Further Investigations on the Metabolism of 2:3-Dimercaptopropanol

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Although 2:3-dimercaptopropanol (British Anti-Lewisite, BAL) was first introduced (Stocken & Thompson, 1941, 1946) as a local antidote for use after contamination of the skin with one of the arsenical vesicants, its application to the treatment of systemic arsenical poisoning in man made it desirable to obtain information concerning the distribution of thiols in the blood and urine after injection of BAL.

In this laboratory (Thompson & Stocken, 1941) it had been shown that shortly after the injection of BAL into rabbits and rats the urine of the animals developed a strong nitroprusside reaction for thiol groups, accompanied by a large increase in the iodine titre of the urine. The nitroprusside reaction, which is ordinarily negative, again disappeared and the iodine titre fell to its previous value within a few hours of the injection.

In order to obtain more precise information regarding the nature of the urinary thiols, the cobalt method for the estimation of BAL (Calvery, 1943; Spray, 1947) has been applied to the urine of rabbits after injection of BAL. Owing to the limited specificity of this reaction (Spray, 1947), a series of experiments was carried out in which the reactions of BAL with salts of other metals were examined, and compared with those given by the thiol appearing in the urine after injection of BAL. These experiments, however, did not by themselves provide conclusive evidence as to the nature of the thiols present in rabbit urine after injection of BAL, and further evidence was therefore sought.

The extraction and purification of the thiols from rabbit urine were investigated, and a procedure was evolved, based on the precipitation of the thiols with the thallos ion in alkaline solution, and subsequent decomposition of the metal complex with H_2S . In this way relatively pure and concentrated preparations of urinary thiols could be obtained.

The reactions with metal salts were then re-examined using the purified thiol, and in addition, through the kindness of Mr E. R. Holiday, a comparison of the absorption spectra of the colours formed by reaction of the cobalt reagent with BAL and with the excreted thiol was made, in order to obtain more precise information regarding the identity of the coloured derivatives.

The thiol was then tested for its ability to protect the pyruvate oxidase enzyme system of brain from inhibition by lewisite. By this means it was hoped to obtain biochemical evidence which would help to characterize the excreted thiol as either a mono- or a dithiol, since it has been shown (Stocken & Thompson, 1941, 1946) that this enzyme system can be completely protected from the toxic action of lewisite oxide by dithiols at concentrations at which all the monothiols that have been studied are completely ineffective (Sinclair, 1940).

EXPERIMENTAL AND RESULTS

The estimation of thiols in rabbit urine after injection of BAL

Male rabbits weighing 1.5–3.5 kg. were catheterized, using a no. 3 soft rubber catheter, and the bladder emptied. After 1½–2 hr. the animals were again catheterized, and a sub-lethal dose of BAL dissolved in propylene glycol was injected intramuscularly into one of the hind legs. The bladder contents were drawn off by catheter at suitable intervals after injection.

One part of each urine sample was titrated with 0.02N-iodine after acidification, and another part was either ultra-filtered or treated with trichloroacetic acid to remove any protein. The protein-free extracts were added to the cobalt nitrate reaction mixture, and the colour that developed was measured in a Pulfrich photometer as described by Spray (1947). The urine sample obtained over a known time interval immediately before injection of the BAL was used to compensate for the interfering effect in the colour estimations of the urinary pigments, and of any products of reaction between the normal urinary constituents and the cobalt reagent. A similar allowance was made for the iodine titre of the pre-injection urine in calculating the BAL output from the iodine titration.

Table 1 gives the results obtained on a series of rabbits, the colour-producing thiols being expressed as mg. BAL. It will be seen that whereas the iodine titration gives a recovery of 10–30 % of the injected BAL during the first 5–8 hr. after injection, the cobalt method accounts for less than 10 %. The recovery of thiols by the cobalt method is of the order of 10–20 % of that by iodine titration.

From the above results it is clear that there is present in the urine of rabbits, after injection of BAL, a thiol capable of reacting with cobalt nitrate

Table 1. *Estimation of thiols in rabbit urine after injection of BAL by the cobalt method and by iodine titration*

A. Cobalt method					
BAL excreted during the interval stated (mg.)					
Rabbit no.	Amount of BAL (mg./kg.)	0-1½ hr.	1½-3½ hr.	3½-5 hr.	Total
1	40	1.6	4.5	0.04	6.1
2	40	0.3	0.9	0.01	2.3
3	40	1.2	2.0	0.01	3.2
4	50	0.3	1.6	0.7	2.6
5	50	0-2 hr.	2-4 hr.	4-8 hr.	
		5.7	0.6	0	6.3
B. Iodine titration					
Rabbit no.	Amount of BAL (mg./kg.)	0-1½ hr.	1½-3½ hr.	3½-5 hr.	5-8 hr.
1	40	6.4	20.0	2.8	—
2	40	3.4	7.5	0.7	—
3	40	5.0	7.5	1.0	—
4	50	5.5	12.5	6.6	2.3
5	50	0-2 hr.	2-4 hr.	4-8 hr.	
		18.9	4.3	1.4	24.6
Percentage amount injected recovered by					Iodine recovery given by cobalt method (%)
Rabbit no.	Cobalt method		Iodine titration		
1	5		24		21
2	1		8		11
3	4		19		24
4	2		18		10
5	7		29		26

to give a colour very similar to that given by BAL itself. A preliminary measurement of the light absorption at different wave-lengths of the colour given by the post-injection urine with the cobalt reagent suggested, however, that the thiol contained in the urine might not be BAL itself, since the maximum light absorption occurred at a different wave-length from that of the BAL colour. In view of this an attempt was next made to isolate and identify the thiol present in post-injection urine.

Extraction and attempted isolation of thiols from post-injection urine

In the hope of obtaining larger amounts of post-injection urine, specimens were collected from rabbits in metabolism cages. It was found, however, that the urine obtained by this method gave only a faint nitroprusside reaction, due possibly to destruction of the thiols by contact of the urine with the metal of the metabolism cages. Waxing of the metal to prevent this contact, however, failed to improve the yield. We reverted therefore to catheterization, which at the same time obviated the necessity for the urine standing for variable periods of time exposed to air.

In the first attempt to isolate the excreted thiols, the acidified urine was saturated with ammonium sulphate and extracted with benzene. This method did not yield satisfactory results, since it was found that the partition coefficient of the urinary thiol differed from that of pure BAL added to urine. Thus,

if post-injection urine is saturated with ammonium sulphate and shaken with 0.25 vol. of benzene, only 20 % of the thiol passes into the benzene layer in the first extraction, instead of 50 % as is found with BAL added to urine.

An attempt was next made to precipitate the thiol with a metal salt. In the course of a qualitative study of mercaptide formation by BAL with metal salts, it was found that while most of the heavy metal mercaptides were insoluble at pH 1, the thallium mercaptide was soluble, precipitation only beginning at pH 5. It was expected therefore that BAL would be regenerated by decomposition of this mercaptide with H_2S .

The method adopted was as follows:

Excess of saturated thallous sulphate solution was added to the acidified urine, followed by $N-NaOH$ drop by drop until precipitation of the yellow mercaptide was complete (pH 7.8-8.0). The solution was then warmed to 50°, with constant stirring, until the precipitate flocculated, after which it was filtered and washed with water. Too intense heating was avoided, since it caused the formation of a compact granular precipitate which was difficult to resuspend in an aqueous phase for decomposition.

The precipitate and filter paper were ground up in a small amount of 0.2N-acetate buffer (pH 5.7), and H_2S was passed until precipitation of the sulphide was complete. The solution was warmed gently, then filtered and the precipitate washed. The filtrate, containing the thiol, was acidified to Congo red, and excess H_2S removed *in vacuo*. In later experiments centrifugation was substituted for filtration at each stage.

Identification of purified thiol in post-injection urine

A comparison was made of the optical densities, at different wave-lengths, of the cobalt colours given by the thallium-purified thiol from post-injection urine and by BAL added to normal rabbit urine, and recovered from it by the thallium procedure. The optical densities are given in Table 2, from which it will be seen that the wave-length of maximum light absorption for the purified excreted thiol differs from that given by BAL.

Table 2. *Optical densities of the cobalt colours given by the purified excreted thiol ('metabolized BAL') and by BAL added to normal rabbit urine and recovered ('recovered BAL')*

Wave-length (Å.)	Optical densities ($E_{1\text{ cm.}}$) for		E/E_{max} ratio	
	'Metabolized BAL' (a)	'Recovered BAL' (b)	a	b
4300	0.11	0.20	1.0	0.97
4700	0.09	0.21	0.79	1.0
5000	0.07	0.17	0.59	0.83
5300	0.03	0.10	0.30	0.48
5700	0.02	0.06	0.18	0.27
6100	0.005	0.03	0.05	0.14
6600	0.0	0.0	0.0	0.0
7200	0.0	0.0	0.0	0.0
7500	0.0	0.0	0.0	0.0

The 'metabolized BAL' solution contained thiols equivalent to 100 $\mu\text{g. BAL/ml.}$ as measured by iodine titration. The 'recovered BAL' solution contained 103 $\mu\text{g. BAL/ml.}$

A solution of the 'metabolized BAL' gave a colour intensity which, for the 4700 Å. band, was c. 2.5 times less than the intensity produced by a solution of BAL giving the same iodine titre; this fact provides some explanation of the differences found in the early experiments between the recoveries of thiol calculated as BAL from the iodine titration and from the cobalt method. Further, if an aqueous solution of the thallium-purified thiol is saturated with $(\text{NH}_4)_2\text{SO}_4$ and shaken with 0.25 vol. of benzene, only 50% of the thiol passes into the benzene layer in the first extraction, as opposed to 90% with aqueous BAL (Peters, Stocken & Thompson, 1944). All these experiments provide

confirmation for the view that the excreted thiol is closely related to BAL, but is not BAL itself.

In view of the limited specificity of the reaction between BAL and cobalt, a survey of the reaction of many thiols (both mono- and 1:2- and other dithiols) with different metal salts was undertaken in the hope of obtaining reactions which would be of use in characterizing thiols of different types. Briefly, no reaction or series of reactions with metal salts was found capable of differentiating between mono- and dithiols, or by which BAL could be conclusively distinguished from other thiols. It was found, however, that the thallium-purified metabolized BAL gave reactions very similar to those given by BAL itself. A summary of the more important reactions is given in Table 3.

Absorption spectra of the colours given by reaction of BAL and the excreted thiol with cobalt nitrate

To confirm the inference drawn from measurements with the Pulfrich photometer that the thiol extracted from post-injection urine was of a slightly

Fig. 1. Chart of the absorption spectra of solutions containing the coloured compounds formed by reaction between BAL and the excreted thiol, and cobalt nitrate. ● absorption spectrum of 'recovered BAL'; ○ Pulfrich estimation with 'recovered BAL' (1 cm. cell, 800 $\mu\text{g./ml.}$); ● absorption spectrum of 'metabolized BAL'; ○ Pulfrich estimation with 'metabolized BAL' (1 cm. cell, 800 $\mu\text{g./ml.}$); all solutions 0.013N by iodine titration.

different nature from BAL, absorption spectrum measurements on the cobalt colours of the two thiols were carried out by Mr E. R. Holiday.

Table 3. *Some metal reactions of BAL and 'metabolized BAL'*

Metal	Reactions (at pH 5.7) of	
	(a) BAL	(b) 'Metabolized BAL'
Fe ⁺⁺	Pink coloration, fading slowly	Pink coloration, fading slowly
Co	Dark brown colour and ppt.	Dark brown colour and ppt.
Ni	Chocolate brown colour	Greenish-brown colour
Hg ⁺⁺	Gelatinous white ppt.	Gelatinous white ppt.
Tl ⁺	Golden yellow ppt.	Yellow ppt., but paler than the BAL ppt.
Cu ⁺⁺	Dark blue colour and ppt., fading to a dirty blue colour	Dark blue colour and ppt., also fading on standing
Pb ⁺	Pale yellow ppt.	Cream ppt.

The thallium method was used to obtain a solution of the excreted thiol present in rabbit urine, and also of BAL when added to normal rabbit urine. The resulting solutions were titrated with iodine and diluted to 0.013N; assuming that only BAL is present this corresponds to 800 $\mu\text{g./ml.}$ Each solution (0.5 ml.) was then added to the usual cobalt reaction mixture, and the visible and ultra-violet absorption spectra measured in the photographic spectroscop.

Fig. 1 shows the variation of intensity of absorption at different wave-lengths calculated for a stratum of 1 cm. thickness. It will be seen that while the colour given by 'recovered BAL' showed a poorly defined maximum in the region of 4700 Å. no such maximum occurred in the case of the 'metabolized BAL'. Also, the colour intensity produced by BAL was considerably greater than that produced by an equivalent amount of the urinary thiol.

For purposes of comparison, the optical densities, as measured in the Pulfrich photometer, for solutions of BAL and the excreted thiol at the same strengths as the solutions used for the absorption spectrum measurement, are also included in Fig. 1.

Ability of the excreted thiol to protect the brain pyruvate oxidase system from inhibition by lewisite

As already pointed out, thiols differ markedly in their capacity to protect the brain pyruvate oxidase system from the toxic effects of lewisite oxide, monothiols giving no protection in concentrations at which dithiols protect the system completely. This finding indicates that experiments with this system might provide useful information regarding the nature of the thiol excreted after injection of BAL.

The manometric measurement of the enzyme inhibition in the presence and absence of the protecting thiol was carried out using the usual Warburg technique (Passmore,

Peters & Sinclair, 1933; Stocken & Thompson, 1946). In the earlier work it was shown that 10 $\mu\text{g.}$ of lewisite/Warburg bottle (containing 100–150 mg. brain in 3 ml. Ringer phosphate-pyruvate solution) gave approximately 50 % inhibition of enzymic activity; the same concentration of lewisite was used in this work.

In the first experiment 0.3 ml. of untreated post-injection urine was added to one pair of bottles to discover whether any protection of the enzyme system was brought about. As controls, further pairs of bottles were set up, containing respectively, 0.3 ml. of pre-injection urine, and the same quantities of pre- and post-injection urine in the absence of lewisite, in order to allow for any possible toxic effect of the urine itself. The post-injection urine (0.3 ml.) had an iodine titre equivalent to 800 $\mu\text{g.}$ of BAL in excess of the pre-injection specimen. This would be equivalent to 80–200 $\mu\text{g.}$ of BAL as determined by the cobalt method.

In the next two experiments, the thiols were extracted from post-injection urine by thallium precipitation, and the regenerated thiol was added to the bottles. BAL, added to normal rabbit urine and recovered by the thallium method, was used as a control in these experiments. Before addition to the bottles, both the 'metabolized BAL' and the 'recovered BAL' were suitably diluted with Ringer phosphate, so that 1 ml. of the final solution (the amount added to the bottles) contained 100 $\mu\text{g.}$ of BAL, calculated from the iodine titre.

The results of these experiments are given in Table 4. It will be seen that both 0.3 ml. of the post-injection urine, and 100 $\mu\text{g.}$ of the extracted urinary thiol (expressed as BAL) almost completely protected the brain pyruvate oxidase system from inhibition by 10 $\mu\text{g.}$ of lewisite. Since no monothiol yet studied has been found to cause any significant protection of this enzyme system at the concentration used here, it may be inferred from these experiments that the greater part of the excreted thiols, extractable with thallium, is dithiol in nature.

The slight toxicity of the thiols in the later stages of the experiments has previously been observed to occur with these amounts of BAL.

Table 4. *Effect of 'metabolized BAL' in protecting the brain pyruvate oxidase system from inhibition by lewisite*

Exp.	Additions	Percentage inhibition of enzyme activity over		
		0–30 min.	30–60 min.	60–90 min.
893	10 $\mu\text{g.}$ lewisite	45	41	—
	0.3 ml. pre-injection urine	17	18	—
	Lewisite + pre-injection urine	45	37	—
	0.3 ml. post-injection urine	0	26	—
	Lewisite + post-injection urine	0	30	—
894	10 $\mu\text{g.}$ lewisite	52	61	55
	100 $\mu\text{g.}$ 'metabolized BAL'	6	14	9
	100 $\mu\text{g.}$ 'recovered BAL'	0	16	16
	Lewisite + 'metabolized BAL'	0	0	0
	Lewisite + 'recovered BAL'	0	3	11
895	10 $\mu\text{g.}$ lewisite	65	69	63
	100 $\mu\text{g.}$ 'metabolized BAL'	0	21	25
	100 $\mu\text{g.}$ 'recovered BAL'	0	20	21
	Lewisite + 'metabolized BAL'	11	6	0
	Lewisite + 'recovered BAL'	7	9	16

DISCUSSION

From the evidence presented above it appears that, after injection of sub-lethal doses of BAL, rabbits excrete in the urine a considerable quantity of thiol or thiols, representing some 20 % of the thiol content of the injected BAL, and that a considerable proportion of this excreted thiol is present as a dithiol closely related to BAL. The evidence suggests that the dithiol is not BAL itself, and the possibility immediately arises that the body may detoxicate BAL either by one of the already familiar detoxication mechanisms or perhaps by some means not so far encountered in the study of detoxication mechanisms.

SUMMARY

1. The thiols present in rabbit urine after injection of 2:3-dimercaptopropanol (BAL) have been estimated by iodine titration and by reaction with cobalt nitrate.

2. Extraction by means of precipitation with

thallous sulphate has yielded a purified preparation of a thiol present in post-injection urine.

3. Examination of the optical densities at different wave-lengths of the cobalt colour given by the purified urinary thiol together with measurements of its absorption spectrum both in the visible and ultra-violet regions of the spectrum suggest that this thiol is closely related to BAL but not identical with it.

4. The urinary thiol is capable of protecting brain pyruvate oxidase preparations from inhibition by lewisite at concentrations at which no monothiol has yet been found to be effective.

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REFERENCES

- Calvery, H. O. (1943). By communication.*
Passmore, R., Peters, R. A. & Sinclair, H. M. (1933). *Biochem. J.* **27**, 842.
Peters, R. A., Stocken, L. A. & Thompson, R. H. S. (1944). Report to Ministry of Supply by Peters, no. 77.*
Sinclair, H. M. (1940). Unpublished observations.
Spray, G. H. (1947). *Biochem. J.* **41**, 360.
Stocken, L. A. & Thompson, R. H. S. (1941). Report to Ministry of Supply by Peters, no. 33.*
Stocken, L. A. & Thompson, R. H. S. (1946). *Biochem. J.* **40**, 535.
Thompson, R. H. S. & Stocken, L. A. (1941). Report to Ministry of Supply by Peters, no. 48.*

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The Effect of Injected British Anti-Lewisite on Urinary Sulphur and Glucuronic Acid Fractions

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In an earlier paper of this series (Spray, Stocken & Thompson, 1947) it was shown that the injection of 2:3-dimercaptopropanol (BAL) into rabbits causes a marked increase in the iodine titre of the urine, the latter also developing a strong nitroprusside reaction for thiol groups. The increase in the iodine titre of the urine could have accounted for some 20 % of the injected BAL, and although the excreted thiol was not isolated, strong indications were obtained that it was not BAL but a closely related dithiol. Aqueous solutions of dithiols are rapidly oxidized by atmospheric oxygen at the pH of urine

and in view of this it was possible that much more than 20 % was excreted in the urine, a proportion of it being in some oxidized form which would not be measured by iodine titration.

The effects of injection of BAL on the urinary excretion of inorganic sulphate, ethereal sulphate and neutral sulphur were therefore studied in rats, to discover how much of the BAL-sulphur was excreted as neutral sulphur, and also to see whether any was oxidized to inorganic sulphate. In addition, if any BAL were detoxicated in the animal body by conjugation with sulphuric acid, as happens with,