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SOME OBSERVATIONS ON THE SOURCE OF CYSTEINE FOR MERCAPTURIC ACID FORMATION

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Abstract—Some types of mercapturic acid precursor have been shown to cause a fall in the total thiol content of rat liver homogenates and evidence is presented that this fall is due largely to a fall in the thiol groups of glutathione. The results given support the view that the initial reaction of mercapturic acid precursors is with glutathione rather than with tissue protein.

SEVERAL types of compound form mercapturic acids (*N*-acetyl-S-substituted-L-cysteines) in the animal body[1] and it is probable that glutathione (GSH) is the immediate source of the cysteine required for conjugation. The evidence for this assumption is (1) there is a fall in liver GSH after administration of mercapturic acid precursors to the rat,[1] (2) several enzymes have been described which catalyse the combination of GSH specifically with mercapturic acid precursors to form S-substituted GSH derivatives[2–5, 12] and (3) rabbit, rat and guinea-pig glutathionase (γ-glutamyl transpeptidase) converts S-(4-chlorobenzyl)glutathione into the corresponding S-substituted cysteine.[6] There is, however, some evidence that the thiol groups of tissue protein may take part in the initial reaction of mercapturic acid precursors. S-iodophenylcysteine peptides have been isolated from the tissues of rats to which labelled iodobenzene had been administered,[7] but it has been suggested[8] that such products may be formed by incorporation of S-substituted cysteines into proteins rather than by simple reaction of mercapturic acid precursors with protein thiol groups.

It was therefore of interest to obtain more information on the relative importance of GSH and protein thiol groups in mercapturic acid formation. In this investigation the reactivities of the two types of grouping was studied by determining the effect of some mercapturic acid precursors on rat liver homogenates, measuring total thiol content amperometrically and GSH by a specific glyoxalase method.

MATERIALS AND METHODS

All materials were purchased, with the exception of 1-nitrobutane, which was prepared as described by Barnes, James and Wood.[1] All aqueous solutions were prepared in deionised water.

Preparation of homogenates. The livers of female rats (200 g body weight) which had been killed by a blow on the back of the neck were quickly removed and immersed in crushed ice. Homogenates (10 per cent w/v) were prepared in 0·1 M-phosphate buffer (pH 7·4) in an M.S.E. homogenizer. Large tissue fragments were separated by centrifuging at 4000 r.p.m. for 20 min.

Preparation of digests. For each experiment 20 ml of homogenate was placed in each of the two glass-stoppered 50-ml conical flasks. One of these served as a control and to the other was added the compound to be examined. In the case of 2,3,5,6-tetrachloronitrobenzene, which is a solid, 1 ml of an absolute ethanolic solution containing the required amount was added, the same volume of absolute ethanol being added to the control. Four samples (1 ml) were removed from each digest as quickly as possible after the additions had been made. One pair was analysed for total thiol groups and the other for GSH. After air had been removed from the flasks by a stream of nitrogen they were stoppered and incubated at 37° with continuous shaking. Further samples were removed for analysis at intervals.

Determination of total thiol content. The amperometric method of Benesch, Lardy and Benesch[9] was used. The sample was added to the titration mixture which consisted of 1·0 M-tris (4·0 ml), 1·0 M-HNO_3 (3·4 ml) and 1·0 M-KCl (0·2 ml) and the volume adjusted to 30 ml with deionised water. It was observed that when the titration was carried out by adding small increments (e.g. 0·2 ml 0·002 M-$AgNO_3$) throughout, somewhat lower values for total thiol content were obtained than when a slight excess (e.g. 1·5 ml) was added and allowed to react with the homogenate for about 30 min before continuing the titration. The latter procedure was adopted.

Since silver nitrate reacts with some groups other than thiol groups in the homogenates, it was necessary to determine the uptake in samples in which the thiol groups had been blocked. Accordingly each titration was paired with a blank titration in which a duplicate mixture containing homogenate which had been treated for 10–15 min at room temperature with a slight excess of 1·0 M-sodium *p*-chloromercuribenzoate (0·1 ml more than the volume equivalent to the $AgNO_3$ added in the 'unblocked' titration). The volume of $AgNO_3$ taken up under these conditions was deducted from the uptake in the titration of the 'unblocked' homogenate to give the volume equivalent to the thiol groups present.

Determination of GSH. The samples were deproteinised by addition of an equal volume of salicylsulphonic acid (2·5 per cent w/v) before application of the method of Martin and McIlwain.[10]

RESULTS AND DISCUSSION

The results are given in Table 1. In assessing the fall in thiol content due to the compounds studied it should be borne in mind that simple subtraction of blank values from experimental values is not permissible except in the very early stages of an experiment, since the extent of the disappearance of thiol groups due to oxidation is reduced in the presence of the reacting compound, due to competition for the thiol groups. Nevertheless, if it is assumed that simple subtraction is permissible for reaction times up to 15 min in order to make an approximate comparison, it is evident that in all cases the loss of GSH thiol groups is much greater than that of other thiol groups. In some instances there seems actually to be an increase in non-GSH thiol groups, but this is probably not significant; it should be noted that these values are obtained by difference. The most reactive of the compounds studied appear to react with all the GSH thiol groups present within 15 min, while in the same period no significant decrease in non-GSH thiol groups occurs. With these compounds the initial values for GSH thiol groups are much lower than those in the blank experiments, indicating that the reaction occurs to a significant extent in the brief interval between mixing

the digests and removal of the sample. The results of the experiment with benzyl chloride in which several samples were taken within the first 8 min show clearly how rapid the reaction can be.

The effects observed in the experiments reported are presumably due to both spontaneous and enzymically-catalysed reactions. However, in the course of metabolism of the compounds *in vivo* both types of reaction will undoubtedly occur, so

TABLE 1. CHANGED IN THE CONCENTRATIONS OF GLUTATHIONE AND NON-GLUTATHIONE THIOL GROUPS IN RAT LIVER HOMOGENATE ON INCUBATION WITH SOME MERCAPTURIC ACID PRECURSORS

Compound added (m-mole per 100 g liver)	Glutathione-SH (m-mole per 100 g liver)				Non-glutathione-SH (m-mole per 100 g liver)			
Minutes	0	15	30	60	0	15	30	60
None	0·80	0·68	0·56	0·47	1·46	1·34	1·11	0·92
Benzyl chloride (53)	0·46	0·04	0·02	—	1·44	1·57	1·43	—
None	0·66	0·58	0·17	0·07	1·71	1·50	1·51	1·36
2,3,5,6-Tetrachloro-nitrobenzene (1·9)	0·54	0	0	0	1·58	1·68	1·50	1·42
None	0·76	0·71	0·50	0·34	1·93	1·68	1·61	1·40
1-Iodobutane (26)	0·65	0·13	0·03	0·04	1·94	2·10	1·86	1·59
None	0·75	0·77	0·61	0·34	1·67	1·53	1·54	1·45
1-Nitrobutane (24)	0·74	0·57	0·21	0·09	1·62	1·54	1·51	1·30
None	0·70	0·69	0·57	0·59	1·74	1·73	1·66	1·60
Bromocyclohexane (53)	0·70	0·57	0·38	0·24	1·72	1·80	1·54	1·42

Minutes	0·5	2·0	4·0	6·0	8·0	0·5	2·0	4·0	6·0	8·0
None	0·88	—	—	—	0·87	1·40	—	—	—	1·34
Benzyl chloride (53)	0·64	0·21	0·09	0·03	0·02	1·64	1·74	1·71	1·65	1·66

Compounds were incubated with rat liver homogenate at pH 7·4 and 37° as described in the Methods section. Glutathione thiol groups were determined by the Martin & McIlwain glyoxalase method[10] and total thiol groups by the amperometric method of Benesch *et al.*[9]

Non-glutathione thiol groups = Total thiol groups—glutathione thiol groups. Values given represent the means of two experiments, except for the first benzyl chloride values which are from six experiments.

that from a practical point of view it is probably irrelevant to distinguish between them.

In summary, these experiments support the view that the initial reaction of mercapturic acid precursors of the types studied is with GSH rather than with tissue protein.

It will be noted that the S-substituted cysteine derivatives formed *in vivo* from the compounds included in this study arise by replacement of a halogen or nitro group.[1, 11] In none of the compounds is mercapturic acid formation associated with the replacement of a hydrogen atom, as with benzene, chlorobenzene and naphthalene. Booth, Boyland and Sims[2] found that compounds of this type did not act as substrates for the enzyme catalysing the transfer of aryl groups to GSH unless microsomes and NADPH were also present. These workers suggest that perhydroxylation first occurs to give a dihydrodihydroxy derivative and that it is either this compound or an epoxide formed from it which is the substrate for the enzyme. It has in fact been shown (unpublished results) that, as would be expected, benzene and similar compounds do not react with GSH under the conditions employed in this investigation.

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