A FURTHER STUDY OF THE INHIBITION OF ACONITASE BY 'INHIBITOR FRACTION' ISOLATED FROM TISSUES POISONED WITH FLUOROACETATE

by

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Recently LOTSPEICH, PETERS AND WILSON[8,9] showed that the fluorotricarboxylic acid (designated as "inhibitor fraction") isolated from kidney tissue treated with fluoroacetate in the presence of fumarate (BUFFA, PETERS AND WAKELIN[2]), inhibited the enzyme aconitase.

This paper deals with an extension of the study of this inhibition, which in these preliminary experiments has proved to be essentially competitive in nature.

EXPERIMENTAL

Aconitase preparation

The preparation of aconitase is the same as that reported previously[8,9]; briefly this was as follows: a water extract of pigeon breast muscle was stabilized with cysteine (0.01 *M*) and ferrous sulphate (0.005 *M*) and stored in the refrigerator under oil. It has been found difficult to obtain reproducible activities from similar quantities of enzyme on different occasions, so that repeated trial runs were often necessary prior to the actual experiment.

Partly purified aconitase was prepared by an ammonium sulphate fractionation method described by DICKMAN AND CLOUTIER[4].

Isocitric estimation

The method of OCHOA[1] using *iso*citric dehydrogenase and measuring the reduction of TPN was used with the addition of KCN (0.005 *M*) to inhibit small traces of aconitase often present. In some experiments *o*-phenanthroline was added to the extract prior to dialysis, which effectively inhibited the aconitase (DICKMAN AND CLOUTIER[4]). The aconitase reaction was stopped with HCl to pH 4.0 approx. and trichloroacetic acid added to 1 %. After centrifugation, amounts of neutralized solution containing 0.01 to 0.1 μmol *iso*citrate were placed in Beckman tubes and estimated using 0.1 μmol TPN per tube. The accuracy was of the order of $\pm$ 0.0015 μmol ($\pm$ 0.3 μg).

Cis-*aconitate estimation*

The method of RACKER[14] was employed, using the absorption of the double bond of *cis*-aconitate in the ultraviolet. Semi-purified aconitase was placed in a 3 ml quartz cell with either citrate or *iso*citrate and the increase in absorption at 240 mμ measured in the Beckman spectrophotometer.

Reagents

As before sodium citrate 3 H_2O (A.R.) was used throughout; D-L *iso*citrate (m.p. 136) was prepared by R. W. WAKELIN. All substances were neutralized to pH 7.2 before use. TPN was prepared by the method of LEPAGE AND MUELLER[7] using a Nuchar C column.

'Inhibitor fractions'

The inhibitor (referred to as 'inhibitor fractions') was isolated by PETERS AND WAKELIN from homogenates exposed to fluoroacetate by the method of BUFFA *et al.*[2] The two fractions used in this study were free from fluoroacetate: 46A contained as impurities citrate and phosphate, 50/51

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was purer containing mainly citrate as impurity. In each fraction there were only a few per cent of other tricarboxylic acids. The quantity of the inhibitor will be expressed in terms of its inhibition of citrate metabolism in guinea pig homogenates[2]. One unit is approximately equivalent to 0.059 μg F and is defined as that amount of "inhibitor fraction", which inhibits the disappearance of 1 μmol of citrate under the conditions of their test system.

Experimental methods for controlling the stability of aconitase

Aconitase when isolated is notorious for its instability, and attempts to stabilize it with glycerol (KREBS AND EGGLESTON)[6] and citrate (BUCHANAN AND ANFINSEN[1]) have been made. In confirmation of the latter workers, no measurable loss of activity occurred in our experiments in 45 min in the presence of one of its substrates.

The recent studies of DICKMAN AND CLOUTIER[3,4] with reducing agents have proved most useful. It was confirmed that ferrous ion and cysteine greatly increase the stability of aconitase and they have been used throughout this work. Undiluted muscle extract in the presence of these reducing agents kept under oil in the refrigerator was quite stable in our experience for two to three months. There are only traces of residual tricarboxylic acids in pigeon breast muscle so that substrate cannot stabilize the enzyme appreciably in the stored material. In the absence of substrate, aconitase solutions, when appropriately diluted for a determination, can lose 75% of their activity in 20 min at 38°, and 40% in 20 min at 17°, even in the presence of reducing agents. In Fig. 1 is shown the type of loss seen in such an experiment. In evacuated Thunberg tubes activity was retained longer (only 20% loss of activity in 15 min); but this method was not thought feasible for experiments involving repeated withdrawals of samples. Aconitase, partially purified by ammonium sulphate fractionation, was found to be much more unstable than the crude extract.

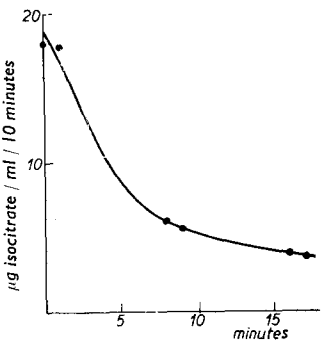

Fig. 1. The enzyme preparation used was a 0.1 *M* phosphate extract of pigeon breast muscle (pH 7.2) stabilized with cysteine (0.01*M*) and ferrous sulphate (0.005 *M*) which had been shown to lose little activity on storage for a number of weeks at 0° under oil. This stock enzyme solution was diluted (1:20 approx.) with 0.01 *M* cysteine and incubated at 38° (in air) for various time intervals prior to the activity determination. Activity was determined by estimating the *iso*citrate produced in 10 min following addition of citrate (1.7 m*M*).

The following precautions were taken in the experiments reported previously[8,9] and those presented here. The diluted enzyme was allowed to stand for 20 min prior to the addition of substrate or inhibitor to allow the initial rapid loss of activity to occur. Reproducibility was considered more important than maximum activity of the enzyme. The quantity of citrate present as an impurity in the "inhibitor fractions" was usually enough to give a concentration of about 60 μg/3 ml of final mixture; this stabilized the enzyme for the incubation period. An equivalent amount of citrate was added to the control tubes. To minimize any loss of enzyme activity during the additions to the first and last tubes the additions of inhibitor or citrate were made alternately at half minute intervals. Following the pre-incubation period the substrate was similarly added to the various tubes at half minute intervals. In control experiments it was found that in practice only a few percent loss of activity occurred in 3 min under these conditions. In experiments shown in Figs. 2a and 2b the inhibitor tube was started first so that any loss of activity in the control would decrease the apparent inhibition rather than increase it.

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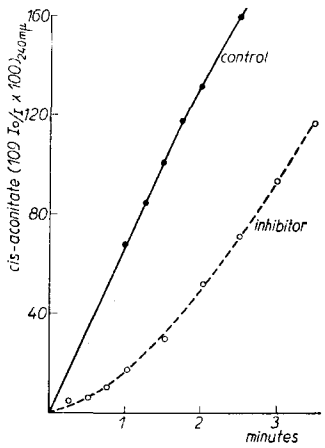

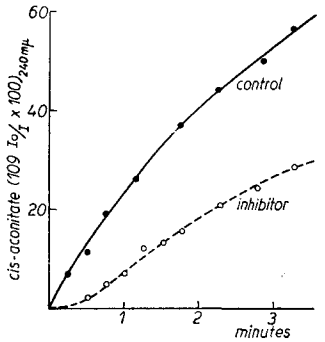

Fig. 2a. Inhibition of *iso*citrate ⟶ *cis*-aconitate reaction by "inhibitor fraction". 9 units of fraction 46A (0.30 ml) were preincubated for 15 min with 2.8 ml of semi-purified aconitase containing 0.02 *M* phosphate pH 7.2 and stabilised with Fe^{++} 0.001 *M* and cysteine 0.01 *M* prior to the addition of substrate. 0.1 ml of D-L *iso*citrate (containing 5.6 μmol) was added to the enzyme solution, placed in a 3 ml quartz cell and the increase in absorption at 240 mμ was followed

Fig. 2b. Inhibition of the citrate ⟶ *cis*-aconitate reaction by the "inhibitor fraction". 4.4 units (0.1 ml as diluted) of "inhibitor fraction" 50/51 were preincubated for 15 min with 2.8 ml of semi-purified aconitase as 2a prior to the addition of substrate. 0.1 ml (5.5 μmol) citrate was added to the enzyme solution, placed in a 3 ml quartz cell and the increase in absorption at 240 mμ was followed

RESULTS

An investigation of the effect of an inhibitor upon the kinetics of the action of aconitase upon the system represented by the equation

$$\text{citrate} \rightleftharpoons \textit{cis}\text{-aconitate} \rightleftharpoons \text{D-}\textit{iso}\text{citrate} \qquad (1)$$

is clearly complicated; it may be necessary to take into account the behaviour of the undissociated acids as well as the dissociated ions. There is also some further complication, which is not yet understood; though OGSTON[12] has pointed out that there is no need to assume the existence of more than one enzyme, ELLIOTT AND KALNITSKY[5] and MARTIUS AND LYNEN[10] have found facts difficult to reconcile with the simple equation[1]; and we ourselves[8,9] were surprised to find that the isolated fluorotricarboxylic acid fractions had less effect quantitatively upon the reactions from *cis*-aconitate than upon those starting from either citrate or *iso*citrate. A complete investigation will require larger amounts of purified inhibitor than are available at present.

Meanwhile, it is essential to decide whether the basic action is competitive. Preliminary experiments of two types are, therefore, reported here in sections a and b, which are thought to demonstrate the existence of competitive inhibition in the sense that the attachment of the inhibitor to the active centre can be modified by the concentration of one of the substrates.

a. One criterion for a competitive inhibitor is that once it has been attached to the enzyme it can be displaced by the substrate. This has been tested by incubating the inhibitor with the enzyme prior to the addition of the substrate. If the addition of substrate then succeeds in displacing the inhibitor, there should be a progressive de-

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crease in the inhibition. The curves given previously, though suggesting this, were indecisive owing to the errors inherent in the measurement of the points at the beginning of the experiments. A more accurate method was therefore sought. It was found possible to take advantage of the very small amounts of *cis*-aconitate which can be estimated accurately by the absorption of its double bond in the ultraviolet region. *Iso*citrate and an aconitase preparation were mixed in a 3 ml cell; after placing in the Beckman photometer, the increase in absorption at 240 μm was recorded. It was necessary to use a partially purified enzyme because of the strong absorption of the crude extract in this region. However, instability of the purified enzyme made it difficult to get uniformly favourable conditions for these experiments. In Fig. 2a, the result of one experiment is shown in which the inhibitor had been pre-incubated with the enzyme at room temperature for 15 min prior to the addition of the substrate *iso*citrate. As compared with the control, an initial inhibition of over 70% was observed at 30 sec as calculated from the slopes of the lines tangent to the curves at this point. This decreased in the next minute to a 25% inhibition which remained constantly at this level throughout the remainder of the experiment, and which represents the equilibrium condition.

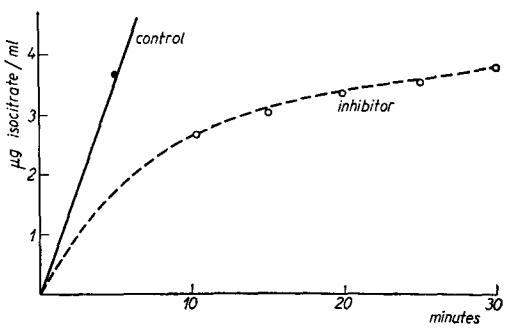

Fig. 3. Inhibition of aconitase by "inhibitor fraction" when inhibitor and substrate added simultaneously. The final mixture (15.89 ml) contained 10 ml of diluted aconitase preparation; 5 ml sodium citrate (1.17 m*M*) and 0.89 ml inhibitor fraction 46A, containing 60 units of inhibitor and 1.56 μmol citrate. In the control 0.89 ml containing only 1.56 μmol citrate replaced the inhibitor. The dilute aconitase extract was a 1:22 dilution of the stock water extract of pigeon muscle, containing finally Fe^{++} 0.006 *M*, cysteine 0.01 *M*, and phosphate buffer pH 7.2, 0.05 *M*.

A similar but less striking experiment of the same type is shown in Fig. 2b where the reaction citrate → *cis*-aconitate was measured. Both experiments support the idea that the inhibitor is displaced by substrate.

b. The type of inhibition can also be studied by measuring the initial velocity of reactions at various substrate concentrations and plotting the results by the LINEWEAVER-BURK method. Experiments were made in which the reaction citrate → *iso*citrate was studied at various concentrations of citrate with and without inhibitor. It proved to be important to allow sufficient time for the development of the equilibrium. Fig. 3 shows that in an experiment where the inhibitor and substrate were added simultaneously, maximum inhibition did not develop for 15 minutes; this was evidently the time required for the equilibrium to be established between the enzyme, substrate and inhibitor. In view of this, the "initial velocity" of the inhibitor curve was taken as the velocity between the fifteenth and thirtieth minute after starting the reaction. In Fig. 4 is shown the information from a complete experiment with several substrate concentrations plotted by the graphic method of LINEWEAVER AND BURK. It demonstrates the competitive nature of the inhibition. In the dotted lower line is shown the data obtained if the actual initial velocities of the inhibitor curves were used, that is before equilibrium conditions were established. This shows the non-competitive type of curve that may be obtained in this type of experiment if care is not taken to allow time for the enzyme, substrate, and inhibitor to come to equilibrium before measuring the initial velocity. It is hoped soon to be able to discuss the reason for the differences between these and the, non-competitive results

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obtained with the kidney homogenates by BUFFA *et al.*[2]

DISCUSSION

In the previous communication LOTSPEICH *et al.*[8,9] showed that four of the reactions of aconitase are inhibited by the "inhibitor fraction". The only other two reactions attributed to this enzyme namely citrate → *cis*-aconitate and *iso*-citrate → *cis*-aconitate have now been shown to

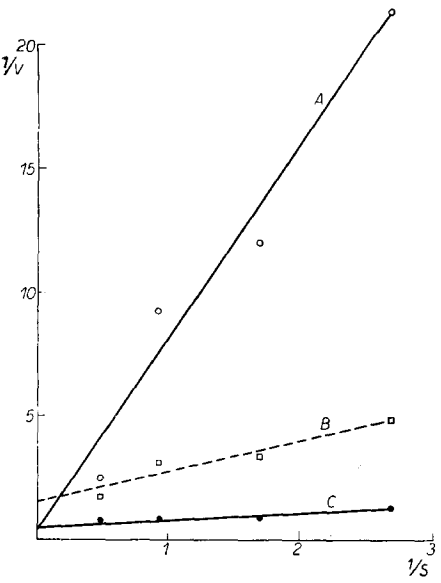

Fig. 4. Inhibition of the citrate → *iso*citrate reaction by "inhibitor fraction" using a LINEWEAVER-BURK plot. Inhibitor fraction 12 units/3 ml in (A) and (B). S = mM concentration of citrate. M = μg *iso*citrate produced in min 1 per ml. (A) velocity after equilibrium was established (B) velocity before equilibrium was established. (C) Control — no inhibitor.

be similarly inhibited by this substance. The competitive nature of this inhibition has now been demonstrated by two different methods.

It may be calculated from the data of Fig. 4 that the inhibitor has twenty times greater affinity than citrate for aconitase.

(Note). The inhibitor is believed to be fluorocitrate (PETERS[13]) though this is not yet proved. Since the above information was obtained it has been possible to test a much purer fraction of inhibitor free from citrate. This gave qualitatively the same effect, as previously, upon the inhibition of aconitase in the aconitase-*iso*citrate dehydrogenase system[8,9].

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SUMMARY

1. The instability of aconitase has been discussed.

2. "Inhibitor fractions" isolated from tissues poisoned with fluoroacetate inhibit the citrate ⟶ *cis*-aconitate, and *iso*citrate ⟶ *cis*-aconitate reactions.

3. The evidence for the competitive nature of the inhibition with the soluble aconitase has been obtained by two different methods.

RÉSUMÉ

1. Une discussion de l'instabilité de l'aconitase est présentée.

2. Des "fractions inhibitrices", isolées à partir des tissus empoisonnés par le fluoroacétate, inhibent la transformation de l'*iso*citrate et du citrate en *cis*-aconitate.

3. Deux méthodes indépendantes ont démontré la nature compétitive de cette inhibition par l'aconitase soluble.

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ZUSAMMENFASSUNG

1. Die Unbeständigheit der Aconitase wird besprochen.

2. Der, aus mit Fluoressigsäure vergifteten Geweben gewonnene, hemmende Bestandteil hindert die Bildung des *cis*-Aconitats aus Citrat oder *Iso*citrat.

3. Die konkurrierende Hemmung, welche die lösliche Aconitase ausübt, wird mit Hilfe zweier unabhängiger Methoden bewiesen.

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