infrared data appear to exclude the presence of an appreciable amount of another compound in the synthetic substance, the differences in the action of the two fluorocitrate preparations on the isolated aconitase must be attributed to the presence of different stereoisomers in each preparation; from the mode of synthesis four stereoisomers should be present. On the other hand, it is unlikely that more than two stereoisomers would be formed enzymically. ,

Peters, Wakelin, Rivett & Thomas (1953) found that there was a difference in the ability of natural and synthetic fluorocitrate to inhibit the disappearance of citrate in kidney particles; in this instance, however, the reverse occurred, the synthetic fluorocitrate being only half as active as the natural fluorocitrate. Admittedly, the results with the kidney particles are not exact, but on the basis that the four stereoisomers were present in equal amounts, these authors concluded that only one optically active centre was concerned in the inhibition. In the experiments with the isolated aconitase, the synthetic is far more active than the natural. It would seem as though the isomers present in the synthetic material, but not present in the natural fluorocitrate, are responsible for the different effects.

Possible explanations for the difference in the toxicity of synthetic fluorocitrate in the kidney-particle test system as found by Peters, Wakelin, Rivett & Thomas (1953) and in the isolated aconitase system are difficult. It might be argued that the 'unnatural' stereoisomers do not penetrate the mitochondria or particles, but it is difficult to see why they should not do so. It might also be suggested that, consistent with the recent photographs of the organization within mitochondria (Palade, 1953; Claude, 1954), the 'unnatural' stereoisomers cannot get at the aconitase owing to structural orientation. On the other hand, if all four stereoisomers 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 synthetic fluorocitrate 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. If this

means that the enzyme behaves differently in the organized and unorganized condition, it raises a matter needing weighty consideration. More conclusive results must await the separation of the stereoisomers of synthetic fluorocitrate and the determination of which stereoisomer(s) is (or are) formed enzymically.

SUMMARY

1. The competitive inhibition of aconitase by natural fluorocitrate has been established.

2. On the other hand, synthetic fluorocitrate has been shown to inhibit aconitase in both a competitive and an apparent irreversible fashion.

3. The dissociation constants of the aconitase–fluorocitrate complexes for both the natural and synthetic fluorocitrates have been measured. The affinity of aconitase for the synthetic compound is much greater than for the natural compound.

4. The possible reasons have been discussed for the difference between the two preparations of fluorocitrate in inhibiting the isolated aconitase and also for 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.

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