AMINO ACID DECARBOXYLASES IN RAT BRAIN AND LIVER

by

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The decarboxylation of cysteine sulphinic, cysteic and glutamic acids by pyridoxal phosphate-requiring enzymes has recently been studied in this laboratory[1]. Mammalian liver cysteine sulphinic and cysteic acid decarboxylases have also been examined by HOPE[2]. As a result of this work he suggests that the two substrates, cysteine sulphinic and cysteic acids, are decarboxylated by the same enzyme in the liver. It is of interest that AWAPARA AND WINGO[3] had earlier discussed this possibility.

In this paper rat liver cysteine sulphinic acid decarboxylase has been studied in some detail. Using a purified enzyme preparation it has been demonstrated that not only is pyridoxal phosphate necessary for enzyme activity but also that a thiol group plays an essential rôle in the decarboxylation of the substrate. Further work supports the hypothesis that cysteine sulphinic and cysteic acids are attacked by the same enzyme in the liver, although this enzyme clearly differs from the cysteine sulphinic acid decarboxylase in the brain.

Evidence is also presented to suggest that, in the brain, cysteine sulphinic, cysteic and glutamic acids are decarboxylated by the same enzyme. The brain decarboxylase, like that of the liver requires pyridoxal phosphate and a free thiol group for the decarboxylation of cysteine sulphinic, cysteic and glutamic acids.

MATERIALS AND METHODS

The materials and methods used were as described in another paper[4]. An accurate measure of relative enzyme activity used throughout the present paper is given by the observed rate of carbon dioxide evolution calculated by the method of least squares. Absolute enzyme activity can be calculated from the fact that 43% of carbon dioxide is retained by phosphate buffer pH 6.8 at 35° C[1] (see Table VI). The values recorded in Tables IV and V for the observed rate of evolution of carbon dioxide by brain suspensions are uncorrected for the small endogenous activity, for reasons which are discussed later.

Cysteine sulphinic acid decarboxylation by rat liver

Separation of rat liver by differential centrifuging shows that the decarboxylase is present in the clear supernatant and not in the particulate fraction. The enzyme activity can be concentrated by adding ammonium sulphate to give 50% (w/v) saturation of the liver supernatant, followed by centrifugation at 8,000 *g* for 10 min. The precipitate after dialysing at 4° C for 3 days can be further purified by centrifuging to give a clear solution containing the enzyme. Fig. 1 shows the reactivation

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TABLE I

LOCALISATION OF RAT LIVER CYSTEINE SULPHINIC ACID DECARBOXYLASE ACTIVITY

Liver from a male rat was suspended 10% w/v in cold 0.25 *M* sucrose and separated into fractions by differential centrifuging at 0° C[5]. Cysteine sulphinic acid decarboxylase activity was measured as described previously[4].

Liver fraction	*Activity as $\mu l CO_2$/250 mg fresh weight of liver/h*
Whole homogenate	359
Nuclei plus cells	40
Mitochondria	60
Supernatant plus microsomes	360
Microsomes	30
Clear supernatant	300

of decarboxylase activity obtained on addition of increasing amounts of pyridoxal phosphate and also the effect of addition of ferrous ions. Cupric ions ($1 \cdot 10^{-3}$ *M*) completely inhibit the activity, while other metal ions tested have little effect. It will be noted that there is a lag of between 20–60 min before decarboxylation commences, and that this lag is reduced by the presence of ferrous ions. Addition of

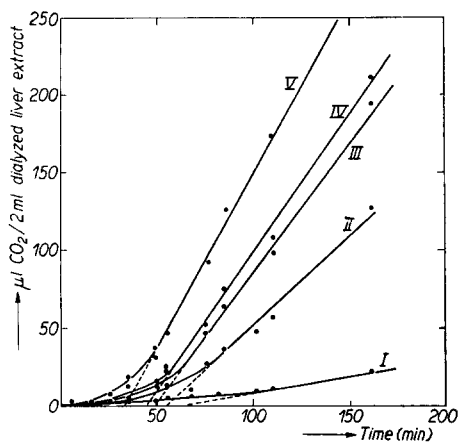

Fig. 1. The effect of the addition of pyridoxal phosphate and ferrous ions to rat liver cysteine sulphinic acid decarboxylase. Each Warburg flask contains a dialysed rat liver preparation (200 mg dry weight) in 0.067 *M* phosphate buffer pH 6.8. Cysteine sulphinic acid concentration is 0.01 *M* and the final volume 3.2 ml. I. Without any addition; II. + 100 µg pyridoxal phosphate; III. + 200 µg pyridoxal phosphate; IV. + 400 µg pyridoxal phosphate; V. + 200 µg pyridoxal phosphate + 100 µg Fe^{++}.

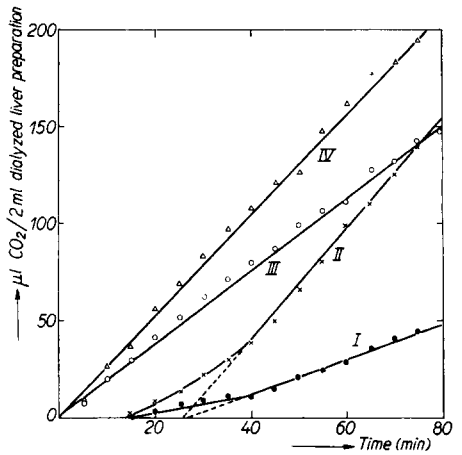

Fig. 2. The effect of adding BAL and pyridoxal phosphate to a dialysed liver preparation. Each flask contained 2 ml of supernatant after centrifuging a dialysed rat liver preparation (12 mg dry weight) and 1 ml of 0.25 *M* phosphate buffer pH 6.8. The enzyme alone (I), with BAL (III), with pyridoxal phosphate (II) and pyridoxal phosphate plus BAL (1 mg) (IV) are shown. Pyridoxal phosphate concentration was 33 µg/ml.

boiled liver extract or cysteine or 2:3 dimercaptopropanol (BAL) completely abolishes this lag (Fig. 2) and results in the linear evolution of carbon dioxide at the same rate as that obtained after 30 min when excess pyridoxal phosphate alone is added. These experiments suggest that not only is pyridoxal phosphate required but that reduction of some group such as a disulphide group is necessary for enzyme activation.

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TABLE II

REVERSAL BY GLUTATHIONE OF *p*-CHLOROMERCURIBENZOATE INHIBITION

1 ml of 25% w/v rat liver suspension or 3 ml of 20% w/v brain suspension plus 100 μg pyridoxal phosphate in phosphate buffer pH 6.8 was incubated for 30 min with *p*-chloromercuribenzoate, the final volume being 3.2 ml. Glutathione was added afterwards as indicated 20 min before adding 0.01 *M* cysteine sulphinic acid.

p-chloromercuribenzoate concentration (M)	*Tissue*	*% Enzyme activity present*	
		No glutathione	*Glutathione* $1 \cdot 10^{-3}$ *M*
Nil	liver	100	99
$4 \cdot 10^{-4}$	liver	44	85
Nil	brain	100	100
$1 \cdot 10^{-4}$	brain	64	94
$4 \cdot 10^{-4}$	brain	34	49

TABLE III

THE ACTION OF INHIBITORS ON CYSTEINE SULPHINIC ACID DECARBOXYLASE ACTIVITY

The inhibition of 1 ml of 25% w/v rat liver suspension in phosphate buffer pH 6.8 was determined from inhibition curves obtained by plotting percentage enzyme inhibition against the negative log of molar concentration.

Inhibitor	*The concentration of inhibitor producing 50% inhibition after 15 min incubation at 35° C (M)*
p-Chloromercuribenzoate	$2.2 \cdot 10^{-4}$
Mercuric chloride	$2 \cdot 10^{-4}$
Iodoacetate	$9 \cdot 10^{-5}$
Sodium fluoride	no inhibition at 10^{-3} *M*
Versene	no inhibition at 10^{-3} *M*
Potassium cyanide	$9 \cdot 10^{-4}$

It was therefore interesting to find that thiol groups are essential for enzyme activity. This is indicated by inhibition by *p*-chloromercuribenzoate, reversal of this inhibition by glutathione (Table II) and inhibition by iodoacetate, mercuric chloride (Table III) and by cupric ions.

The 30 min lag shown in Figs. 1 and 2 therefore can be accounted for by slow reduction of the disulphide bond by cysteine sulphinic acid (or its decarboxylation product hypotaurine). This hypothesis seems to be true because the dialysed enzyme with added pyridoxal phosphate and cysteic acid (where the reducing group $R.SO_2H$ is replaced by the non-reducing group $R.SO_3H$) as substrate, shows no activity, although with the addition of BAL there is immediate and linear evolution of carbon dioxide. Shaking the preparation in air for 10 min before gassing produces a lag of about 45 min instead of 20–30 min. Finally, addition of substrate 30 min before addition of pyridoxal phosphate results in rapid and linear evolution of carbon dioxide whereas when pyridoxal phosphate is added for 30 min followed by the substrate there is a lag in the reactivation of the enzyme. Preliminary experiments suggest that under these conditions only 10 μg of pyridoxal phosphate is necessary with BAL for 100% activation and that pyridoxal may be replaced by higher concentrations of pyridoxamine but not by pyridoxine phosphate. Inhibition of the decarboxylase by keto reagents and the results of deficiency experiments[4,1,2], also suggests that pyridoxal phosphate is present as a co-enzyme.

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TABLE IV

COMPARISON OF THE EFFECT OF THERMAL INACTIVATION OF RAT LIVER AND BRAIN DECARBOXYLASE ACTIVITIES

Tissue suspensions were heated for 10 min at the temperature indicated. After cooling residual activity of 1 ml of liver suspension or 3 ml of brain suspension plus 100 μg of pyridoxal phosphate was determined. Substrate concentration was 0.01 M.

Tissue (Temp. °C)	*Enzyme activity* { $\mu l\ CO_2$/min/200 mg wet weight (liver); $\mu l\ CO_2$/2 min/1 g wet weight (brain) }					
	Cysteine sulphinic acid		*Cysteic acid*		*Glutamic acid*	
	$\mu l\ CO_2$	% *Act.*	$\mu l\ CO_2$	% *Act.*	$\mu l\ CO_2$	% *Act.*
Liver control at 35	3.9	100	0.54	100	0	0
Liver heated to 45	3.9	100	0.51	95		
Liver heated to 50	2.02	52	0.22	41		
Liver heated to 55	0.9	23	—*	—		
Liver heated to 60	0.25	—	—	—		
Brain control at 35	2.07	100	1.23	100	2.94	100
Brain heated to 45	1.22	59	0.72	59	1.93	66
Brain heated to 50	0.23	11	0.18	15	0.35	12

* too small to measure accurately.

TABLE V

THE DISTRIBUTION OF RAT LIVER AND BRAIN DECARBOXYLASE ACTIVITY AFTER CENTRIFUGATION OF A SUSPENSION

Female rat liver (25% w/v) and brain (20% w/v) suspensions in phosphate buffer pH 6.8 have been centrifuged for 10 min at approximately 8,000 g. The precipitate and supernatant were adjusted to the original volume and the decarboxylase activity of each fraction was determined using 1 ml of liver suspension (2 ml with cysteic acid as substrate) and 3 ml of brain suspension. Substrate concentrations were 0.01 M.

Preparation	*Enzyme activity* { $\mu l\ CO_2$/min/250 mg wet weight (liver); $\mu l\ CO_2$/2 min/600 mg wet weight (brain) }					
	Cysteine sulphinic acid		*Cysteic acid*		*Glutamic acid*	
	$\mu l\ CO_2$	% *Act.*	$\mu l\ CO_2$	% *Act.*	$\mu l\ CO_2$	% *Act.*
Whole liver suspension	3.7	100	0.5	100	—	—
Re-suspended precipitate	0.64	17	0.05	10	—	—
Supernatant	3.6	97	0.5	100	—	—
Whole brain suspension	3.92	100	1.8	100	4.1	100
Re-suspended precipitate	1.74	45	0.76	42	1.86	45
Supernatant	1.12	28	0.48	27	1.17	29

Comparative decarboxylation of cysteine sulphinic and cysteic acids by rat liver

Evidence has been obtained to support the concept that cysteine sulphinic and cysteic acids are decarboxylated by the same enzyme. Thus, both substrates are decarboxylated by the soluble fraction of a liver suspension (Table V) and the loss of activity obtained by heating a liver suspension drops in the same manner for each of the two substrates (Table IV). Furthermore there is no indication of the existence of two separate enzymes after purification, for cysteine sulphinic acid is decarboxylated 7 times as fast as cysteic acid by the purified preparation while cysteine sulphinic acid is decarboxylated 7.5 times as fast as cysteic acid in whole liver suspensions. Finally, there is a good correlation between the inhibition of cysteic and cysteine sulphinic acid liver decarboxylase activity by *p*-chloromercuribenzoate (Fig. 3).

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Comparative decarboxylation of cysteine sulphinic, cysteic and glutamic acids by rat brain

Glutamic, cysteine sulphinic and cysteic acids are decarboxylated by brain suspensions. The possibility that all three substrates are decarboxylated by the same enzyme is suggested by the absence of any summation of activity when any two substrates are together added to a brain suspension (Table VI). Further experiments have therefore been designed to support this possibility. Thus, Table IV shows that suspensions of brain heated to 45° C and 50° C for 10 min lose the same percentage activity with cysteine sulphinic, cysteic and glutamic acids as substrate, at each temperature. It has also been observed that after centrifugation of a suspension the activity with the three substrates is distributed in the precipitate and supernatant in exactly the same manner (Table V). Finally, the existance of only one enzyme decarboxylating cysteine sulphinic, cysteic and glutamic acids is also suggested by the similarity of the inhibition curves by *p*-chloromercuribenzoate. Since this inhibition by *p*-chloromercuribenzoate is reversed by glutathione (Table II) and the enzyme can also be inhibited by iodoacetate (1 and $5 \cdot 10^{-4} M$ incubated with the enzyme for 15 min at 35° C inhibits by 61% and 95% respectively) it is probable that as for the liver a thiol group is necessary together with pyridoxal phosphate for brain decarboxylase activity.

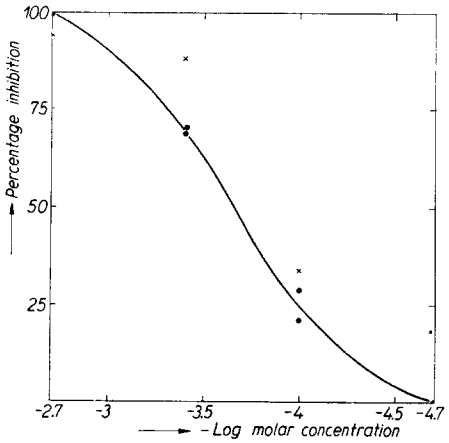

Fig. 3. The inhibition of liver decarboxylase activity by *p*-chloromercuribenzoate. Each flask contained 1 ml of 25% w/v rat liver suspension in 0.067 *M* phosphate buffer pH 6.8 and varying concentrations of *p*-chloromercuribenzoate, the final volume being 3 ml. The inhibitor was incubated with the liver suspension for 15 min at 35° C before adding the substrate (0.01 *M*), cysteine sulphinic (•) and cysteic (×) acids.

TABLE VI

SUMMATION OF SUBSTRATE BRAIN DECARBOXYLASE ACTIVITIES

3 ml of female rat brain suspension (20% w/v) in 0.067 *M* Sørensen's phosphate buffer with excess pyridoxal phosphate (100 μg) was pipetted into Warburg flasks. 0.2 ml of each of the substrates (0.01 *M*) was placed as indicated in one side arm and 0.2 ml of 5 *N* sulphuric acid in the other. The activity was determined at 35° C as described.

Substrate (added to brain suspension)	*μl CO_2/2 min/600 mg wet weight of brain*	
	uncorrected for CO_2 retention	*absolute value determined by acid tip*
No substrate	1.66	2.37
Glutamic acid	6.03	12.4
Cysteine sulphinic acid	4.47	9.1
Cysteic acid	2.44	5.35
Glutamic + cysteine sulphinic acids	5.56	11.7
Cysteine sulphinic + cysteic acids	4.14	8.45
Glutamic + cysteic acids	5.16	10.7
Glutamic acid (0.001 *M*)	2.24	5.23
Cysteic (0.01 *M*) + Glutamic (0.001 *M*) acids	2.70	5.7

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DISCUSSION

It has been shown in this paper that cysteine sulphinic acid decarboxylase activity is located in the supernatant obtained by centrifugation of a liver suspension. Further purification of the enzyme was effected by dialysing a precipitate obtained by treatment of the supernatant with ammonium sulphate. The dialysed preparation was reactivated, after a lag period of 20–60 min by addition of pyridoxal phosphate—not effectively replaced by pyridoxamine or pyridoxine. At first sight it therefore seemed possible that this lag was due to a slow combination of pyridoxal phosphate and apoenzyme and that as a result of treatment with ammonium sulphate[6] and dialysation most of the co-enzyme had been removed.

However, it has been found that addition of boiled liver extract or cysteine or 2:3 dimercaptopropanol (BAL) abolishes the lag period (Fig. 2) most probably by reduction of the disulphide groups formed during the purification of the enzyme. It will be noted that reactivation of the dialysed enzyme with added BAL alone (curve III) is more than the residual enzyme activity (curve I). This work therefore suggests that a thiol group—not linked directly to the co-enzyme—is essential for enzyme activity and that the method used results in only partial removal of the pyridoxal phosphate from the enzyme. It has been shown in an earlier paper[4] that the reaction between *iso*nicotinyl hydrazide and pyridoxal phosphate either in the presence or absence of the enzyme has about the same energy of activation. This suggests that the co-enzyme is not activated by the enzyme and hence that the thiol group plays an important part in the energy transfer between substrate and enzyme. It is interesting that other decarboxylases, lysine[7], DOPA[8], and diaminopimelic acid[9] decarboxylases, beside the cysteine sulphinic acid decarboxylase of liver and brain have been shown to require pyridoxal phosphate and thiol groups for their activity. Thus it is possible that a thiol group together with pyridoxal phosphate may be a general requirement for amino acid decarboxylase activity.

HOPE[2] has presented evidence based on competition experiments and observations on pyridoxine-deficient rats, to show that cysteine sulphinic and cysteic acids are decarboxylated by the same enzyme. This hypothesis is fully confirmed in this paper, by experiments on localisation, thermal inactivation and inhibition by *p*-chloromercuribenzoate of decarboxylase activity.

The quantitative study of brain decarboxylase activity is complicated by the fact that the enzyme activity is low and that in the absence of added substrate (even after dialysing) brain suspensions continue to evolve small amounts of carbon dioxide (about 1 μl CO_2/2 min/600 mg) and thus in these respects differ from the liver. Since glutamic and γ-amino butyric acids with traces of cysteine sulphinic and aspartic acids are the only free amino acids so far detected in the brain[10], it may be supposed that the endogenous carbon dioxide output is chiefly due to decarboxylation of glutamic acid. This view is strongly supported by the work of WINGO AND AWAPARA[11] and observations made in the course of this work. Thus, the localisation of endogenous activity, its thermal inactivation and inhibition has been found to be the same as that for the glutamic, cysteine sulphinic and cysteic acid decarboxylases. Since the amount of substrate added is very much greater than the glutamate already present in the brain ($3 \cdot 10^{-4}$ *M*)[10] and addition of 0.001 *M* glutamate did not significantly increase the decarboxylation of 0.01 *M* cysteic acid, decarboxylase activities can best

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be represented by the carbon dioxide evolution without correction for blank activity.

Comparison of liver and brain cysteine sulphinic acid decarboxylases shows quite clearly that these two enzymes are different. Thus BERGERET, CHATAGNER AND FROMAGEOT[1] have shown that after feeding rats on a diet deficient in vitamin B_6, brain cysteine sulphinic acid decarboxylase activity disappears but can be reactivated by addition of pyridoxal phosphate, whereas in the liver the activity also disappears but cannot be reactivated[1,2]. Furthermore, in the brain cysteine sulphinic and cysteic acids are decarboxylated at different relative rates to those in the liver. Liver cysteine sulphinic acid decarboxylase appears to be readily soluble whereas that of the brain is largely associated with the particulate matter. The fact that glutamic acid is not decarboxylated by rat liver, while there is evidence to suggest that glutamic, cysteine sulphinic and cysteic acids are decarboxylated in the brain by the same enzyme, again emphasises the difference between the brain and liver decarboxylases.

Indeed, as a result of experiments on summation of substrate activity, localisation of enzyme activity, thermal inactivation and the inhibition by *p*-chloromercuribenzoate it appears that, in the brain, glutamic, cysteine sulphinic and cysteic acids are decarboxylated by the same enzyme. The pH optima, requirement for pyridoxal phosphate as co-enzyme and the action of inhibitors on the brain decarboxylases, as reported in the literature, support the same view.

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SUMMARY

1. Liver cysteine sulphinic acid decarboxylase is found in the clear supernatant of a liver suspension after fractional centrifugation, while brain cysteine sulphinic acid decarboxylase is more or less associated with particulate matter.

2. Thiol groups and pyridoxal phosphate are shown to be necessary for the activity of liver and brain decarboxylases.

3. Further evidence is presented to suggest that cysteine sulphinic and cysteic acids are decarboxylated by the same enzyme in the liver.

4. Evidence is also given that glutamic, cysteine sulphinic and cysteic acids are decarboxylated by the same enzyme in the brain.

RÉSUMÉ

1. La cystéinesulfinique décarboxylase du foie est retrouvée dans le surnageant limpide d'une suspension de foie après centrifugation fractionnée, tandis que la cystéinesulfinique décarboxylase du cerveau est plus ou moins associée à des particules.

2. Des groupes thiols et le phosphate de pyridoxal sont nécessaires à l'activité des décarboxylases du foie et du cerveau.

3. De nouvelles observations, qui suggèrent que les acides cystéinesulfinique et cystéique sont décarboxylés par le même enzyme dans le foie, sont décrites.

4. Une preuve, que les acides glutamique, cystéinesulfinique et cystéique sont décarboxylés par le même enzyme dans le cerveau, est également apportée.

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ZUSAMMENFASSUNG

1. Cysteinsulfinsäuredekarboxylase wurde in der klaren überstehenden Flüssigkeit einer Lebersuspension nach fraktioniertem Abschleudern gefunden, während dasselbe Enzym aus dem Gehirn mehr oder weniger an die Partikelsubstanz gebunden ist.

2. Es wird über die Beobachtung berichtet, dass Thiolgruppen und Pyridoxalphosphat für die Aktivität der Leber- und Gehirndekarboxylasen notwendig sind.

3. Weitere Hinweise werden angeführt, welche annehmen lassen, dass Cysteinsulfinsäure und Cysteinsäure in der Leber durch dasselbe Enzym dekarboxyliert werden.

4. Es werden ferner Beweise dafür erbracht, dass Glutaminsäure, Cysteinsulfinsäure und Cysteinsäure im Gehirn durch dasselbe Enzym dekarboxyliert werden.

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