being reversed completely by BAL and only partially by glutathione. Lewisite inhibited also the synthesis of carbohydrate from pyruvate by kidney slices.

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The Action of British Anti-lewisite (BAL) on Enzyme Systems*

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It has been shown by Peters and others that 2:3-dimercaptopropanol (BAL) can be used as an antidote to lewisite, and can prevent vesication of human skin by lewisite if applied within 1 hr. after contamination. It was also shown not only to protect the arsenic-sensitive pyruvic oxidase from the inhibitory action of the arsenical *in vitro*, but to reverse poisoning which had already occurred, owing to its high affinity for arsenic. The work on BAL has been briefly reviewed by Peters, Stocken & Thompson (1945). BAL is itself toxic in large doses, with an LD_{50} of 110 mg./kg. by subcutaneous injection in rats. In an attempt to study the cause of this toxicity the action of BAL on a large number of enzymes was investigated *in vitro*.

EXPERIMENTAL

Methods. For each enzyme the conditions of estimation (amount of enzyme, etc.) were so arranged that the enzyme concentration was limiting, i.e. such that the rate of reaction was proportional to the amount of active enzyme present.

A pure specimen of BAL, obtained from Prof. Peters, was used throughout this work as a stock M/4 solution in ethylene glycol monoethyl ether. The required concentration of BAL was added to the enzyme solution in buffer and incubated with it for 15 min. before starting the reaction by addition of the substrate. Zymohexase and creatine phosphokinase† were tested in veronal buffer at pH 7·4, pyrophosphatase in veronal buffer at pH 7·9, and *d*-amino-acid oxidase in phosphate buffer at pH 7·7. All other experiments were carried out at pH 7·4 in phosphate or bicarbonate buffer.

Enzymes. These were prepared from the following materials: polyphenol oxidase from mushrooms (Keilin & Mann, 1938); carbonic anhydrase from mammalian erythrocytes (Keilin & Mann, 1940); catalase from ox liver (Sumner & Dounce, 1939); peroxidase from cow's milk (Elliott, 1932); aldehyde mutase from ox liver (Dixon & Lutwak-Mann, 1937); phosphorylase from potato (Hanes, 1940); xanthine oxidase from milk (Dixon & Thurlow, 1924); *d*-amino-acid oxidase from ox kidney (Krebs, 1935); cholinesterase from horse serum (Stedman & Stedman, 1935) and from rabbit brain (a strained tissue-suspension in bicarbonate-Ringer); glyoxalase from ox liver (a dialyzed extract of acetone-dried tissue); pyro-

* This work formed a part of the research programme carried out for the Ministry of Supply by an Extra-Mural Research team under the direction of Dr M. Dixon, and was reported to the Ministry in 1943 (Webb & van Heyningen, 1943).

† The term phosphokinase has been suggested by Needham & Dixon (1942) as a general name for enzymes of the hexokinase group which catalyze reactions of the type

$$X + \text{Adenosinetriphosphate} \rightleftharpoons X\text{-phosphate} + \text{Adenosinediphosphate}.$$

phosphatase from rabbit kidney (an extract in 0·5 M-KCl, dialyzed); hexokinase from baker's yeast (van Heyningen, unpublished). Pepsin, trypsin and papain were commercial preparations (British Drug Houses Ltd.); creatine phosphokinase, zymohexase and the glycolysis system were studied in a dialyzed extract from an acetone precipitate of rabbit muscle extract; and cytochrome oxidase and succinic dehydrogenase were studied in an exhaustively washed ground pig-heart suspension (Keilin & Hartree, 1938).

RESULTS

The inhibitory action of various concentrations of BAL on the enzymes listed above is shown in Table 1. The maximum concentration of BAL used was 0·01 M. Seven enzymes were strongly inhibited: polyphenol oxidase, carbonic anhydrase, catalase, peroxidase, aldehyde mutase, phosphorylase and

Table 1. *Inhibition of various enzymes by 2:3-dimercaptopropanol (BAL)*

(Incubated with BAL for 15 min. before addition of substrate.)

Enzyme	Substrate	Method of estimation	Temp. of incubation (°)	BAL (M)	Inhibition (%)
Polyphenol oxidase	Catechol	Oxygen uptake	17	0·0025	95–100
			17	0·001	95
Carbonic anhydrase	$NaHCO_3$-Na_2CO_3	CO_2 production	17	0·005	75
			17	0·0025	60
			17	0·00125	50
Catalase	H_2O_2	O_2 production	17	0·0044	90
			17	0·0022	85
			17	0·0008	45
Peroxidase	(*a*) H_2O_2 and excess $NaNO_2$	Excess H_2O_2 estimated by catalase	17	0·0025	70
	(*b*) Pyrogallol and H_2O_2	Purpurogallin formation	17	0·00125	60
Cytochrome oxidase	Cytochrome *c* and catechol	Oxygen uptake	37	0·005	0
Aldehyde mutase*	Acetaldehyde	CO_2 production	37	0·0042	90
Phosphorylase	Cori ester	Formation of inorganic phosphate	25	0·0025	40
Glyoxalase*	Methylglyoxal	CO_2 production	37	0·0028	80
Hexokinase*	Glucose and adenosine-triphosphate	CO_2 production in $NaHCO_3$ buffer	37	0·01	0 (*a*)
			37	0·01	40 (*b*)
Pyrophosphatase	$Na_4P_2O_7$	Formation of inorganic phosphate	25	0·008	0
Succinic dehydrogenase	Succinate	Oxygen uptake (with methylene blue as carrier)	37	0·00125	0
Xanthine oxidase	Hypoxanthine	Oxygen uptake	37	0·005	0
d-Amino-acid oxidase	*dl*-Alanine	Oxygen uptake	37	0·0025	0
Creatine phosphokinase	Creatine and adenosine-triphosphate	Formation of creatine phosphate	37	0·0125	0
Cholinesterase (serum)	Acetylcholine	CO_2 production	20	0·005	0
Cholinesterase (brain)	Acetylcholine	CO_2 production	37	0·0042	0
Pepsin	Casein	Formol titration	37	0·0042	0
Trypsin	Casein	Formol titration	37	0·0042	0
Papain	Casein	Formol titration	Some activation by BAL, the amount depending on previous treatment of the enzyme		
Zymohexase	Hexosediphosphate	Formation of alkali-labile phosphate	37	0·0025	Activated 20%
Glycolysis system	Cori ester	Acid production	37	0·005	Activated 60%

* *Aldehyde mutase*: excess coenzyme I was added with the substrate. *Glyoxalase*: 0·004 M-glutathione was added as activator, and the same result was obtained whether this was added with the substrate or with the BAL. *Hexokinase*: (*a*) 0·0033 M-$MgCl_2$ added with the enzyme; (*b*) no $MgCl_2$ added. All oxygen uptakes and gas outputs were measured in Barcroft manometers.

glyoxalase. Some of these effects were studied in more detail.

Polyphenol oxidase. This enzyme is almost completely inhibited by concentrations of BAL as low as 0·001 M. In an experiment designed to test whether this is due to combination with the copper prosthetic group (Keilin & Mann, 1938), the enzyme was incubated with M/400 BAL for 15 min. at 17°, and then $CuSO_4$ was added to give a final concentration of 0·01 M and the incubation continued for a further 15 min. The result, illustrated in Fig. 1, shows that the activity was almost completely restored by addition of Cu^{++}.

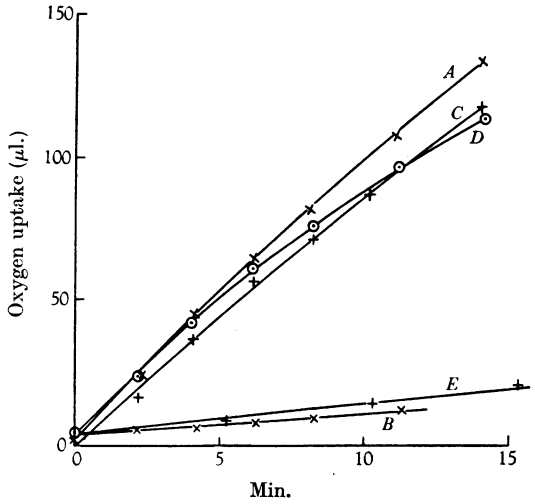

Fig. 1. Inhibition of polyphenol oxidase by BAL, and its reversal by Cu^{++}. Temp. 17°; pH 7·4. Reaction started by tipping in 6 mg. catechol. *A*, enzyme only (control). *B*, enzyme incubated with M/400 BAL for 30 min. *C*, enzyme incubated with M/400 BAL for 15 min., then for a further 15 min. after addition of M/100 $CuSO_4$. *D*, enzyme incubated in M/100 $CuSO_4$ for 15 min. *E*, no enzyme; oxidation in M/100 $CuSO_4$ only (blank for *C* and *D*).

Hexokinase. In the standard conditions for estimation of hexokinase worked out by van Heyningen (1942), $MgCl_2$ is added to give a concentration of M/300. Under these conditions BAL does not inhibit hexokinase at a concentration of 0·01 M. When no Mg^{++} is added, the hexokinase gives an activity about 17 % of the maximum, presumably due to traces of Mg^{++} in the enzyme preparation (an undialyzed solution of an ethanolic precipitate of autolyzed baker's yeast). This low activity was decreased by 40 % after incubation with 0·01 M-BAL. This is not due to an action on the enzyme itself, for if excess $MgCl_2$ is added after 20 min., the activity of the treated enzyme becomes equal to that of the control (Fig. 2).

The cytochrome system and oxidation of BAL. In dilute aqueous solution BAL undergoes atmospheric oxidation, each molecule taking up one atom of oxygen. The time for half-oxidation of an 0·005 M-solution in phosphate buffer pH 7·4, vigorously shaken in air, was about 70 min. at 17° and 20 min. at 37° (Fig. 3).

A solution of cytochrome *c* was rapidly reduced on addition of a few drops of BAL solution, the resulting liquid showing the normal absorption spectrum of reduced cytochrome *c*. It was not autoxidizable but on addition of a cytochrome oxidase preparation an absorption spectrum appeared which

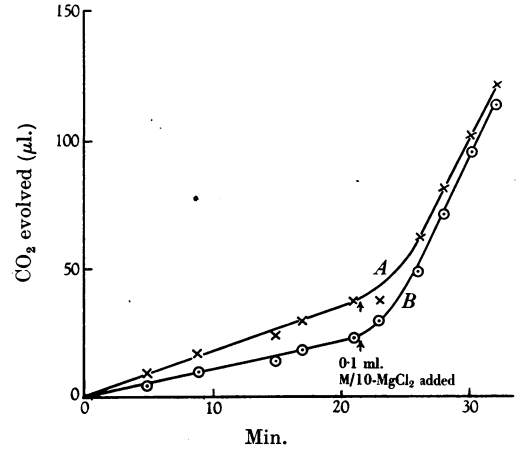

Fig. 2. Action of BAL on hexokinase: with and without addition of Mg^{++}. Temp. 38°; bicarbonate buffer pH 7·4. *A*, enzyme only (control); *B*, enzyme in M/400 BAL. Both incubated for 15 min. (in M/150 glucose for stabilization) before starting the reaction by addition of M/8-glucose and adenosinetriphosphate (1·5 mg. '7 min.' P). After 20 min., $MgCl_2$ was added to give a final concentration of M/300; the difference between the activity of the treated enzyme and the control disappeared.

was identical with that of the original oxidized cytochrome. Thus there was no evidence that BAL combined with cytochrome *c*, or interfered with its action as a hydrogen carrier.

In a manometric experiment, the amounts of cytochrome *c* and of cytochrome oxidase (a washed suspension of ground heart muscle) were such that the oxidase concentration was limiting. (This was investigated in a preliminary experiment with varying amounts of oxidase, the rate of oxygen uptake on addition of an excess of catechol being measured.) When BAL was tipped in, there was a rapid oxygen uptake, the rate remaining constant until almost one atom of oxygen/molecule of BAL had been taken up (Fig. 3, *A*). Since this initial rate was independent of the concentration of BAL remaining, the cytochrome system must have been

working at its maximum speed, i.e. the rate of oxygen uptake depended only on the active concentration of cytochrome oxidase. In another manometer containing the same amounts of cytochrome and oxidase, catechol was tipped from a Keilin tube. In this case the initial rate of oxidation was the same as that obtained with BAL (Fig. 3, *B*). Since catechol is not known to affect cytochrome oxidase, it was concluded that BAL likewise has no effect on the enzyme.

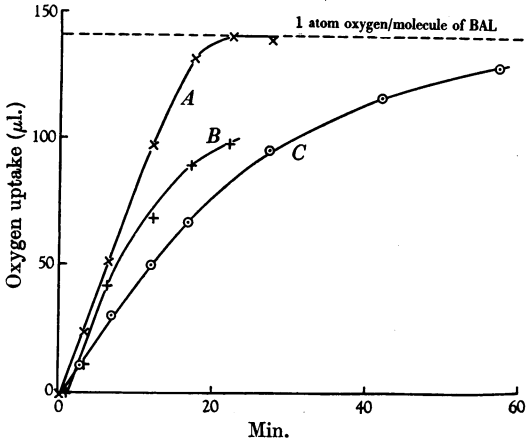

Fig. 3. Oxidation of BAL in M/200-aqueous solution. Temp. 37°; phosphate buffer pH 7·4. *A*, oxidation of 0·0125 mmol. of BAL by the cytochrome system, with limiting cytochrome oxidase. *B*, oxidation of 6 mg. catechol by cytochrome system similar to that in *A* (control for cytochrome oxidase activity). *C*, atmospheric oxidation of 0·0125 mmol. of BAL shaken in M/15 phosphate buffer.

Formation of BAL-metal complexes. The existence of stable compounds of BAL with copper, zinc, magnesium and iron was investigated qualitatively. On addition of excess $CuSO_4$ solution to a fresh 0·005 M-solution of BAL in water, a dark blue precipitate was produced. The precipitate was filtered off and the residual Cu^{++} estimated by titration with thiosulphate after addition of KI and H_2SO_4. The results showed the insoluble compound to contain one atom of Cu/mol. of BAL. The compound is a dark green powder, insoluble in water, HCl, ethanol, acetone or ether, and only slightly soluble in boiling 50 % HCl. It is much less readily oxidized than BAL and remains apparently unchanged after several months.

BAL (in 0·005 M-solution) gave a blue-green precipitate with ferric salts, but no precipitate with ferrous salts. (The ferric-BAL compound was soluble in strong ammonia to give a stable blood-red solution; addition of ammonia to an aqueous mixture of BAL and ferrous salt gave a similar result.) No insoluble compound of BAL with Zn^{++} or Mg^{++} was formed at neutral pH.

DISCUSSION

Of the seven enzymes inhibited by BAL, four are known to be metallo-proteins. Polyphenol oxidase contains Cu^{++}, carbonic anhydrase Zn^{++} (Keilin & Mann, 1940), and catalase and peroxidase an iron-containing prosthetic group (Keilin & Hartree, 1936; Elliott, 1932; Keilin & Mann, 1937). This strongly suggests that BAL specifically inhibits metal-enzymes and that this inhibition is due to a combination with the metals, for which BAL is known to have a high affinity. The only enzyme examined which is believed to be a metal enzyme and is not inhibited by BAL is cytochrome oxidase, which is probably part of the insoluble cytochrome complex (Keilin & Hartree, 1939). BAL is, however, rapidly oxidized by this enzyme. Cytochrome *c* itself is also unaffected.

Three enzymes other than the known metal enzymes were inhibited by BAL: glyoxalase, aldehyde mutase, and potato phosphorylase. Aldehyde mutase and phosphorylase have not been shown to be metal-containing enzymes but this possibility is perhaps not excluded. Glyoxalase needs glutathione as coenzyme (Lohmann, 1932), and in this case the inhibition may be competitive, the thiol grouping of the BAL having a higher affinity for the enzyme than has glutathione.

Papain, and the glycolytic system of muscle acetone powder, were both activated by BAL. Cysteine also activates these systems; the effect is probably due to the reducing properties of thiol groups. Zymohexase was also slightly activated; since this enzyme is known to be inhibited by traces of heavy metals (Herbert, Gordon, Subrahmanyan & Green, 1940), the apparent activation was probably due to the removal by BAL of traces of these metals in the undialyzed solution of muscle acetone powder used.

In the case of polyphenol oxidase, the effect of BAL is certainly due to formation of an insoluble copper-BAL compound, and the enzyme can be reactivated by the addition of excess copper salt. BAL probably acts as a general inhibitor of metal-containing enzymes and as an antagonist of metal activation of enzymes, even interfering with the activation of Mg^{++}-activated enzymes (e.g. hexokinase) by suboptimal concentrations of Mg^{++}. Whether the toxicity of the substance is due to this inhibition of metal-containing enzymes must remain an open question at present. BAL may be of value in future work for indicating whether an enzyme contains a metal prosthetic group or is dependent for its activity on traces of metal ions present in crude preparations.

SUMMARY

1. 2:3-Dimercaptopropanol (BAL) inhibits seven enzymes of a large number examined. Four of these,

polyphenol oxidase, carbonic anhydrase, catalase and peroxidase, are known to be metal-containing enzymes. The inhibition of glyoxalase is due to competition with glutathione, the activator of the enzyme. Aldehyde mutase and phosphorylase are also inhibited.

2. The inhibition of mushroom polyphenol oxidase by BAL is reversed by addition of excess of copper salt. This inhibition is due to the formation of an insoluble cupric-BAL compound.

3. The activity of hexokinase in the presence of suboptimal concentrations of Mg^{++} is diminished by BAL, an effect immediately reversed by addition of excess Mg^{++}.

4. BAL can be oxidized by the cytochrome system without any effect on the activity of the latter.

5. BAL may be considered as a potent inhibitor of metal-containing enzymes with the exception of the cytochrome system.

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The Effect of 2:3-Dimercaptopropanol on the Activity of Enzymes and on the Metabolism of Tissues

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The brilliant work of Peters, and co-workers (see Peters, Stocken & Thompson, 1945), on the reversal of lewisite action by dithiol compounds which possess the property of forming stable cyclic arsenical rings has been one of the great achievements in the treatment of certain war-gas casualties. In fact, dithiols have already found a place among the useful drugs for the treatment of a number of intoxications (arsenicals or heavy metals). It was therefore necessary to study carefully the effect of dithiols on the activity of enzyme systems and on the metabolism of tissues in order to understand the toxic effects observed when dithiols in large amounts are injected into animals. Such a reducing agent, with rather negative oxidation-reduction potential, reacting readily with heavy metals, might produce enzyme inhibitions, either by maintaining in the reduced state the electron transfer catalysts of the oxidation enzyme systems, or by combining with the heavy metals which act as the prosthetic groups of the protein moiety of some enzymes. Webb & van Heyningen (1943) have, in fact, shown that 2:3-dimercaptopropanol can combine with the metals acting as prosthetic groups. The experiments presented in this paper demonstrate that the reducing power of dimercaptopropanol is also responsible for some of the toxic effects.

EXPERIMENTAL AND RESULTS

The effect of dimercaptopropanol on the activity of enzymes

Webb & van Heyningen (1943) have investigated the effect of dimercaptopropanol on a number of oxidative and hydrolytic enzymes. Enzymes possessing heavy metals as prosthetic groups were