The Role of Sulphydryl Groups of Yeast Carboxylase

By A. O. M. STOPPANI, A. S. ACTIS, J. O. DEFERRARI and E. L. GONZALEZ
Department of Biochemistry, School of Medicine, University of Buenos Aires

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Thiol groups play an essential role in most of the enzymes of pyruvate metabolism (Barron, 1951). In yeast, pyruvate oxidation by living cells is strongly inhibited by thiol reagents such as ethyl iodoacetate or chloroacetophenone, which, nevertheless, do not apparently affect pyruvate decarboxylation by carboxylase (Stoppani, 1951). These observations have led us to make a detailed study of the role of thiol groups in yeast carboxylase.

Thiol groups of enzymes are usually detected by selective reaction with mercaptide-forming, alkylating or oxidizing agents (Barron, 1951). The action of thiol detectors on carboxylase has been studied previously, but with contradictory results. *p*-Aminophenyldichloroarsine (Barron & Singer, 1945), *p*-chloromercuribenzoate and iodoacetate (Meyer, 1945), cupric ions (Green, Herbert & Subrahmanyan, 1941; Meyer, 1945), mercuric and silver ions (Green *et al.* 1941), iodine (Meyer, 1945) and quinone (Kuhn & Beinert, 1943) apparently inhibit carboxylase. However, in other experiments, this enzyme is not significantly affected by lewisite oxide (Barron, Miller, Bartlett, Meyer & Singer, 1947; Peters, Sinclair & Thompson, 1946), ethylarsenoxide (Peters *et al.* 1946), iodoacetate (Green *et al.* 1941) or ethyl iodoacetate (Mackworth, 1948). Inactivation by a single type of thiol detector may not be conclusive evidence of the presence of thiol groups, as even the very selective mercurial compounds can inactivate enzymes like cytochrome oxidase or catalase which in all probability are not thiols (Kreke, Schaefer, Seibert & Cook, 1950; Seibert, Kreke & Cook, 1950). On the other hand, the effect of some thiol reagents may be minimized or nullified by the denatured proteins present in impure enzyme preparations (cf. Hopkins & Morgan, 1938) a fact that has been overlooked in some of the studies quoted. It seemed advisable to make a systematic analysis of the effect of several types of thiol detectors on highly purified yeast carboxylase, and in this paper evidence will be given showing that carboxylase is a thiol enzyme and that the thiol group has an essential role in the catalysis of pyruvate decarboxylation. A preliminary account of this work has been given elsewhere (Stoppani, Actis, Deferrari & Gonzalez, 1952).

EXPERIMENTAL

Enzyme preparations. Carboxylase with a Q_{CO_2} (μl. CO_2/hr./mg. dry wt.) about 12 000–13 000 was obtained from yeast, as described by Green *et al.* (1941). The protein concentration of each preparation was determined by its absorption at 280 mμ., in a Beckman spectrophotometer. The enzyme was stored at 4°, suspended in 0·05 M-sodium citrate, pH 6·0, 50% saturated with $(NH_4)_2SO_4$. The nitroprusside test was negative with the purified protein.

Measurement of carboxylase activity. Measurements were made manometrically, in duplicate. In most of the experiments, carboxylase dissolved in phosphate buffer was placed in the main compartment of the manometer flask and after 5–15 min. of equilibration at 30°, was mixed with 0·1 ml. M-sodium pyruvate added from the side bulb. The final volume of fluid was 1·0 ml. and the final concentration of buffer (phosphate, unless stated otherwise), 0·05 M, pH 6·0. The inhibitors were placed with the enzyme solution in the flask main compartment. Carboxylase activity was usually read 5 and 10 min. after adding the substrate. Phosphate buffer has been preferred to citrate (recommended by Green *et al.* 1941) as the latter forms complexes with heavy metals. In our experiments carboxylase had the same specific activity in citrate or phosphate buffer; the smaller activity of carboxylase in phosphate reported by Green *et al.* (1941) and Cajori (1942) can perhaps be attributed to metal impurities in the reagent.

Reagents. The following have been used (in parentheses are given the source or method of preparation): methyldichloroarsine (Baeyer, 1898) b.p. 132–134°; 3-amino-4-hydroxyphenylarsenoxide hydrochloride (Parke Davis and Co.), 3-amino-4-hydroxyphenyldichloroarsine hydrochloride (E. R. Squibb and Sons); *p*-chloromercuribenzoic acid (Whitmore & Woodward, 1941) 99·5% pure, as determined by chlorine estimation; L-cystine (E. Merck, Darmstadt); iodoacetic acid (Schering-Kahlbaum); ethyl iodoacetate (Tiemann, 1898); chloroacetophenone and bromobenzyl cyanide (Rojahn & Giral, 1946); *o*-iodosobenzoic acid (Askenasy & Meyer, 1893), 99·5% pure by iodometric titration; alloxan (Eastman-Kodak Co.); DL-cysteine hydrochloride, and glutathione (Eastman-Kodak Co.); 2:3-dimercaptopropanol (Boots Pure Drug Co., Ltd.); cocarboxylase (Hoffman-La Roche) and sodium pyruvate (Robertson, 1942). All solutions were made up with glass-distilled water.

Cysteine, glutathione, 2:3-dimercaptopropanol, and the arsenical compounds, were dissolved in water and the pH adjusted to 6·0 immediately before use. Dichloroarsines hydrolyse and give the respective arsenoxides and the results are attributed to the latter. Bromobenzyl cyanide

and chloroacetophenone are sparingly soluble in water and therefore were first dissolved in dioxan (peroxide free) and 4·0 μl. of the solution added per flask. Controls received the same volume of dioxan; this does not significantly modify the activity of carboxylase.

Abbreviations. The following have been introduced when necessary: 3-amino-4-hydroxyphenylarsenoxide and 3-amino-4-hydroxyphenyldichloroarsine, mapharside; *p*-chloromercuribenzoic acid, ClHgB; *o*-iodosobenzoic acid, IOB; iodoacetic acid, IA; ethyl iodoacetate, EtIA; bromobenzyl cyanide, BBC; chloroacetophenone, CAP; DL-cysteine, CySH; reduced glutathione, GSH; 2:3-dimercaptopropanol, BAL; glycine, Gly; DL-alanine, Ala and DL-lysine, Lys.

pH. This was measured with a glass electrode.

Expression of results. As the activity of purified carboxylase preparations was constant, only the amount of purified protein used in each experiment and the percentage inhibition due to the SH reagent has been recorded.

Thiol compounds may prevent carboxylase inhibition (protection experiments, *P*) or reverse it (reactivation experiments, *R*). *P* and *R* will be calculated with expressions (1) and (2) where *I* is the percentage change of activity due to the inhibitor alone and, I_p and I_r the same in the presence of thiol added before (I_p) or after (I_r) carboxylase has reacted with the inhibitor. When the thiol does not afford complete protection (3) gives a more accurate expression of the enzyme reactivation. For reactivation experiments, manometer vessels with double side arms were used, one for pyruvate and the other for the thiol solution. Pyruvate and thiol were mixed simultaneously with the content of the flask main compartment.

$$P = 100\,\frac{I - I_p}{I}\,(\%); \qquad (1)$$

$$R = 100\,\frac{I - I_r}{I}\,(\%); \qquad (2)$$

$$R' = 100\,\frac{I - I_r}{I - I_p}\,(\%). \qquad (3)$$

Cysteine, glutathione and BAL do not modify the activity of freshly prepared carboxylase, but, after several weeks' storage in the cold, the Q_{CO_2} can slightly diminish and coincidently the thiols may show a reactivating action. For this reason the effect of inhibitors in the presence of thiols has always been calculated with reference to a control treated with the same amount of thiol compound.

RESULTS

Experiments with arsenical compounds

Effect of amount of inhibitor and rate of inhibition. Methylarsenoxide and mapharside inhibit carboxylase, the latter being about 6 times more powerful. When the percentage inhibition is plotted against $-\log_{10}$ arsenoxide molarity (*p*I), curves of similar shape are obtained (Fig. 1). The toxicity of the arsenicals (1·8 mM-mapharside or 2·5 mM-methylarsenoxide) does not vary when the enzyme concentration changes from 530 to 14·0 μg. protein/ml. The rate of carboxylase inhibition is fairly rapid as it is fully developed after 10 min. of contact with the arsenical and is the same for both compounds. The inhibition is scarcely reversible. If arsenicals in the amount required to produce 70 or 60 % inhibition are added to carboxylase, and after 15 min. incubation the mixture is diluted to reduce significantly the arsenical concentration, the activity of the enzyme does not increase.

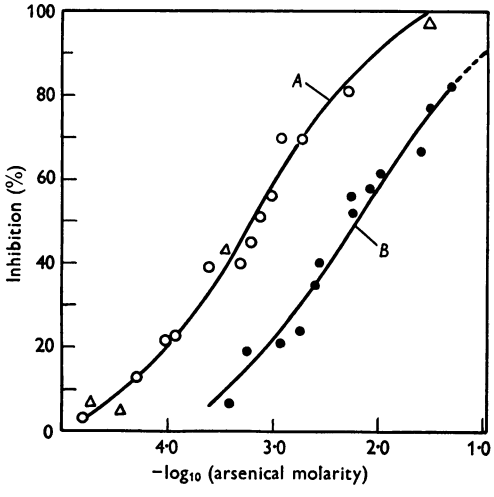

Fig. 1. Effect of arsenicals on carboxylase activity. Carboxylase (30·0 or 70·0 μg./ml.) treated for 15 min. with the arsenical, at 30°, before the addition of pyruvate. Curve *A*, mapharside (○, 3-amino-4-hydroxyphenylarsenoxide; △, 3-amino-4-hydroxydichlorophenarsine). Curve *B*, methylarsenoxide.

Effect of arsenicals in the presence of thiol compounds. The results with methylarsenoxide differ in part from those obtained with mapharside. Cysteine, glutathione and BAL prevent carboxylase inactivation by methylarsenoxide, but only BAL and cysteine (in a much smaller degree) can reactivate the enzyme poisoned with the arsenical (Table 1). BAL is more effective than cysteine as with a ratio of SH:As of 5:1, the protection is 100 % with BAL and 65 % with cysteine, whereas the reversion is 40 % with BAL and 1 % with cysteine. When the SH:As ratio is about 1 the toxicity of methylarsenoxide increases with either thiol compound.

Cysteine, glutathione and BAL protect carboxylase against mapharside, cysteine being more effective than BAL (Table 1 and Fig. 2); on the other hand, cysteine is less active than BAL in reversing mapharside inhibition. When the SH:As ratio is about 10, the protection is 94 % with cysteine and 68 % with BAL, while BAL reverses the inactivation by 40 % and cysteine none; equivalent amounts of mapharside and thiols are

Table 1. *Effect of thiol compounds on carboxylase inactivation by arsenicals*

(Inactivation experiments (*I*): carboxylase treated for 15 min. with the arsenical. Protection experiments (*P*): arsenical added to carboxylase + thiol. Reversal experiments (*R*): thiol added to carboxylase treated with arsenical for 15 min. 45·0 or 67·0 μg./ml. carboxylase.)

Experiment	Arsenical (mM)	Thiol compound	Inhibition of carboxylase activity (%)	Prevention or reversal of inhibition (%)
	Methylarsenoxide			
I	4·5	None	60·5	—
P	4·5	17·0 mM-GSH	11·2	*P* 81·5
R	4·5	17·0 mM-GSH	63·5	*R* −4·7
I	2·8	None	40·0	—
P	2·8	5·0 mM-BAL	0·0	*P* 100·0
R	2·8	5·0 mM-BAL	24·2	*R* 39·4
	Mapharside			
I	0·6	None	59·0	—
P	0·6	17·0 mM-GSH	5·6	*P* 90·5
R	0·6	17·0 mM-GSH	63·5	*R* −7·6
I	0·6	None	49·5	—
P	0·6	10·0 mM-BAL	16·2	*P* 67·3
R	0·6	10·0 mM-BAL	14·7	*R* 70·4

more toxic than the arsenical (Fig. 2). The reversal of mapharside poisoning with BAL does not depend on the time carboxylase was incubated with the arsenical, the completeness of the latter combination being granted. With methylarsenoxide, the reactivation by BAL is somewhat delayed when the time of its incubation with carboxylase increases from 15 to 45 min.

Experiments with heavy metals

Inhibition of carboxylase by p-chloromercuribenzoate, mercuric, cupric and silver ions. The activity of carboxylase diminishes in the presence of small concentrations of heavy metals within a range of 0·01–0·001 mM. The toxicity of equivalent concentrations decreases in the order ClHgB, Hg^{2+}, Ag^{+}, Cu^{2+}, and the pI_{50} are 5·85, 5·85, 5·60 and 4·35, respectively (Fig. 3). These values are significant only in regard to purified carboxylase, in the amounts and conditions described. Carboxylase reacts quickly with heavy metals as after 2 min. incubation with the enzyme (88·0 μg.) there is no further increase in the effect of Hg^{2+} (2·0 μM), Ag^{+} (5·0 μM), Cu^{2+} (0·01 mM) and ClHgB (0·01 mM).

Stability of carboxylase metal mercaptides. Carboxylase forms scarcely dissociated compounds with metals as proved by: (*a*) the dependence of the inhibition intensity on the protein concentration (Table 2), and (*b*) the stability of the inhibition when the carboxylase-mercaptide is diluted to reduce significantly the inhibitor concentration.

Effect of heavy metals in the presence of thiol compounds. Thiols can prevent carboxylase inactivation by metals, or reverse it (Table 3). When the SH:metal ratio is 2·5 or 5·0:1, cysteine inactivates silver ions and *p*-chloromercuribenzoate, reduces the action of mercuric ions but increases the toxicity of cupric ions. On the other hand, carboxy-

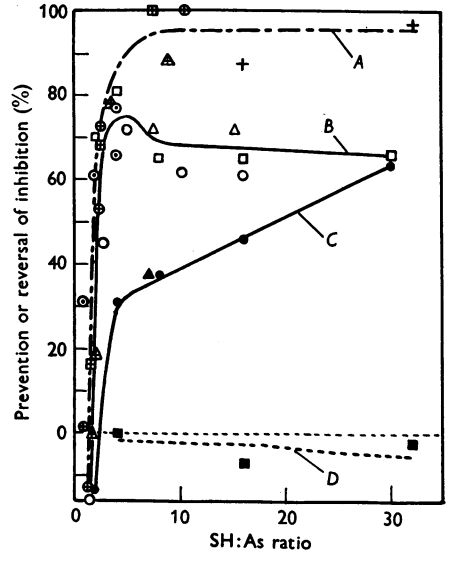

Fig. 2. Effect of cysteine and BAL on mapharside toxicity. Protection experiments: *A*, with cysteine and *B* with BAL. Reversal experiments: *C*, with BAL and *D* with cysteine. The ratios SH:As reported in the abscissa have been obtained with the following concentrations of mapharside (or thiol): *A*: +, 0·6 mM-mapharside; ⊕, 4·4 mM-CySH; ⊞, 8·8 mM-CySH; ⊞, 1·7 mM-mapharside. *B*: □, 0·6 mM-mapharside; △, 4·4 mM-BAL; ⊙, 1·2 mM-mapharside. *C*: ●, 1·2 mM-mapharside; ▲, 4·4 mM-BAL. *D*: ■, 0·6 mM-mapharside. For further details see Table 1.

lase inhibited by *p*-chloromercuribenzoate, mercuric and silver ions is about half reactivated by cysteine. BAL almost nullifies the toxicity of *p*-chloromercuribenzoate, mercuric, cupric and silver ions and also reverses their effect, although to a lesser degree. The ability of cysteine and BAL to reactivate carboxylase poisoned with *p*-chloromercuribenzoate and silver ions is about the same, but BAL is more effective with carboxylase poisoned with mercuric and cupric ions (Fig. 4). Cysteine, even in large excess, did not completely reactivate carboxylase treated with heavy metals. Thus, in experiments as described in Table 3, the effects of 3·0 μM-ClHgB, 4·0 μM-Ag^+ and 10·0 μM-Hg^{2+}, were reversed 83·0, 78·0 and 83·0 % by

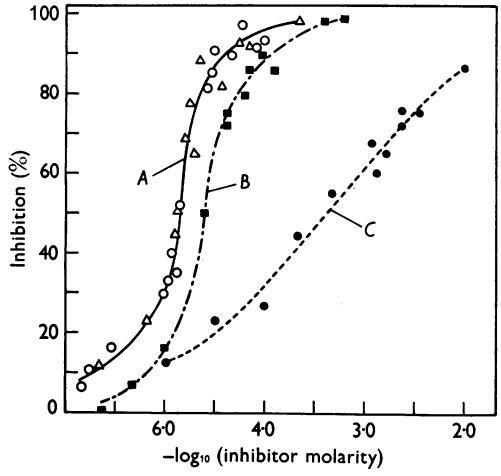

Fig. 3. Effect of *p*-chloromercuribenzoate, mercuric, silver and cupric ions on the activity of carboxylase. Carboxylase (45·0 μg./ml.) treated for 10 min. at 30° with ClHgB (*A*, ○), Hg^{2+} (*A*, △), Ag^+ (*B*) or Cu^{2+} (*C*).

Table 2. *Effect of heavy metals on several amounts of carboxylase*

(Carboxylase treated with the inhibitor for 10 min. before the addition of pyruvate.)

	Percentage inhibition with			
Carboxylase (μg./ml.)	ClHgB (1·2 μM)	Hg^{2+} (1·1 μM)	Ag^+ (2·4 μM)	Cu^{2+} (0·12 mM)
61·0	7·0	15·9	0·0	24·2
30·5	33·0	43·0	15·7	31·8
15·7	75·0	—	51·0	54·2
7·8	95·0	85·0	87·5	72·5

Table 3. *Effect of thiol compounds on carboxylase inactivation by heavy metals*

(*I*, *P* and *R*, same as in Table 1, metal reagent instead of arsenical. 66·0 μg./ml. carboxylase.)

Experiment	Inhibitor (μM)	Thiol compound	Inhibition of carboxylase activity (%)	Prevention or reversal of inhibition (%)
	ClHgB			
I	10·0	None	89·9	—
P	10·0	50·0 μM-CySH	14·2	*P* 84·2
R	10·0	50·0 μM-CySH	59·9	*R* 33·3
R	10·0	25·0 μM-CySH	62·6	*R* 30·4
P	10·0	25·0 μM-BAL	4·4	*P* 95·2
R	10·0	25·0 μM-BAL	50·5	*R* 44·0
R	10·0	12·5 μM-BAL	49·4	*R* 45·1
	Hg^{2+}			
I	10·0	None	91·0	—
P	10·0	50·0 μM-CySH	22·1	*P* 75·8
R	10·0	50·0 μM-CySH	51·6	*R* 43·3
P	10·0	25·0 μM-BAL	9·5	*P* 89·5
R	10·0	25·0 μM-BAL	28·9	*R* 68·6
	Ag^+			
I	10·0	None	91·5	—
P	10·0	50·0 μM-CySH	6·6	*P* 92·8
R	10·0	50·0 μM-CySH	31·6	*R* 65·4
R	10·0	25·0 μM-CySH	40·2	*R* 56·2
P	10·0	25·0 μM-BAL	5·8	*P* 93·6
R	10·0	25·0 μM-BAL	33·9	*R* 62·9
R	10·0	12·5 μM-BAL	48·0	*R* 47·5
	Cu^{2+}			
I	50·0	None	56·7	—
P	50·0	0·250 mM-CySH	76·8	*P* −35·5
R	50·0	0·250 mM-CySH	56·5	*R* 0·3
P	50·0	0·125 mM-BAL	6·0	*P* 89·5
R	50·0	0·125 mM-BAL	26·2	*R* 53·8

16·0, 48·0 and 4·8 mM-cysteine, respectively. The toxicity of cupric ions increases in the presence of cysteine, at a ratio SH:Cu^{2+} of 7:1 or less (Fig. 4), whereas larger concentrations of cysteine prevent cupric ion inhibition, or partially reverse it. The reactivation, however, is never complete even to a concentration of 50 mM of cysteine. Similar results may be obtained with glutathione. BAL is more effective than monothiols and does not potentiate the effect of cupric ions. The potentiation of the toxicity of cupric ions by cysteine or glutathione is due to the formation of a cuprous mercaptide

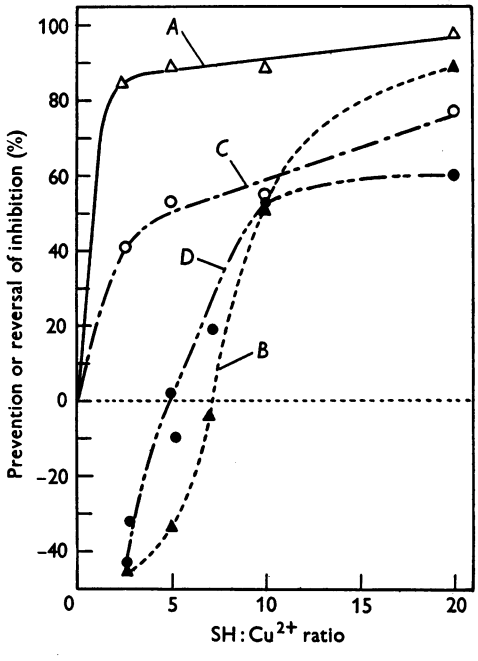

Fig. 4. Effect of cysteine and BAL on cupric ions toxicity. 66·0 μg./ml. carboxylase. 50·0 μM-Cu^{2+}. Protection experiments: curve *A*, with BAL and *B*, with cysteine. Reversal experiments: curve *C*, with BAL and *D*, with cysteine. Thiol in the concentration required for the SH:Cu^{2+} ratio represented in the abscissa. For further details see Table 3.

(Pirie, 1931) more active than the equivalent amount of copper (Stoppani, Actis & Deferrari, 1952).

Prevention with heavy metals of carboxylase inactivation by arsenicals. If heavy metals and the arsenical compounds react with the same group of carboxylase, it may be assumed that carboxylase combined with the metal will not react with the arsenical, as the metal mercaptides are about a thousand times more stable than the corresponding thioarsenites. Addition of cysteine or glutathione to the mixture will inactivate the arsenical and, on the other hand, it will reactivate the enzyme by dissociating its metal mercaptide. A control treated with arsenical alone will be scarcely reactivated by the thiol, if the latter concentration is properly chosen. A quantitative expression of the interference of the heavy metal with the arsenical may be derived as follows. P_M (4) is the difference between R_{MAs} (5), the relative activity of carboxylase treated with metal $_{(M)}$ arsenical $_{(A)}$ and thiol $_{(SH)}$, and R_{As} (6), the activity of the control treated only with arsenical and thiol.

$$P_M\,(\%) = R_{MAs}\,(\%) - R_{As}\,(\%), \qquad (4)$$

R_{MAs}, and R_{As}, are calculated with expression (5) and (6) respectively, where I_{As}, I_{AsSH} and I_{MAsSH} are the diminution of carboxylase activity in the presence of arsenical, arsenical + thiol, and metal + arsenical + thiol, respectively.

$$R_{MAs} = 100\,\frac{I_{As} - I_{MAsSH}}{I_{As}}\,(\%); \qquad (5)$$

$$R_{As} = 100\,\frac{I_{As} - I_{AsSH}}{I_{As}}\,(\%). \qquad (6)$$

As the metal effect is not completely reversed by the thiol compounds, P_M must be corrected in order to obtain P'_M (7),

$$P'_M = 100\,\frac{P_M}{R_M}\,(\%), \qquad (7)$$

where R_M represents the percentage reversal (by the thiol) of the metal toxic effect. Moreover, if the arsenical is not completely inactivated by the thiol, R'_{As} (3) will be used instead of (6). P'_M expresses the interference of the metal in the reaction of carboxylase with the arsenical.

As postulated, the reversal with cysteine or glutathione of carboxylase inactivation by arsenicals increases significantly when the enzyme has been previously treated with *p*-chloromercuribenzoate (Table 4) or metal ions (Table 5); the protection afforded by the latter reagents cannot be attributed to their combination with the arsenical compounds as the mixture of arsenical and metal inactivates carboxylase to a larger extent than each component alone. However, heavy metals may not completely prevent the reaction of carboxylase with the arsenicals, even in a concentration saturating all the enzyme active groups.

Experiments with alkylating agents

Effect of iodoacetate, ethyl iodoacetate, bromobenzyl cyanide and chloroacetophenone upon carboxylase. When allowed enough time to react, alkylating agents inactive carboxylase (Table 6). Maximal effect is obtained after 10 min. of contact with ethyl iodoacetate; 20 min. with iodoacetate

Table 4. *Effect of* p-*chloromercuribenzoate on carboxylase inactivation by arsenicals*

(*p*-Chloromercuribenzoate is added to carboxylase 2 min. before the arsenical. The mixture is incubated at 30° for 15 min. and pyruvate and thiol are added separately. Carboxylase activity is measured 10 min. later. The figures enclose the respective controls.)

Additions	Inhibition of carboxylase activity (%)	Reversal of inhibition (%)	Percentage interference of *p*-chloromercuribenzoate with arsenical (P'_M)
2·9 mM-Methylarsenoxide + 16·0 mM-CySH	48·8	−1·8	—
5·6 μM-ClHgB + 2·9 mM-methylarsenoxide + 16·0 mM-CySH	21·4	82·3	104·5
5·6 μM-ClHgB + 16·0 mM-CySH	18·6	80·5	—
1·1 mM-Mapharside + 10·0 mM-GSH	59·5	0·8	—
5·6 μM-ClHgB + 1·1 mM-mapharside + 10·0 mM-GSH	36·2	39·7	56·0
5·6 μM-ClHgB + 10·0 mM-GSH	29·0	69·5	—

Controls. Percentage inhibition of carboxylase by 2·9 mM-methylarsenoxide (*a*) 48·2; by 1·1 mM-mapharside (*b*) 60·0; by 5·6 μM-ClHgB (*c*) 94·7; by *a* + *c*, 97·0; by *b* + *c*, 97·0; inactivation (%) of *a*, by 16·0 mM-CySH, 67·0; same of *a* + *c*, 68·2; same of *b* and *c*, and *b* + *c* by 10·0 mM-GSH, 100·0; same of *c* by 16·0 mM-CySH, 100·0. 30·0 μg./ml. carboxylase.

Table 5. *Effect of mercuric, cupric and silver ions on carboxylase inactivation by mapharside*

(Experiments carried out as in Table 4.)

Carboxylase (μg./ml.)	Additions	Inhibition of carboxylase activity (%)	Reversal of inhibition (%)	Percentage interference of metal with marpharside (P'_M)
280·0	1·1 mM-Mapharside + 15·0 mM-CySH	61·0	3·2	—
280·0	10·0 μM-Hg^{2+} + 1·1 mM-mapharside + 15·0 mM-CySH	25·0	60·3	69·3
280·0	10·0 μM-Hg^{2+} + 15·0 mM-CySH	16·6	82·4	—
30·0	1·1 mM-Mapharside + 10·0 mM-GSH	65·0	−3·2	—
30·0	0·2 mM-Cu^{2+} + 1·1 mM-mapharside + 10·0 mM-GSH	39·6	37·2	62·0
30·0	0·2 mM-Cu^{2+} + 10·0 mM-GSH	28·2	65·2	—
194·0	1·1 mM-Mapharside + 15·0 mM-CySH	62·0	1·6	—
194·0	10·0 μM-Ag^{+} + 1·1 mM-mapharside + 15·0 mM-CySH	29·0	54·0	57·4
194·0	10·0 μM-Ag^{+} + 15·0 mM-CySH	7·0	91·3	—

Controls. Percentage inhibition of carboxylase by 1·1 mM-mapharside (*a*) 63·0; by 10·0 μM-Hg^{2+} (*b*) 94·5; by 0·2 mM-Cu^{2+} (*c*) 81·0; by 10·0 μM-Ag^{+} (*d*) 81·0. Inactivation (%) of *a*, *b* and *d*, by 15·0 mM-CySH, and *c* by 10·0 mM-GSH, 100·0; inactivation (%) of *a* + *b* and *a* + *c* by 15·0 mM-CySH, and *a* + *d* by 10·0 mM-GSH, 100·0.

Table 6. *Inactivation of carboxylase by alkylating agents*

(45·0 μg. of carboxylase treated with inhibitor for 15 (*a*) or 30 (*b*) min., before the addition of pyruvate.)

Iodoacetate		Ethyl iodoacetate		Bromobenzyl cyanide		Chloroacetophenone		
	Inhibition (%)		Inhibition (%)		Inhibition (%)		Inhibition (%)	
mM	(*a*)	mM	(*a*)	mM	(*a*)	mM	(*a*)	(*b*)
10·5	89·0	13·8	64·0	15·0	88·2	10·6	37·8	—
7·3	68·0	7·0	44·1	3·8	78·0	9·0	33·2	81·5
5·2	60·9	3·5	37·6	1·9	63·0	8·6	24·2	—
3·0	57·2	0·7	14·5	1·0	40·5	4·5	17·3	68·0
1·1	45·0	0·3	3·2	0·5	23·8	2·2	17·0	62·0
0·5	28·0	—	—	—	—	1·1	—	41·0
0·1	10·6	—	—	—	—	0·4	—	15·0

and 30 min. with bromobenzyl cyanide or chloroacetophenone.

Effect of alkylating agents in the presence of thiols. Thiols prevent but do not reverse carboxylase inactivation by alkylating agents. Cysteine and BAL afford complete protection against iodoacetate and ethyl iodoacetate, but only protect partially against bromobenzyl cyanide or chloroacetophenone (Table 7). The reaction of bromine and chlorine compounds with cysteine is slow (Barron, 1951) and therefore bromobenzyl cyanide and chloroacetophenone may react with the enzyme

Table 7. *Effect of thiol compounds and simple amino-acids on carboxylase inactivation by alkylating agents*

(*I*, *P* and *R*, same as in Table 1. Alkylating agent instead of arsenical. 45·0 μg./ml. carboxylase.)

Experiment	Inhibitor (mM)	Thiol compound or amino-acid	Inhibition of carboxylase activity (%)	Prevention or reversal of inhibition (%)
	IA			
I	3·0	None	52·5	—
P	3·0	8·0 mM-CySH	0·0	*P* 100·0
R	3·0	8·0 mM-CySH	63·0	*R* −20·0
P	3·0	4·0 mM-BAL	16·0	*P* 69·5
P	3·0	8·0 mM-Gly	43·5	*P* 17·1
P	3·0	8·0 mM-Ala	52·0	*P* 0·9
P	3·0	4·0 mM-Lys	50·5	*P* 3·8
	EtIA			
I	3·4	None	34·0	—
P	3·4	8·0 mM-CySH	0·0	*P* 100·0
R	3·4	8·0 mM-CySH	32·8	*R* 3·5
P	3·4	8·0 mM-Gly	16·7	*P* 50·9
R	3·4	8·0 mM-Ala	21·8	*R* 36·0
	BBC			
I	1·9	None	59·5	—
P	1·9	8·0 mM-CySH	28·2	*P* 52·6
R	1·9	8·0 mM-CySH	62·0	*R* −4·2
I	1·1	None	33·2	—
P	1·1	20·0 mM-BAL	16·6	*P* 50·0
R	1·1	20·0 mM-BAL	43·5	*R* −31·0
	CAP			
I	2·0	None	22·0	—
P	2·0	8·0 mM-CySH	11·3	*P* 48·6
R	2·0	8·0 mM-CySH	31·0	*R* −41·0

Table 8. *Effect of* p*-chloromercuribenzoate and mercuric ions on carboxylase inactivation by alkylating agents*

(Experiments as in Table 4. Iodoacetate instead of arsenical. 79·2 μg./ml. carboxylase.)

Additions	Inhibition of carboxylase activity (%)	Reversal of inhibition (%)	Percentage interference of mercurial with iodoacetate (P'_M)
3·0 mM-IA + 8·0 mM-CySH	63·0	4·5	—
20·0 μM-Hg^{2+} + 3·0 mM-IA + 8·0 mM-CySH	21·0	68·0	73·0
20·0 μM-Hg^{2+} + 8·0 mM-CySH	12·7	87·0	—
10·0 μM-ClHgB + 3·0 mM-IA + 8·0 mM-CySH	35·0	47·0	64·7
10·0 μM-ClHgB + 8·0 mM-CySH	32·8	65·7	—

Controls. Percentage inactivation of carboxylase by 3·0 mM-IA (*a*) 66·0; by 20·0 μM-Hg^{2+} (*b*) 98·0; by 10·0 μM-ClHgB (*c*) 95·5; by *a* + *b*, 92·5; by *a* + *c*, 95·5. Inactivation (%) of *a*, *b*, *c*, *a* + *b* by 8·0 mM-CySH, 100·0.

notwithstanding the presence of thiol. As alkylating agents can also react with amines some attempts at protection were made with glycine, alanine and lysine. Glycine protects carboxylase against iodoacetate or ethyl iodoacetate and alanine and lysine against the latter, but the simple amino-acids are less effective than the equivalent amount of cysteine. In the reactivation experiments, cysteine rather increases bromobenzyl cyanide and chloroacetophenone toxicity.

Effect of metals on carboxylase inactivation by alkylating agents. Cysteine reactivates to a large extent carboxylase treated successively with *p*-chloromercuribenzoate (or mercuric ions) and iodoacetate, but does not activate a control treated with the same amount of the latter (Table 8). The mercurial compounds have been chosen for these experiments on account of the easier reversal of their effect. The interference of ClHgB or Hg^{2+} with the alkylation of carboxylase, which has been calculated as in the similar case with the arsenical compounds, shows that iodoacetate and ClHgB (or Hg^{2+}) react to a considerable extent with the same group of the enzyme. In similar experiments,

ClHgB and Hg^{2+} do not interfere, however, with ethyl iodoacetate, bromobenzyl cyanide and chloroacetophenone.

Experiments with oxidizing agents

Effect of o-*iodosobenzoate.* At pH 7·0 this agent oxidizes SH groups to disulphide (Hellerman, Chinard & Ramsdell, 1941), the reaction having a pH parameter, as with higher hydrogen-ion concentration the oxidation may proceed further. In the experiments already reported, the pH was 6·0 and therefore comparative experiments at pH 6·0 and 7·0 were carried out, *o*-iodosobenzoate being somewhat more effective at pH 7·0. In no case was complete inactivation of carboxylase obtained (Table 9). The effect does not depend on the protein concentration (from 158 to 1180 μg./ml.; IOB 1·0 mM) and is complete at pH 6·0 after 5 min. of contact with carboxylase. At pH 7·0 the rate of activation is rapid but still increases after 5 min.

Table 9. *Inactivation of carboxylase by* o-*iodosobenzoate*

(136–218 μg./ml. carboxylase treated with *o*-iodosobenzoate for 15 min. at 30°. pH 6·0.)

IOB (mM)	Inactivation (%), at pH 6·0	Inactivation (%), at pH 7·0
1·7	78·0	—
1·0	54·0	66·0
0·5	47·8	—
0·1	27·5	45·0
0·01	6·6	10·0

Effect of thiol compounds on o-*iodosobenzoate toxicity.* Cysteine and BAL prevent carboxylase oxidation by this agent, BAL being more active than cysteine. At an SH:IOB ratio of 2·5:1, BAL prevents 80 % and cysteine 34 % of the carboxylase inactivation; apparently reduction of *o*-iodosobenzoate by dithiols is easier than by monothiols. Carboxylase oxidized at pH 7·0 can be partly reactivated by thiols (Table 10). This fact must be attributed to a reduction of the inactive protein rather than to removal of the inhibitor, as carboxylase, treated with *o*-iodosobenzoate, does not increase its activity on reduction of the inhibitor concentration by dilution. BAL is as effective as cysteine, at the same SH concentration. At pH 6·0 the effect of IOB is not reversed by thiols, which suggests that the oxidation of enzyme SH proceeds beyond the disulphide stage.

Table 10. *Reactivation with cysteine or BAL of carboxylase oxidized with* o-*iodosobenzoate*

(1·92 mg. carboxylase in 8·0 ml. 0·06M-phosphate are treated for 5 min. at 30° with 1·0 mM-*o*-iodosobenzoate. 0·7 ml. mixture is mixed with 0·2 ml. thiol solution, and after 5 min. equilibration at 30°, with 0·1 ml. M-pyruvate.)

pH	Thiol compound	Inhibition of carboxylase activity (%)	Reversal of inhibition (%)
7·0	None	52·5	—
7·0	20·0 mM-CySH	39·2	25·4
7·0	10·0 mM-BAL	39·0	25·7
6·0	None	46·5	—
6·0	20·0 mM-CySH	54·0	−16·1
6·0	10·0 mM-BAL	45·0	3·2

Prevention by p-*chloromercuribenzoate and mercuric ions of carboxylase oxidation with* o-*iodosobenzoate.* If all these agents react with the same sulphydryl of carboxylase it may be assumed that the mercaptides will not be oxidized by *o*-iodosobenzoate, and therefore at pH 6·0, cysteine will reactivate carboxylase treated with metal and oxidant, but will not reactivate the enzyme treated with the latter alone. This is confirmed to a large extent by the experiments reported in Table 11.

Effect of other oxidizing agents. Carboxylase is inhibited by iodine, and alloxan, but not by L-cystine. Carboxylase treated with alloxan shows a pink colour. The effect of 0·0125 mM-iodine (63·0 % inhibition of activity) is not reversed by an

Table 11. *Effect of* p-*chloromercuribenzoate or mercuric ions on carboxylase inactivation by* o-*iodosobenzoate*

(218 μg./ml. carboxylase. Experiments as in Table 4. *o*-Iodosobenzoate instead of arsenical.)

Additions	Inhibition of carboxylase activity (%)	Reversal of inhibition (%)	Percentage interference of mercurial with IOB (P'_M)
1·0 mM-IOB + 10·0 mM-CySH	49·0	5·3	—
10·0 μM-Hg^{2+} + 1·0 mM-IOB + 10·0 mM-CySH	18·6	70·5	71·1
10·0 μM-Hg^{2+} + 10·0 mM-CySH	8·3	91·7	—
1·0 mM-IOB + 10·0 mM-CySH	54·9	−7·3	—
10·0 μM-ClHgB + 1·0 mM-IOB + 10·0 mM-CySH	32·3	41·1	60·3
10·0 μM-ClHgB + 10·0 mM-CySH	19·8	80·2	—

Controls. Percentage inactivation of carboxylase by 1·0 mM-IOB, 51·5; by 10·0 μM-Hg^{2+}, 100·0; by 10·0 μM-ClHgB, 100·0; by IOB + Hg^{2+}, 91·5; by IOB + ClHgB, 94·5. Inactivation (%) by 10·0 mM-CySH of 1·0 mM-IOB, 93·0; of 10·0 μM-Hg^{2+} or 10·0 μM-ClHgB, 100·0; of IOB + Hg^{2+}, and IOB + ClHgB, 94·0.

Table 12. *Inhibition of carboxylase by heavy metals in the presence of pyruvate*

(0·1 ml. carboxylase solution (45·0 μg.) mixed with 0·9 ml. 0·05 M-phosphate buffer containing pyruvate and inhibitor. Carboxylase activity read 5 min. later.)

3·3 μM-ClHgB		1·7 μM-Hg^{2+}		0·3 mM-Cu^{2+}		12·0 μM-Ag^{+}	
Pyruvate concentration (mM)	Carboxylase inhibition (%)	Pyruvate concentration (mM)	Carboxylase inhibition (%)	Pyruvate concentration (mM)	Carboxylase inhibition (%)	Pyruvate concentration (mM)	Carboxylase inhibition (%)
0·0*	88·0	0·0*	79·2	0·0*	58·6	0·0*	85·0
25·0	71·0	26·0	58·5	27·0	46·3	—	—
51·5	58·7	52·0	51·0	54·0	40·0	50·0	86·0
108·5	33·8	104·0	30·1	108·0	28·1	100·0	77·6
217·0	24·5	236·0	17·8	216·0	27·8	200·0	75·8

* Pyruvate (0·1 ml., M) added to carboxylase 15 min. after the inhibitor.

excess of cysteine or BAL (10·0 mM), and moreover, mercuric ions or *p*-chloromercuribenzoate (10·0 μM) do not prevent carboxylase oxidation by iodine (0·0125 mM).

Effect of pyruvate on the combination of SH *reagents with carboxylase*

The effect of pyruvate on carboxylase inactivation has been studied (*a*) by adding the enzyme to the inhibitor in the presence of pyruvate and (*b*) by adding increasing amounts of pyruvate to carboxylase already combined with the inhibitor.

Experiments with p*-chloromercuribenzoate and metal ions.* When *p*-chloromercuribenzoate, mercuric or cupric ions are added to carboxylase in the presence of pyruvate, their effectiveness diminishes in relation to the amount of pyruvate (Table 12). The ability of pyruvate to reduce the degree of inhibition is not dependent on variations of the solution molarity (cf. Burk, 1943) as equivalent amounts of sodium chloride or acetate neither diminish the inhibitor's toxicity nor increases the protection afforded by pyruvate. The rate of combination of *p*-chloromercuribenzoate with carboxylase is considerably delayed by the presence of pyruvate (Fig. 5), as 20 min. after the addition of mercurial with pyruvate, the inhibition was 48·0 %, while the same amount of mercurial alone, in 2 min. (or less) gave 90·0 % inhibition. Similar results are obtained with mercuric ions. If inhibition in the presence of substrate is calculated according to the xpression (8) derived (Hunter & Downs, 1945) from che Michaelis-Menten theory of substrate-inhibitor competition, for each concentration of inhibitor, (8) satisfies the experimental values of $[S]$ and α (Fig. 6), where $[S]$ is the pyruvate molarity and α equals $100-I$ (I, percentage of inhibition). On the other hand, if $[S]$ is kept constant and the inhibitor molarity $[i]$ varies, the percentage inhibition values

$$[i]\frac{\alpha}{100-\alpha}=k\,[S] \qquad (8)$$

do not fit the expression (8) (Table 13). Furthermore, the protection afforded by increasing amounts of pyruvate is no longer observed when the substrate is mixed with carboxylase after the enzyme has reacted with the mercurial; thus, with 10·0 μM-ClHgB and 55·0 mM-pyruvate added to 140 μg.

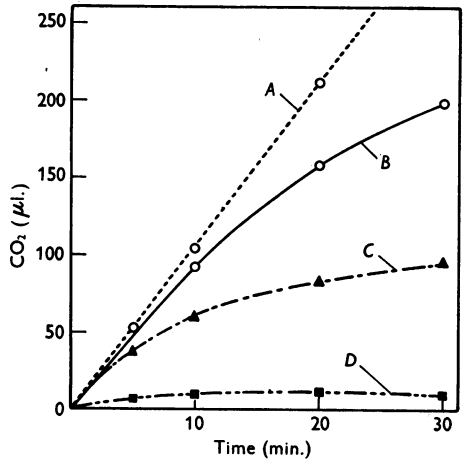

Fig. 5. Rate of carboxylase inhibition by *p*-chloromercuribenzoate, in the presence of pyruvate. 45·0 μg./ml. carboxylase. *A*, extrapolated initial activity; *B*, carboxylase activity; *C*, 3·6 μM-ClHgB, added to carboxylase with pyruvate; *D*, 3·6 μM-ClHgB added to carboxylase 5 min. before pyruvate.

carboxylase 10 min. after the mercurial, the inactivation (measured 5 min. after the addition of pyruvate) was 89·9 %; with 100, 200 or 400 mM-pyruvate the percentage inhibition was 89·0, 92·0 and 93·4 respectively.

Experiments with arsenicals. When methylarsenoxide or mapharside is added to carboxylase in the presence of pyruvate, inhibition diminishes with increasing pyruvate concentration (Table 14). The protection afforded by pyruvate is independent

of variation in salt concentration. However, when the ion concentration is increased by addition of similar amounts of sodium chloride or acetate to a mixture of arsenical and carboxylase before addition of pyruvate, then the salt causes increased inhibition. The interference of pyruvate with arsenical inhibition must also be differentiated from

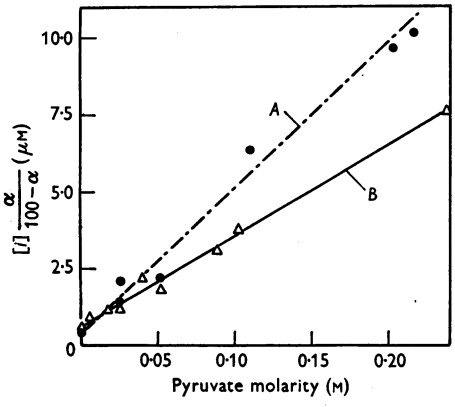

Fig. 6. Interference of pyruvate with *p*-chloromercuribenzoate and mercuric ions, at constant inhibitor concentration. *A*, 3·3 μM-ClHgB; *B*, 1·7 μM-Hg^{2+}. $\alpha = 100 - I$. *I*, percentage inactivation of carboxylase (45·0 μg./ml.); *i*, inhibitor molarity.

true competition as, after the arsenical has reacted with carboxylase, the addition of increasing concentrations of pyruvate rather exaggerates the inhibition, instead of diminishing it. Thus, with 5·0 mM-mapharside, and 66·0 mM-pyruvate added to carboxylase 15 min. after the arsenical the inactivation was 65·0 %, while with 500 mM-pyruvate, it was 71·0 %.

Experiments with o-*iodosobenzoate and iodoacetate.* When these agents react with carboxylase in the presence of pyruvate their toxicity diminishes considerably (Table 15). The effect of pyruvate is again independent of the change in the medium molarity, as shown by controls where pyruvate has been replaced by sodium chloride or acetate. Carboxylase oxidized by *o*-iodosobenzoate is not activated by increasing pyruvate concentrations. Thus, with 1·0 mM-IOB and 32·0 mM-pyruvate, added to carboxylase 10 min. after the inhibitor, the inactivation was 47·0 %, while with 160 or 320 mM-pyruvate it was 47·5 and 46·0 %, respectively.

Table 13. *Interference of pyruvate with* p-*chloromercuribenzoate and mercuric ions, at constant pyruvate concentration*

(0·1 ml. carboxylase solution (70·0 μg. protein) is mixed with 0·9 ml. phosphate containing pyruvate and inhibitor. Final concentration of pyruvate, 200 mM; of phosphate, 0·05 M. Carboxylase inhibition (%) by 15·0, 10·0 or 7·5 μM-ClHgB alone, 96·5, 92·0 and 92·0 respectively; same, by 10·0, 6·0 or 4·0 μM-Hg^{2+} alone, 96·0, 92·5 and 91·0, respectively.)

Inhibitor	*I*, inhibition of carboxylase activity (%)	$[i] = \frac{\alpha}{100-\alpha}$ (μM)
15·0 μM-ClHgB	88·0	2·04
10·0 μM-ClHgB	66·7	5·00
7·5 μM-ClHgB	53·5	6·50
10·0 μM-Hg^{2+}	83·0	2·05
6·0 μM-Hg^{2+}	71·5	2·39
4·0 μM-Hg^{2+}	48·6	4·22

$\alpha = 100 - I$.

The relation of thiol reagents to cocarboxylase

The effect of iodoacetic acid, *p*-chloromercuribenzoate and mercuric or cupric ions is not affected when added to carboxylase in the presence of an excess of cocarboxylase and/or magnesium ions

Table 14. *Inhibition of carboxylase by arsenicals in the presence of pyruvate*

(45·0 or 31·0 μg./ml. carboxylase. Experiments as in Table 12.)

Methylarsenoxide				Mapharside			
(5·4 mM)		(2·2 mM)		(5·0 mM)		(0·7 mM)	
Pyruvate concentration (mM)	Carboxylase inhibition (%)	Pyruvate concentration (mM)	Carboxylase inhibition (%)	Pyruvate concentration (mM)	Carboxylase inhibition (%)	Pyruvate concentration (mM)	Carboxylase inhibition (%)
0·0*	57·2	0·0*	37·2	0·0*	65·0	0·0*	47·6
5·4	47·2	5·2	29·1	—	—	18·0	31·0
11·0	44·6	7·8	25·0	13·0	37·0	25·0	22·9
16·0	37·8	15·6	15·1	33·0	28·2	33·0	19·0
27·0	30·7	16·5	13·7	—	—	41·0	12·8
54·0	22·0	55·0	12·8	—	—	62·0	11·2
108·0	13·3	83·0	4·3	160·0	14·5	103·0	5·8
192·0	3·1	110·0	2·1	330·0	3·9	330·0	0·0

* Pyruvate (0·1 ml., M) added to carboxylase 15 min. after the arsenical.

(Table 16). Citrate buffer has been used in these experiments, as recommended by Green *et al.* (1941).

Table 15. *Inactivation of carboxylase by iodoacetate and o-iodosobenzoate in the presence of pyruvate*

(218·0 μg./ml. (with IOB) or 45·0 μg./ml. (with IA) carboxylase. Experiments as in Table 12.)

1·0 mM-*o*-Iodosobenzoate		10·0 mM-Iodoacetate	
Pyruvate (mM)	Inactivation (%)	Pyruvate (mM)	Inactivation (%)
0·0*	47·5	0·0*	75·2
19·0	33·0	10·0	54·0
32·0	19·3	25·0	42·1
64·0	16·1	50·0	39·6
160·0	5·7	100·0	27·6
320·0	5·0	200·0	7·2

* Pyruvate (0·1 ml., M) added to carboxylase 15 min. after the inhibitor.

DISCUSSION

Among all the SH reagents, *p*-chloromercuribenzoate and mercuric or silver ions are the most powerful inhibitors. Carboxylase is at least as sensitive towards heavy metals as typical thiol enzymes such as urease (Hellerman, Chinard & Deitz, 1943) and DL-glyceraldehyde dehydrogenase (Barron & Dickman, 1949) and, with the exception of iodine, the selectivity of which is uncertain (cf. Anson, 1945), the remaining SH reagents are about a hundredth to a thousandth as effective. Mapharside is more active than methylarsenoxide. Barron found inhibition with *p*-aminophenylarsenoxide (Barron & Singer, 1945) and none with lewisite oxide (Barron *et al.* 1947). The lesser toxicity of the aliphatic arsenicals explains the latter observation and those of Peters *et al.* (1946) with lewisite oxide and ethylarsenoxide. The alkylating agents are not very effective inhibitors of carboxylase which, however, is inactivated to a larger degree than other thiol enzymes such as urease (Hellerman *et al.* 1943) or *iso*citrate dehydrogenase (Lotspeich & Peters, 1951). The slight action attributed by Mackworth (1948) to ethyl iodoacetate is now explained by the insufficient concentration of reagent used. The sensitivity of carboxylase towards *o*-iodosobenzoate is about the same as that of urease (Hellerman *et al.* 1943) or succinoxidase (Barron & Singer, 1945), but no complete inactivation is obtained such as observed by Bailey & Perry (1947) on myosin.

Thiols modify the effect of arsenical compounds on carboxylase, as well as on the other enzyme systems (cf. Stocken & Thompson, 1946; Barron, 1951). Their action may be explained by reversible formation of thioarsenites. The difference between the protecting and reversing action of each thiol shows, however, the existence of some irreversible step in the whole process, very likely the combination of carboxylase with the arsenical. BAL is less effective against mapharside than against methylarsenoxide, which may be attributed to the stronger affinity of carboxylase for mapharside and to the comparatively lesser stability of the mapharside-BAL compound (Peters & Stocken, 1947). Thiols also reverse the poisoning of carboxylase by heavy metals which is a weighty argument for reaction through SH groups (cf. Barron & Singer, 1945) as non-SH enzymes (cytochrome oxidase or catalase) inactivated by mercurials are not reactivated by addition of thiols (Kreke *et al.* 1950; Seibert *et al.* 1950). The reactivation is never complete, even with *p*-chloromercuribenzoate which on other SH enzymes such as urease (Hellerman *et al.* 1943), produces fully reversible inhibitions; this indicates that some slightly irreversible reactions must be associated with carboxylase mercaptide formation.

In the presence of pyruvate, SH detectors are less effective inhibitors of carboxylase. This protection by the substrate indicates reaction with the enzyme active group, as in competitive inhibition. Although *p*-chloromercuribenzoate and mercuric ions are the inhibitors that best fit in with this assumption, their competition with pyruvate cannot be fully represented according to the classical theory of competitive inhibition. This competition is mainly due (Ackermann & Potter, 1949) to the large difference between the dissociation constants (K) of the complexes formed by the enzyme with the substrate

Table 16. *Effect of heavy metals and iodoacetic acid in the presence of excess of cocarboxylase and/or magnesium*

(45·0 μg./ml. carboxylase in 0·05 M-citrate buffer, pH 6·0, for 10 min. with cocarboxylase or magnesium before adding the inhibitors, and 15 min. with the latter before the addition of pyruvate.)

Additions	Percentage inactivation with			
	2·0 mM-IA	2·2 μM-ClHgB	1·4 μM-Hg^{2+}	0·1 mM-Cu^{2+}
None	23·1	32·2	86·0	30·3
0·5 mM-Cocarboxylase	—	30·8	94·5	—
2·0 mM-Cocarboxylase	27·5	—	—	—
6·0 mM-Mg^{2+}	31·0	46·0	85·6	34·8
0·5 mM-Cocarboxylase + 6·0 mM-Mg^{2+}	—	47·2	100·0	—
2·0 mM-Cocarboxylase + 6·0 mM-Mg^{2+}	24·3	—	—	35·2

(K_s about 10^{-2} M), and the inhibitor (K_i about 10^{-5} M). If the data for *p*-chloromercuribenzoate and mercuric ions are calculated with Ackermann & Potter equations, no quantitative agreement is found with the experimental values, the difference probably being due to the partial irreversibility of these inhibitions. With the arsenicals, iodoacetate and *o*-iodosobenzoate, the apparent dissociation of their complexes is nearer (K_i about 10^{-3} M) to the enzyme-substrate complex dissociation, but the mechanism of the reaction of these agents with carboxylase is scarcely or not at all reversible and therefore no quantitative attempts can be made at present to deal with their interference with pyruvate. Nevertheless, the protection afforded by *p*-chloromercuribenzoate and mercuric ions against arsenicals, iodoacetate and *o*-iodosobenzoate confirms that the three latter react with the enzyme group involved in the activation of pyruvate.

The interference of pyruvate and SH reagents can be explained by assuming that substrate and inhibitor react either with the same group of carboxylase or with two groups placed close enough so that the combination of pyruvate with one of them makes the other inaccessible to the inhibitor (and vice versa). This latter mechanism is scarcely probable, as *o*-iodosobenzoate does not form a stable compound with the SH group and therefore in carboxylase oxidized by this agent, no spatial hindrance for the reaction with pyruvate would be likely. It must be then concluded that a sulphydryl is essential for the formation of the pyruvate-carboxylase complex. This SH belongs to the sluggish type described by Barron (1951) or to the group *b* of Hellerman *et al.* (1943) since it is very sensitive to metals but far less to oxidizing or alkylating agents. It is also negative in the nitroprusside test, and is involved in substrate activation. The 'sluggishness' of this SH, however, is limited. Its reactions with alkylating agents are slow but reaction with metals proceeds at a fairly rapid rate. The existence of this group provides a sounder basis for the interpretation of the enzymic decarboxylation of pyruvic acid and, in this connexion, it must be pointed out that pyruvate reacts with thiols to form mercaptals (Schubert, 1936) easily decarboxylated by heavy metals (Cavallini, 1951).

Meyer (1945) has put forward the view that the function of SH in carboxylase is to bind apo- and co-enzyme, as the latter protects apocarboxylase (an alkali-washed yeast preparation) against SH reagents. Our results neither confirm nor disprove Meyer's contention, as at pH 6·0 purified carboxylase seems to be an undissociated compound (Green *et al.* 1941; Kubowitz & Lüttgens, 1941), but in any case, if carboxylase, magnesium and the protein were bound by a sulphide link, this potential SH should not be important for the catalytic properties of carboxylase in its physiological environment, which is about pH 6·0–6·3 (Conway & Downey, 1950).

SUMMARY

1. Highly purified carboxylase is sensitive towards thiol reagents such as heavy metals, trivalent arsenicals and oxidizing and alkylating agents.

2. Heavy metals are the fastest reacting inhibitors. Alkylating agents are the slowest.

3. Heavy metals form slightly dissociated mercaptides with carboxylase, the degree of inhibition depending on the enzyme concentration. Arsenicals also form stable mercaptides, but the inhibition is not dependent on the concentration of carboxylase.

4. Cysteine, glutathione or 2:3-dimercaptopropanol protect carboxylase against sulphydryl reagents. 2:3-Dimercaptopropanol is more effective towards *o*-iodosobenzoate, methylarsenoxide, iodine, cupric and mercuric ions. Cysteine is more effective against mapharside or iodoacetate. *p*-Chloromercuribenzoate or silver ions are detoxicated by cysteine, glutathione and 2:3-dimercaptopropanol in about the same degree.

5. Cysteine, glutathione or 2:3-dimercaptopropanol reactivate to a considerable extent carboxylase poisoned by arsenicals, heavy metals and *o*-iodosobenzoate but not by iodine or alkylating agents. 2:3-Dimercaptopropanol is more effective than cysteine or glutathione in reversing the inhibition by arsenicals, cupric or mercuric ions, but is no more effective than the other thiols on enzyme treated with *p*-chloromercuribenzoate, silver ions and *o*-iodosobenzoate.

6. Pyruvate interferes with inhibition by arsenicals, heavy metals, *o*-iodosobenzoate and iodoacetate.

7. Cocarboxylase does not prevent carboxylase inactivation by iodoacetate, *p*-chloromercuribenzoate, mercuric and cupric ions.

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Studies in Detoxication

50. THE ISOLATION OF METHYL AND ETHYL GLUCURONIDES FROM THE URINE OF RABBITS RECEIVING METHANOL AND ETHANOL

BY I. A. KAMIL, J. N. SMITH AND R. T. WILLIAMS
Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

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The literature on the metabolism of ethanol has been reviewed recently by Jacobsen (1952*a*, *b*), and it seems clear that ethanol is mainly oxidized in the body to carbon dioxide via acetaldehyde and acetic acid. Methanol is also oxidized to formaldehyde and formic acid (Lund, 1948*a*, *b*) and according to Du Vigneaud & Verly (1950) (cf. also Du Vigneaud *et al.* 1951) it can serve indirectly as a precursor of labile methyl groups. It is well known, however, that methanol is more slowly eliminated from the body than ethanol (cf. Bartlett, 1950; Leaf & Zatman, 1952).

Recently we showed that in rabbits most primary aliphatic alcohols were conjugated to a small extent with glucuronic acid (Kamil, Smith & Williams, 1953). The conjugations of methanol and ethanol, however, were so small as to be almost within the experimental error of the quantitative method used. It was therefore uncertain whether these alcohols were conjugated. In the present paper, we show by the isolation of the glucuronides that these two alcohols do, in fact, conjugate with glucuronic acid in the rabbit.

EXPERIMENTAL

Glucuronic acid was determined by the method of Hanson, Mills & Williams (1944). All animals were kept on a constant diet and the pure alcohols, diluted with water, were administered by stomach tube.

Ethanol was fed at three levels, 2·9, 7·2 and 14·4 ml./3 kg., or 0·05, 0·125 and 0·25 mole/3 kg. rabbit, and methanol at two levels, 3 and 10 ml./3 kg. or 0·075 and 0·25 mole/kg., each dose being studied in three animals. A number of