3. Nevertheless, under these conditions, some maltotriose, maltose and glucose are formed by fission of longer chains. The yield of glucose is far below that expected from a random fission.

4. The action of these enzymes on dilute amylopectin yielded, in the early stages, negligible traces of fission products of shorter chain length than 6–8 units. Salivary and fungal (*Aspergillus oryzae*) amylases under the same conditions yielded products of chain length of 3 units and upwards.

5. Dilute amylose in corresponding reactions yielded appreciable quantities of fission products of chain length of 2 units and upwards (malt and salivary) or 3 units and upwards (bacterial and fungal).

6. Salivary amylase does not readily attack the first two, and the bacterial and malt α-amylases the first five linkages from the non-reducing end.

7. Salivary and malt α-amylases readily attack the second and bacterial amylase the third linkage from the reducing end, other linkages nearer this end with increasing difficulty. It is concluded that linkages other than those near the ends here specified are attacked at random.

8. The action of the α-amylases on shorter linear chains, such as maltohexaose and maltotriose, is restricted as regards linkages near the non-reducing end so that fission must often take place nearer to the reducing end than is normal, and more glucose may be formed.

9. A hypothesis is suggested to explain the actions of the α-amylases.

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The Purification of Aconitase

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The enzyme which catalyses the reaction citric acid ⇌ *cis*-aconitic acid ⇌ *iso*citric acid was discovered by Martius & Knoop (1936), and named aconitase by Breusch (1937). Although this enzyme has been known for some 16 years, it has not been isolated in the pure state. Ochoa (1948) was able to concentrate the enzyme by ammonium sulphate fractionation, but there was no increase in the specific acitivity and no further attempts were made to purify it. Buchanan & Anfinsen (1949) obtained a 23-fold purification of aconitase by low-temperature ethanol and ammonium sulphate fractionation. Electrophoretic analysis showed that the preparation consisted of 'three non-homogeneous components', and the purity of the enzyme was estimated to be 30%.

The purification of aconitase has been greatly hampered by its apparent instability. Krebs & Eggleston (1944) found that glycerol stabilized crude enzyme extracts, but Buchanan & Anfinsen (1949) reported that glycerol was without effect in stabilizing purified preparations. They also found

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that whilst cysteine stabilized the crude enzyme, it strongly inhibited the purified enzyme. Citrate or *cis*-aconitate were found to be the most effective stabilizers of aconitase.

An important finding for the further study of the purification of aconitase was made by Dickman & Cloutier (1950). They found that crude solutions of aconitase could be both stabilized and activated by the addition of Fe^{2+} and cysteine. Further work (Dickman & Cloutier, 1951) showed that these agents were also effective in stabilizing and activating more highly purified solutions of the enzyme. Fe^{2+} was the only cation which gave consistent activation of aconitase. Ascorbic acid was as effective as cysteine as a reducing agent, whilst glutathione was only one-half as effective.

As part of a project to extend the preliminary investigations of Peters & Wilson (1952) on the effect of fluorocitrate on aconitase, an attempt was made to purify the enzyme further in the light of the findings of Dickman & Cloutier (1950, 1951). For a preliminary communication see Morrison (1953).

EXPERIMENTAL

Reagents. cis-Aconitic anhydride (m.p. 77°) 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. DL-*iso*Citric acid (m.p. 136°) was prepared by hydrolysis of the lactone. Trichloromethylparaconic acid was prepared by the method of Fittig & Miller (1889) and converted into *iso*-citric lactone by the method of Krebs & Eggleston (1944). Sodium *iso*citrate was formed by neutralizing the acid to pH 7·4 with N-NaOH. Both compounds were prepared by Mr R. W. Wakelin. The ethanol was taken to be 100%. L-Cysteine hydrochloride used in the later work was free from iron and obtained from Roche Products Ltd. The ammonium sulphate was British Drug Houses Ltd. Special Grade, and 2:2′-dipyridyl was a British Drug Houses Ltd. Spot Test reagent. All other reagents were A.R.

Estimation of citrate. The method of Pucher, Sherman & Vickery (1936), as modified by Buffa & Peters (1949), was used. Samples ranging from 1·0 to 3·0 ml. and containing up to 200 μg. of citric acid were used, as within this range the colour of the final solution was proportional to the amount of citric acid present.

Estimation of protein. The protein concentration of aconitase solutions was determined by measurement of the absorption in the Beckman spectrophotometer at a wavelength of 280 mμ. after the appropriate dilution with 0·1N-NaOH. Under these conditions, a solution containing 1 mg. protein/ml. was found to have an absorption of 1·5.

Activation of aconitase. A suitable sample of the enzyme solution was measured into a test tube and placed in an ice bath. Non-neutralized ferrous ammonium sulphate and neutralized cysteine were added, so that on dilution of the solution with cold water to 10 ml., the final concentration of Fe^{2+} was 5×10^{-4} M and cysteine was 0·01 M. The mixture was then neutralized with N-NaOH to pH 7·4, using a glass electrode, and incubated in the ice bath for 1 hr. before the aconitase activity was determined. Except where otherwise stated, the various fractions were activated before an activity determination was carried out.

Determination of aconitase activity. To a series of Pyrex test tubes, 4·4 ml. or 4·3 ml. of water were added; 0·5 ml. of a solution containing 20 μmoles of *cis*-aconitate at pH 7·4 was then added and the test tubes were placed in a water bath at 30°. After an equilibration period of 5 min., the reaction was started by the addition of 0·1 ml. or 0·2 ml. of enzyme solution. The reaction was stopped after 15 min. by the addition of 0·5 ml. of 50% (w/v) trichloroacetic acid. If a visible precipitate formed, it was filtered off through a no. 30 Whatman filter paper, otherwise the citrate estimation was carried out on the acidified solution without further treatment. It was found that small amounts of protein did not interfere with the estimation. Under these conditions, the amount of citric acid formed from *cis*-aconitic acid was proportional to the amount of enzyme added. The activity determinations were carried out at two enzyme levels to ensure that the substrate concentration was not limiting.

Definition of a unit of aconitase activity. One unit of aconitase activity was taken to be the amount of enzyme which formed one μmole of citric acid from *cis*-aconitic acid in 15 min. at pH 7·4 and 30° in the absence of buffer.

Specific activity. This was taken to be the units of aconitase activity/mg. of protein.

*Other enzyme activity determinations. iso*Citric dehydrogenase activity was determined according to the method of Ochoa (1951). One unit was defined as a change of $\log I_0/I$ of 0·01 per min. Fumarase activity was determined by the method of Racker (1950). One unit was defined as a change of $\log I_0/I$ of 0·001 per min.

RESULTS

Activation of aconitase

As the activation of aconitase by Fe^{2+} and cysteine was of importance, the work of Dickman & Cloutier (1951) was repeated using their purified preparation of aconitase. The results were in good agreement with those obtained by these authors. However, the activating effect of cysteine alone is open to criticism. It was found that the preparation of cysteine which gave similar results to those of Dickman & Cloutier (1951) contained appreciable amounts of iron. Nevertheless, the importance of Fe^{2+} and cysteine as activators of aconitase was confirmed. The cysteine used in the later experiments described here was completely free from iron.

Inhibition of aconitase

Having confirmed that Fe^{2+} was an integral component of the system, it seemed likely that the presence of ions, such as phosphate, which are capable of forming insoluble complexes with iron, would lead to inactivation of the system as a whole. In order to show this experimentally, the same enzyme preparation was activated and pre-incubated at 30° with 0·05 M phosphate buffer (pH 7·4) for various periods of time. Substrate was then added and the reaction allowed to proceed for

15 min. In the control system, water replaced the buffer. Fig. 1 shows that phosphate caused a rapid inactivation of the activated enzyme when substrate was absent. When phosphate and substrate were added simultaneously, the enzyme activity was greater than that obtained in the absence of phosphate. This increased enzyme activity was shown to be due to the influence of the ions present in solution on the pH optimum of the enzyme. The enzyme activity was not increased by the simultaneous addition of excess of Fe^{2+} (5×10^{-3} M) and substrate, following pre-incubation for various periods in the presence of phosphate. Thus, phosphate is capable of inactivating the activated enzyme. This finding is in contrast to the results of Dickman & Cloutier (1951), for they found that phosphate did not decrease the activity of an activated preparation. As similar results were obtained with veronal—acetate, borate and glycerophosphate buffers at pH 7·4, enzyme solutions were not diluted with buffers and activity determinations were carried out in the absence of buffer. A check of the pH of the system before and after incubation showed that although there was a small alteration of pH in the absence of buffer, the results were perfectly reproducible.

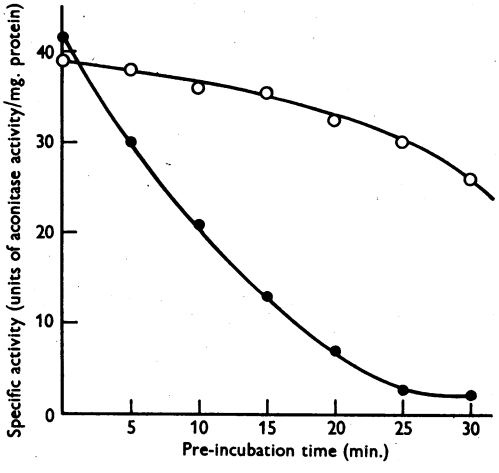

Fig. 1. Effect of incubating aconitase in the absence of substrate. The enzyme was activated with Fe^{2+} and cysteine and incubated at 30° for various periods of time. *cis*-Aconitate was added and the reaction was allowed to proceed for 15 min. ○—○, no buffer present, reagents adjusted to pH 7·4; ●—●, 0·05 M phosphate buffer (pH 7·4).

The inactivating effect of phosphate in the absence of substrate can at least partially explain the instability of the enzyme found by Krebs & Eggleston (1944) and by Peters & Wilson (1952). These workers used phosphate buffer in the test systems. It also renders the assay figures of Johnson (1939) of the aconitase activity of various animal organs open to criticism, as the organs were extracted with phosphate buffer. Phosphate was therefore not used during the course of the purification of the enzyme.

Preparation of aconitase by the method of Buchanan & Anfinsen (1949)

As Fe^{2+} and cysteine had not been used by Buchanan & Anfinsen (1949) in their test system for aconitase activity, it was probable that their final enzyme preparation was, in fact, of a higher specific activity than reported. In order to determine whether or not this was the case, aconitase was prepared according to the method of these workers. A study was then made of the activity of various fractions in the presence and absence of Fe^{2+} and cysteine. Although Buchanan & Anfinsen (1949) carried out their tests at 38°, in the present work the tests were made at 30° and the activities were multiplied by 1·5, the temperature coefficient found for 38°/30°.

The results of Table 1 clearly show that in the absence of Fe^{2+} and cysteine, the specific activities of the intermediate fractions are much lower than those claimed by Buchanan & Anfinsen (1949). It was only in the presence of these two agents that

Table 1. *Comparison of aconitase activities in the presence and absence of* Fe^{2+} *and cysteine*

(Fractions were prepared according to the method of Buchanan & Anfinsen (1949). Activation by Fe^{2+} and cysteine was carried out as described in the text. Temp. 30°; pH 7·4. The results obtained were multiplied by 1·5, the temperature coefficient for 38°/30°, so that they were equivalent to activities at 38°. The specific activity is the units of aconitase activity/mg. protein.)

	Results of Buchanan & Anfinsen (1949)		Present results				
	No additions		No additions		Fe^{2+} + cysteine added		Ratio of activities of activated enzyme
Fraction	Total units	Specific activity	Total units	Specific activity	Total units	Specific activity	non-activated enzyme
Extract	310 000	10·0	261 000	10·5	278 000	11·3	1·07
First ethanol ppt.	133 000	18·9	144 000	26·9	220 500	41·1	1·53
Second ethanol ppt.	102 000	68	100 500	29·3	175 000	51·5	1·74
Third ethanol ppt.	63 000	132	45 000	45	105 000	107	2·35

similar activities could be obtained. A comparison of the activities of the activated and non-activated fractions shows that the degree of activation by Fe^{2+} and cysteine increases as purification proceeds, indicating that the prosthetic group of the enzyme is removed to some extent by the fractionation procedure. It will also be noted that the relative purification of the enzyme in each step differs markedly from that obtained by Buchanan & Anfinsen (1949). Results for the ammonium sulphate step are not given, as it was found that the enzyme was completely precipitated when the solution was brought to 66% saturation. There was no increase in the specific activity.

Method of isolation

The following fractionation procedure was adopted after repeated trials. The superscripts refer to the notes given later.

Extraction of tissue. Immediately after the death of the pig, the heart was removed, cut into strips of about 0·25 in. thickness, freed from fat and connective tissue and placed in ice. At the laboratory, the hearts were minced through a coarse mincer and stored at −15°. Under these conditions, pig heart could be stored for some months without loss of aconitase activity. A 1 kg. sample of frozen heart was allowed to thaw at room temperature and then comminuted in 200 g. lots for 2 min. in a pre-cooled Waring Blendor with 600 ml. of 0·004M citrate buffer (pH 4·7) and 130 ml. of $CHCl_3$.[1] The product was centrifuged at −1° for 15 min. at 1000*g*. The clear red-amber supernatant (pH 5·7)[2] was poured off and filtered through a fluted filter paper (Whatman no. 531).

First ethanol fractionation. For convenience, the supernatant was divided into two equal amounts and fractionated simultaneously. From this stage onwards, all operations were carried out in the coldroom. The supernatant was brought to 0° in a dry-ice : ethanol bath and 90% (v/v) ethanol[3] was added with mechanical stirring at the rate of 250–300 ml./hr., to a concentration of 15% (v/v). During the addition of ethanol, the temperature was lowered to −5°.[4] Hyflo Supercel was added to the solution (5 g./l.) and filtration was carried out on a Büchner funnel under slight pressure (Whatman no. 30 filter paper). To the clear red-amber filtrate, saturated NaCl (1·25 ml./100 ml. of filtrate) was added. The ethanol concentration of the solution was brought to 45%, the temperature being gradually lowered to −10°. The mixture was allowed to stand overnight at −10°.[5]

Second ethanol fractionation. The precipitate was centrifuged off at −10° and stored at this temperature, whilst another 1 kg. of pig heart was treated in the same fashion. The two precipitates were combined and dissolved in 800 ml. of ice-cold water. A small amount of insoluble matter was centrifuged off to yield a clear deep red-amber solution. The solution was brought to 0° and ethanol added to a concentration of 10% (v/v),[6] the temperature being lowered to −4°. A small amount of a white precipitate was centrifuged off at −4°. The pH of the supernatant, which was about 5·6, was adjusted to pH 6·8 with 5% (w/v) sodium carbonate solution. Ethanol was added to a concentration of 23% (v/v), whilst the temperature was lowered to −10°. The precipitate was centrifuged off at −10° and dissolved in 100 ml. of ice-cold water.

Ammonium sulphate fractionation. The aqueous solution was diluted 2·5 times with 0·125M citrate buffer (pH 6·8). $(NH_4)_2SO_4$ (36·7 g./100 ml. of solution) was added slowly, the temperature being lowered to −6°. The yellow precipitate was centrifuged off at −6° for 10 min. at 5000*g*, or alternatively filtered on a Büchner funnel with the aid of Hyflo Supercel. To the supernatant, $(NH_4)_2SO_4$ (9·2 g./100 ml. of supernatant) was added slowly and the temperature was lowered to −10°. The precipitate was centrifuged off at −10° for 15 min. at 5000*g* and dissolved in 90 ml. of 0·004M citrate buffer (pH 5·7) to give a deep-amber solution. This solution was dialysed overnight with stirring against the same buffer.[7]

Heat fractionation. The dialysed solution in 20–25 ml. lots was heated in a water bath for 15 min. at 50° and cooled rapidly in an ice bath. The white flocculent precipitate was centrifuged off at 0° and discarded.

Ethanol fractionation at pH 8·0. The supernatant was cooled to 0° and ethanol was added to a concentration of 20% (v/v), the temperature being lowered to −5°. A small precipitate was centrifuged off at −5° and discarded. The supernatant was adjusted to pH 8·0 with a saturated solution of $NaHCO_3$ and ethanol was added to a concentration of 50% (v/v).[8] During the addition, the temperature was lowered to −12°. The reddish precipitate was centrifuged off from a cloudy supernatant (due to the presence of $NaHCO_3$) at −12° and dissolved in 20 ml. of 0·004M citrate buffer (pH 5·7).

Ammonium sulphate fractionation at pH 8·5. After adjustment of the solution to pH 8·5 with ammonia, a saturated solution of $(NH_4)_2SO_4$ (pH 8·5)[9] was added dropwise until the saturation was 0·65. The temperature was gradually lowered to −10°. The precipitate was centrifuged off at −10°, dissolved in 10 ml. of 0·004M citrate buffer (pH 5·7) and dialysed against the same buffer overnight at 0°. The final solution was amber in colour.

Notes. (1) $CHCl_3$ reduced the haemoglobin content of the extract. There was no denaturation of the enzyme and the specific activity of the extract was increased.

(2) Marked loss of enzyme activity occurred when the pH of the extract was adjusted below pH 5·0.

(3) 90% (v/v) ethanol was used throughout the fractionation.

(4) It was important that the solution should not freeze at this stage, as freezing leads to loss of enzyme activity.

(5) Contact of the aconitase with ethanol for this period had no harmful effect, for the specific activity was the same whether the solution was centrifuged immediately, or allowed to stand overnight.

(6) The ethanol already present was neglected.

(7) The enzyme could be dialysed against citrate buffer without loss of activity, whereas large losses of activity occurred when the dialysis was carried out against water and salt solutions.

(8) During the fractionation at alkaline pH values, care was taken to prevent CO_2 from distilling over from the dry-ice : ethanol bath into the protein solution.

(9) A saturated solution of $(NH_4)_2SO_4$ at room temperature was neutralized with strong ammonia, so that on dilution of 1 in 5, the pH was 8·5.

Attempts to purify the enzyme further were unsuccessful. Large amounts of calcium phosphate

gel were required to adsorb the enzyme from solution between pH 5·7 and 6·8 and no differential adsorption or elution could be obtained. Although the enzyme could be stirred with butanol for 30 min. at room temperature, this treatment did not alter the precipitation pattern after further ethanol fractionation. No protein was precipitated by the nucleic acid precipitants manganese sulphate and protamine sulphate. Fractionation with acetone precipitated the enzyme between 30 and 50% (v/v) acetone concentration, but marked loss of activity occurred even though the fractionation was carried out between $-5°$ and $-10°$. This finding is in contrast to that of Buchanan & Anfinsen (1949), but is consistent with the fact that acetone powders of pig heart are devoid of aconitase activity. The enzyme was not precipitated by sodium chloride or ammonium acetate. Ethanol fractionation in the presence of zinc was ineffective, presumably because the zinc reacted with the citrate present.

Although the enzyme constituted the major portion of the final solution, it could not be induced to crystallize. The slow addition of ethanol always gave rise to an amorphous product. Attempts were also made to crystallize the enzyme from ammonium sulphate solutions by the addition of an amount just insufficient to cause precipitation, followed by adjustment of the pH. Within the pH range 5·7–8·5, both in the presence and the absence of Fe^{2+} and cysteine, the enzyme precipitated only in small amounts and in an amorphous form.

In Table 2 is given a summary of the yields and degrees of purification of aconitase during the fractionation procedure. This procedure brings about a 24-fold increase in purity of aconitase, with an overall recovery of about 12%. The final product has a specific activity of about 265 when the test is carried out in the absence of buffer. If the test is carried out in 0·05 M phosphate buffer (pH 7·4), the specific activity is increased to 336. This increase is due to the fact that the pH optimum of aconitase is dependent upon the ions present in solution. The variation of the pH optimum in different buffers will be reported later. Thus, at 38° the specific activity is about 500, which corresponds to a value of 235 as claimed by Buchanan & Anfinsen (1949) for their final preparation.

General properties of aconitase

The enzyme in dilute citrate buffer at pH 5·7 retained its activity for many weeks when stored in the frozen state and was unaffected by repeated freezing and thawing. It was not found necessary to add Fe^{2+} and cysteine as stabilizing agents, although in the absence of both or either of these agents the enzyme activity was markedly reduced. (Details of the activation of aconitase will be reported later.) The final enzyme preparation could also be freeze-dried without loss of activity, but it was found more convenient to keep it in the frozen state. Fig. 2 shows that aconitase is stable to heat until a temperature of 51° is attained, after which there is a sharp loss of activity. A characteristic feature of the enzyme was its wide range of precipitation. Aconitase activity was found in every fraction obtained with all solvents and salts used over the pH range from 5·7 to 8·5.

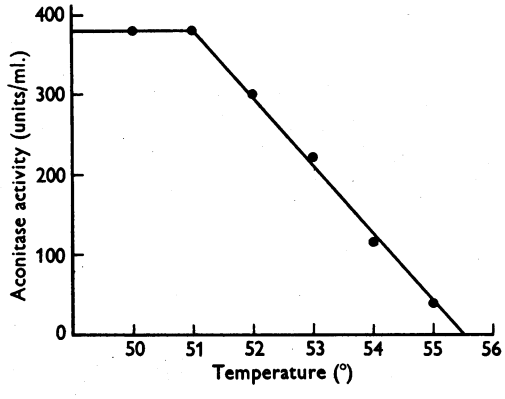

Fig. 2. The effect of temperature on aconitase activity. The protein concentration was 9 mg./ml. Heating was carried out in a water bath for 15 min., using 2·0 ml. samples of a solution of the dialysed $(NH_4)_2SO_4$ precipitate. Aconitase activity was determined after activation with Fe^{2+} and cysteine as described in the text. Temp. 30°, pH 7·4.

Table 2. *Summary of yields and specific activities of fractions obtained during the purification of aconitase from* 2 kg. *pig heart*

(The specific activity is the units of aconitase activity/mg. protein. For other details see text.)

Fraction	Volume (ml.)	Total protein (g.)	Total units	Specific activity	Yield (%)
Extract	6240	39·3	437 000	11·1	100
First ethanol ppt.	832	5·3	260 000	49	60
Second ethanol ppt.	112	2·2	212 000	96	49
Dialysed ammonium sulphate ppt.	116	0·98	157 000	168	36
Supernatant after heating at 50°	102	0·72	135 000	188	31
pH 8·0 ethanol ppt.	25	0·41	96 000	232	22
pH 8·5 ammonium sulphate ppt.	15	0·19	50 400	265	12

Electrophoretic analysis

The electrophoretic pattern of the final preparation of aconitase is illustrated in Fig. 3. All the components migrated to the anode when electrophoresis was carried out at pH 8·6, but at pH 5·7 at least two of the minor components still migrated to the anode whilst the main component migrated to the cathode. pH values below 5·0 could not be used owing to denaturation of the enzyme.

In order to determine whether or not the main component was responsible for the aconitase activity, a second electrophoretic run was carried out after dialysis of a 1% solution of the protein against veronal buffer at pH 8·6 (ionic strength 0·075) which contained sodium citrate (ionic strength 0·025). A similar electrophoretic pattern was obtained. Enzymic analysis of the main component after separation showed that this component was responsible for the aconitase activity, whilst the other components were devoid of activity. Both fractions were dialysed overnight against 0·004 M citrate buffer (pH 5·7) and activated with Fe^{2+} and cysteine before the aconitase activity was determined. Unfortunately, the enzyme lost activity during the dialysis and electrophoresis. The value obtained for the specific activity of the electrophoretically homogeneous protein was less than that of the original solution. The main component was completely free from the amber pigment which was associated with the enzyme during the stages of purification. The mobility of the amber protein was somewhat similar to that of aconitase, so that electrophoresis had to be continued for a long period to effect separation. It may be calculated that the purity of the electrophoretically homogeneous protein which possesses aconitase activity in this preparation is 75–80%. If this component consists entirely of aconitase, it would appear that aconitase represents a large proportion of the total protein of heart muscle.

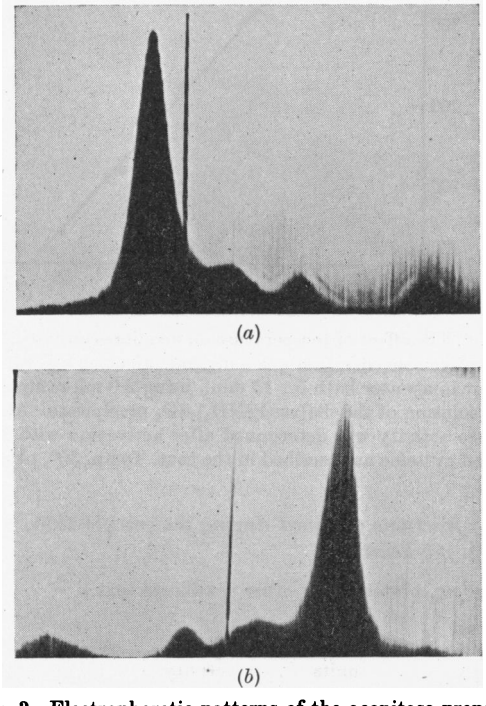

Fig. 3. Electrophoretic patterns of the aconitase preparation obtained after fractionation as described in the text. Veronal buffer: pH 8·6; ionic strength, 0·1; temperature, 1°; current, 10 mA; protein concentration, 1%; time, 168 min. (*a*) Descending boundary; (*b*) ascending boundary.

Conversion of cis-*aconitate and* iso*citrate to citrate*

Jacobsohn, Soares & Tapadinhas (1940), on the basis of kinetic measurements, have postulated that aconitase might consist of two distinct enzymes, α-aconitase catalysing the reaction *cis*-aconitate $\rightleftharpoons$ *iso*citrate, and β-aconitase catalysing the reaction *cis*-aconitate $\rightleftharpoons$ citrate. Martius & Leonhardt (1943) questioned this finding, and up to the present time there has been no definite evidence for the existence of two aconitases. Buchanan & Anfinsen (1949) found that the ratio of the activity of the enzyme in forming citrate and *iso*citrate from *cis*-aconitate remained practically constant during the fractionation procedure. They also found that the two activities were not separated by electrophoresis. However, the active fraction was contaminated by a coloured protein which was considered to be a haemoglobin or myohaemoglobin or both.

In this work it has been shown that the coloured component does not possess aconitase activity. The electrophoretically homogeneous protein which showed aconitase activity was devoid of colour and was capable of converting both *iso*citrate and *cis*-aconitate into citrate. Thus it is established that a single protein which is homogeneous in the electrophoresis apparatus can bring about those reactions associated with aconitase activity. This finding is consistent with the hypothesis advanced by Ogston (1951) as a result of theoretical considerations. It is, of course, possible that the protein is not homogeneous in the ultracentrifuge and this point will be investigated later.

Activation of aconitase fractions by Fe^{2+} *and cysteine*

During the purification, various fractions were tested for the ability to convert both *iso*citrate and *cis*-aconitate into citrate. Table 3 shows that the ratio of the two activities remains constant within the limits of experimental error. The addition of Fe^{2+} and cysteine increased the two activities equally, so that there was no alteration in the ratio. The activating effect of Fe^{2+} and cysteine during the

Table 3. *Comparison of the rates of citrate formation from* isocitrate *and* cis-*aconitate by activated and non-activated fractions of aconitase*

(Fractions were obtained and activated as described in the text.)

	Specific activities (units of aconitase activity/mg. protein)						Ratio of activities of activated enzyme / non-activated enzyme	
	Non-activated			Activated				
Fraction	(*a*) *cis*-Aconitate	(*b*) *iso*-Citrate	Ratio *a* : *b*	(*c*) *cis*-Aconitate	(*d*) *iso*-Citrate	Ratio *c* : *d*	*cis*-Aconitate	*iso*-Citrate
Extract	7·0	4·35	1·61	11·4	7·2	1·59	1·63	1·66
First ethanol ppt.	31	17·5	1·78	49	31	1·58	1·6	1·8
Second ethanol ppt.	50	32·5	1·54	96	56	1·71	1·9	1·7
Ammonium sulphate ppt.	72·5	45·5	1·59	151	97	1·56	2·1	2·1
Dialysed ammonium sulphate ppt.	7·0	4·7	1·49	142	85	1·67	20	18
Supernatant after heating at 50°	6·7	4·2	1·60	174	113	1·54	26	27

early stages of purification was small and remained reasonably constant. After dialysis the activation was greatly enhanced. Fe^{2+} and cysteine can, therefore, completely replace the dialysable prosthetic group. The addition of 10^{-4} M 2:2′-dipyridyl to the dialysis medium caused the formation of ferrous tridipyridyl inside the dialysis sac, and this complex slowly diffused out into the medium. No tests have, as yet, been carried out for the identification of a reducing substance in the dialysis medium. It would appear that not only is Fe^{2+} capable of activating aconitase, but it is also the metal with which the enzyme is associated *in vivo*. The results also suggest that the Fe^{2+} is bound to the enzyme sufficiently strongly to resist splitting as a result of ethanol and ammonium sulphate fractionation, but the linkage is readily broken by dialysis.

Tests for the presence of other enzymes in the final preparation

The final preparation contained 196 units of *iso*citric dehydrogenase activity/mg. of protein, and 1100 units of fumarase activity/mg. of protein. No evidence was found for the presence of lactic dehydrogenase. Thus it is possible that two of the three minor components of the electrophoretic pattern are due to the presence of *iso*citric dehydrogenase and fumarase.

SUMMARY

1. The activation of aconitase by Fe^{2+} and cysteine has been confirmed. Phosphate was shown to inactivate rapidly the activated enzyme in the absence of substrate.

2. Aconitase has been purified 24-fold by low-temperature ethanol and ammonium sulphate fractionation combined with heat fractionation. The purity of the final preparation was estimated to be 75–80 %.

3. The final aconitase preparation was contaminated to a small extent by *iso*citric dehydrogenase and fumarase.

4. Aconitase has been isolated from the final preparation as an electrophoretically homogeneous protein. This protein was free from pigment and was capable of converting both *iso*citrate and *cis*-aconitate into citrate.

5. During the purification, the degree of activation of aconitase by Fe^{2+} and cysteine increased, especially after dialysis.

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