BBA 66193

ACONITATE HYDRATASE

SOME NEW OBSERVATIONS WITH ESPECIAL RELATION TO ITS USE AS A TEST SYSTEM FOR FLUOROCITRATE, TOGETHER WITH STUDIES ON THE ELECTROPHORETIC SEPARATION OF COMPONENTS OF THE CRUDE ENZYME

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(Received June 29th, 1970)

SUMMARY

1. Preparations of aconitate hydratase (citrate (isocitrate) hydro-lyase, EC 4.2.1.3) from pig heart, purified to the first ethanol stage have been studied, and evidence supporting the existence of two centres has been confirmed; wide variations in the ratio of the isocitrate hydro-lyase to that of the citrate hydro-lyase have been found. Additive effects occur on the enzymatic action when one of these substrates is added to the other, both additions being at v_{max}. Fluorocitrate never inhibits these mixed enzymes completely.

2. Our crude preparations are relatively stable at −19°; their activity is not usually increased by addition of ferrous salts with or without cysteine. There is no inhibition by adding Fe^{2+} *plus* oxaloacetate.

3. Attempts have been made to fractionate our basic preparation with electrophoresis on starch gel, polyacrylamide gel, and the latter *plus* ampholine pH 7–9. With starch gel 15 proteins separated towards anode, 5 towards cathode, 3 gave activity for isocitrate hydro-lyase and 2 for citrate hydro-lyase. Using ampholine and polyacrylamide gel, approx. 19 protein bands were seen; 9 bands stained for isocitrate hydro-lyase activity, and 6 for citrate using sucrose gradient (ampholine) there was wide separation; some fractions separated at the anode and cathode, respectively. The behaviour of these fractions was variable. Some gave both hydro-lyase activities; in others one or the other predominated. Some were inhibited by fluorocitrate, and others were comparatively insensitive to this.

4. Activation of the crude enzymes has been seen with various purines and with thiamine, and inhibition with tannic acid. The hypothesis is advanced that the activation is related to some conformational change in the active centres.

Abbreviation: TEMED, *N,N,N',N'*-tetramethylethylamine diamine.

INTRODUCTION

In this communication some new facts are recorded about aconitate hydratase (citrate (isocitrate) hydro-lyase, EC 4.2.1.3) found while testing for fluorocitrate. The enzyme preparation used from pig heart was purified to the first ethanol stage in the method of MORRISON[1] as used by PETERS[2]; this stage does not require activation. We have found more facts to support our view[3] that the enzyme is a complex with two active centres, citrate hydro-lyase and isocitrate hydro-lyase, which can age at different rates. Hence we have reverted to earlier views[4,5]. GUARRIERA-BOBYLEVA AND BUEFA[6] have found recently that intramitochondrial and extramitochondrial aconitate hydratase are not the same enzyme; the latter is much less inhibited *in vitro* by fluorocitrate. *In vivo* the citrate hydro-lyase is specifically poisoned by the monofluoro compounds. These facts indicate that there may be at least 4 enzymatic centres concerned in aconitate hydratase activity, *viz.* a citrate hydro-lyase, an isocitrate hydro-lyase, specific for both mitochondria and cytoplasm. At this stage we cannot exclude on logical grounds that there may be more; and new facts obtained by ourselves recorded in this communication as well as by KOEN AND GOODMAN[7] support this. Other facts difficult to reconcile with current views[8] are that PALMER[9] purified an aconitate hydratase from plants which appeared to contain no essential Fe^{2+}, and again that synthetic fluorocitrate was 100% less inhibitory to the aconitase in single-cell cultures of sycamore than to a heart preparation[10].

This communication also describes some instances of activation and inhibition, not previously described[2,11], as well as an unsuccessful attempt to confirm with our preparations an inhibition with oxaloacetate and Fe^{2+} found by BRITTEN[12].

METHODS

Chemicals

We are grateful to Dr. Vickery for the sample of potassium D,L(+)-isocitrate used. Caffeine was AnalaR. Theophylline and theobromine were British Pharmacopaeia, adenosine, Roche; ADP and ATP (sodium salts) from Sigma. Thiamine (synthetic) was presented by the Ministry of Food. Thanks are due to Mr. Brown for sodium fluorocitrate (synthetic). Other chemical substances were AnalaR.

Enzymatic tests

Observations were made with the Beckman D.K. spectrophotometer, of the formation of *cis*-aconitate, (double bond at 240 mμ). Concentrations and conditions are given in the legends to the tables; these follow those used earlier[2], except that the temperature was 30° instead of 22°. Amounts of the substrates giving v_{max} were employed; an increase of 20% in the concentration of substrate gave no increase in the initial velocity. When the inhibitory power of a fluorocitrate preparation was tested, it was incubated with the enzyme for 10 min before the addition of the substrate. This produced larger inhibitions than the method described earlier[2].

There is evidence now that fluorocitrate may inhibit reversibly as well as irreversibly[2,13]. In this connection it is noticeable that the initial rate of formation of the double bond after incubation of the enzymes with fluorocitrate is slower for 0.5–1 min, than the later activity. This can be interpreted as the reversible reaction.

Isoelectric focussing in gels

The method used of setting up the tubes was as follows (Personal communication from Dr. A. J. BARRETT): Stock solutions: (i) Acrylamide stock. Acrylamide (recrystallized) 28.0 g, bis-(*N*,*N*-methylene bisacrylamide) 0.70 g. Water to 100 ml. (ii) Catalyst stock. Riboflavin 7.0 mg; TEMED (*N*,*N*,*N'*,*N'*-tetramethylethylamine diamine) 0.70 ml. Water to 100 ml (in a brown bottle). (iii) Acrylamide stock 3.2 ml, catalyst stock 0.9 ml, ampholine (40%) 0.4 ml.

Make up freshly in a brown bottle, when required. Degass the gel stock before use in a desiccator connected to a filter pump, and also degass the samples made up to 1 ml each. Each sample should contain 50–300 μg protein.

Mix 0.5 ml gel stock with each sample, transfer to the running tubes (6.5 cm × 0.5 cm internal diameter). Polymerize gels in the cold, by exposure to fluorescent tube, after layering with water.

The electrode runs are set up so that the top (+) has 0.2% H_3PO_4 and the bottom (−) 2.0% TEMED.

The gels are run overnight with a potential of 75 V, and then stained for protein or enzyme activity.

For proteins, fix after the run in 5% trichloroacetic acid for 1 h; stain with coomassie brilliant blue.

For detection of enzyme activity, transfer the gels to small test tubes containing the following: phosphate buffer (pH 8.0) containing 0.05% $MgCl_2$, 20.0 ml; 3 mg $NADP^+$ in 0.3 ml water; 100 μg isocitrate dehydrogenase (Boehringer) in 0.1 ml; 0.25 ml nitroso blue tetrazolium (2 mg/ml) and 0.025 ml phenazine methosulphate 1%. 1 mg sodium *cis*-aconitate (0.1 ml) or 10 mg sodium citrate (0.1 ml). Total volume is 20.8 ml. Of this 5 ml is used per gel with or without 100 μg sodium fluorocitrate.

The technique used for isoelectric focussing in bulk is given below.

RESULTS

Enzyme experiments

The crude preparations after purification to the first ethanol stage[1], were stored after division into test tubes at −19°, some with water and some with citrate (0.004 M); the latter were more stable, up to 10 months; and the isocitrate hydro-lyase activity was more stable than the citrate. (For examination by electrophoresis, see below.)

Dual character of the aconitate hydratase complex

Further support was obtained for the presence of two enzymatic centres. When citrate at v_{max} was added to isocitrate acting upon the enzyme preparation also at v_{max}, there was an increased rate of enzymatic activity, and *vice versa* (Fig. 1). When the two substrates were added together both at a concentration giving v_{max}, there was an increased formation of double bond.

In one case, some of our usual preparation was taken to a further stage[1]; after dialysis, citrate hydro-lyase activity had disappeared. Usually the citrate hydro-lyase activity was less than the isocitrate hydro-lyase after storage. However, we once found a preparation giving a greater citrate hydro-lyase activity in a further precipitate coming down on cold storage from the first ethanol stage.

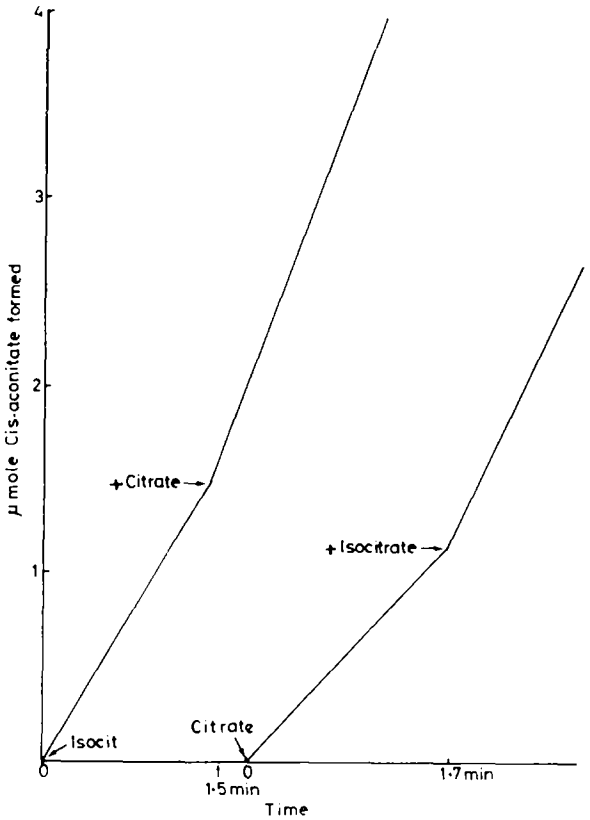

Fig. 1. Observations were made on a Beckman D.K. spectrophotometer at 240 mμ. The cuvettes contained in a total volume of 2.0 ml: 0.05 M Tris buffer (pH 7.4); 0.01 ml enzyme (110 μg protein); either 0.05 ml 30 μM potassium D$_s$(−)-isocitrate or 0.05 ml 600 μM sodium citrate was added to start the reaction after equilibration for 10 min at 30°. Additions of the compounds mentioned were made in volumes of 0.01–0.1 ml, before the incubation, which were incubated in the cuvette during the 10-min period. After this the Tris buffer was added, followed by the substrate. The enzyme was purified as far as the first ethanol stage of the method of MORRISON[1] as described by PETERS[2]; the Fe^{2+} *plus* cysteine activation is not necessary.

Iron activation

In the past, the presence of Fe^{2+} has been found to be specifically connected with pig heart aconitase[14,12]. In 16 experiments we have examined the effects of Fe^{2+} and Fe^{3+} on our enzymes at the first ethanol stage with virtually negative results. Only in one instance was an increase of 28% observed with ferrous ammonium sulphate and cysteine. In this case, we kept the nucleus and cell debris in the deep freeze for 11 days previous to extraction, as recommended by DICKMAN AND SPEYER[15] for liberating mitochondrial aconitase. This observation suggests that we may have mainly the extramitochondrial aconitate hydratase of GUARRIERA-BOBYLEVA AND BUFFA[6].

Following BRITTEN[12], we have tried to find an inhibitory effect of Fe^{2+} *plus* oxaloacetate, without success. Dr. J. S. BRITTEN (personal communication) has told us that he has found no evidence for an inhibition by oxalomalate[16]. Furthermore we

could not find oxaloacetate present in our enzymes by enzymatic tests for this.

There is no doubt that care must be used in interpreting small effects of Fe^{2+} at 240 mμ, because we found that when Fe^{2+} and citrate are added together there is a very rapid absorption, which is due to the formation of ferrous citrate. Curiously this does not happen in the presence of the enzyme preparation.

Another difference between our enzymes and those of others is in the reaction to phosphate. When this is introduced into our crude enzymes before the addition of substrate, there is no evidence of inhibition, even when working with enzymes stored in water. Phenanthroline[11] also has no action in a period of 10 min. If this had been removing an essential Fe^{2+}, we should have expected a quicker action.

Except in the one instance (above) we were unable to get Fe^{2+} activation, until we tried this on some of our fractions from the LKB ampholine focussing (see below).

Electrophoretic separation on gels

This was attempted in the hope of finding enzymatic centres specially sensitive to fluorocitrate. Our crude preparations were subjected to electrophoresis on starch

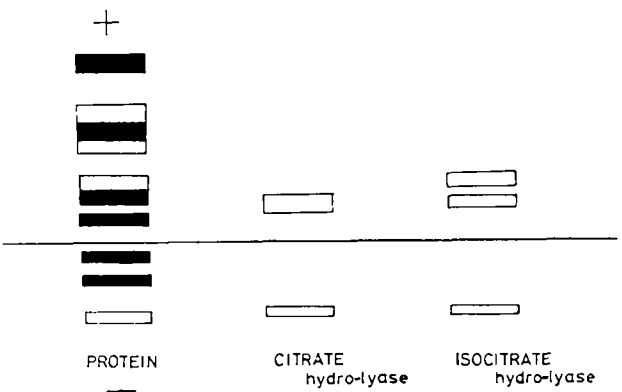

Fig. 2. Separation of proteins, isocitrate hydro-lyase and citrate hydro-lyase from crude enzyme (pig heart), using starch gel electrophoresis, 18.5 h at 4° and 300 V.

gel, polyacrylamide gel at pH 8.0, and polyacrylamide gel containing LKB ampholine pH 7–9. Many protein bands were found, up to 19, staining with amido black or coomassie brilliant blue. Several bands of enzyme activity appeared. With starch gel (Fig. 2) 5 proteins went towards the anode and 3 towards the cathode. When enzymatic activity was measured using additions of isocitrate dehydrogenase, $NADP^+$ and tetrazolium, the further addition of *cis*-aconitate is a test for isocitrate hydro-lyase activity, and of citrate for citrate hydro-lyase activity. Fig. 3 shows an experiment with ampholine and acrylamide. It is interesting that much enzymatic activity was present in the anodic region as well as in that of the cathode, even though the pH's were somewhat extreme (pH 3 and pH $\geq$ 9). It was possible to detect 9 areas of activity for isocitrate hydro-lyase and 6 areas for citrate hydro-lyase; in the presence of fluorocitrate, those in the intermediate pH range were much diminished or eliminated with isocitrate; with citrate there was also much diminution in the cathode region.

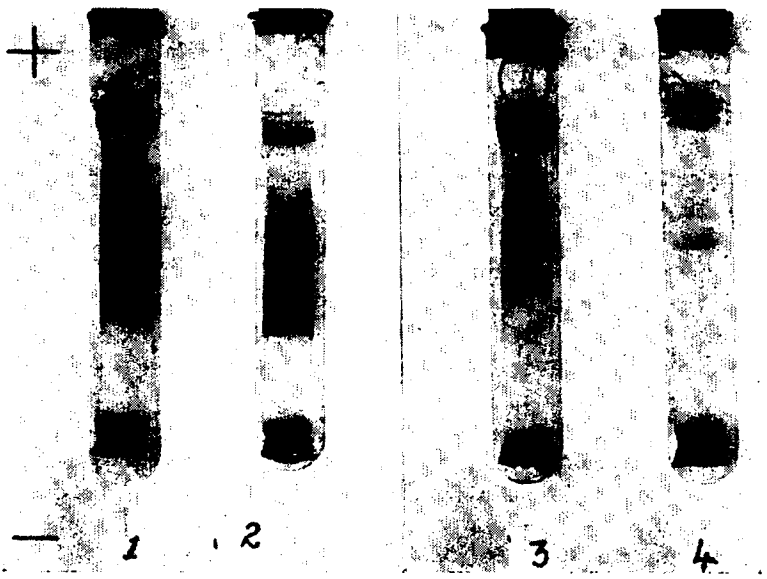

Fig. 3. Ampholine focussing, pH 7–9, of crude enzymes using polyacrylamide gel. 17 h at 4°, 75 V. Bands of isocitrate hydro-lyase and citrate hydro-lyase were stained for enzyme activity (see text). 1 and 2, isocitrate hydro-lyase; 3 and 4, citrate hydro-lyase; 2 and 4, fluorocitrate present.

Attempted fractionation with isoelectric focussing, using LKB ampholine

Before leaving the problem, 3 experiments were performed on the larger scale with the LKB ampholine technique using their 110-ml glass electrophoretic apparatus; of these the first experiment was discarded. The gradient was set up with ampholine (pH 7–9) and sucrose gradient, as recommended in the manufacturers instructions. The running time varied, always at 4° or less. 2-ml (Expt. 2) or 1.25-ml (Expt. 3) samples were withdrawn starting from the anodic end, so that 55 samples were obtained from Expt. 2, and 80 from Expt. 3. Each sample was kept rigorously cold (on ice), as it came from the column. It was evident that most of the coloured material travelled to the anode; even at 4° or less, redistribution of the bands occurred rapidly after discontinuing the current, *i.e.* within 2 h. As soon as possible, assays for 'aconitase' activity were made; the effect of fluorocitrate was tested, as also that of activation with Fe^{2+} and cysteine. The Fe^{2+} activation succeeded in Expt. 2 in several fractions; but it did not succeed in Expt. 3. Any Fe^{2+} activation, however, did not last even for 24 h at −19°; the activated preparations became completely inactive.

We could not find a suitable method of removing the ampholine and sucrose sufficiently quickly. Owing to the instability, we were forced to abandon the attempt to isolate possible isoenzymes, especially because we could find no satisfactory stabilizing agent. Cysteine alone had no effect and dithiothreitol with or without Fe^{2+} was ineffective. As our observations had to be made in presence of the focussing compounds, controls were necessary to determine the effects of ampholine with or without sucrose on the enzyme activity. Ampholine alone had virtually no effect; ampholine + sucrose (50%) produced a 34 and a 37% decrease, respectively, in the measurement of isocitrate and citrate hydro-lyase activity; and the same agents caused a 40% loss of activity after standing overnight at −19°. In spite of these dif-

TABLE I

SOME RESULTS OF AMPHOLINE FOCUSSING, SELECTED FROM 2 EXPERIMENTS

The results are expressed as specific activity, units/mg protein. Protein estimated by light absorption at 280 nm. One unit of enzyme activity is the amount of enzyme forming 1 μmole *cis*-aconitate in 3 min at 30°. Initial specific activity in crude enzymes: isocitrate hydro-lyase, 7; citrate hydro-lyase, 4.

Expt.	*Sample*	*Hydro-lyase*		*After Fe^{2+}*		*Fluorocitrate***	
		Isocitrate	*Citrate*	*Isocitrate hydro-lyase*	*Citrate hydro-lyase*	*Isocitrate hydro-lyase*	*Citrate hydro-lyase*
2	9	10	2	19	6		
	10	70	35	130	70		
	16	6	6	0	9		
	18	7	7	0	4		
	20	6	4	6	16		
	22	0	5	21	21		
	25	5	5	21	50		
	29	1	5	26	25		
	30	0	4	100	100		
3	10	1	0.2	0.7	0	—	—
	13	1.5	3.5	0	2.6	1.3	—
	14	0.7	1.1	0.7	1.1	0.9	—
	22/3	0.47	0.4	0.3	0.3	0	—
	45	0.45	0.13	0.3	0	0	—
	46	0.36	0.36	0.1	0	0	—
	75	0.5	0.5	0*	0*	0	—
	80	0.25	0.27	0*	0*	0	—

* A decrease in absorption was actually seen.
** Before Fe^{2+}-attempted activation.

ficulties, some observations were made; a selection of these showing especial points are recorded in Table I. Instances are given where fractions showed different activities with citrate and isocitrate, of effects of Fe^{2+} *plus* cysteine activation and of the action of fluorocitrate.

Expt. 2 was run with ampholine pH 7–9 for 1.5 h at 400 V and 22 h at 1000 V. A coarse separation was made by removing 5 × 35-ml fractions of which fractions 2 and 3 (*i.e.* 50 ml) were run again—a new density gradient with no extra ampholine added—for 21.5 h at 1000 V; 2-ml samples were collected.

Expt. 3 was run with ampholine pH 7–9 for 1.5 h at 400 V and 30 h at 1000 V at 4°. 1.25-ml fractions (80) were then withdrawn and kept on ice until tested. The attempted activation with Fe^{2+} *plus* cysteine was 24 h after removal from the column. There was little Fe^{2+} activation in Expt. 3.

Facts observed in Expts. 2 and *3*

(a) Cases showing different activities for isocitrate hydro-lyase and citrate hydro-lyase. Isocitrate hydro-lyase > citrate hydro-lyase: Expt. 2, samples 9, 10; Expt. 3, samples 10, 45. Citrate hydro-lyase > isocitrate hydro-lyase: Expt. 2, samples 22, 29, 30; Expt. 3, samples 13, 14. Fe^{2+} activation: (1) isocitrate hydro-lyase > citrate hydrolyase: Expt. 2, samples 9, 10; Expt. 3, sample 10. (2) Citrate hydro-lyase > isocitrate hydro-lyase: Expt. 2, samples 16, 18, 20, 25; Expt. 3, sample 13.

(b) Cases showing the same activities for isocitrate hydro-lyase and citrate hydro-

lyase. Expt. 2, samples 16, 18, 25; Expt. 3, samples 22/23, 46, 75, 80. After Fe^{2+} activation: Expt. 2, samples 22, 29, 30; Expt. 3, sample 22/23.

(c) Cases explored with fluorocitrate, isocitrate hydro-lyase. Expt. 3, samples 14, 22/23, 45, 46, 75, 80, completely inhibited. Sample 13, only slightly inhibited.

Interpretations

(*b*) Results as they stand support the idea that there is one centre for isocitrate hydro-lyase and citrate hydro-lyase, which is fully activated by Fe^{2+}. On the other hand, a closer scrutiny shows that in samples 22, 29 and 30 there were originally different activities.

In (*a*), there are several examples given of discrepancies in the activities of the two centres.

In (*c*), suggestion of samples responding differently to fluorocitrate are given.

It can be concluded that Fe^{2+} activation is necessary; but that too long an exposure to the ampholine changes the active centres, so that they cannot be reactivated. In Expt. 2 Fe^{2+} activation succeeded for a short time. In fact, sample 10 showed a specific activity much more active than the purest fraction described by MORRISON[1]; and this did not allow for the depression caused by the presence of the focussing agents. In Expt. 3, the prolonged ampholine treatment gave a preparation, which was not activated by Fe^{2+} *plus* cysteine.

The fact that Expt. 2 needed Fe^{2+} *plus* cysteine for activation is consistent with the LKB statement that ampholine removes metals, if we support the classical view that Fe^{2+} is part of the active enzyme. Even so, activity was found in some samples after ampholine treatment. Cogent as the evidence is for the necessity of Fe^{2+}, it is still indirect evidence and therefore the possibility still exists that the Fe^{2+} is needed for some special activation of a sulphur group, and is not actually part of the enzymes.

Our experiments with ampholine focussing show many instances of discrepancy between citrate and isocitrate hydro-lyase activity, and therefore reinforce the suggestion that there are two enzymes. Where the samples showed equal hydro-lyase activity, it has still to be decided whether this is the result of complex formation of two enzymes, or is a separate enzyme with two activities.

Activators and inhibitors

During the course of tests for the presence of fluorocitrate in various animal and plant extracts we have found some instances of apparent activation and inhibition not previously described. Following on the report by HSU AND MILLER[17], that fluoroacetate and fluorocitrate could be detected in certain forage plants exposed to fluoride fumes, and by our knowledge that WARD AND HUSKISSON[18] had found the formation of fluorocitrate in lettuces treated with fluoroacetate, we examined extracts of tea for fluorocitrate, because of its well-known content of fluoride. During this work we became aware that our extracts of tea contained something which increased the activity of the enzymes. In searching for a possible compound, we found that small amounts of caffeine could have an activating effect, and extended our observations to some other purine and pyrimidine compounds. We were surprised by these activating effects, as 11 years ago in a series of experiments (unpublished) with various compounds the only compounds inducing activation were certain malonates at high concentration. Further, no activating substances were mentioned by DICKMAN[11].

Examples of our results for activation by caffeine and thiamine are given in

TABLE II

ACTIVATORS AND INHIBITORS OF ACONITATE HYDRATASE

Values given = increase or decrease in % of rate without addition. Experiments (a) on one line were performed with same enzyme preparation and (b) with the same in each experiment.

Activation. (a) Instances of activation by caffeine and thiamine. Similar results were obtained with theophylline, adenosine, ADP and ATP.

Expt.	*Addition*	*Concn. (μM)*	*% Change substrate*			
					Fluorocitrate (69.9 μM)	
			Isocitrate	*Citrate*	*Isocitrate*	*Citrate*
1	Caffeine	51.4	+ 297*	+ 97	33	− 37
		129.7	+ 32	−		
2	Thiamine	74.1	+ 400**	+ 220	+ 58	—
		74.1	+ 82			

Activation. (b) Instances of activation by one compound only.

Expt.	*Addition*	*Concn. (μM)*	*% Change*	
			Isocitrate	*Citrate*
3	Caffeine	26.5	+ 5	+ 6
	Thiamine	14.8	+ 62	+ 37
4	Caffeine	26.5	+ 12	
	Thiamine	29.6	+ 38	
5	Caffeine	128.5	− 2.5	− 4.7
	Thiamine	74.1	+ 55	+ 44

Inhibition or no effect.

Addition	*Concn.*	*% Change*** *Isocitrate*
Tannic acid	10 μg/2.0 ml	61.4
Gallic acid	50 μg/2.0 ml	74.5
Kinetin	100 μg/2.0 ml	100.0
Cu^{2+}	2 μg/2.0 ml	51.0
Mo^{3+}	2 μg/2.0 ml	116.0
Sodium oxaloacetate	30 μg/2.0 ml	100.0

* The figure given for caffeine and isocitrate was the highest seen. An increase of approximately 40% was the usual amount observed.

** The highest figure for thiamine is quoted; + 30–40% is more usual.

*** No change = 100.0%.

Table II (a) and for some inhibitors in Table II (b). In addition to these, activations have been seen with theophylline, theobromine, adenosine, ADP and ATP. Tannic acid was a powerful inhibitor and gallic acid a weaker one.

The activation has been variable and was in some cases substantial. There were also instances in which it occurred with one compound and not the other, and in one instance it happened with one enzyme and not with the other. In several cases, the

activation of the two enzymatic centres did not run parallel. Some preparations after storage in the deep freeze lost the property of potentiation.

It is important to be certain that the activation is a genuine effect upon the active centre. In this connection, we proved that it is not due to an interaction with the 'Tris' buffer as it occurs when the buffer is phosphate. It does not happen if the enzyme has been previously inactivated by boiling. The only compound showing some small effects in absence of the enzyme was theobromine. When the aconitate hydratase activity is blocked by fluorocitrate the extra activity is also inhibited.

PETERS[2] has shown that additions of NaCl or of KCl produce substantial activations, a fact confirmed by THOMSON *et al.*[19]. The KCl effect in these experiments was cut short at 12.1 M; but since the publication, this inhibition has been shown to be due to an impurity in the specimen of KCl used. This salt effect must be considered in experiments with aconitate hydratase; in our experiments the addition of salts with the activators was small enough to exclude its effect. The fact that either citrate or isocitrate is necessary for the activation excludes the action of any malate dehydrogenase present, and any isocitrate dehydrogenase is inactive in the absence of $NADP^+$ or NAD^+. Hence we can conclude that the potentiation is a genuine effect on the enzymatic centres of aconitate hydratase. This is also consistent with the inhibition of the potentiation by fluorocitrate.

There is another possibility, *viz.* that the apparent activation is due to the removal of an inhibitor, which would be consistent with the fact that small concentrations were often active. The inhibition induced by Cu^{2+} (ref. 20) was confirmed; it could not be removed with caffeine or adenosine.

The only workable hypothesis left appears to be that the potentiating effect is due to the induction of some conformational change in the protein centre involved. With thiamine, it does not seem to be due to the kind of thiamine effect, described by COOPER AND ITAKAWA[21] in nerve, as it is not inhibited by pyrithiamine. It appears to be associated with the presence of a pyrimidine ring. We do not know whether these activating effects occur *in vivo*; but in so far as they induce large increases of activity upon occasion, they show that the enzymes concerned are capable of much greater activity than is normally observed.

DISCUSSION

The facts reported by us show that the problem of aconitate hydratase is more complex than has been believed hitherto, and make clear that much more work will be needed to solve all the facets raised. Progress in understanding the true nature of the various proteins with enzymatic activity revealed by electrophoretic methods will depend upon the discovery of methods of stabilizing the enzymes concerned. Whichever way we look at the problem in the light of our facts, we are forced to conclude that there exist several different types of enzymes in a preparation from pig heart. We have found separate activities for isocitrate and citrate hydro-lyases, as well as proteins with enzymatic centres which can be activated by Fe^{2+} *plus* cysteine, and which behave with equal activity to exposure to isocitrate and citrate. Although some of these behave as one centre after isoelectric focussing, the fact that the unfractionated preparation shows marked increases upon addition of either of the sub-

strates at v_{max} to the other one, suggests that they may actually be a complex of two centres.

So far as the behaviour to fluorocitrate is concerned, some of the fractions separated are much less sensitive to this. Hence there is evidence of enzymatic centres both sensitive and rather insensitive to the inhibitor. This is consistent with the presence of intramitochondrial and extramitochondrial aconitase found by GUARRIERA-BOBYLEVA AND BUFFA[6] to behave differently. It is also consistent with the fact that in earlier work PETERS[2] and in our present work, we never obtained a complete inhibition of the enzymes by fluorocitrate.

ACKNOWLEDGEMENTS

We are grateful to the Wellcome Trustees for grants in aid of this work to Professor F. G. Young, F.R.S. for facilities provided, to Dr. Barrett, Strangeways Research Laboratory for extensive advice and help with ampholine focussing and to many colleagues in the Department for advice. We also thank Dr. B. Hartley and the LKB Company for their co-operation in the loan of apparatus, and Miss B. Tucker for advice on starch gel electrophoresis.

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