British Anti-lewisite

4. ANTIDOTAL EFFECTS AGAINST THERAPEUTIC ARSENICALS

By L. A. STOCKEN, R. H. S. THOMPSON AND V. P. WHITTAKER

Department of Biochemistry, Oxford

(Received 17 June 1946)

Since the original objective of this research was the provision of an antidote for arsenical vesicants, the earlier work (Stocken & Thompson, 1941) with 2:3-dimercaptopropanol (British Anti-lewisite or 'BAL') was carried out with arsenoxides derived from chemical warfare agents, or with sodium arsenite.

After demonstrating the effectiveness of BAL in the local treatment of skin contaminated with lewisite, it was shown that systemic intoxication arising from heavy contamination with lewisite could also be satisfactorily treated in laboratory animals. It was therefore obviously desirable to determine whether BAL was also effective against the therapeutic arsenicals, with a view ultimately to the treatment of patients suffering from the toxic manifestations that sometimes occur during the course of arsenotherapy. About the same time that this experimental work was commenced a start was made in the collection and study of a series of patients showing toxic reactions developing during treatment with therapeutic arsenicals. The results of this clinical study are reported elsewhere (Carleton, Peters, Stocken, Thompson, Williams, Storey, Levvy & Chance, 1946). The only previous work in man in which lesions other than purely experimental contaminations due to chemical warfare agents had been studied was that of Longcope, Wintrobe & Luetscher (1943) on the use of BAL in the treatment of arsenical dermatitis occurring in factory operatives. The problem of the therapeutic arsenicals was simultaneously begun in this Department and in the U.S.A. by Eagle, who, in 1943, reported on the effectiveness of BAL in resuscitating trypanosomes from the effects of mapharside, and succeeded in saving rabbits by the injection, 5 min. after lethal doses of mapharside, of BAL dissolved in propylene glycol.

The work described here deals with four arsenicals, representative of the various chemical types used in therapy—mapharside, arsphenamine, neoarsphenamine and a pentavalent arsonic acid—and has already been reported to the Ministry of Supply (Stocken, Thompson & Whittaker, 1944; Whittaker, 1945). Earlier work on the inhibitory action of therapeutic and other arsenicals on the respiratory

enzyme system of cells has already been reviewed (Peters, Sinclair & Thompson, 1946).

Two types of experimental evidence have been obtained which demonstrate the effectiveness of BAL against these therapeutic arsenicals: (a) experiments to demonstrate the protection and reactivation of the brain pyruvate oxidase system from their inhibitory action. (b) A study of the survival rate of rats treated with BAL after poisoning by these arsenicals.

EXPERIMENTAL

Arsenicals. Freshly prepared solutions of the following arsenicals were used:

Mapharside (3-amino-4-hydroxyphenylarsenoxide hydrochloride): samples of 'undiluted' mapharside were kindly provided by Parke, Davis and Co.

Arsphenamine (3:3'-diamino-4:4'-dihydroxyarsenobenzene hydrochloride): this was prepared from 3-amino-4-hydroxyphenylarsonic acid (cf. Raiziss & Gavron, 1923).

Neoarsphenamine (sodium 3:3'-diamino-4:4'-dihydroxyarsenobenzene-*N*-methylenesulphoxylate): the commercial product of May and Baker Ltd. The dry solid arsenical was kept in a nitrogen-filled desiccator, and no increase in toxicity was observed over an interval of many months.

3-Amino-4-hydroxyphenylarsonic acid: kindly supplied by May and Baker Ltd.

Thiols. The sample of BAL used throughout was from a water-purified batch described by Peters, Stocken & Thompson (1944). Its thiol content was 99–100% of the theoretical iodine titration. 2-Mercaptoethanol was a commercial preparation and had a thiol content of 87% theoretical (iodine titration); this was allowed for in the enzyme experiments.

Enzyme experiments. Two types of pyruvate oxidase preparation have been used, activity being determined by the Warburg technique in each case.

(1) Rat brain *brei*. The minced brain (Passmore, Peters & Sinclair, 1933) was added to Ringer-phosphate solution (pH 7.3) in tared Warburg bottles containing sodium pyruvate in a final concentration of 0.02 M. Respiration was measured in air for at least 1 hr. at 38°.

(2) Pigeon brain dispersion, prepared by the method of Banga, Ochoa & Peters (1939). The brain, pounded to a homogeneous cream in an ice-cold mortar, was ground with ice-cold 0.9% KCl. The dispersion was then pressed through muslin, 1.3 ml. 0.5 M-phosphate buffer (pH 7.3) added, and the volume adjusted to 16.5 ml. with more 0.9% KCl. 2 ml.

of this mixture were added to each of 8 bottles, standing in ice, containing 0.006 M-pyruvate, 0.004 M-fumarate and appropriate amounts of thiol and arsenical, and enough water to a final volume of 3 ml. Measurements, again in air, were usually carried out for 30 min., oxygen consumption being expressed as μ l. O_2 /2.0 ml. dispersion/30 min. Determinations were made in duplicate with both types of preparation.

The percentage inhibitions due to the arsenical alone, or in the presence of thiol, or, in reactivation experiments, the percentage reactivation of enzyme by thiol, are given by the following expressions:

If x_0 = mean rate of O_2 consumption in control bottles,
 x = mean rate in bottles containing thiol,
 y = mean rate in bottles containing arsenical,
 z = mean rate in bottles containing arsenical + thiol,

$$\text{Inhibition due to arsenical } (I_0) = 100 \frac{(x_0 - y)}{x_0} \%,$$

$$\text{Inhibition due to arsenical + thiol } (I) = 100 \frac{(x_0 - z)}{x_0} \%,$$

$$\text{Percentage reactivation of enzyme} \\ = 100 \frac{(I_0 - I)}{I_0} = 100 \frac{(x_0 z - xy)}{x(x_0 - y)}.$$

RESULTS

Enzyme experiments

Mapharside. Using the rat brain *brei*, a final concentration of 0.07 mM-mapharside caused under our conditions approximately 50% inhibition of the total respiration in the presence of pyruvate. In the protection experiments, the BAL, freshly dissolved in Ringer-phosphate, was added to the brain sample dispersed in the Ringer-pyruvate mixture, the mapharside being added 2–3 min. later.

Table 1 shows the mean percentage inhibition of pyruvate oxidation brought about by mapharside in the absence of and in the presence of 1.7 and 3.4 equiv. of BAL (1 mol. BAL $\equiv$ 1 mol. mapharside). Three experiments are also included to show that 1.8 and 3.6 equiv. of 2-mercaptoethanol confer

Table 1. *Protection of rat brain brei pyruvate oxidase system from inhibition by mapharside*

(Conc. mapharside 0.07 mM.)

Exp.	Thiol	Thiol. concentration (mM.)	Inhibition over 1 hr. (%)	
			Mapharside	Thiol + Mapharside
1	BAL	0.12	50	17
2	"	0.12	59	34
3	"	0.12	55	21
1	"	0.24	50	2
4	"	0.24	56	0
5	"	0.24	37	0
6	"	0.24	59	0
7	"	0.24	62	13
6	2-Mercaptoethanol	0.26	59	66
2	"	0.51	59	78
7	"	0.51	62	80

no protection; in each case the inhibition was actually increased by the monothiol, though the interpretation of this is not clear.

A similar result was obtained using the brain dispersion (Table 2); a considerable measure of protection was again provided by 1.5 equiv. of BAL, 3 equiv. affording complete or almost complete protection. It will be noted from Table 2 that

Table 2. *Effect of BAL on the mapharside ('Maph.') inhibition of pyruvate oxidation by brain dispersions*

(Conc. mapharside 0.014 mM.)

Equiv. of BAL	O_2 uptake (μ l. O_2 /30 min./2 ml. dispersion)				Inhibition (%)	
	Control	Maph.	BAL	BAL + Maph.	Maph.	Maph. + BAL
1.5	333	216	459	416	35	9
1.5	288	142	336	301	52	10
1.5	314	148	407	336	53	17
3.0	335	144	272	277	57	0
3.0	437	232	512	495	47	3
3.0	327	154	444	424	53	5

the addition of BAL causes a considerable increase in the oxygen uptake of the dispersion. This effect, which is not obtained with the brain *brei*, is further discussed in an Appendix to this paper.

A few experiments have been carried out with the brain *brei* to determine whether the addition of BAL to the system, already poisoned with mapharside, can bring about any reversal of the inhibition of enzyme activity.

In these experiments (Table 3) the BAL was placed in the side-bulbs, and the mapharside added to the unprotected brain sample in the main compartment of the flask. The manometers were then attached to the thermostat, and 15 min. allowed for temperature equilibration at 38°, after which the side-bulb contents were tipped into the main compartment, and the inhibition measured as before over two succeeding half-hour periods. The total period of poisoning before the addition of the thiol was 20 min., of which the last 15 min. were spent in the thermostat at 38°. In each experiment, bottles with no added thiol were included, so that a direct comparison could be obtained between the degree of inhibition in the completely unprotected system and that remaining in the bottles to which a thiol was added after 20 min. poisoning.

Although in the experiments summarized in Table 3 no measurements were made of the mapharside inhibition before the addition of BAL, the experiments, in our opinion, do provide true evidence of reversal, in that our earlier work has indicated that inhibition of this system by mapharside (or arsenite or lewisite oxide) is rapid, and remains constant over three or four succeeding 15 min. periods.

Table 3. *Reactivation by BAL of rat brain brei pyruvate oxidase system after poisoning by 0.07 mM-mapharside*

Exp. no.	Inhibition over 1 hr. (%)		Reactivation of enzyme (%)
	Maph. alone	Maph. + BAL	
0.24 mM-BAL			
1	58	21	64
2	58	21	64
3	54	17	69
4	53	16	70
Mean	56	19	67
0.48 mM-BAL			
1	58	26	55
2	58	8	86
3	54	7	87
4	53	16	70
Mean	56	19	72

this time BAL was tipped into four of the bottles (two controls and two with neoarsphenamine), and the measurements of oxygen uptake resumed in all bottles from 10 to 30 min. Since the brain dispersion had been added 15 min. before zero reading (20 min. in the case of the first exp.) the system had been poisoned for a total of about 20 min. before the addition of the thiol. Under these conditions BAL is able to effect a mean reactivation of the poisoned enzyme of about 60 % in the course of the first 20 min. after its addition to the system.

Arsphenamine. This arsenical was found to have very nearly the same toxicity towards pyruvate oxidation in either brain *brei* or dispersion as has neoarsphenamine. Table 7 shows that BAL is also effective in detoxicating this arsenical, whereas a concentration of 2-mercaptoethanol equivalent to twice that of BAL is almost completely inactive.

Table 4. *Relative sensitivities of pigeon brain brei and dispersion to therapeutic arsenicals*

Arsenical	<i>Brei</i> (0.02 M-pyruvate)			Dispersion (0.006 M-pyruvate)			Ratio of As concentrations
	No. of exp.	Concentration of arsenical (mM)	Mean inhibition (%)	No. of exp.	Concentration of arsenical (mM)	Mean inhibition (%)	
Mapharside	7	0.07	61	8	0.014	53	5
Neoarsphenamine	7	0.22	61	7	0.07	72	3
Arsphenamine	4	0.21	50	6	0.07	49	3

Neoarsphenamine. Preliminary experiments with the rat-brain *brei* showed that BAL was also able to protect this system from inhibition by neoarsphenamine, 2-mercaptoethanol being again, under our conditions, ineffective. Relatively high concentrations (of the order of 0.2 mM) of neoarsphenamine were, however, required to produce 50 % inhibition of oxygen uptake, and a concentration of BAL equal to or greater than this produced by itself a variable but marked effect on the respiration, often increasing the oxygen uptake slightly during the first 30 min., and inhibiting it to the extent of about 20 % during the second 30 min. period. For these reasons the pigeon-brain dispersion was investigated as an alternative test system, since it was known from earlier work (Sinclair & Thompson, 1940) that dispersions are more sensitive to arsenicals than the brain *brei*. A comparison of the relative sensitivities of the pigeon-brain *brei* and dispersion to three types of therapeutic arsenical is given in Table 4.

A comparison of the relative effectiveness of BAL and 2-mercaptoethanol in protecting the pyruvate oxidation system present in brain dispersion from the inhibitory effect of neoarsphenamine is given in Table 5.

Table 6 shows the results of reactivation experiments with BAL. In these experiments the inhibition produced by the neoarsphenamine was measured for the first 5 min. in all the bottles. At the end of

Table 5. *The effect of BAL and 2-mercaptoethanol on the inhibition by neoarsphenamine ('Neo.') of pyruvate oxidation in pigeon brain dispersions*

(Concentration of neoarsphenamine 0.07 mm.)						
Ratio SH:As	O ₂ uptake (μl. O ₂ /30 min./2 ml. dispersion)				Inhibition (%)	
	Control	Neo.	Thiol BAL	Thiol + Neo.	Neo.	Neo. + thiol
6	301	55	460	477	82	0
6	314	91	501	517	71	0
7	410	159	534	534	61	0
2-Mercaptoethanol						
12	309	89	336	149	71	56
12	243	73	305	91	70	70
12	263	70	255	93	73	64

Table 6. *The reactivation of pyruvate oxidation in brain dispersions by BAL after poisoning with 0.07 mM-neoarsphenamine*

Ratio SH : As	Inhibition (%)			Reactivation by BAL (%)
	Neo. (0-5 min.)	Neo. (10-30 min.)	Neo. + BAL (10-30 min.)	
6	69	76	33	58
6	46	56	30	45
6	56	70	21	70
12	55	75	35	53
12	66	80	18	77
12	64	80	21	74

White rats of about 200 g. weight were injected with an aqueous solution of neoarsphenamine intramuscularly into a hind-leg, the dose being contained in 0.2 ml. of solution. In animals receiving BAL the dose of arsenical was followed 15 min. later by one of BAL of approx. 70 mg./kg. in 0.15 ml. propylene glycol injected intramuscularly into the other hind-leg, and repeated every 24 hr. for three successive days. The animals were kept under observation for 1 month. The results are shown in Table 10.

Table 10. *Effect of BAL on the survival of rats after lethal doses of neoarsphenamine*

Exp.	Dose of neoarsph. (mg./kg.)	Dose of BAL (mg./kg.)	No. of rats used	Deaths		Survivors	
				No.	Time of survival (days)	No.	%
Control							
1	126-134	—	4	—	—	4	100
2	161-166	—	4	2	4	1	25
				1	9		
3	175-188	—	4	2	3	1	25
				1	5		
4	188-214	—	10	4	4	3	30
				2	5		
				1	6		
Treated							
3	184-194	77-81	3	—	—	3	100
4	182-212	66-80	10	—	—	10	100

DISCUSSION

The results described here with the pyruvate oxidase system of rat and pigeon brain show that good protection against the inhibitory action of three therapeutic arsenicals, mapharside, neoarsphenamine and arsphenamine, can be obtained by the addition to the system of small amounts of BAL. In the case of mapharside almost complete protection is afforded by the presence of 3 equiv. of BAL. With neoarsphenamine 6 mol. of BAL/mol. were effective. With neither type of arsenical was the one monothiol investigated (2-mercaptoethanol) effective, although 24 mol. were used in the case of

neoarsphenamine. In this connexion it should be noted that a high relative concentration of glutathione (10 : 1) was required by Voegtlin, Rosenthal & Johnson (1931) to prevent the reduction in total respiration of animal tissue by arsenoxide; when sodium arsenite was used instead of arsenoxides, 40 mol. of glutathione were required, a ratio of 10 : 1 being entirely without effect.

Experiments with rats following the injection of lethal amounts of these arsenicals has shown that BAL can act as an effective detoxicant. It was concluded from this work that a strong case existed for the testing of BAL as a therapeutic agent in man against the toxic effects of arseno-therapy.

Considering the narrower point of the relative toxicities of the arsenicals used here it is interesting to note that the results obtained with the brain pyruvate oxidase system are in general agreement with those described by Voegtlin and his colleagues for other tissues, thus supporting the view that arsenicals exert their toxic effect through conversion to an 'active' arsenoxide form.

SUMMARY

1. In extension of earlier work with sodium arsenite and the chemical warfare arsenicals, British Anti-Lewisite (BAL) has been tested as an antidote to the toxic action of mapharside, neoarsphenamine and arsphenamine.

2. Using the pyruvate oxidase system of brain it has been shown that BAL can protect effectively against these three arsenicals, and can also bring about a significant degree of reactivation of the already poisoned enzyme system.

3. BAL is also effective in bringing about the survival of rats after the injection of lethal doses of mapharside or neoarsphenamine.

This work was carried out for the Ministry of Supply as part of a programme of research under the direction of Prof. R. A. Peters, M.C., F.R.S. Our thanks are due to the Chief Scientific Officer, Ministry of Supply, for permission to publish, and to Miss M. R. Kempson for technical assistance.

REFERENCES

- Banga, I., Ochoa, S. & Peters, R. A. (1939). *Biochem. J.* **33**, 1109.
- Carleton, A. B., Peters, R. A., Stocken, L. A., Thompson, R. H. S., Williams, D. I., Storey, I. D. E., Levvy, G. A. & Chance, A. C. (1946). In preparation.
- Eagle, H. (1943). By communication.*
- Longcope, W. T., Wintrobe, M. M. & Leutscher, J. A. (1943). By communication.*
- Passmore, R., Peters, R. A. & Sinclair, H. M. (1933). *Biochem. J.* **27**, 842.
- Peters, R. A., Sinclair, H. M. & Thompson, R. H. S. (1946). *Biochem. J.* **40**, 516.
- Peters, R. A., Stocken, L. A. & Thompson, R. H. S. (1944). Report to Ministry of Supply by Peters, no. 77.*
- Raiziss, G. W. & Gavron, J. L. (1923). *Organic Arsenical Compounds*. New York: The Chemical Catalog Co.
- Sinclair, H. M. & Thompson, R. H. S. (1940). Report to Ministry of Supply by Peters, no. 4.*
- Stocken, L. A. & Thompson, R. H. S. (1941). Report to Ministry of Supply by Peters, no. 33.*
- Stocken, L. A., Thompson, R. H. S. & Whittaker, V. P. (1944). Report to Ministry of Supply by Peters, no. 78.*
- Voegtlin, C., Rosenthal, S. M. & Johnson, J. M. (1931). *U.S. Publ. Hlth Rep.* **46**, 339.
- Voegtlin, C. & Smith, H. W. (1920). *J. Pharmacol.* **15**, 479.
- Whittaker, V. P. (1945). Report to Ministry of Supply by Peters, no. 88.*

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

Appendix. The Effect of BAL on Pyruvate Oxidation in Pigeon Brain Dispersions

By V. P. WHITTAKER, *Department of Biochemistry, Oxford*

It will be noted in Tables 2, 5 and 7 of the preceding paper that BAL and to a lesser extent 2-mercapto-ethanol cause a considerable increase in the oxygen uptake of pigeon brain dispersions in the presence of pyruvate. A similar effect was observed by Peters & Wakelin (1941) with kerateine. It was obtained so consistently with all concentrations of BAL used in these experiments that it was thought worth while to find out whether it was actually due to an increased oxidation of pyruvate.

In Table 1, the measured difference in the pyruvate concentrations at the end of the experiment between bottles containing pyruvate alone and pyruvate with BAL is compared with the difference in pyruvate concentration calculated by means of McGowan's quotient (McGowan, 1937; 1 mg. pyruvate = 450 μ l. O₂) from the increase in oxygen uptake in the BAL bottles over the controls.

Brain dispersions were prepared as described in the previous paper. At the end of each experiment (duration 40 min.), protein was precipitated with 2 ml. 25% (w/v) trichloroacetic acid and the bisulphite-binding capacity (B.B.C.) estimated by the method of Clift & Cook (1932). The difference in B.B.C. is expressed as mg. pyruvate (1 ml. N/200 iodine $\equiv$ 0.275 mg. pyruvate).

Table 1. *The effect of BAL on the pyruvate oxidation of pigeon brain dispersions*

(a) = 2 ml. dispersion + 0.006 M-pyruvate + 0.004 M-fumarate.

(b) = (a) + 0.5 mM-BAL.

(Numbers in brackets are number of bottles averaged.)

O ₂ uptake (μ l.) in 40 min.		Increase in O ₂ uptake (b) - (a) due to BAL		Difference (a) - (b) in bisulphite-binding capacity after 40 min. (mg. pyruvate)
(a)	(b)	μ l. O ₂	mg. pyruvate	
320(2)	510(1)	190	0.42	0.44
276(2)	333(1)	57	0.13	0.18
332(3)	530(4)	198	0.44	0.57

In each case the B.B.C. of the bottles containing BAL was lower than that of the controls, the difference running parallel with the extra oxygen uptake in the BAL bottles. In the second experiment, the effect of added BAL was less than usual on this occasion (the BAL solution having stood for over half an hour before addition of the dispersion); significantly, the difference in bisulphite binding is less marked also.

Table 2 shows that the increased oxygen uptake is not due to the oxygen uptake of BAL alone, resulting from oxidation of the BAL during incubation at 38° in air, or to an effect of BAL on the residual respiration (in presence of 0.004 M-fumarate but no pyruvate), since both are small and of the same order of magnitude.

Table 2. *The effect of 0.5 mM-BAL on the residual respiration of pigeon brain dispersions*

(O₂ uptake expressed as μ l./40 min./2 ml. dispersion.)

Dispersion	Dispersion + BAL	Increase due to BAL	BAL alone
153	177	24	21
141	163	22	3

The conclusion drawn from these experiments, that BAL stimulates the oxidation of pyruvate by pigeon brain dispersions, is not unexpected. Thiol groups are believed to be essential to the activity of one or more of the protein components of the pyruvate oxidase system (cf. Peters & Wakelin, 1946); the addition of thiol would be expected to increase the number of these, thereby increasing also the enzymic activity of the whole system.

SUMMARY

The increase in oxygen uptake by pigeon brain dispersion in the presence of pyruvate brought about by low concentrations of BAL has been shown to be due to a stimulation of the enzymic activity of the pyruvate oxidase system.

REFERENCES

- Clift, F. P. & Cook, R. P. (1932). *Biochem. J.* **26**, 1788.
 McGowan, G. K. (1937). *Biochem. J.* **31**, 1627.
 Peters, R. A. & Wakelin, R. W. (1941). Report to Ministry of Supply by Peters, no. 39.*
 Peters, R. A. & Wakelin, R. W. (1946). *Biochem. J.* **40**, 513.

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