stability of the 'tissue acceptor' and antidote-mercaptides, and the relative ease of penetration of inhibitor and antidote to the active centres.

The increased oxygen uptake of brain dispersions brought about by the presence of BAL has been observed before, and has been shown to be due to a true stimulation of the enzyme activity (Stocken *et al.* 1947).

SUMMARY

The ability of 2:3-dimercaptopropanol (British Anti-Lewisite or 'BAL') to detoxicate compounds of

antimony, gold and mercury has been demonstrated, using the pyruvate oxidase system of pigeon brain as a test system.

This work was carried out for the Ministry of Supply as part of a larger programme under the direction of Prof. R. A. Peters. Our thanks are due to Dr L. A. Stocken for his helpful suggestions in the course of this work, to the Chief Scientific Officer, Ministry of Supply, for permission to publish, to Messrs May and Baker for a sample of myocrisin, and to the Wellcome Bureau of Scientific Research for samples of stibophen, stibacetin and aurothioglucose.

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* Information available on application to Ministry of Supply, London.

The Inhibition of Succinoxidase by Heavy Metals and its Reactivation with Dithiols*

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Many heavy metals either combine with —SH groups to form mercaptides or act as oxidizing agents. Their combination with the —SH groups of those enzymes requiring the presence of these groups for activity results in enzyme inhibition. Since a large number of enzymes are sulphydryl enzymes (Barron & Singer, 1945; Singer & Barron, 1945), it is reasonable to assume that the toxicity of these heavy metals must be largely due to inhibition

of —SH enzymes. Mercaptides are in general easily dissociable, i.e. the metals leave the —SH groups to which they were originally attached when in the presence of other —SH groups with which they form more stable compounds. A typical example of such reactions is that given by Hellerman (1937) when he introduced *p*-chloromercuribenzoic acid (Hg-Cl-benzoate) as an inhibitor of —SH enzymes; the inhibitions produced by it were easily reversed on addition of thiols (cysteine or glutathione). In spite, however, of the knowledge that some heavy metals form mercaptides, very little has been done to see if they combine with the —SH groups of proteins. If

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago.

such combinations were found to exist, they would explain the mechanism of heavy metal intoxication. Furthermore, it would then be possible to search for the appropriate thiol compounds able to dissociate the enzyme-heavy metal complex.

Of the sulphydryl enzymes, succinoxidase was chosen as a test system because of its easy preparation and reproducible results. The effects produced by those heavy metals expected to combine with the —SH groups of the enzyme were studied and reactivations of the inhibited enzyme with different thiols were attempted.

EXPERIMENTAL

Enzyme preparation. Pigeon breast muscle was used as source of the enzyme. The chopped muscle was poured into a Waring blender and shaken twice in 200 ml. of water plus 200 g. of cracked ice for 3 min. After each washing the mixture was centrifuged for 30 min. at 3000 r.p.m. and the supernatant fluid was discarded. An equal volume of 0.1M-Na₂HPO₄ was added to the remaining solid portion and the whole was shaken for 2 hr. at 27°. After centrifugation for 40 min., the supernatant fluid was used for the experiments. This colloidal suspension showed no O₂ uptake in the absence of succinate; in the presence of succinate (0.03M) it gave a Q_{O₂} value of about 350 (μl. O₂/mg./hr.). The suspension was then diluted so that 0.6 ml. of the diluted suspension showed about 200 μl. O₂ uptake in 30 min. at 38° at pH 7.0. The heavy metals were added in aqueous solutions and kept in contact with the enzyme for 20 min. previous to the measurement of the O₂ uptake. In reactivation studies the thiols were added, as a rule, after the 20 min. of contact between enzyme and heavy metal.

Mercury. Mercury, in the form of an organic mercurial preparation (*p*-chloromercuribenzoate), was introduced by Hellerman (1937) as a mercaptide-forming reagent, the reaction being:

Mercuric chloride is extremely toxic to the animal body and has long been considered toxic to enzymes, but the mechanism of its action has not been studied. Haarmann (1943) believes that inorganic Hg is not specific for —SH groups and maintains that it combines primarily with the peptide groups in proteins and secondarily with the N of the imidazole group. In Fig. 1 can be seen the effect of HgCl₂ on the activity of succinoxidase where, on plotting concentration of inhibitor against percentage inhibition a typical S-shaped curve was obtained. Hg⁺⁺ at concentrations c. 10⁻⁵M inhibited succinoxidase, half inhibition being obtained at 1.25 × 10⁻⁵M concentration of Hg. If Hg⁺⁺, like the organic mercurial Hg Cl-benzoate, forms reversible mercaptides with —SH groups of proteins, it will be possible to reactivate the enzyme on addition of thiol compounds at the concentration necessary to dissociate the Hg from the protein —SH groups. Effective reversals of Hg inhibition were obtained on addition of BAL and the dithiols 2, 3, 10 and 18 (for nomenclature

see Barron, Miller & Kalnitsky (1947)). At a ratio of HgCl₂ : dithiol compound (in terms of molar concentration) of 1 : 10, dithiols 3, 2 and 10 reactivated the enzyme 70–85 %. BAL produced a reactivation of 63 %, and glutathione (at equivalent SH concentration) of only 21.5 %. When the ratio was

Fig. 1. Inhibition of succinoxidase by HgCl₂.

diminished to 1 : 5, dithiols 2, 3 and 10 were still effective reversal agents while the reversal effect of BAL and dithiol 18 diminished to half, i.e. 30 % reactivation. At a ratio of 1 : 3.3 and 1 : 2.2 the dithiols 2 and 3 were still effective (Table 1). The choice reversal agents are therefore dithiols 2, 3 and possibly 10 and their trial in the treatment of HgCl₂ poisoning seems warranted. Dr Longcope (Longcope & Luetscher, 1946), has used BAL successfully in the treatment of Hg poisoning.

Table 1. Reactivation by dithiols of succinoxidase inhibited with HgCl₂

(Original inhibition of enzyme activity, 87.6 %. Enzyme, 0.6 ml.; phosphate buffer, 0.05M, pH 7.0; succinate, 0.02M; HgCl₂, 3 × 10⁻⁵M; dithiols at concentrations 10, 5, 3.3 and 2.2 times the concentration of HgCl₂ added 15 min. after addition of Hg. Vol. 3 ml.; temp. 38°; duration of exp. 30 min.)

Dithiol*	Reactivation (%) at ratios of Hg : dithiol			
	1 : 10	1 : 5	1 : 3.3	1 : 2.2
2	70.0	60.2	46.0	25.5
3	85.8	63.2	42.2	15.0
1	62.7	32.7	0	0
21	66.0	—	—	—
18	67.4	27.3	16.3	5.1
10	81.0	64.6	29.4	6.3
16	44.6	—	—	—
Glutathione (ratio 1 : 20)	21.5	—	—	—

* For this numbering, see Barron, Miller & Kalnitsky (1947); also Glossary, p. 351.

Bismuth. The insolubility of inorganic bismuth salts, and the use of the soluble organic compound NaK-bismuthyl tartrate for therapeutic purposes prompted us to use this compound in our work on the inhibiting effect of this metal. The compound contains 41% Bi, but its exact structural formula is unknown. A concentration of Bi of approximately 10^{-5} g.-atoms/l. was sufficient to produce inhibitions of succinoxidase, half inhibition being reached at 1.2×10^{-5} g.-atoms/l. (Fig. 2). This inhibition, like that of Hg, is a reversible inhibition and presumably

Fig. 2. Inhibition of succinoxidase by bismuth.

due to mercaptide formation. Complete reactivation of the enzyme was attained with dithiols 2 and 3 at a ratio of Bi : dithiol of 1 : 4. At the same ratio, dithiol 10 produced a reactivation of 87%; BAL

Table 2. *Reactivation by dithiols of succinoxidase inhibited with bismuth*

(Original inhibition by bismuth, 86.5%. Enzyme, 0.6 ml.; succinate, 0.02M; phosphate buffer, 0.05M, pH 7.0; Bi (in form of NaKBi tartrate), 2.5×10^{-5} M; total vol. 3.0 ml.; duration 25 min.; temp. 38°; duration of exp. 30 min. Dithiol added 20 min. after addition of Bi.)

Dithiol	Reactivation (%) at ratios of Bi : dithiol					
	1 : 10	1 : 4	1 : 3.2	1 : 1.6	1 : 0.8	1 : 0.4
2	—	100	92.5	66.3	27.6	—
3	—	100	100	78.2	47.8	37.9
1	—	45.3	25.5	19.8	—	—
21	72.4	—	—	—	—	—
18	—	14.9	2.1	1.0	—	—
10	—	86.9	58.4	23.1	30.6	0.0
16	56.4	—	24.4	—	—	—

reversed the inhibition by only 45%. When the concentrations of dithiols were reduced to a ratio of Bi : dithiol of 1 : 0.8, dithiol 3 was able to produce half-reactivation (Table 2). The affinity of this dithiol for Bi is so great that its use in the treatment of Bi poisoning is strongly recommended.

Cadmium. It was shown in this laboratory in collaboration with Dr Gerard's co-workers that cadmium is an SH enzyme inhibitor, and Tobias, Lushbaugh, Patt, Postel, Swift & Gerard (1946) have shown that mice poisoned with this metal can be successfully treated by the administration of BAL. Although it has been reported that metallic Cd and CdO are more toxic than $CdCl_2$ (Irwin & Semmons, 1946) this last salt was used in these experiments. Enzyme inhibition was obtained at concentrations around 10^{-6} M-Cd, half inhibition requiring 7×10^{-6} M (Fig. 3). Dithiols 2, 3 and 10

Fig. 3. Inhibition of succinoxidase by $CdCl_2$.

were more effective than BAL in producing reactivation of the enzyme. Thus, at a ratio of Cd : dithiol of 1 : 10 there was almost complete reactivation with dithiol 3 while BAL produced only half-reactivation. At this ratio, glutathione produced almost no reactivation. When the ratio was diminished to 1 : 1, dithiol 3 was able to produce half-reactivation while BAL had no effect at all (Table 3). It is possible to obtain complete reactivation of the enzyme with glutathione when the ratio Cd : SH compound is increased to 1 : 500.

Table 3. *Reactivation by dithiols of succinoxidase inhibited with cadmium*

(Original inhibition by Cd, 80.7%. Enzyme, 0.6 ml.; succinate, 0.02M; phosphate buffer, 0.05M, pH 7.0; $CdCl_2$, 1×10^{-5} M; total vol. 3.0 ml.; temp. 38°; duration of exp. 30 min. Dithiol added 20 min. after addition of Cd.)

Dithiol	Reactivation (%) at ratios of Cd : dithiol			
	1 : 20	1 : 10	1 : 2	1 : 1
2	—	81.6	64.8	14.6
3	—	92.2	55.1	49.4
1	—	57.8	20.5	0.0
21	—	65.4	—	—
18	—	15.7	0.0	0.0
10	—	78.2	59.2	10.2
16	—	19.8	—	—

Vanadium. Vanadium in the form of VCl_2 produced inhibition of succinoxidase at concentrations around 10^{-4} M. Half inhibition of enzyme activity

was obtained with $1.1 \times 10^{-4} \text{ M}$ (Fig. 4). Reversal of vanadium inhibition was obtained only on addition of dithiol 3, a reversal which was obtained even at a ratio of V : dithiol of 1 : 2.2. At this ratio, dithiol 3 produced a reactivation of 79 %; dithiol 2 reactivated the enzyme by only 9 %; while the remaining dithiols, including BAL, had no effect at all on the vanadium inhibition of succinoxidase (Table 4).

Fig. 4. Inhibition of succinoxidase by vanadium.

Table 4. *Reactivation by dithiols of succinoxidase inhibited with vanadium*

(Original inhibition by VCl_2 , 78.2%. Enzyme, 0.6 ml.; succinate, 0.02 M; phosphate buffer, 0.05 M, pH 7.0; VCl_2 , $1.5 \times 10^{-4} \text{ M}$; vol. 3.0 ml.; temp. 38° . Dithiol added 20 min. after addition of V to enzyme.)

Dithiols	Reactivation (%) at ratios of V : dithiol		
	1 : 8.5	1 : 7	1 : 2.2
2	—	16.6	9.0
3	80.0	78.0	79.0
1	—	9.0	0
18	—	0	0
10	—	12.0	3.0

Selenium. Collett (1924, 1933) found that selenium and tellurium inhibit the activity of succinoxidase. This inhibition is undoubtedly due to their action on the —SH groups of the activating protein. Selenium also inhibits the activity of another —SH enzyme, papain (Bersin & Logerman, 1933). The inhibiting effect of selenite was quite different from that produced by heavy metals. In fact, concentrations of selenite c. 10^{-5} M produced a slight inhibition of the enzyme, which did not increase on addition of increasing amounts of selenite until a concentration of 10^{-4} M was reached. Around this concentration the inhibition rose with the concentration of inhibitor. Half inhibition was obtained at $1.1 \times 10^{-4} \text{ M}$ -selenite (Fig. 5). Reversal of inhibition was not as effective as with heavy metal inhibition. Dithiols 2 and 3 at a ratio of Se : dithiol

of 1 : 7 produced about half-reactivation, while BAL and other dithiols used had no effect at all (Table 5). A possible explanation of this failure may be due to the large amount of dithiols required for reactivation for we have already shown that dithiols at concentrations above $1 \times 10^{-3} \text{ M}$ inhibit succinoxidase.

Fig. 5. Inhibition of succinoxidase by SeO_2 .

Table 5. *Reactivation by dithiols of succinoxidase inhibited by selenium*

(Original inhibition by SeO_2 , 75.4%. Enzyme, 0.6 ml.; succinate, 0.02 M; phosphate buffer, 0.05 M, pH 7.0; SeO_2 , $4 \times 10^{-4} \text{ M}$; vol. 3.0 ml.; temp. 38° . Dithiol added 20 min. after addition of Se to enzyme.)

Dithiols	Reactivation (%) at ratios of Se : dithiol		
	1 : 7	1 : 5	1 : 2.3
2	41.4	32.6	0
3	54.3	45.6	15.4
1	0	0	0
18	0	0	0
10	0	10.9	0

Zinc. Zinc inhibition of succinoxidase was obtained only with relatively high concentrations of ZnCl_2 , half inhibition requiring $1.5 \times 10^{-3} \text{ M}$ -Zn. Reactivations were complete on addition of 0.03 M-glutathione. Gilman & McNamara (1946) have shown that BAL is of value in preventing the development of pulmonary lesions after inhalation of zinc fumes.

Lead. Since Pb is closely related to Hg and Bi by its position in the periodic table, there is a possibility that this metal is also an inhibitor of —SH enzymes. However, because of the insolubility of lead phosphate (the enzyme is extracted by 0.1 M- Na_2HPO_4), the experiments on lead were performed after dialysis of the enzyme in running water and resuspension in acetate buffer. Under these conditions, at pH 6.3, lead inhibited succinoxidase. Half-inhibition was reached at a concentration of $2.5 \times 10^{-4} \text{ M}$.

Effect of inhibitor concentration on extent of enzyme reactivation. In the experiments reported above, the inhibitor was used at a concentration sufficient to

produce from 75 to 87 % inhibition. When the concentration of the inhibitor is increased so as to produce complete inhibition, reactivation becomes much more difficult, possibly because of irreversible denaturation. This effect was tested with Bi as the heavy metal inhibitor. Up to a certain concentration (1.2×10^{-5} M-Bi) the inhibition was reversed on addition of dithiol 3 by 75 %; when the concentration was increased (2.5×10^{-5} M) to produce 95 % inhibition, the reversal on addition of dithiol dropped to only 13.5 % (Table 6).

Table 6. *Reversal of inhibition with increasing concentration of inhibitor*

(Enzyme, 0.6 ml.; succinate, 0.02 M; phosphate buffer, 0.05 M, pH 7.0; Bi (NaKBi tartrate) in indicated concentrations; dithiol 3, 1.6 times concentration of Bi; vol. 3.0 ml.; temp. 38°. Dithiol added 20 min. after addition of Bi to enzyme.)

Concentration of Bi (M)	Inhibition (%)	Dithiol added.	
		Ratio Bi : dithiol	Reactivation (%)
1.2×10^{-5}	87.0	1 : 1.6	74.7
1.9×10^{-5}	88.0	1 : 1.6	28.0
2.5×10^{-5}	95.3	1 : 1.6	13.5

Effect of time on enzyme reactivation. It has been pointed out that the protein component of —SH enzymes tends to be irreversibly denatured when the —SH groups have undergone chemical change. In order to determine the minimum time for complete reactivation of —SH enzymes (in this case succinoxidase) after addition of heavy metal inhibitors, experiments were performed where the selected dithiols were added at different intervals of time after addition of the heavy metal (Table 7).

Table 7. *Effect of addition of dithiol at various times after contact of enzyme with bismuth*

(Enzyme, 0.6 ml.; succinate, 0.02 M; phosphate buffer, 0.05 M, pH 7.0; Bi (in form of NaKBi tartrate) in indicated concentrations; concentration of dithiol 3, 1.6 times concentration of Bi; temp. 38°.)

Concentration of Bi (M)	Reactivation (%) on addition of dithiol at various times (min.) after contact of enzyme with Bi					
	0	15	20	30	45	60
3.1×10^{-5}	—	45	—	34	27	2.7
2.5×10^{-5}	87.6	—	87.6	76.8	74.5	—

On addition of heavy metal in sufficient quantity to produce 88 % inhibition (2.5×10^{-5} M-Bi) the enzyme was effectively reactivated (88 %) on addition of dithiol 3 at a ratio of 1 : 1.6, 20 min. after Bi addition. After 45 min., there was still good reversal (74 %). When the concentration of Bi was raised to 3.1×10^{-5} M-Bi, addition of dithiol 30 min. after metal addition produced a reactivation of only 34 %;

the extent of reactivation decreased to 27 % when the dithiol was added 45 min. after the Bi. At the end of 1 hr. the enzyme had been irreversibly inhibited.

Table 8. *Comparison of toxicity of heavy metals for succinoxidase system*

(Succinoxidase, 0.6 ml.; succinate, 0.01 M; phosphate-HCl buffer, 0.033 M, pH 7.0; metals in indicated concentrations. Total vol. 3.0 ml.; temp. 38°.)

Metal inhibitor	Half-inhibition (M)	Best reversal agent*
Bi	1.2×10^{-5}	3, 2, 10, BAL
Hg	1.25×10^{-5}	3, 3, 10, BAL
Cd	7×10^{-6}	3, 2, 10, BAL
V	1.1×10^{-4}	3, BAL ineffective
Se	1.1×10^{-4}	2 = 3
Zn	1.5×10^{-3}	—
Sb	2.0×10^{-3}	—

* Numbers refer to thiols listed in the glossary of Barron, Miller & Kalnitsky (1947).

In Table 8 there is given a summary of the toxicity of heavy metals studied as well as the dithiols found to be the best reversal agents. It must be emphasized that in all cases of heavy metal inhibition BAL was not as effective as dithiols 2 and 3. It may be noted that Dr Harry Eagle (personal communication) has reported that BAL was not effective in the treatment of Bi intoxication produced in rabbits.

Antimony. To study the effect of this metal on the activity of succinoxidase the organic antimony compound Fuadin* (sodium salt of sodium antimony biscatechol disulphonate) was used. According to the distributors of this drug 1 ml. of this solution contains 8.5 mg. of antimony. The reducing substances contained in the solution were oxidized with 0.1 N-iodine. Under those conditions the aqueous solutions of Fuadin inhibited the activity of succinoxidase; 2×10^{-3} M-Sb produced half inhibition. The inhibition was reversed on addition of 4×10^{-2} M-glutathione. Attempts to reactivate the enzyme with BAL or dithiol 2 were unsuccessful because of the relatively large concentration of dithiol theoretically required to achieve reactivation.

DISCUSSION

It is very difficult to decide without further experimentation the kind of linkage formed between the heavy metal and the —SH groups of the protein molecule and to give any quantitative treatment to the data on degree of inhibition with increasing concentrations of metal. The heavy metal (M) may combine with two —SH groups: Protein $\begin{matrix} \diagup S \\ \diagdown S \end{matrix} M$

* Fuadin was generously donated by Winthrop and Company.

forming cyclic compounds; it may combine with only one —SH group: Protein-S-MR', as seems to happen with the organic mercurial complexes. If only the first reaction occurs, the dissociation constant of the reaction between the —SH groups of the protein and the metal would be obtained from equation

$$K = \frac{(-SH)^2 (M)}{\left(M \begin{array}{c} \diagup S \\ \diagdown S \end{array} \right)}$$

The second type of compound would give rise to the dissociation constant

$$K = \frac{(-SH) (MR)}{(M-S-R')}$$

If both reactions took place, the two equations would have to be extended and the contributions of one or the other would be difficult to determine.

There is, however, indirect evidence in favour of the opinion that heavy metals might combine with the —SH groups of the protein, forming metal cyclic compounds. This evidence is given by the rapid reactivation of heavy metal inhibition on addition of dithiols while monothiools (glutathione) were poor agents for reactivation.

Selenite possibly acts as an oxidizing agent. This would explain the lack of complete reactivation on addition of dithiols, the partial reactivation being possibly due to the reducing power of these compounds. However, it is possible that Se might form the compound R—S—Se—S—R (Painter, 1941).

In conclusion we must make a plea for studying in more detail the therapeutic properties of the dithiols.

BAL has proved to be an efficient agent for the treatment of lewisite poisoning and Rhoads and co-workers have reported that dithiol 9 is more efficient than BAL for the treatment of arsine poisoning. The work here presented is an indication that the dithiols 2 and 3 might be more efficient than BAL in the treatment of heavy metal poisoning.

SUMMARY

1. A large number of heavy metals has been found to inhibit the enzyme activity of succinoxidase. The inhibition is due to combination of the metal with the —SH groups of the protein moiety. Bi, Cd and HgCl₂ had the greatest inhibitory power, producing 90 % inhibition at *c.* 10⁻⁵ M concentration. V, Zn and Pb produced effective inhibitions at about 10⁻⁴ M. Zn and Sb required higher concentrations, half-inhibition being attained at about 1.5 × 10⁻³ M.

2. The inhibition produced by these heavy metals was reversed on addition of dithiols. The most effective compounds for the reversal of Bi, Hg and Cd inhibition were 1:3-propanedithiol, 1:3-dimercaptopropanol and 2:3-dimercaptopropionic acid. 1:3-Propanedithiol was the only effective agent for the reversal of V inhibition. 1:3-Dimercaptopropanol and 1:3-propanedithiol were effective against Se inhibition. Reversal by BAL (2:3-dimercaptopropanol) occurred only at higher concentrations. Glutathione at the concentrations used with dithiols was generally ineffective; at higher concentrations it produced reactivation.

3. Reversal of inhibition became increasingly difficult when the concentration of inhibitor was raised so as to produce complete inhibition.

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GLOSSARY

Compound	Name	Compound	Name
1	2:3-Dimercaptopropanol (BAL)	10	2:3-Dimercaptopropionic acid
2	1:3-Dimercaptopropanol	16	bis- <i>S</i> (<i>N</i> -Ethylacetamidomethyl) ether of BAL
3	Propane-1:3-dithiol	18	2:3-Dimercaptopropyl propionate
9	2:3-Dimercaptopropyl ethyl ether	21	3:4-Dimercaptobutanol-1