greater than for 2 mM-2-naphthylamine. Its apparent affinity for 3 β -methoxyandrost-5-en-17-one is high, K_i being 1.5 μ M.

5. The corresponding values for guinea-pig-liver arylamine sulphokinase are: K_m , 0.5 mM-2-naphthylamine; K_i , less than 0.2 μ M-oxo steroid.

6. An explanation of these effects is suggested. It is based on a mechanism formally similar to the partial competitive inhibition of Dixon & Webb (1958), in which a ternary enzyme-steroid-substrate complex (dissociation constant K'_i) can be formed. The rate of breakdown of this complex to give the reaction products is identical with that of the breakdown of the normal enzyme-substrate

complex (dissociation constant K_s). If K'_i is greater than K_s in such a system then the steroid will be an inhibitor of the enzyme; if K'_i is less than K_s the steroid will be an activator.

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Biochem. J. (1961) **79**, 261

Further Experiments on the Inhibition of Aconitase by Enzymically Prepared Fluorocitric Acid

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(Received 14 July 1960)

Peters, Wakelin, Rivett & Thomas (1953) found that fluorocitric acid made by enzymic action inhibits the aconitase in kidney particles more powerfully than does synthetic fluorocitric acid. This finding was in line with the fact that the synthetic fluorocitric acid should be a mixture of four isomers, of which only one or two might be expected to be active. When Morrison & Peters (1954) compared the effects of enzymically and chemically prepared fluorocitrate on a highly purified soluble aconitase, the behaviour of the two was very different. Synthetic fluorocitric acid was a very strong inhibitor, whereas the enzymic fluorocitric acid was much less inhibitory. The inhibition obtained was reversible even after preincubation in the absence of substrate.

These differences were difficult to explain and led to the suggestions either that the synthetic fluorocitric acid did not penetrate properly into the particle, or alternatively that there was some difference in mitochondrial aconitase. The use of another preparation of soluble aconitase has now made it possible to get an inhibition with fluorocitrate which is of the same order as that found in the particle preparation. In addition, a new

method of testing the activity of specimens of fluorocitric acid has been devised. Some of these results have already been published in a preliminary form (Peters, 1959).

There are several problems involved: (1) that of the discrepancy between the effect of enzymically prepared fluorocitrate on mitochondrial and soluble aconitase; (2) that of the best method of testing the relative activity of specimens of fluorocitric acid with soluble aconitase; (3) that of the discrepancy between the activity of enzymically prepared and synthetic fluorocitric acid on soluble aconitase; (4) finally, whether the inhibition is truly reversible with enzymically prepared or synthetic fluorocitric acid.

Of these problems only (1), (2) and (4) are considered here. Problem (3) forms the subject of a separate paper (Ward & Peters, 1961).

METHODS AND PREPARATIONS

Chemicals. These were AnalaR where possible; DL-iso-citric acid (as Na salt at pH 7.4) was prepared from the lactone essentially by the method of Krebs & Eggleston (1944) or Ochoa (1948). Glass-distilled water was used throughout. Phosphate buffer was made by neutralizing KH_2PO_4 solutions with NaOH.

Fluorocitric acid. This was neutralized to pH 7.4, usually

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with NaOH. Where possible the preparations used were made biosynthetically. In some cases, where it was a matter of comparison, to save such preparations, synthetic specimens were used; that these were generally only half as active did not matter in such experiments. During the progress of this work, it was discovered that the maximum activity of specimens of synthetic fluorocitric acid was obtained after an activation by alkali, which is described by Ward & Peters (1958, 1961). The final and crucial experiments were made with enzymically prepared fluorocitrate purified by Dr P. F. V. Ward on silicic acid columns and treated with alkali.

Aconitase. The aconitase preparations were made by the method of Morrison (1954). They were either taken to the final stage of purification (M) or used after the first ethanol precipitation (E). When required they were activated by Morrison's (1954) method based on Dickman & Cloutiers' (1950) discovery; in this an appropriate amount of enzyme (0.2 ml.) was treated with ferrous ammonium sulphate (1.0 mM, 0.3 ml.), to this was added L-cysteine hydrochloride (63.6 mM, 0.75 ml.) neutralized, to pH 7.4, immediately before addition, and the mixture then made up to 2.0 ml. with water. After standing at 0° for 1 hr. amounts of 0.1–0.25 ml. of enzyme solution, depending on the activity, were taken for each cuvette in the experiment. When used without activation 0.3 ml. of enzyme solution was made up to 2.0 ml. with water and 0.25 ml. of this dilution put into each cuvette. The experiments were made by observing the increase of absorption at 240 m μ , which represents the development of the double bond, and so of the formation of *cis*-aconitate from *isocitrate*. The cuvettes were filled in the following order: water (2.0 ml.) or the very dilute *isocitrate* if used for stabilization; buffer solution (0.3 ml.), which was initially tris (2-amino-2-hydroxy-methylpropane-1:3-diol), pH 7.7, or phosphate of the same pH; latterly tris (pH 7.4) was used exclusively; fluorocitric acid (neutralized), enzyme solution 0.1–0.25 ml.; and finally water to a total volume of 2.75 ml. By adding the enzyme last, it was exposed to the required concentration of fluorocitrate. The whole was mixed by passing air through the solution. After incubation, sodium *isocitrate* was added as mentioned below.

In addition to the water blank, when the usual carrier is used, three experimental cuvettes can be studied at a time, e.g. the enzyme alone, and the enzyme with two concentrations of fluorocitrate. The solutions were kept in the constant-temperature room in the spectrophotometer usually for 10 min., during which time an equilibrium temperature of $21.9 \pm 0.2^\circ$ was reached. It was better not to use a water-jacketed cuvette holder, as with this only two cuvettes could be used at one time. It was thus more difficult to allow for the gradual loss of activity of the enzyme. After an initial reading, the experiment was best started by adding 0.01 ml. of DL-*isocitric* acid (containing 1.38 μ moles of D-isomer) with an Agla syringe. If this amount is put carefully on the top of the solution in the cuvette, not much interaction takes place until stirring, which was done by passing air. By this method duplicates agreed to within ± 0.01 . The extinction at 240 m μ was read at 0.5, 1, 2, 3 and 4 min. As described by Morrison & Peters (1954) an initial disturbance, which was non-enzymic, occurred on adding the reagent and largely finished in 0.5 min. The effects were compared between 0.5 and 4 min.

RESULTS

Some observations on the stability of different preparations of aconitase

Purified aconitase. A specimen of Morrison's (1954) purified aconitase (M) at the final stage, which had been kept at -16° , remained active for over four years, though towards the end of this time there was some loss of activity. The specimen had been repeatedly thawed and refrozen. There was little activity in this specimen without activation with Fe^{2+} ions and cysteine, in agreement with Morrison's (1954) observations.

In our hands preparations taken further than the 'first ethanol' precipitate to various intermediate stages towards a final purification lost their activity on freezing and thawing. We have made no attempt to find the reason for this; but we suggest that it is connected with the volume in which the final stage tested is dissolved. Two further preparations taken to the first ethanol stage behaved similarly to the one on which most work was done.

Aconitase at the first ethanol-precipitation stage (E). This was used in the majority of the experiments. As Morrison (1954) showed, purification was accompanied by loss of activity due to removal of Fe^{2+} ions. Hence the purest preparation (M) was almost inactive without treatment with Fe^{2+} ions and cysteine. At stage E, about 50 % of the enzyme is still active without treatment with Fe^{2+} ions and can therefore be used with a simple dilution. When dissolved in water, it was clear and stable for 5 months at -19° , and could be thawed and refrozen with only slight loss of activity. After 6 months' storage, the decrease in activity was under 20 %, but larger losses occurred upon longer storage. It was best to store the preparations in 10 ml. tubes at -19° , which avoids thawing the whole bulk.

The kinetics of the enzyme reaction with aconitase preparation E are considered below. In all the experiments (unless specially mentioned) only observations during the first 0.5–4 min. are reported. After 4.0 min. the observations became progressively vitiated by *cis*-aconitate accumulation, which alters the rate of reaction (see below). The time given was a convenient compromise.

Inhibition of soluble aconitase preparations

Morrison's purified preparation (M). Tests with fluorocitrate specimens in which the activated enzyme was used gave essentially the same results as those of Morrison & Peters (1954); high concentrations of enzymic fluorocitric acid were required to give a substantial inhibition; but the inhibition was somewhat variable; thus 5 and 15 μ g. in one case reduced activity by 10 % and in another case

30 $\mu\text{g.}$ was needed for a decrease of 60%; it is a common experience with this type of preparation to find that an increase of fluorocitrate concentration beyond a certain value gives little or no change in the inhibition.

When tris buffer was substituted for phosphate, though the activity of the enzyme alone was re-

duced (about 22% during the 4 min. period), the inhibition for 5 $\mu\text{g.}$ of fluorocitric acid was increased from 15 to 52% (Figs. 1a and 1b).

Morrison (1954) found that with M preparations activity was lost in phosphate-buffered solution unless some *isocitrate* was present during the pre-incubation period. With tris buffer this loss did not occur, and in one experiment, when no *isocitrate* was added during incubation, there was a slight increase in inhibition from 22 to 37%. But the effect of changing to tris buffer seemed to be much less than with the enzyme E.

First ethanol stage (E). This contained much of the enzyme in a form not requiring activation by Fe^{2+} -cysteine. Fig. 1c shows that when activated with Fe^{2+} ions and cysteine, the effects of fluorocitric acid were much greater than in M, i.e. 71% for 2 $\mu\text{g.}$ of fluorocitrate. As, differing from M, there was no noticeable loss of activity upon incubation for 10 min. without *isocitrate*, this was omitted, and the enzyme was studied also in tris buffer without activation. The effect of as little as 0.002 $\mu\text{g.}$ of biosynthetic fluorocitric acid could be detected under the conditions of test; 0.02 $\mu\text{g.}$ gave an inhibition of 64% similar to that of 10 $\mu\text{g.}$ or more with the M enzyme, i.e. the activity was at least 500 times greater than previously (Fig. 2). Accepting the values as they stand, 50% of the maximum inhibition found experimentally was given by $6.5 \times 10^{-9} \text{ M}$ (6.5 μmM ; 0.0035 $\mu\text{g./cuvette}$), a value which is to be compared with the 58% inhibition induced previously with the M enzyme by 0.16 mM

Fig. 1. Comparison of the inhibition induced by 'enzymic' fluorocitric acid on Morrison's purified aconitase (M) and aconitase at the first ethanol stage (E) in phosphate or tris buffer, pH 7.7. Both preparations were activated with Fe^{2+} ions and cysteine as in Morrison's method. The enzyme was preincubated in the presence or absence of fluorocitrate for 10 min. in the presence of dilute DL-*isocitrate* (0.19 mM). The experiment was started by adding DL-*isocitrate* (2.76 μmoles) and observing for 4 min. Comparisons of absorption at 240 $\text{m}\mu$ due to formation of *cis*-aconitate from 0.5 to 4.0 min. are: (a) M in phosphate buffer; (b) M in tris buffer; (c) E in tris buffer. Δ , No fluorocitrate; $\circ$, 2 $\mu\text{g.}$ of fluorocitrate; $\bullet$, 5 $\mu\text{g.}$ of fluorocitrate.

Fig. 2. Inhibition of aconitase at the first ethanol stage in tris buffer (pH 7.4) by enzymic fluorocitric acid (activated). The enzyme was preincubated in the presence or absence of fluorocitrate for 10 min. without *isocitrate*. The experiments were started by adding DL-*isocitrate* (2.76 μmoles), and observing for 4 min. The ordinate scale represents the change in absorption at 240 $\text{m}\mu$ from 0.5 to 4.0 min., as percentage inhibition.

(Morrison & Peters, 1954). Instead of being less, the inhibition here may be even greater than that on the aconitase in the mitochondrial particles, though the concentration inside such particles *in vitro* is not accurately known. Hence it is clear that a soluble form of aconitase can behave like 'particulate' aconitase, and it is no longer necessary to invoke differences in permeability or in orientation to explain the different concentrations formerly found necessary for particulate and soluble forms of the enzyme.

In a search for the factors which decrease the inhibitory activity of fluorocitrate on the soluble aconitase E, we have investigated the effects of: (1) substituting phosphate for tris buffer which gave a reduction in inhibition to 15% from 47% (Figs. 3a and b); (2) activation of the aconitase in the usual manner as compared with the non-activated enzyme. This reduced the inhibition. For instance, the percentage inhibition for 0.04 μ g. of biosynthetic fluorocitrate (69 μ M) was 57.6% for non-activated and 25% for the preparation treated

Fig. 3. Aconitase (E, not treated with Fe^{2+} ions). Comparison of the effect of (a) phosphate, and (b) tris buffer (pH 7.4) on the inhibition by fluorocitrate. The substrate was DL-isocitrate. Δ , Control; $\circ$, 0.2 μ g. of synthetic fluorocitrate.

with Fe^{2+} ions and cysteine; (3) the addition of Fe^{2+} ions and cysteine alone or separately to the cuvettes. The effect of these was variable. In our first preparation they had no appreciable effect, e.g. in the presence of Fe^{2+} alone 0.1 μ g. of fluorocitrate inhibited 55%, 0.05 μ g. of fluorocitrate inhibited 34%; with cysteine alone 0.1 μ g. of fluorocitrate inhibited 43% and 0.2 μ g. of fluorocitrate, 62%. With cysteine + Fe^{2+} , 0.1 μ g. of fluorocitrate inhibited 39% and 0.2 μ g. of fluorocitrate inhibited 55%. In a later preparation, Fe^{2+} and cysteine much reduced the effects of fluorocitric acid. This point evidently requires more work; but it can be stated that the addition of the small amounts of Fe^{2+} ions and cysteine, which might be carried over from an activation, had no effect; (4) the addition of small amounts of isocitrate (0.19 mM) during the incubation period. This reduced the inhibition with 0.1 μ g. of fluorocitrate from more than 60 to 6%.

The general effect of (1), (2) and (4) is to decrease the inhibition induced by a given amount of fluorocitric acid, though none of them can be responsible for the very large difference in inhibition between M and E. It may be concluded that, practically, for the greatest sensitivity, tests should be done with a preparation at the first ethanol stage of purification, diluted with water and not activated with Fe^{2+} ions, in the presence of tris buffer after a 10 min. incubation with the fluorocitrate in the absence of isocitrate.

Effect of temperature. The variation of inhibition with temperature has been studied to make certain that differences of $\pm 0.2^\circ$ do not influence the results. With a jacketed cuvette, the change for $\pm 3.0^\circ$ to $\pm 4.0^\circ$ was not great, from -69% at 21.9° to -56% at 27° and -52% (at 17°) respectively or about 4% per degree (Table 1). In another experiment at 27° the change was to -48%.

Table 1. Effect of temperature on the inhibition of aconitase for period 0.5-4 min. after addition of DL-isocitrate

The enzyme control was read against the water blank, then against the enzyme containing 0.01 μ g. of fluorocitrate and finally again against water to ensure that there had been no change in activity. For other experimental details, see text.

Temp.	Conditions	Diff. in absorption ($10^3 E_{240 \text{ m}\mu}$)	Inhibition* (%)
27°	Control	70, 68	56.5
	Fluorocitrate	30	
17°	Control	59, 53	52
	Fluorocitrate	27,	

* 100 - % difference.

Effect of pH. A fall of 10% in the activity of the enzyme was observed on changing from pH 7.7 to 8.3. With tris buffer at pH 7.95 instead of pH 7.4 a change in the inhibition was found, from 69 to 66%. With tris buffer after bringing to pH 6.8,

there was an increase of 4% in enzymic activity, i.e. within experimental error. The inhibition was changed from 67 to 63%. It was considered that the effect of smaller variations in pH could be neglected.

Kinetic behaviour. Fig. 4 gives the apparent K_m for the aconitase E for the reaction *isocitrate* $\rightarrow$ *cis*-aconitate in tris buffer (pH 7.4) plotted in the conventional way as $100/V$ against $100/S$. Reckoned as DL-*isocitrate*, K_m is 4.73 mM, making 2.36 mM for the D-*isocitrate* component. This is to be compared with Morrison's value of 0.48 mM, which was done in the presence of phosphate at pH 7.7. It is to be noted that the value for the lowest concentration of the substrate falls off the line. This has not been explained.

Effects of variations in the salt content. Though this has not been investigated extensively, some facts about the effects of salt concentration on the degree of inhibition by fluorocitrate were obtained, because it is sometimes necessary to assay tissue extracts with variable salt contents. In general (Fig. 5) an increase of both NaCl and KCl lowered the inhibition observed with a small amount of fluorocitrate; the presence of 0.0624M-NaCl and 0.0485M-KCl reduced this almost completely, and about 50 times the amount of fluorocitrate was then needed for the same degree of inhibition.

There were interesting differences between NaCl and KCl in their effects on aconitase alone; increasing amounts of NaCl increased the activity, whereas KCl gave a more complicated picture, first increasing and then decreasing activity. It is clear that the salt content must be controlled when testing for fluorocitrate concentration with aconitase, and that more work is needed to understand what is happening.

Reversal of the inhibition by fluorocitrate and the effect of pre-formed *cis*-aconitate and citrate on the reversal.

Many experiments were done similar to those of Morrison & Peters (1954) in which more *isocitrate* was added after 4–5 min. to ascertain the amount by which the inhibition was reduced on increasing the concentration of substrate. This is important because the possibility of finding an antidote to fluoroacetate poisoning turns to some extent on whether the inhibition by fluorocitrate is reversible. The experiments made in this series are not given in detail here; in all experiments there was indication of reversal by a closer approximation of the curves of reaction, and sometimes the reversal was complete. But the results were variable, and this led to a closer examination of the phenomena.

In our original experiments, the effect on the subsequent behaviour of any *cis*-aconitate or citrate

Fig. 4. Apparent K_m for aconitase E. Reaction *isocitrate* $\rightarrow$ *cis*-aconitate in tris buffer, pH 7.4.

Fig. 5. Effect of KCl and NaCl on the inhibition of aconitase E produced by enzymic fluorocitrate (0.01 μ g.) and on the enzymic activity. Each point represents the difference in the activity or the amount of *cis*-aconitate formed from DL-*isocitrate* (2.78 μ moles) for the period 0.5–4.0 min. in the presence and absence of the salts indicated. Preparations were preincubated for 10 min. (a) KCl, 10 mg. (48.5 mM); (b) NaCl, 10 mg. (62.4 mM). —, Effect on aconitase activity; - - -, change in percentage inhibition.

formed during the initial period of exposure to the lower *isocitrate* concentration was neglected. If, however, small amounts of *cis*-aconitate (or citrate) have an appreciable inhibitory effect on the subsequent course of the reaction *isocitrate* $\rightarrow$ *cis*-aconitate when more *isocitrate* is added, then the reversal of the inhibition might be only apparent. The greater amount of *cis*-aconitate formed in the control might slow the reaction enough to make the rate identical with that of the cuvette containing fluorocitrate. This would mean that the inhibition was less competitive than we had thought. It could also account for some of our variable results. The matter has been tested by starting the reaction with some *cis*-aconitate or citrate present.

An addition of 38.7 μ moles of citrate had little effect on the rate of reaction of *isocitrate*; but it was different with *cis*-aconitate. Fig. 6 shows that 15 μ g. (0.086 μ mole) and 25 μ g. (0.14 μ mole) of *cis*-aconitic acid (neutralized with NaOH) induce large effects on the rate of reaction of 2.6 μ moles of DL-*isocitrate* with aconitase. It can be calculated that at the end of the 4 min. incubation there may sometimes be a difference between the sample inhibited by fluorocitrate and the control sample of approx. 0.052 μ mole (10 μ g.) of *cis*-aconitic acid. The extra *cis*-aconitic acid in the control sample could slow the rate of the subsequent reactions occurring after the further addition of *isocitrate*, so that the control rate became identical with that of the inhibited reaction; the reversibility described above would be then only an apparent reversibility. The degree of inhibition for 0.005 μ g. of fluorocitrate was 50 or 54 %, i.e. unchanged within the limits of error by the effect of *cis*-aconitate.

Fig. 6. Effect on the rate of action of aconitase during the first 4 min. of adding initially small amounts of DL-*cis*-aconitate. Experimental details were as for Fig. 2. All contained 2.76 μ moles of DL-*isocitrate*. A, Control; B, 0.086 μ mole of *cis*-aconitate; C, 0.086 μ mole of *cis*-aconitate + 0.005 μ g. of fluorocitrate; D, 0.144 μ mole of *cis*-aconitate; E, 0.144 μ mole of *cis*-aconitate + 0.005 μ g. of fluorocitrate.

In view of these results it became essential to study the inhibition in the presence of increasing concentrations of *isocitrate* added initially. This should show decisively whether increasing concentrations of substrate reversed the effect. An amount of fluorocitrate (0.005 μ g.) was used which would produce an inhibition of 50 % of the maximum (see Fig. 6). In Fig. 7 the rates of formation of *cis*-aconitic acid over the 3.5 min. period in the presence and absence of fluorocitrate have been plotted against the initial amounts of DL-*isocitrate* added. This method of plotting was found more satisfactory than any other. With increasing *isocitrate* concentration there was a decreased inhibition; but the reversal was much less marked than in our earlier experiments. It appears therefore that the suspected effect of small amounts of *cis*-aconitate formed during the first few minutes was influencing the previous experiments. In fact in the presence of 208 μ moles of *isocitrate*, added initially, there is still an inhibition of 8 % under these conditions. Hence it is much more difficult to reverse the inhibition than was previously thought. *In vivo* the necessary concentration of citrate for reducing the inhibition to 8 % could not

Fig. 7. Effect of fluorocitrate on initial rates of action of aconitase with increasing substrate concentration. The enzyme was incubated in tris buffer for 10 min. with and without fluorocitrate, after which amounts of DL-*isocitrate* were added varying from 2.76 to 55.2 μ moles. With increasing concentration of *isocitrate* there was an increase of the initial non-specific change in absorption in the first 0.5 min.; for instance, whereas with addition of 5.52 μ moles this was 40, with 27.6 μ moles it became 150 and with 110.4 μ moles 250. After disregarding the first 0.5 min. the readings became linear and the two curves show the differences in absorption for amounts of *isocitrate* up to 55.2 μ moles over the remaining 3.5 min. between the controls (●) and samples treated with 0.005 mg. of fluorocitrate (○). It is clear that there is a reversal, but that this is not so marked as was thought previously. Even with 208 μ moles the reversal was not complete. (10 mg. of *isocitrate* $\equiv$ 52.1 μ moles.)

be reached, as this would have to be some nine times that of *isocitrate*.

Fig. 7 indicates that it is difficult to reverse the inhibition *in vitro* under these conditions. However, it must be stressed that the reactions with aconitase are complex. This is shown by the fact that if the amount of enzyme is much increased the amount of *cis*-aconitate formed reaches a lower maximum, and then actually decreases as the reaction proceeds, owing presumably to the formation of citrate, which does not absorb at 240 $m\mu$.

A few experiments were made on the amount of citrate formed after 4 and 7 min. of reaction. The amounts of citrate present were small, not more than 0.2 μ mole/cuvette; such amounts did not increase in this time with increasing concentrations of *isocitrate*. The *cis*-aconitate is evidently not always converted into citrate immediately.

$L_s(+)$ -*isocitrate* as substrate. As all the previous experiments were done with DL-*isocitrate*, some of the main points were checked with a specimen of $L_s(+)$ -*isocitrate* for which we are indebted to Dr H. B. Vickery. In every case tried the reaction rate for a given amount of $L_s(+)$ -*isocitrate* was approximately double that with the DL-compound, so that the unnatural isomer did not influence these results. There was no difference in the degree of inhibition with fluorocitrate. From this we conclude that the use of the DL-*isocitrate* has not affected our results.

DISCUSSION

The results appear to dispose of some of the difficult discrepancies between the inhibition by fluorocitrate of mitochondrial and of soluble aconitase, as they show that a form of soluble aconitase can be very sensitive to fluorocitrate inhibition. The values reached in these experiments are of the order of those found with the phosphorus-containing insecticides and cholinesterase. On the other hand, an exact evaluation of the conditions inside the mitochondria is difficult. Since with the particle preparations we worked in 1% KCl so far as the bulk solution was concerned, the inhibitory action of the fluorocitrate would be much diminished if the salt concentration inside the mitochondrion was the same. Within the mitochondrion again the fluorocitrate concentration is unknown; but the enzyme *in vivo* could be more sensitive even than in these tests. A calculation indicated that citrate metabolism in pigeon-brain tissue *in vivo* was blocked by about 0.1 μ g. of fluorocitrate/g. of wet tissue. *In vivo* also, accumulation of citrate reduced the inhibition, which could explain the reduction reported by Chenoweth (1949) in the effect of a second dose of fluorocitrate when given 24 hr. later than the first. Amounts of citrate higher than 20.8 μ moles/g. have not been found after poisoning

animals with these compounds, and such concentration would not be enough to cause reversal if the behaviour were the same as with aconitase E.

So far as the action of aconitase itself is concerned, these experiments raise more problems than they solve, because they have been oriented solely to the problem of how to test for fluorocitrate. Some of the problems may, however, be mentioned. First, there seems to be a real difference so far as inhibition is concerned between aconitase M and E, which is not just a matter of the amount of Fe^{2+} and cysteine present. If this is so, then the reconstituted enzyme would differ in some way from that in the tissues.

Secondly, it is curious that within the first few minutes of the reaction the amount of citrate formed does not correspond with the increase of *cis*-aconitate when the concentration of *isocitrate* is increased. This is consistent with the idea of the intermediate carbonium ion in aconitase, as set forth most recently by Dickman & Speyer (1954) and England & Colowick (1957); but it does not seem consistent with the idea that some citrate can be formed directly without the formation of *cis*-aconitate, unless we take the view that the addition of water to the intermediate complex is more difficult than elimination to the anhydrous *cis*-aconitate.

The changes of activity induced by salts are of theoretical interest and deserve much more study, certainly with change in pH and with other ions. It is possible indeed that there might appear changes in optimum pH similar to those found in different buffers for aconitase (Morrison, 1954) or for fumarase with different inhibitors (Massey, 1953). In order to get a proper picture, however, simultaneous observations will be needed of the amounts of all three tricarboxylic acids, taking into account the large influence of small amounts of *cis*-aconitate.

SUMMARY

1. A new method has been worked out for testing the activity of fluorocitrate on soluble aconitase. In this a cruder enzyme preparation is used, taken to an intermediate stage of fractionation, which does not require reactivation with ferrous salts and cysteine.

2. When this preparation was used in 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer solution instead of phosphate solution, 50% inhibition was observed with fluorocitrate (enzymically synthesized) at a concentration of 6.5 μ mM.

3. This large increase in the inhibitory activity observed means that there is no longer a discrepancy between the effects of fluorocitrate upon aconitase in particle preparations and in a soluble form. It makes unnecessary the hypothesis of

specific concentration or orientation previously proposed.

4. The inhibitory effects were much reduced by the presence of phosphate or by preincubation with 5 mM-*isocitrate*.

5. In the presence of sodium chloride the activity of the aconitase increased. With potassium chloride there was an increase followed by a fall. An increase in the concentration of both salts much reduced the inhibition of fluorocitrate.

6. The percentage inhibition with fluorocitrate was the same for $L_1(+)$ -*isocitrate* (D), though the rate for a given amount of *isocitrate* was doubled. There was no indication that the inactive isomer was an inhibitor.

7. Variation of temperature within $\pm 4^\circ$ was about 4% per degree. Variations in pH of ± 0.6 caused little change in fluorocitrate inhibition, though a fall in activity was found on making more alkaline.

Thanks are due to Miss P. Ivall for efficient technical assistance, and to Miss R. Shawdon for some of the preliminary work; to Dr P. F. V. Ward for the final purification of the fluorocitrate used, to others in the Depart-

ment for advice and to Dr D. H. Treble for reading the MS. Thanks are also due to Professor F. G. Young, F.R.S., for providing facilities for the finish of this work.

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Biochem. J. (1961) **79**, 268

The Metabolites of Piperic Acid and Some Related Compounds in the Rat

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(Received 18 October 1960)

Human urine contains many compounds which give coloured derivatives with Altman reagent on paper chromatograms (Acheson & Dearnaley, 1960; Smith, Paul, McGeer & McGeer, 1959; Armstrong, Shaw & Wall, 1956). This reagent (4-dimethylaminobenzaldehyde in acetic anhydride) reacts with aroyl-glycines and similar compounds to give coloured oxazolones, a number of which have been synthesized and their absorption spectra measured (Acheson, Booth, Brettle & Harris, 1960). 5-Phenylpenta-2:4-dienoylglycine gives, when heated with the reagent on paper chromatograms, a red-purple colour similar to those given by compounds 54 and 55, which were found in human urine by Acheson & Dearnaley (1960), but the three compounds had different R_f values. It was possible that compounds 54 and 55 were derived from a substituted 5-phenylpenta-2:4-dienoic acid which is a dietary constituent and

excreted as the glycine conjugate. In order to test this in connexion with piperic acid, which occurs as derivatives in pepper, piperoylglycine was prepared and the metabolism of piperic acid has been investigated in the rat.

MATERIALS AND METHODS

All melting points are uncorrected, and the absorption spectra were determined in methanol solution unless otherwise specified.

Piperonylic acid, m.p. 226-227°, λ_{max} 293 and 256 m μ (ϵ 6400 and 7500 respectively), was prepared by the oxidation of piperonal with neutral potassium permanganate (*Organic Syntheses*, 1943). 3:4-Methylenedioxyhippuric acid, m.p. 178°, was prepared as described previously by Acheson *et al.* (1960), and 3:4-methylenedioxy-cinnamic acid, m.p. 238°, was obtained from malonic acid and piperonal (*Organic Reactions*, 1942).

3:4-Methylenedioxy-cinnamoylglycine. The cinnamic acid (1.0 g.) was covered with ethyl aminoacetate (0.8 g.) in dry