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.

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; to Dr V. P. Whittaker for providing us with some of the thiols used in investigating the reactions with heavy metal salts, to Mr E. R. Holiday for carrying out the ultra-violet absorption measurements, and to Miss M. R. Kempson for skilled assistance.

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

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

BY G. H. SPRAY, Department of Biochemistry, Oxford

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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,
many other hydroxyl compounds, there would be an increase in ethereal sulphate excretion after injection.

As it is known that compounds containing hydroxyl groups can also be detoxicated by con-
jugation with glucuronic acid the urinary excretion of glucuronides has also been followed after admin-
istration of BAL.

EXPERIMENTAL

Urinary sulphur partition

Groups of six male rats weighing 200–400 g. each were used. The animals were placed in metal metabolism cages, the funnels of which were coated with paraffin wax to minimize contact of the urine with metal. The rats were transferred to other cages for 2 hr. each day for feeding, urine being collected for the remaining 22 hr. This external feeding eliminated the possibility of contamination of the urine with food, and ensured that a constant amount of food was eaten every day. A ‘synthetic’ diet of constant com-
position was used throughout.

Each morning, the funnels of the metabolism cages over which the urine passed were washed with distilled water, the washings being added to the urine. Urine and washings were made up to the smallest convenient volume, filtered clear, and divided into six equal portions for duplicate estimations of the three sulphur fractions.

After several daily determinations of the normal excre-
tions of sulphur fractions had been made, the animals were injected with BAL and the determinations were continued for at least 10 days after injection. In order to vary the injection route, the rats were divided into four groups, groups 1 and 2 receiving 100 mg. BAL/kg. body wt., intramuscularly into a hind leg, and groups 3 and 4 receiving 80 mg./kg. body wt., subcutaneously into the flanks. The BAL was freshly dissolved in 0-9% saline, and the strengths of the solutions were estimated by iodine titration immediately before use.

Inorganic and total sulphate were estimated by the methods of Folin (1905) and total sulphur by the method of Benedict (1909), as modified by Givens (1917). The latter method gave theoretical yields of barium sulphate with known amounts of pure BAL.

The results obtained with the first two groups are shown in Figs. 1 and 2. It will be seen that the injections caused a big rise in neutral sulphur excretion on the day following injection, after which it returned to normal. The increases in neutral sulphur over the means of the normal values repre-
sented some 60% of the sulphur injected as BAL. The injections had no significant effect on ethereal sulphate excretion, but there was a marked rise in inorganic sulphate excretion occurring on the day following injection and persisting for at least 10 days. The cumulative increases in inorganic sulphate over the pre-injection levels were far greater than could be accounted for by oxidation of the remaining 40% of the BAL to inorganic sulphate and excretion as such. Thus, in group 1, a total of 77.5 mg. of sulphur was injected as BAL, and in group 2, 72.8 mg. Yet the cumulative excess excretions of inorganic sul-
phate, apart from the neutral sulphur increase, accounted for c. 220 mg. and 270 mg. sulphur re-
spectively. This extra sulphur, which must have ar-
isen from some source other than BAL, could not have come from the diet, since this was constant before and after the injection.

Cuthbertson (1930, 1931), and Cuthbertson, McGirr & Robertson (1939) have found increased excretions of N, S and P following injury in both rats and human beings. Cuthbertson (1931) showed that the increased sulphur excretion was due mainly to an increase in the inorganic sulphate fraction of the urine. It seemed possible, therefore, that the increased inorganic sulphate excretions observed in these experiments were due to increased tissue catal-
bolism caused by injury arising directly or indirectly from the injection. Calvery (1944) has shown that injection of BAL can cause considerable muscle necrosis and other damage, both gross and micro-
scopic.

With this possibility in view, BAL was injected subcutaneously into two further groups of rats, as it was thought that this injection route might cause less tissue damage than that following intramuscular injection. The results of these experiments are given in Figs. 3 and 4. The general course of the excretion rates was similar to that with groups 1 and 2, the rise in neutral sulphur accounting this time for c. 40% of the injected BAL. Again there was a rise in inorganic sulphate, not as marked as previously, but still too great to be due simply to day-to-day physiological variation. In these experiments esti-
mations of urinary nitrogen were carried out, and a slight rise in nitrogen excretion after injection was detected, confirming the view that the enhanced excretion of inorganic sulphate is probably largely due to tissue damage caused by the injection.

Glucuronic acid excretion

In preliminary qualitative experiments, in which the urine of rats and rabbits was tested for glucuronic acid by Tollens’s (1908) reaction before and after injection of BAL, some evidence was obtained of increased glucuronide excretion after the injection. A quantitative study of the glucuronic acid excretion of animals before and after injection of BAL was therefore undertaken.

For the experiments with rats, groups of three (or in one case, six) large male rats (200–400 g. each) were placed in metal metabolism cages over glass funnels, and were fed outside the cages. The feeding technique, and the treatment of the urine, were the same as already described. The total sulphate, total sulphur and glucuronic acid contents of the urines were determined each day. After two determinations of the normal levels had been made, the animals were given 80 mg. BAL/kg. body wt. dissolved in 0-9% saline, by various routes. Measurements were continued for some days after injection.
Figs. 1-4. Urinary sulphate and neutral sulphur excretions of rats before and after injections of BAL.

- Inorganic sulphate (as $\text{SO}_4^{2-}$);
- Ethereal sulphate (as $\text{SO}_3^-$);
- Neutral sulphur (as S).
A few experiments with rabbits were also made. Large male rabbits were catheterized and their bladders emptied. They were then given an intramuscular injection of BAL and were catheterized at intervals afterwards. The increases in iodine titre and in glucuronic acid content of the urine after injection were measured.

*The estimation of glucuronic acid.* Maughan, Evelyn & Browne (1938) and Mozolowski (1940) estimated glucuronic acid by measuring the intensity of the colour developed on heating glucuronic acid with naphthoresorcinol in aqueous solution in the presence of HCl. A modification of this method was used here.

The quantities of reagents and the heating time were the same as those used by the earlier workers. A detailed study of the effects of HCl concentration, naphthoresorcinol concentration, and heating time on the colour intensity confirmed the earlier findings that these quantities and heating time gave the best results. After cooling, the reaction mixtures were diluted with equal volumes of water and were extracted first with 5 ml. and then with 2 ml. ethyl acetate, the ethyl acetate layers being removed by capillary pipettes to graduated 10 ml. cylinders. The combined ethyl acetate extracts were made up to 6 ml. with pure ethyl acetate, and dried with anhydrous Na₂SO₄. The colour intensity was measured in a Pulfrich photometer using a 1 cm. cell and the 5700 A. filter, against a blank obtained by heating and extracting a tube containing water, naphthoresorcinol, and HCl only. The amount of glucuronic acid in the original solution was read off from a calibration curve obtained with known amounts of pure borneol glucuronic acid.

The earlier workers used ether for extracting the colour, and they did not employ the double extraction technique or dry their extracts. It was found that the method used here gave clearer extracts and more complete extraction than could be obtained in any other way.

The method gave a linear relationship between colour intensity and glucuronic acid concentration in the range 10–60 μg. glucuronic acid, and the results were reproducible at intervals of several months. Other constituents of urine did not affect the method at the dilutions necessary to bring the glucuronic acid content within the range of accuracy.

Table 1 shows that in rats there is a marked rise in the urinary glucuronic acid output, accompanied again by an increase in neutral sulphur excretion, during the 24 hr. following the injection of BAL, after which both these excretions return almost to the normal level. The rise in glucuronic acid, relative to neutral sulphur, is much less following intraperitoneal than intramuscular or subcutaneous injection. If the excess excretions of glucuronic acid and sulphur (reckoned as BAL) during the 24 hr. following injection be calculated, it is found that there may be either more or less glucuronic acid excreted than would appear to be required for complete detoxication of the BAL (see molar ratios, Table 1).

In the experiments with rabbits, on the other hand, it was found that the increases in iodine titre, reckoned as BAL, were far in excess of the increases in glucuronic acid in the post-injection urine than in the pre-injection sample. There was also a large excess of thiol over glucuronic acid in the extract obtained by thallium precipitation (Spray, Stocken & Thompson, 1947) of the post-injection urine.

### Table 1. The daily neutral sulphur (S) and glucuronic acid (G.A.) excrections (in mg.) of rats before and after BAL injections

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Injection route</th>
<th>Excretions of</th>
<th>Days before injection</th>
<th>Injection day</th>
<th>Days after injection</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>Subcutaneous</td>
<td>S</td>
<td>21.5</td>
<td>18.1</td>
<td>42.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G.A.</td>
<td>68.6</td>
<td>70.7</td>
<td>155.7</td>
</tr>
<tr>
<td>2</td>
<td>Intraperitoneal</td>
<td>S</td>
<td>11.6</td>
<td>8.5</td>
<td>28.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G.A.</td>
<td>48.7</td>
<td>42.8</td>
<td>71.4</td>
</tr>
<tr>
<td>3*</td>
<td>Intraperitoneal</td>
<td>S</td>
<td>7.6</td>
<td>7.3</td>
<td>23.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G.A.</td>
<td>35.7</td>
<td>33.9</td>
<td>35.7</td>
</tr>
<tr>
<td>4</td>
<td>Intramuscular</td>
<td>S</td>
<td>6.9</td>
<td>6.9</td>
<td>20.0</td>
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<tr>
<td></td>
<td></td>
<td>G.A.</td>
<td>31.3</td>
<td>32.4</td>
<td>123.0</td>
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<tr>
<td>5</td>
<td>Intramuscular</td>
<td>S</td>
<td>10.5</td>
<td>10.9</td>
<td>24.6</td>
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<tr>
<td></td>
<td></td>
<td>G.A.</td>
<td>59.5</td>
<td>60.0</td>
<td>201.8</td>
</tr>
<tr>
<td>6</td>
<td>Subcutaneous</td>
<td>S</td>
<td>7.3</td>
<td>8.9</td>
<td>24.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G.A.</td>
<td>49.2</td>
<td>53.4</td>
<td>152.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group no.</th>
<th></th>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molar ratio of excess G.A./excess S (as BAL)</td>
<td>1.2</td>
<td>0.4</td>
<td>0.2</td>
<td>2.2</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* One rat in this group died from the injection. To make some allowance for this, the pre-injection figures have been multiplied by two-thirds and are included in parentheses. The post-injection values refer to two rats only.
The above results indicate that the injection of BAL into rats produces an increased excretion of glucuronic acid in the urine; with rabbits, on the other hand, no such increase can be shown. This provides support for the view that the rat excretes BAL at least partially as a glucuronide, though in the absence of a constant ratio between the excess excretions of BAL and glucuronic acid, it is impossible to say definitely that the excreted compound is a BAL glucuronide.

In view of the large increases in glucuronic acid excretion in rats, the absence of any increased excretion in the rabbit following the injection of BAL is not easy to understand.

SUMMARY

1. Following the injection of BAL into rats, a sharp rise in the urinary excretion of neutral sulphur occurs in the first 24 hr. after injection, the increases representing 40–60% of the sulphur injected as BAL.

2. There is no increase in ethereal sulphate excretion after injection, so that rats do not conjugate BAL with sulphuric acid. However, a marked and persistent rise in inorganic sulphate excretion occurs. This appears to be due rather to increased breakdown of tissues caused by the injury produced by the injection of BAL, than to oxidation of BAL-sulphur to sulphate.

3. Injections of BAL cause increased excretions of glucuronic acid in the urine of rats in the first 24 hr. after injection, suggesting that BAL is excreted as a glucuronide by this species. No such increase was found in the rabbit.

I should like to express my thanks to Prof. R. A. Peters, M.C., F.R.S., Dr R. H. S. Thompson and Dr L. A. Stocken for their help and advice in connexion with this work.

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

The Use of British Anti-Lewisite Containing Radioactive Sulphur for Metabolism Investigations


(Received 23 December 1946)

Various methods have been used for investigating the metabolism of BAL. The nitroprusside reaction and iodine titration (Stocken & Thompson, 1941, 1946) showed that following the injection of BAL there was a rapid increase in the urinary excretion of thiol compounds. An examination of the coloured metallic derivatives suggested the presence of a dithiol, a view which was confirmed by enzyme protection experiments. On the other hand, the ultraviolet and visible absorption spectra of the excreted dithiol showed that this was not unmodified BAL (Spray, Stocken & Thompson, 1947). In the rat, but not in the rabbit, Spray (1947) obtained evidence indicating the possibility of conjugation of the urinary thiol with glucuronic acid but not with sulphuric acid. At the same time, he found an increased excretion of inorganic sulphate which was presumed to be due to increased tissue catabolism caused by local damage at the site of injection. In an attempt to establish this last point with more certainty and to learn more about the distribution, BAL containing S35 was tried. Further, since no satisfactory method has been found for the estimation of BAL in blood, it was considered that the "tracer" technique could provide some information as to the rate of absorption, persistence and amount