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The Activation of Aconitase by Ferrous Ions and Reducing Agents

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Dickman & Cloutier (1951) showed that aconitase is dependent on the presence of ferrous ions and a reducing agent for maximum activity. Qualitative studies indicated that the ferrous ion is capable of forming complexes with the substrates of aconitase and with the enzyme under the conditions of optimum enzyme activity. The authors explained the action of ferrous ion in terms of the hypothesis advanced by Smith (1951), namely, that the metal is responsible for the linkage between the apoenzyme and the substrate in the aconitase–tricarboxylic acid complex. They assumed that the reducing agents played a double role in maintaining the iron in the ferrous state and in keeping the reducing groups of the protein in the reduced state.

The high residual activity of the aconitase preparation of Dickman & Cloutier (1951) in the absence of ferrous ion and reducing agents did not permit of a more detailed investigation of the function of these substances in the aconitase system. The aconitase preparation obtained from pig heart by the method of Morrison (1954*a*) provided a means of doing this. The preparation showed little activity in the absence of ferrous ion and a reducing agent, whereas it showed considerable activity on the addition of these substances. This preparation was used for testing the effect of ferrous ion and reducing agents on the aconitase activity. The results reported in this paper are consistent with the idea that both ferrous ion and the reducing agents act as activators or prosthetic groups of aconitase and are thus concerned in the formation of the active enzyme complex. It is suggested that the active form of the enzyme may be an enzyme–Fe^{2+}–reducing agent or an enzyme–Fe^{2+}–activator complex. For a preliminary communication, see Morrison (1954*b*).

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EXPERIMENTAL

Methods

Details of the methods for the preparation and activation of aconitase as well as the determination of enzymic activity have been previously described (Morrison, 1954*a*). Electrophoretic analysis of the final enzyme preparation showed that the major component, which possessed aconitase activity, formed approximately 75% of the total protein present. After dialysis against 0·004M citrate buffer, pH 5·7, the enzyme could be stored in the frozen state for many weeks with only slight loss of activity. After activation with 5×10^{-4}M-Fe^{2+} and 10^{-2}M cysteine, 1 mg. of this preparation was capable of forming 230 μmoles of citric acid from *cis*-aconitic acid in 15 min. at 30° and pH 7·4 in the absence of buffer.

Aconitase was activated by adding a sample of the enzyme to a solution containing the indicated amounts of ferrous ammonium sulphate and cysteine, or other reducing agent. The mixture was then neutralized to pH 7·4 with N-NaOH, using a glass electrode and, except where otherwise stated, was incubated in an ice bath for 1 hr. before the determination of aconitase activity. Activation could not be carried out in the presence of a buffer on account of the ability of buffers to inactivate aconitase in the absence of substrate (Morrison, 1954*a*).

The conditions used for the determination of aconitase activity are described in the legends to the figures. Buffers were used in the later experiments for it was found that when the enzyme was added to a mixture of substrate and buffer, there was no loss of enzyme activity over the period of the test.

In all instances the reaction *cis*-aconitate → citrate was studied. Citric acid was estimated by the method of Pucher, Sherman & Vickery (1936) as modified by Buffa & Peters (1949).

Materials

cis-Aconitic anhydride was prepared from *trans*-aconitic acid by the method of Malachowski & Maslowski (1928). Sodium *cis*-aconitate was formed by neutralizing the anhydride to pH 7·4 with N-NaOH. Ascorbic acid and cysteine were obtained from Roche Products Ltd., thioglycollate was a British Drug Houses Ltd. product and glutathione was a gift from the Distillers Co. (Biochemicals) Ltd., Liverpool. As determined by thiol titration, the glutathione was 99% pure (Cecil & McPhee, to be published). All these compounds were free of iron. The other reagents were A.R.

RESULTS

Activation of aconitase by ferrous ion and cysteine

Fig. 1 shows the effect of Fe^{2+} and cysteine on the activity of aconitase. The purified, dialysed preparation possesses little activity; cysteine alone does not increase the activity; Fe^{2+} brings about a 15-fold increase in the activity whilst the addition of Fe^{2+} and cysteine increases the activity 70-fold. The investigation of the activation of aconitase by Fe^{2+} alone involved some technical difficulties.

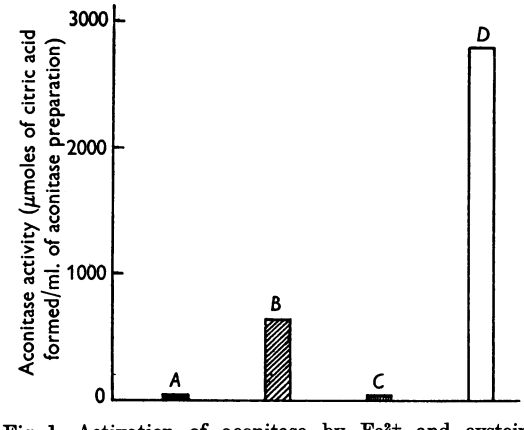

Fig. 1. Activation of aconitase by Fe^{2+} and cysteine. Activation was carried out by incubating samples of the aconitase preparation, which contained 12 mg. of protein/ml., with the indicated solutions in a final volume of 10 ml. for 1 hr. at 0° after adjustment of the pH to 7·4. The samples were equivalent to 1·0 ml. of the aconitase preparation, except when the enzyme was incubated with both Fe^{2+} and cysteine. In this instance, a 0·1 ml. sample was used. The enzymic activity was determined by adding 0·1 ml. samples of the above enzyme solutions to media containing 0·5 ml. of *cis*-aconitic acid (final concentration, 4×10^{-3} M) at pH 7·4 and 4·4 ml. of water. The reaction was stopped after 15 min. at 30° by the addition of 0·5 ml. of 50% (w/v) trichloroacetic acid. Citric acid was estimated as described in the text. *A*, no additions; *B*, 5×10^{-4} M-Fe^{2+}; *C*, 0·01 M cysteine; *D*, 5×10^{-4} M-Fe^{2+} + 0·01 M cysteine.

Because of the small amount of protein present in solution, it showed little or no buffering capacity. The ferrous ammonium sulphate solution was acidic so that if it were not partly neutralized the pH of the Fe^{2+}–enzyme solution fell below 5·0 with a result that there was denaturation of the enzyme. The adjustment of the pH of this solution to 7·4 was difficult and at this pH value at least some of the Fe^{2+} was in the colloidal state. On the other hand, there was no formation of colloidal iron when a reducing agent was also present and in the presence of cysteine, the solution was buffered at pH 7·4. Therefore it is not possible to state that the above value represents an accurate estimation of the activation of aconitase by Fe^{2+}. Nevertheless, it is clear that Fe^{2+} and cysteine bring about a very marked activation of the highly purified aconitase preparation. The activation was due to Fe^{2+} ions, as ammonium and sulphate ions were inert.

Factors affecting the activation of aconitase

It is clear from Table 1 that there is an initial rapid activation of aconitase by Fe^{2+} and cysteine. The activity is increased by further incubation. At 30 min. the enzyme activity is maximal. This activity is retained for at least 4 hr., after which there is a slow decrease; at 24 hr. the enzyme possesses only 60% of its maximum activity. The loss of enzyme activity could not be ascribed to alterations in the pH of the enzyme solution or to an accumulation of the end-products of the oxidation of cysteine catalysed by Fe^{2+} in the presence of oxygen. The pH was constant over the 24 hr. period of the test. When the non-activated enzyme was added to a mixture of Fe^{2+}, cysteine and water which had previously been incubated at 0° for 24 hr., the activity following a 30 min. incubation at 0° was

Table 1. *Effect of pre-incubation time on the activation of aconitase by ferrous ion and cysteine*

Aconitase was incubated at pH 7·4 and 0° with 5×10^{-4} M-Fe^{2+} and 10^{-2} M cysteine. Samples (0·2 ml.), equivalent to 12 μg. of the final aconitase preparation, were removed at intervals and added to test tubes containing 4·8 ml. *cis*-aconitate (final concentration 4×10^{-3} M), pH 7·4. Reaction was stopped after 15 min. by the addition of 0·5 ml. of 50% (w/v) trichloroacetic acid and the mixture analysed for citrate. Temp. 30°.

Time of activation (hr.)	Aconitase activity (μg. citrate formed/15 min.)
0	285
0·5	335
1·0	320
2·0	320
3·0	320
4·0	318
5·0	295
6·0	278
24·0	192

maximal. It is likely that the loss of enzyme activity is due to denaturation of the enzyme in dilute solution.

The usual method of activating aconitase consisted of incubating the enzyme for 1 hr. at 0° in the presence of 5×10^{-4}M-Fe^{2+} and 10^{-2}M cysteine; a sample of the activated enzyme was then added to the test medium. (This method of activation will be referred to as method 1.) Thus when 0·1 ml. of the activated enzyme solution was added to 5 ml. of medium, the concentrations of Fe^{2+} and cysteine in the medium were reduced to 10^{-5}M and 2×10^{-4}M, respectively. When non-activated aconitase was added to a medium containing the lower concentrations of Fe^{2+} and cysteine and incubated for 5 min. at 30° before the addition of substrate, the enzyme showed no activity. When the concentrations of Fe^{2+} and cysteine in the medium were increased to 5×10^{-4}M and 10^{-2}M, respectively, the enzyme showed the same activity over the initial period of the reaction as it did after activation by method 1. (The activation of aconitase for 5 min. at 30° in the presence of 5×10^{-4}M-Fe^{2+} and 10^{-2}M cysteine in the test medium will be referred to as method 2.) Fig. 2 shows the effect of adding the non-activated enzyme directly to a mixture of Fe^{2+}, cysteine and substrate. It will be noted that there is a distinct lag period of about 3 min. and that the reaction rate is slower than that of the control in which the enzyme was pre-incubated with the Fe^{2+} and cysteine before the addition of substrate. The fall in the control rate after the first 10 min. was a characteristic feature of the reaction after activation of the enzyme by method 2.

Activation of aconitase under strictly anaerobic conditions did not increase the enzyme activity, but when the enzyme–Fe^{2+}–cysteine mixture was shaken in air during the activation period, the enzyme showed no activity on the addition of substrate. The activated enzyme could also be inactivated by shaking in air during the course of the reaction; the degree of inactivation was dependent on the method of activation. Fig. 3 shows that there is a slight loss of enzyme activity when the medium is rapidly shaken in air after the addition of the enzyme activated by method 1. On the other hand, there is a marked loss of activity under these conditions when the enzyme is activated in the medium by method 2 before the addition of substrate. It would seem likely that the inactivation of aconitase is due to the absorption of the enzyme by suspended cystine formed as a result of the catalytic oxidation of cysteine by Fe^{2+} in presence of oxygen (Mathews & Walker, 1909). Although the addition of cystine to the medium in an amount less than that required for a saturated solution did not cause any inhibition, the above idea is consistent with the finding that the presence of high concentrations of cysteine which give rise to suspended cystine, caused more marked inhibition of aconitase. These results are of importance in so far as artificial systems for the oxidation of citrate are concerned. Activation of aconitase in such a way that the concentrations of Fe^{2+} and cysteine in the test medium are minimal is clearly preferable if aconitase is to act at a maximum rate for long periods of time under aerobic conditions.

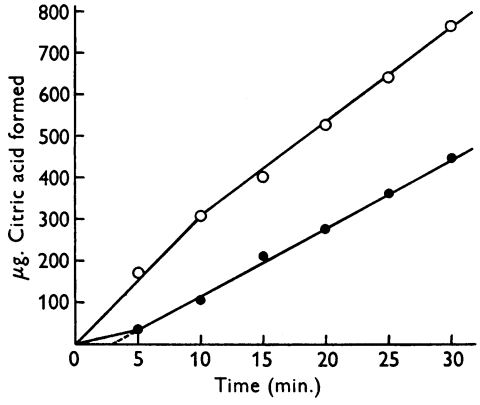

Fig. 2. The activation of aconitase by the addition of Fe^{2+} and cysteine to the test medium. 12 μg. of the non-activated aconitase preparation were added to the test medium which contained 5×10^{-4}M-Fe^{2+}, 10^{-2}M cysteine and 4×10^{-3}M *cis*-aconitate. Total volume 5·0 ml., temp. 30°, no buffer, pH 7·4. ○, aconitase incubated 5 min. with Fe^{2+} and cysteine before the addition of substrate; ●, aconitase added to a mixture of Fe^{2+}, cysteine and substrate without pre-incubation.

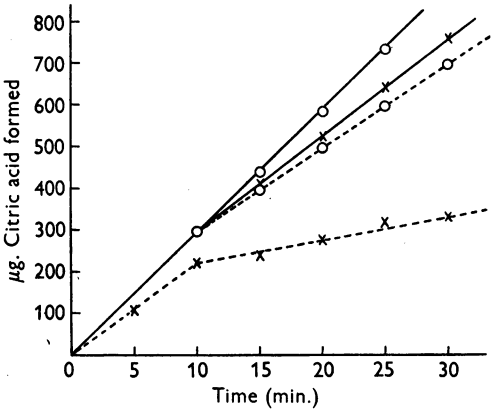

Fig. 3. The effect of rapid shaking in air on the activity of activated aconitase. The amount of aconitase added was equivalent to 12 μg. of the final aconitase preparation. Temp. 30°, no buffer, pH 7·4. ○, aconitase activated by method 1 as described in the text; ×, aconitase activated by method 2 as described in the text; ——, non-shaken; ----, shaken.

Specificity of ferrous ion

There was no relationship between the ability of a metal to oxidize cysteine catalytically and the ability of the system to activate aconitase. It was found that Cu^{2+} and Mn^{2+} at concentrations of 5×10^{-4}M did not bring about any activation of aconitase. Moreover, when this concentration of Cu^{2+} was added to the usual activation system containing Fe^{2+} and cysteine, the activity of the enzyme was inhibited by 50%. Krebs & Eggleston (1944) also found that aconitase was inhibited by Cu^{2+}. It is of interest that Cu^{2+} and Mn^{2+}, which belong to the same transition series as Fe^{2+} and which therefore have similar properties, do not activate aconitase.

Activation of aconitase by reducing agents

Table 2 shows the relative effectiveness of four reducing agents in activating aconitase in the presence of a fixed amount of Fe^{2+}. At a level of 0·01 M, cysteine brings about the greatest activation of aconitase, whilst thioglycollate, ascorbic acid and glutathione are decreasingly effective in the given order. When the concentration of reducing agent was increased to 0·025 M, the degree of activation was increased in all cases, being most marked with glutathione. Cysteine is still the most effective, but the activation by glutathione is increased to such an extent that it is as effective as thioglycollate. The higher concentration of ascorbic acid caused only a small increase in the enzyme activity, so that at this level it was the least effective. It was found that when the concentration of cysteine and ascorbic acid was increased to 0·1 M, the enzymic activity was slightly less than that obtained at a concentration of 0·01 M.

Table 2. *Effect of reducing agents on aconitase activity*

Aconitase was incubated with the reducing agent and 5×10^{-4}M-Fe^{2+} for 1 hr. at 0°. Samples (0·25 ml.), equivalent to 15 μg. of the final aconitase preparation, were added to media containing 1·5 ml. of 0·1 M phosphate buffer (pH 7·7) and 1·25 ml. of *cis*-aconitate (final concentration $6{\cdot}7 \times 10^{-3}$M). Reaction was stopped after 15 min. by the addition of 0·5 ml. of 50% (w/v) trichloroacetic acid and the mixture analysed for citrate. Temp. 22°.

	Aconitase activity (μg. citrate formed) at concentration of reducing agent of	
Reducing agent	0·01 M	0·025 M
Cysteine	178	265
Ascorbic acid	100	114
Glutathione	23	182
Thioglycollate	129	182

Role of ferrous ion in the action of aconitase

In order to elucidate the role played by Fe^{2+} in the action of aconitase, a quantitative study was made of the effect of the Fe^{2+} concentration on the activity of aconitase. The observations on the relationship between the Fe^{2+} concentration and the reaction velocity in the presence of cysteine and ascorbic acid, as shown in Fig. 4, conform to a simple Michaelis–Menten relationship, i.e. to the equation $v = Vx/(K_x + x)$, where v = initial velocity of the reaction at a standard concentration of substrate in the presence of a reducing agent, V = maximum reaction velocity, x = concentration of Fe^{2+}, and K_x = dissociation constant of the Fe^{2+}–enzyme complex formed according to the equation

$$Fe^{2+} + \text{enzyme} \rightleftharpoons Fe^{2+}\text{–enzyme}.$$

These results are consistent with the hypothesis that Fe^{2+} combines with aconitase in the ratio of one ferrous ion to one active centre to form the Fe^{2+}–enzyme complex, which is at least partially responsible for enzyme activity.

This interpretation is valid only because it was known from previous experiments that the above equilibrium was established during the preincubation of the enzyme with Fe^{2+} and the reducing agent for 1 hr. at 0°. The substrate was not added and the reaction rate was not determined until

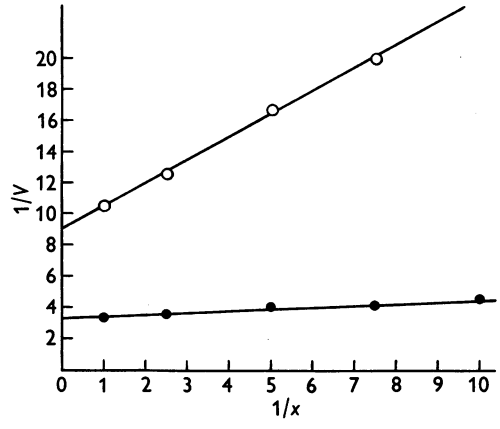

Fig. 4. The effect of Fe^{2+} concentration on aconitase activity. The graphs are plotted according to the method of Lineweaver & Burk (1934). Aconitase was activated in the presence of 10^{-2}M cysteine or ascorbic acid and various concentrations of Fe^{2+} at pH 7·4 for 1 hr. at 0°. Samples (0·25 ml.) of the enzyme solutions, containing 15 μg. of the final aconitase preparation, were added to media containing 1·5 ml. of 0·1 M phosphate buffer (pH 7·7) and 1·25 ml. of neutralized *cis*-aconitate (final concentration $6{\cdot}7 \times 10^{-3}$M). The reaction was stopped after 15 min. by the addition of 0·5 ml. of 50% (w/v) trichloroacetic acid and the solutions analysed for citrate. Temp. 22°. x, concentration of $Fe^{2+} \times 10^4$M; V, μg. of citrate formed/15 min. $\times 10^{-3}$; ●, cysteine; ○, ascorbic acid.

after the equilibrium was established, so it can be taken that that reaction rate was proportional to the amount of the Fe^{2+}–enzyme complex formed. As the results conform to a Michaelis–Menten relation, the reaction between Fe^{2+} and the reducing agents (see p. 690) apparently does not effectively lower the Fe^{2+} concentration.

As the final enzyme preparation was subjected to prolonged dialysis against citrate buffer (pH 5·7) before use, it was assumed that there was little or no Fe^{2+} remaining in the preparation. It was on this basis that the dissociation constants were calculated to be $3{\cdot}9 \times 10^{-6}$ M in the presence of cysteine and $1{\cdot}7 \times 10^{-5}$ M in the presence of ascorbic acid. The above values may not be precise in view of the fact that the enzyme did possess slight residual activity in the absence of both Fe^{2+} and a reducing agent (see Fig. 1). The values for the dissociation constants may vary from one preparation to another. However, it is clear that aconitase has a very high affinity for Fe^{2+}. It can also be seen in Fig. 4 that the absolute value of the maximum rate in the presence of cysteine is over twice as great as that obtained in the presence of ascorbic acid.

The relationship between the enzyme activity and the concentration of Fe^{2+} in the presence of thioglycollate was also determined. In this case, the enzyme activity fell off so sharply that it was not possible to determine accurately the reaction velocities under the same conditions as used for cysteine and ascorbic acid. The enzyme activity at a Fe^{2+} concentration of 5×10^{-5} M was only about 8 % of the activity at a Fe^{2+} concentration of 10^{-4} M.

Role of reducing agents on the action of aconitase

The above experiments indicate that reducing agents as well as Fe^{2+} are concerned in the formation of the active aconitase complex. Therefore, an investigation was made of the effect of varying concentrations of reducing agents on the aconitase activity in the presence of 5×10^{-4} M-Fe^{2+}.

Cysteine, thioglycollate and ascorbic acid. The observations on the relationship between the concentration of cysteine, thioglycollate and ascorbic acid and the reaction velocity, as shown in Fig. 5, conform to a simple Michaelis–Menten relation over the concentrations used, i.e. to the equation $v = Vc/(K_c + c)$, where v = initial velocity of the reaction at a standard concentration of substrate, in the presence of 5×10^{-4} M-Fe^{2+}; V = maximum reaction velocity; c = concentration of the reducing agent, and K_c = dissociation constant of the reducing agent–enzyme complex, formed according to the equation

$$\text{enzyme} + \text{reducing agent} \rightleftharpoons \text{enzyme–reducing agent}.$$

It should be pointed out that the results obtained with cysteine and thioglycollate were somewhat variable. The points obtained in a number of experiments were scattered around, rather than lying directly on a straight line. The results illustrated in Fig. 5 are typical of a number of experiments where the points fell on a straight line. In all experiments the values obtained for the reciprocals of the reaction velocities at each concentration of reducing agent were within + 10 % and − 5 % of the values shown. At lower concentrations of these reducing agents (below 2×10^{-3} M), the points obtained fell well above the straight line passing through the points obtained with higher concentrations. It would seem as though these variations could be related to the catalytic oxidation of cysteine and thioglycollate by Fe^{2+} in the presence of dissolved oxygen. With low concentrations of these substances the relative reduction in the concentration would be marked. On the other hand, with ascorbic acid the points obtained at all concentrations invariably fell on a straight line. This substance is not catalytically oxidized by Fe^{2+}. However, it was clear that the activity of aconitase is dependent on the concentration and nature of the reducing agent. The results can be interpreted to indicate that each reducing agent combines with aconitase in the presence of Fe^{2+} in the ratio of one molecule of the reducing agent to one active centre of the enzyme to form an enzyme–reducing agent

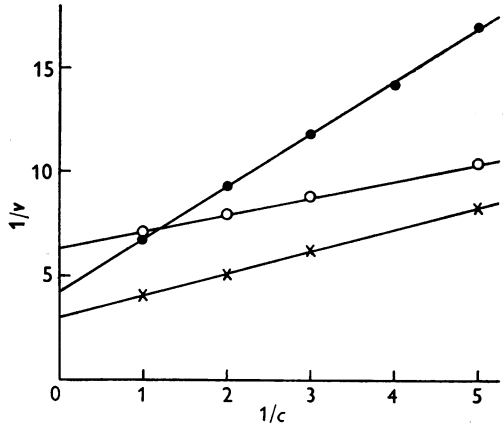

Fig. 5. The effect of cysteine, thioglycollate and ascorbic acid concentrations on aconitase activity in the presence of Fe^{2+}. The graphs are plotted according to the method of Lineweaver & Burk (1934). Aconitase (60 μg. of the final preparation/ml.) was incubated with 5×10^{-4} M-Fe^{2+} and various concentrations of the reducing agents for 1 hr. at 0° and pH 7·4. The same enzyme solution was used in all experiments. The enzyme activity was determined as described in Fig. 4. c, concentration of reducing agent $\times 10^2$ M; v, μg. of citrate formed/15 min. $\times 10^{-2}$; ×, cysteine; ○, ascorbic acid; ●, thioglycollate.

complex. No activities were obtained unless Fe^{2+} was also present. Again this interpretation is valid only because the system had attained equilibrium before the reaction rates were determined.

The dissociation constants of the enzyme–reducing agent complexes were calculated to be $2{\cdot}3-3{\cdot}6\times10^{-3}$M for cysteine, $4-6\times10^{-3}$M for thioglycollate and $1{\cdot}2\times10^{-3}$M for ascorbic acid. These values indicate that the affinity of the enzyme for ascorbic acid is greater than for cysteine, which in turn is greater than that for thioglycollate. There is no relationship between the affinities of the enzyme for the reducing agent and the maximum activity of the enzyme in the presence of the same reducing agents. Cysteine gives the greatest maximum velocity, ascorbic acid the lowest, whilst thioglycollate gives an intermediate value.

Glutathione. When glutathione was tested for its ability to activate aconitase, it was seen (Table 2) that very little activation was obtained at a concentration of 0·01M. However, an appreciable activation of the enzyme was obtained with 0·025M glutathione. A study of the effect of glutathione concentration on the enzyme activity, as shown in Fig. 6, indicates that over the range of concentrations required for enzyme activity there is no simple Michaelis–Menten relation as obtained with cysteine, thioglycollate and ascorbic acid. Thus the mechanism of the activation must differ in some respect from that obtained with the other reducing agents.

DISCUSSION

The marked activation of aconitase by Fe^{2+} in the presence of a reducing agent and the failure of other metal ions to activate, indicate that Fe^{2+} is a specific integral component of the aconitase system. The observations on the relationship between the Fe^{2+} concentration and the enzyme activity are consistent with the idea that Fe^{2+} reacts with the enzyme in the ratio of one ferrous ion to one active centre of the enzyme. Thus the Fe^{2+}–enzyme complex can be considered as an active component of the system. If this complex only were concerned in the activation, then when the Fe^{2+} concentration rises sufficiently the limiting concentration of the Fe^{2+}–enzyme complex should be independent of the nature of the reducing agent. As the activation of the enzyme is further increased by the presence of a reducing agent and influenced by the type of reducing agent, this cannot be the only reaction concerned in the activation. This idea is strengthened by the finding that the dissociation constant of the Fe^{2+}–enzyme complex varies according to the reducing agent present.

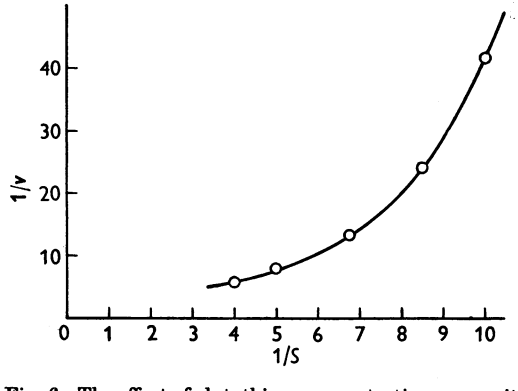

Fig. 6. The effect of glutathione concentration on aconitase activity. The graph is plotted according to the method of Lineweaver & Burk (1934). The conditions were the same as those described in Fig. 5. *S*, concentration of glutathione × 10M, and *v*, μg. of citrate formed/15 min. × 10^{-3}.

The function of the reducing agents is not likely to be related to their ability to maintain the iron in the ferrous state as suggested by Dickman & Cloutier (1951). The concentration of the reducing agents was always in excess of that of Fe^{2+} and the oxidation–reduction potentials are such that they favour the reduction of iron, so only a small amount of Fe^{3+} must be present under the conditions of these experiments. Therefore, it is unlikely that a Michaelis–Menten relationship between the concentration of the reducing agents and the enzyme activity would be found if this were the sole function of the reducing agents. Moreover, if this were the case, the maximum velocity of the reaction should be independent of the nature of the reducing agent. It is also unlikely that the activation of aconitase by the reducing agents is concerned with the reduction of disulphide linkages on the surface of the enzyme (Dickman & Cloutier, 1951). The Michaelis–Menten relations indicate that only one molecule of reducing agent reacts with each active centre, and preliminary studies with arsenical inhibitors indicate that aconitase is a monothiol enzyme (unpublished work). If this is the case and provided that there was no steric hindrance, the active monothiol enzyme molecules could give rise to inactive dimers as a result of the formation of disulphide linkages. However, if the reducing agents were concerned in splitting disulphide linkages, it would be expected that the maximum enzyme activity would be independent of the nature of the reducing agent.

It seems as though the results with cysteine, thioglycollate and ascorbic acid can best be interpreted by considering that they react with aconitase in the ratio of one molecule of the reducing agent to each active centre of the enzyme. The reducing agent can then be considered as taking part in the formation of the active enzyme complex.

The situation is that one molecule of reducing agent and one ferrous ion react with aconitase to form the active complex. Schubert (1932) showed

that cysteine and thioglycollate react with Fe^{2+} to form a ferrous–biscysteine or a ferrous–bisthioglycollate complex, and possibly ascorbic acid can form the same type of complex. Therefore, it seems as though Fe^{2+} might react with aconitase and that the Fe^{2+}–enzyme reacts in turn with one molecule of reducing agent to form the active complex. The nature of this complex would be determined by the reducing agent. The results clearly show that the equilibria

$$\text{enzyme} + Fe^{2+} \rightleftarrows Fe^{2+}\text{–enzyme}$$

and

$$\text{enzyme} + \text{reducing agent} \rightleftarrows \text{enzyme–reducing agent}$$

are not independent.

A similar pattern for the activation of arginase by Fe^{2+}–cysteine and Fe^{2+}–ascorbic acid was reported by Purr & Weil (1934). These authors concluded that the activation mechanism is probably connected with a specific oxidation–reduction potential. However, the activation of this enzyme must differ from that of aconitase in so far as activation can also be brought about by other metallic ions alone, e.g. Co^{2+} and Mn^{2+} (Mohamed & Greenberg, 1945). With aconitase, it remains to be determined whether or not the organic activator must be a reducing agent and/or a compound capable of forming a complex with Fe^{2+}. Until this point is decided, it may be better to consider the active form of aconitase as being an enzyme–Fe^{2+}–activator complex.

Since the substrates of aconitase are also capable of forming complexes with Fe^{2+} (Dickman & Cloutier, 1951) it is tempting to suggest that the remaining free valencies of Fe^{2+} are concerned in linking the substrate to the enzyme. On the other hand, if Fe^{2+} alone were concerned with linking the substrate to the enzyme and the reducing agent were capable of reacting with the Fe^{2+} bound to the enzyme, there would be in effect competition between the activators of the enzyme and the substrates. At the same time, it must be borne in mind that the Fe^{2+} and reducing agents could function by fixing the degrees of freedom of the enzyme, so that the substrate could be linked to some other part of the enzyme molecule.

The results with glutathione do not completely fit in with the above hypothesis. Glutathione does activate aconitase, but if an enzyme–Fe^{2+}–glutathione complex is required for activity, the glutathione dissociation differs from that with the other activators. Moreover, the concentration of glutathione required for maximum activation is very much higher than that required with the other reducing agents. It is interesting to compare this result with those obtained by Kubowitz (1935) on the reaction between Fe^{2+} and glutathione. He found that it was possible to form a carbon monoxide complex of ferrous glutathione only in the presence of high concentrations of glutathione and concluded that this was due to the high dissociability of the ferrous–glutathione complex. With a Fe^{2+} concentration of 2×10^{-3} M, he found that the concentration of glutathione required to form the carbon monoxide complex of ferrous glutathione was 10^{-1} M. On this basis, a Fe^{2+} concentration of 5×10^{-4} M would require a glutathione concentration of $2{\cdot}5 \times 10^{-2}$ M to form the complex. This is in fact the concentration of glutathione found to be required for maximum activation of aconitase. It seems to follow from these results that the activation of aconitase by glutathione is related to its ability to form a Fe^{2+} complex.

The problem of the activation of aconitase is indeed a complex one. Although the reducing agents increase the activity of the enzyme above that obtained with Fe^{2+} alone, it is clear that they are not essential for aconitase activity; the addition of Fe^{2+} alone greatly enhances the activity of the purified enzyme. The complexities of the equilibria between the Fe^{2+} and the enzyme, activators and substrates of aconitase further complicate the problem and make the kinetics of the system difficult. Perhaps further light may be thrown on the problem by the determination of the Michaelis constants and maximum velocities of aconitase for its three substrates in the presence of different organic activators. It would also be of interest to determine whether or not Fe^{2+} can form mixed complexes with each of the activators and the substrates of aconitase.

SUMMARY

1. It was shown that the final purified aconitase preparation possessed little activity in the absence of cofactors. It was not activated by cysteine alone; the addition of Fe^{2+} increased the activity 15-fold, whilst the addition of Fe^{2+} and cysteine gave a 70-fold increase in activity.

2. The optimum conditions required both for activating aconitase and for the estimation of enzymic activity were determined.

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

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

5. A Michaelis–Menten relationship was also found to exist between the enzyme activity and the concentration of the reducing agents cysteine, thioglycollate and ascorbic acid. It was concluded that these compounds form an enzyme–reducing agent complex which is partly responsible for enzyme

activity. Only higher concentrations of glutathione appreciably activated aconitase; there was no simple Michaelis–Menten relationship between the concentration of glutathione and the enzyme activity.

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

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The Relationship of Quaternary Ammonium Salts to the Anionic Sites of True and Pseudo Cholinesterase

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In recent experiments on serum cholinesterase (Bergmann & Wurzel, 1954) evidence for the presence of an anionic site near the esteratic site of the enzyme was obtained and the conclusion was reached that the active surface of this enzyme is essentially similar to that of true cholinesterase. Since the differences between these two esterases have in the past been ascribed to the absence or unimportance of a negative site in the pseudo enzyme (Adams & Whittaker, 1950), it now becomes necessary to explain on a new basis the specific substrate affinity to either enzyme.

Paton & Zaimis (1949), in their study on alkane bistrimethylammonium salts ('methonium' compounds), reported only little inhibitory activity towards serum cholinesterase, but pronounced activity against laked erythrocytes of rabbits. The results of Bergmann & Wurzel (1954), obtained under different experimental conditions, were not in accord with their findings and indicated that the ratios of affinities of inhibitors of the methonium series towards the two enzymes reverse with increasing chain length. It was expected that a quantitative study of this problem would not only clarify the differences reported, but also contribute to a more precise definition of the active surface of the two cholinesterases and to an understanding of their physiological role. We have therefore investigated the inhibitory effect of two homologous series of ammonium derivatives, viz. monoacidic and diacidic quaternary ammonium bases with the nitrogen attached to the ends of unbranched paraffin chains, in order to evaluate quantitatively the factors determining the intermolecular forces between these enzymes and their inhibitors.

MATERIALS AND METHODS

Substrates. Commercial acetylcholine bromide (ACh) was used. Benzoylcholine chloride (BCh) was obtained through the courtesy of Dr Aeschlimann, Hoffmann-La Roche, Nutley, New Jersey. Diacetin was supplied by British Industrial Solvents Ltd., London.

Inhibitors. Butane-1:4-bistrimethylammonium dibromide ('tetramethonium') and heptamethonium were a gift of Dr H. J. Barber of May and Baker Ltd., Dagenham, Essex, and decamethonium of Messrs Allen and Hanburys Ltd., Manchester, England. All other methonium compounds were synthesized in our laboratory by a method to be published elsewhere. Monoacidic quaternary ammonium salts were prepared from trimethylamine and alkyl halides in dilute ethanolic solutions. The lower members, methyl to *n*-pentyl, were crystallized as bromides. However, the halides of higher members of the series proved to be too hygroscopic for purification. It was, therefore, necessary to use the perchlorates, the properties of which are summarized in Table 1.