Studies on Glutathione *S*-Alkyltransferase of the Rat

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(Received 20 April 1965)

1. A rat-liver enzyme catalysing the *S*-alkylation of glutathione by iodomethane and various other alkyl compounds has been identified and partially purified; its stability, specificity and response to inhibitors and activators and to changes in reaction pH have been studied. 2. The enzyme is distinct from glutathione *S*-aryltransferase, but both enzymes respond similarly to various inhibitors. 3. A similar enzyme has been found in the kidney and adrenal of rat and in the liver and kidney of numerous species. 4. The identity and the physiological role of the enzyme are discussed.

In the preceding paper (Johnson, 1966) the rapid conversion of rat-liver GSH into *S*-methylglutathione after an oral dose of iodomethane was reported and brief evidence was stated for the fact that this reaction is enzymically catalysed. The present paper is devoted to a study of the enzyme. Some of this work has been briefly reported (Johnson, 1963).

METHODS

Materials. Besides those listed in the accompanying paper (Johnson, 1966) the following were obtained from the sources noted: bromomethane (May and Baker Ltd., Dagenham, Essex); all other alkyl halides (Hopkin and Williams Ltd., Chadwell Heath, Essex): they were fractionally distilled before use; chloroacetic acid, chloroacetamide and iodoacetic acid (British Drug Houses Ltd., Poole, Dorset); chloroacetaldehyde [aq. 40% (v/v) solution], chloroacetonitrile, 2-bromopropionic acid, 3-bromopropionic acid, 2-mercaptopropionic acid, 2-mercaptoethylamine, β -propiolactone and 3,4-dichloronitrobenzene (L. Light and Co. Ltd., Colnbrook, Bucks.); ^{14}C -labelled compounds (The Radiochemical Centre, Amersham, Bucks.); ^{14}C -labelled bromoethane was synthesized from labelled ethanol by a small-scale version of the standard preparation described by Vogel (1948): 5g. of bromoethane (330 μC) was obtained from 4 ml. of ethanol (500 μC).

Enzyme assays. Unless specifically noted, assays were at pH 7.0 to obtain low rates of non-enzymic reaction. GSH solutions normally contained EDTA (2 mM) and were purged with N_2 before use: even at pH 9 no loss of free thiol occurred in such solutions during 1 hr. at 20°. Alkyl halides were added from stock solutions in acetone, which did not influence the reaction at concentrations below 3% (v/v).

(a) By disappearance of iodomethane. Iodomethane (about 0.5 mM) and GSH (1–2 mM) were incubated in 50 mM-phosphate buffer, pH 7.0, or 50 mM-triethanolamine hydrochloride buffer, pH 7.0, with tissue homogenate at 37°. The reaction was stopped by the addition of cold HClO_4 (final concn. 6%, w/v). Residual iodomethane was extracted

from the deproteinized solution and assayed by a modification of the method of Epstein, Rosenthal & Ess (1955). Results were rather variable but the method was useful for screening purposes.

(b) By disappearance of thiol group. The use of the 5,5'-dithiobis-(2-nitrobenzoic acid) reagent and its sensitivity has been described in the preceding paper (Johnson, 1966). GSH (0.5–2 mM) and alkyl halide (0.5–6 mM) were incubated with enzyme preparations at 25° in 66 mM-phosphate buffer, pH 7.0, containing EDTA (2 mM). Samples were withdrawn and quenched each minute up to the sixth minute. The manner of quenching depended on the purity of enzyme. In experiments with partially purified enzyme, it was sufficient to transfer rapidly a sample (0.16–0.45 ml., accurately measured with a syringe pipette) of the reaction mixture to a tube containing 6 ml. of 0.3 M- Na_2HPO_4 , pH 7.7, and 1 ml. of 5,5'-dithiobis-(2-nitrobenzoic acid) reagent (0.04% solution in 0.3 M- Na_2HPO_4 , pH 7.7). The tube was stoppered and contents were mixed quickly. The reaction was stopped instantly by the conversion of residual GSH into a mixed disulphide with liberation of the yellow reagent thiol anion: the extinction was read at 410 m μ (the soluble protein in the mixture had no effect). When tissue extracts were used to supply enzyme, the reaction mixture was opaque and not suitable for direct assay; 2 ml. samples were removed and blown into 4 ml. of cold 3% (w/v) sulphosalicylic acid to stop the reaction. Protein was removed by centrifuging and portions (0.5–2 ml.) of the deproteinized supernatant were assayed with the reagent as above. Some reactive thiol groups of proteins in rat-liver supernatant reacted with the reagent and contributed an appreciable blank. This blank declined during the course of reaction as these groups were rapidly attacked by iodomethane; it was usually found that a steady rate of reaction was obtained after an apparent surge during the first 2 min. while this non-enzymic attack on protein thiol groups was being measured in addition to the transferase reaction: the protein of stock purified enzyme (see below) produced no appreciable blank. At the concentrations of GSH usually studied, the removal of GSH equivalent to an extinction change of more than 0.08 (about 15% of the

total) significantly decreased the rate of reaction: the method was therefore of greatest use in qualitative and comparative experiments rather than in precise kinetic determinations.

(c) By production of H^+ ion. The production of H^+ ion in 16 ml. of solution containing GSH (6 mM) and alkyl halide (2–6 mM) plus purified enzyme in triethanolamine hydrochloride (0.5 mM) and EDTA (0.2 mM) was followed at pH 7.0 and 20° with the Radiometer pH-stat.

(d) By production of I^- ion. This method has not been described previously. Street (1960) described an assay of serum bromide based on a similar principle. A 25 ml. reaction mixture containing GSH and iodomethane (both 6 mM) and tissue was incubated at 25° for up to 16 min. in 66 mM-phosphate buffer, pH 7–8, or 66 mM-triethanolamine buffer, pH 7–8, both containing EDTA (1–2 mM). Samples (4 ml.) were taken every 2 min. and quenched and treated according to their protein content; when it was low, as in assays with iodomethane and purified enzyme, the sample was blown into a cold (10°) mixture of 1 ml. of 10% (w/v) $Na_2Cr_2O_7$ solution in 6N- H_2SO_4 plus 4 ml. of cyclohexane in a centrifuge tube. The stoppered tube was shaken vigorously for 1 min. to oxidize I^- ion and to extract I_2 from the deproteinized solution, and then centrifuged at 1500g for 20 min. to separate the phases. The small volume of protein remained at the interface. The extinction of a portion of the upper organic phase was measured at 520 m μ . The I_2 solution produced from 4 ml. of 1 mM-KI solution under these conditions had an extinction of 0.42 (0.405–0.43 in several experiments). With larger quantities of protein, phase separation was not so clear and loss of I_2 occurred by adsorption; it was necessary to deproteinize a 4 ml. reaction sample with 1 ml. of 60% (v/v) $HClO_4$ and to assay I^- ion in a 4 ml. portion of protein-free supernatant obtained after centrifuging. Residual iodomethane or iodoethane in the reaction mixture yielded no measurable I_2 . The $Na_2Cr_2O_7$ reagent was stable indefinitely. Reaction rates linear with time and enzyme concentration were obtained when purified enzyme was used. Because of the method's low sensitivity, high substrate concentrations were used and appreciable non-enzymic blanks were incurred during the comparatively long reaction periods studied. The method was not suitable for assays in which Br^- ion was liberated because the extinction coefficient of Br_2 solution is only about one-fifth that of I_2 .

(e) By production of non-volatile radioactive glutathione derivative. This method was only used with purified enzyme preparations and required ^{14}C -labelled alkyl halide. Tracers were diluted with stock concentrated solutions of alkyl halide in acetone and stored at 0°. The reaction was studied at pH 7.0 and 25° in 66 mM-phosphate buffer containing EDTA (2 mM). GSH (0.4–3 mM), alkyl halide (0.4–6 mM; up to 15 μ C) and purified enzyme were present. In individual experiments the total volume was sometimes varied to obtain a range of final concentrations from a fixed quantity of alkyl halide added from stock; this led to some anomalous results and it seemed possible that losses of alkyl halide by evaporation varied according to the surface/volume ratio. Subsequent work used a fixed reaction volume (usually 6 or 8 ml.) in each experiment and a range of radioactive stock solutions was prepared to supply different final substrate concentrations. Four samples (0.5 ml.) were withdrawn with an accurate syringe pipette at fixed intervals (usually 1 min.) during the reaction and quenched

immediately by blowing them into 0.5 ml. of 1% (v/v) Br_2 solution in methanol, which oxidized GSH and denatured protein; the quenching was performed in scintillation-counter vials. The Br_2 , water, methanol and unchanged volatile substrate were evaporated from the vial in a fumecupboard by blowing air over the surface for several hours. The non-volatile solid residue (except protein) was dissolved in hot water (0.5 ml.), and 14 ml. of a scintillation medium capable of holding water (the XDC medium of Bruno & Christian, 1961) was added. The radioactivity of the sample was measured by a 10 min. count in a Packard series 3000 Tri-Carb counter. Under optimum conditions (25% gain, window set to 100–1000 v), the efficiency of counting was 70–74% and was not influenced by the traces of insoluble salts and denatured protein in the mixture. The rate of reaction was determined from the slope of the line through points on a graph plotted of sample counts against time of sampling; good linear plots were obtained but these did not pass through the origin (Fig. 1). One cause was the presence of traces of non-volatile material in the tracer supplied (particularly with iodoethane); however, with some but not all enzyme preparations the zero blank was considerably greater with alkyl halide incubated with both GSH and enzyme than the sum of the blanks from halide plus GSH and halide plus enzyme. It seemed probable that GSH interacted with proteins (possibly by reduction of disulphides) to form in them reactive chemical groups available for alkylation. Provided that substrate of reasonable specific activity was used, it was not necessary to utilize more than 5% of the substrate. There was a linear relationship of rate to tissue concentration. When reaction times were brief, blanks were negligible (less than 2%) except with iodomethane, where the blank was up to 10% of the total reaction at high substrate concentration. Non-enzymic rates were usually determined over longer periods

Fig. 1. Enzyme assay method *e*: rate of production of non-volatile radioactivity. The assay was carried out at pH 7.0 and 25° as described in the text; the concentration of both substrates was 2 mM, the specific activity of iodomethane being 41 μ C/m-mole. ▲, Enzyme + iodomethane; ○, GSH + iodomethane; ●, enzyme + GSH + iodomethane.

(8 min. for iodomethane; up to 2 hr. for the others). This assay method was undoubtedly the most accurate of the five and was used for the determination of kinetic constants whenever labelled substrates were available.

Enzyme purification. Rat liver (6g.) was homogenized with a smooth Perspex pestle (Aldridge, Emery & Street, 1960) in ice-cold unbuffered 2% (w/v) NaCl solution (23 ml.), and 12 combined homogenates (fraction A; see Table 3) were centrifuged at 2° in the angle 30 head of the Spinco model L ultracentrifuge at 60000g for 30 min. The cloudy supernatants were combined and diluted to 320 ml. with 2% NaCl containing concentrated triethanolamine hydrochloride and hydroxylamine-acetate buffers to give a final 10 mM concentration of each buffer species. The pH at this stage was 6.3–6.8 in different preparations. The supernatant (fraction B) was kept at 2° in an ethanol–solid CO₂ bath and stirred magnetically: 64 ml. of 90% (v/v) ethanol precooled to –5° was added rapidly to give a final ethanol concentration of 15% (v/v) and the pH was adjusted to read 6.0 at 2°. No attempt was made to compensate for the effect of low temperature on the performance of the pH-meter electrodes. It was found throughout these fractionations that the pH of the suspensions altered noticeably in the first few minutes after the addition of a precipitation reagent, and checking and readjustment of pH was performed as a routine 5 min. after any pH adjustment: no further adjustment was then needed. The pH adjustments were made with solutions of acetic acid or triethanolamine. After 20 min. at 0–2°, the grey precipitate was removed by centrifuging at 5000g for 5 min. and discarded. A 0.2M-ZnSO₄ solution was added to the deep-red supernatant (fraction C) to bring the concentration to 2.2 mM. After the mixture had been stirred at 0–2° and pH 6.0 for 20 min., the red precipitate was removed by centrifuging at 5000g for 5 min. and discarded. A 0.2M-ZnSO₄ solution was added to the pale-golden-yellow supernatant (fraction D) to increase the concentration by 12.8 mM (i.e. final concn. 15 mM if the Zn²⁺ removed with the first precipitate is ignored). After the mixture had been stirred at 0–2° and pH 6.2 for 20 min., the brown precipitate containing most of the activity was removed by centrifuging at 5000g for 5 min. The white opalescent supernatant was discarded. The pellet was suspended in 2% NaCl made 10 mM with respect to each of hydroxylamine-acetate and triethanolamine buffers, pH 7.0, and containing EDTA (4 mM) (referred to as NaCl/H/T/V) and rapidly dissolved by the addition of 8 ml. of 0.2M-EDTA (slow dissolution could be achieved with about 5 ml.): the orange-yellow solution (fraction E) was diluted with NaCl/H/T/V to 178 ml. at pH 7.0. Then (NH₄)₂SO₄ (52.2 g.) was added to bring the solution to 47% saturation and the suspension was stirred at 0–2° and pH 7.0 for 30 min. The tan-coloured precipitate was removed by centrifuging at 5000g for 10 min. and discarded. The pale-yellow solution (199 ml.) was brought to 60% saturation with (NH₄)₂SO₄ (an extra 16.6 g.) and stirred at 0–2° and pH 7.0 for 30 min. The active precipitate (fraction F) was removed by centrifuging at 5000g for 10 min. and dissolved in NaCl/H/T/V, which was adjusted to a volume of 25 ml. at pH 7.0. The solution was pale yellow. For comparative assay all precipitates were dissolved in NaCl/H/T/V (with extra EDTA as needed) and all solutions were adjusted to pH 7.0. For storage of the active fraction F, (NH₄)₂SO₄ was added to give 70% saturation without adjustment of pH (which was usually about 6.5): the white

suspension (fraction G) was stored at –5° and was stable for many weeks. Samples were usually prepared for study by centrifuging down the protein and redissolving the pellet in suitable buffer: much of the remaining glutathione aryltransferase activity of fraction G remained in the supernatant and was discarded, so that the final ratio of activity towards the two substrates was about 25–30 compared with the value of 10 achieved with fraction F (Table 3). Fractions obtained in the purification process were assayed by method (b) and a deproteinization step was included for samples up to the end of the ZnSO₄ fractionation, but not thereafter. Protein was determined by the biuret method (Robinson & Hogden, 1940) on early fractions and by the extinction at 280 mμ in later steps. As performed the biuret method gave an extinction of 1.0/38 mg. of protein in a final 11 ml. (calibrated with bovine serum albumin) and E₂₈₀ gave an extinction 1.0/2–2.2 mg. in 3 ml. (roughly calibrated by comparison with biuret samples).

Chromatography. Paper chromatography with solvent descending for 16 hr. was with butan-1-ol-acetic acid-water (11:4:5, by vol.). Dried papers were stained by dipping in ninhydrin (0.25% in acetone) and heating at 55° for 30 min.

RESULTS

The disappearance of iodomethane was catalysed by homogenates of liver, kidney and adrenal of the rat in the presence of GSH (Table 1). The enzymic nature of the reaction is shown by the tissue specificity and the lack of reaction in the absence of GSH or when denatured liver homogenate was used (Table 1). That GSH is a second substrate and not a cofactor was shown by incubating purified enzyme with both compounds (6 mM). Consumption of thiol and production of I[–] ion was equivalent [26.8 and 26.9 μmoles respectively in 6 min. (assays

Table 1. *Enzymically catalysed reaction of glutathione and iodomethane*

Tissue was homogenized in the balanced saline medium of Rodnight & McIlwain (1954) diluted threefold with 2% NaCl (high ionic strength stabilizes the enzyme). The disappearance of iodomethane was followed by assay (a). * Signifies that the loss was not significant.

Homogenate	Iodomethane lost (μmoles/min./g. of tissue)
Liver	1.7
Liver (heated at 70° for 4 min.)	< 0.25*
Liver (GSH omitted from assay)	< 0.25*
Kidney	1.4
Adrenal	0.6
Heart	< 0.25*
Lung	< 0.25*
Spleen	< 0.25*
Brain	< 0.25*
Blood	< 0.25*

b and d)], and a single product was found corresponding to authentic *S*-methylglutathione on chromatographic investigation: this completely replaced the GSH spot if the reaction was allowed to run to completion (R_f of GSH 0.38; R_f of *S*-methylglutathione 0.42). The non-enzymic reaction was about 8% of the total.

Subcellular distribution. Differential centrifugation of liver and kidney homogenates in 0.3M-sucrose showed that about 80% of the activity was in the supernatant fraction (assay *a*). Supernatants obtained from homogenates prepared in 2% sodium chloride solution and centrifuged at 60000g for 30 min. were highly active (85% of the whole homogenate activity in one sample assayed with the pH-stat) and were used for many studies, as the starting material for purification of enzyme, and also as the standard preparation for inter-species comparisons. Such supernatants are referred to below as 'extracts'.

Cofactors. No diffusible cofactors were found. Dialysis of liver extracts against deionized water for 5 hr. at 2° caused a heavy flocculent precipitate to form, and only 28% of the activity was recovered from the soluble protein and almost none from the precipitate, which was mostly insoluble in 2% sodium chloride. A similar effect was observed with kidney extract. This loss of activity was due to denaturation of the protein in the solution of low ionic strength rather than to loss of cofactor, since dialysis for the same time against 2% sodium chloride solution in deionized water led to only a slight precipitation of protein and retention of 72–95% of the activity in solution in several experiments.

Assayed rates were unaltered by the presence or absence in the medium of EDTA (2mM), which was normally added to protect GSH against oxidation catalysed by heavy-metal ions.

Stability. The activity of supernatants from sucrose homogenates was extremely variable from experiment to experiment and declined rapidly at 2°. Supernatants in 2% sodium chloride appeared fairly stable for 5 hr. at 2°, but both supernatants lost all activity if stored for 18 hr. at 2° and both retained full activity when stored at –20° for 48 hr. Activity was destroyed by the addition of acetone (15–40%, v/v) but not by ethanol in concentrations up to about 25% (v/v). Ammonium sulphate (70% saturation) precipitated the enzyme in a form stable for months at –5°.

Distinction from glutathione *S*-aryltransferase. That the enzyme coupling iodomethane to GSH was distinct from that coupling 3,4-dichloronitrobenzene, sulphobromophthalein and some other aromatic compounds to GSH (Combes & Stakelum, 1961; Booth, Boyland & Sims, 1961; Al-Kassab, Boyland & Williams, 1963; Grover & Sims, 1964)

was shown by the different proportions of these activities in liver and kidney, and by their different responses to dialysis, to heat inactivation and to ammonium sulphate fractionation, as shown in Table 2. J. Booth, E. Boyland & P. Sims (personal communication) have shown that their crude preparations of glutathione *S*-aryltransferase from liver (which are stored and survive indefinitely at –2°) have negligible activity with iodomethane as substrate.

Separation and partial purification. For the study of substrate specificity it was essential to use an enzyme preparation substantially free from the glutathione *S*-aryltransferase, and partially purified enzyme also simplified assay procedures by eliminating blanks. Since most of the work *in vivo* had been concentrated on the liver it seemed preferable to continue with a study of the enzyme separated from rat liver, which also is available in greater quantity than kidney although the latter tissue would be convenient since it does not contain glutathione *S*-aryltransferase. Purification procedures were limited by the instability of the enzyme in solutions of low ionic strength such as are necessary for adsorption of proteins on ion-exchange celluloses and calcium phosphate gels. However, a simple procedure that could be completed in 1 day was developed which gave a preparation stable for many weeks at –5° and which provided negligible

Table 2. *Distinctions between enzymes coupling iodomethane and 3,4-dichloronitrobenzene to glutathione*

Test	With iodomethane	With 3,4-dichloronitrobenzene
1. Activity/g. of liver (arbitrary units)	100	100
2. Activity/g. of kidney (arbitrary units)	70	< 5*
3. Liver/kidney activity ratio	1.4:1	> 20:1*
4. Activity remaining after 5 hr. dialysis against deionized water:		
(a) Liver extract	19%	73%
(b) Kidney extract	21%	—
(c) Extract of Chester Beatty male rat liver	19%	95%*
5. Activity remaining after heating at 46° for 8 min. (liver)	41%	94%
6. Activity precipitated by (NH ₄) ₂ SO ₄ (60% saturation) at pH 7.5 (liver)	70%	10%*

* Agrees with Booth *et al.* (1961).

assay blanks: this material was used in many studies of the enzyme characteristics. The specific activity with respect to protein was sufficient to obviate the deproteinization step in all assay methods except (*d*). Fractionation of liver extract was according to the solubility of zinc proteinates in 15% ethanolic solution: it is based on methods developed by Cohn *et al.* (1950) for plasma fractionation but apparently seldom used by enzymologists in spite of several practical advantages. The only drawback was that buffers commonly used in the range pH 6–8 were unsuitable because of their power to precipitate or form complexes with zinc salts. A mixture of hydroxylamine hydrochloride, sodium acetate and triethanolamine provided a fair buffer over the range pH 3.6–8.6 without interfering with the zinc-protein interaction. Ammonium sulphate fractionation followed the zinc sulphate fractionation: use of Sephadex G-100 gel achieved a very similar fractionation to that with ammonium sulphate but was slower and required rather careful monitoring.

The preparation of a typical batch of purified enzyme is described in the Methods section and summarized in Table 3.

The activity of a typical specimen of fraction G was 0.7 μ mole/min./ml. at pH 7.0 and 25° (GSH, 1.4 mM; iodomethane, 2.0 mM).

Activity with respect to pH. Fig. 2 shows the pH-activity curve for the enzyme-catalysed reaction of GSH (1.6 mM) with iodomethane (2.05 mM). The maximum enzymic rate is observed between pH 8 and 9, above which it dropped sharply. This contrasts with the non-enzymic rate, which increases steadily to a maximum at about pH 10. It seems probable that the non-enzymic reaction involves the molecular species GS^- , which will increasingly

predominate over GSH as pH is raised. The fact that the enzymic rate does not increase up to pH 10 in a parallel fashion indicates that the protein is significantly affected at high pH. The fact that change of pH influences the K'_m of glutathione (see below) means that a plot of V_{max} against pH may well be significantly different from Fig. 2, which refers to an arbitrarily chosen concentration of GSH.

Substrates. The results below indicate that both the GSH and alkyl moieties are bound to the

Fig. 2. Relationship of rate of reaction to pH at the chosen substrate concentration. The reaction of GSH (1.6 mM) with iodomethane (2.05 mM) was assayed for 4 min. at 25° by method *b* with purified enzyme as described in the text. Rates are corrected for non-enzymic reaction (negligible below pH 7.8).

Table 3. *Partial purification of glutathione S-alkyltransferase*

Separation was as described in the text. Both enzymes were assayed by method *b* (iodomethane at pH 7.0 and 25°, 3,4-dichloronitrobenzene at pH 8.0 and 37°, in order that low activity could be detected). Activities are expressed as percentages of that in fraction B, which had activities of 3.9 and 3.0 μ moles of substrate/min./g. of liver for alkyltransferase and aryltransferase respectively.

Fraction	Vol. (ml.)	Activity (% of B)	Protein (% of B)	Activity/ protein ratio	Activity of GSH aryl- transferase (% of B)	Alkyltransferase/ aryltransferase activity ratio
A 2% NaCl homogenate*	350	(110–120)	252	0.45	115	1.0
B 2% NaCl supernatant (extract)	320	100	100	1.0	100	1.0
C Ethanol supernatant	330	100	59	1.7	—	—
D First ZnSO_4 supernatant	320	68	30	2.7	61	1.1
E Second ZnSO_4 ppt. (redissolved)	178	50	16	3.1	35	1.4
F $(\text{NH}_4)_2\text{SO}_4$ ppt.:						
(i) (48–56% saturation)	25	20	3.1	6.5	<3	approx. 7
or (ii) (47–60% saturation)	25	28	4.5	6.2	<3	approx. 10
G $(\text{NH}_4)_2\text{SO}_4$ ppt. (0–70% saturation)	—	approx. 25	—	—	<1	approx. 30

* Values derived from separate experiments.

enzyme during reaction. It is convenient to consider them separately.

(a) Thiol substrate. (i) Specificity. None of the following compounds reacted with iodomethane at increased rate under influence of the enzyme: L-cysteine; L-histidine; 2-mercaptopropionic acid; 2-mercaptoethylamine; 2-mercaptoethanol; 2,3-dimercaptopropan-1-ol (BAL). However, some of these compounds reacted non-enzymically with iodomethane at a greater rate and some at a lower rate than did GSH. It therefore seems impossible that the enzyme mechanism involves merely direct reaction of an 'activated iodomethane' with thiols, which themselves remain uninfluenced by the enzyme.

(ii) Kinetics. Fig. 3 shows that with iodomethane (1 mM) a linear Lineweaver-Burk plot was obtained for GSH at pH 7.0: K'_m (the apparent K_m at this non-saturating concentration of iodomethane) was 2.2 mM. At pH 6.2, K'_m was 6.0 although V'_{max} was virtually the same as that at pH 7.0. This is in accord with a postulated mechanism in which the ionized form GS^- rather than GSH is bound to the enzyme. If GSH were bound V'_{max} would be expected to increase with pH while K'_m remained

constant. These deductions are only valid if the comparatively small pH change has not significantly affected the protein.

(iii) Inhibitions. Besides being ineffective as a substrate, cysteine (2 mM) had no influence on the reaction with GSH (2 mM) as substrate (assay e). However, both GSSG and *S*-methylglutathione inhibited the reaction competitively (Figs. 4 and 5).

No investigation was made on the effect of varying the pH or the concentration of iodomethane on the inhibitions.

(b) Alkyl substrate. (i) Specificity. Potential substrates were screened by measuring disappearance of GSH when this was incubated with the substrate in the presence and absence of purified enzyme. Many were also screened with liver extract, but non-specific blanks masked the small rates of truly enzymic reaction. Table 4 shows that some compounds other than simple alkyl halides were substrates. However, not all reactive halogen compounds were substrates (e.g. iodoacetate) and the fact that the ratio of enzymic to non-enzymic rate (A/B) under fixed conditions varied widely over the range of substrates indicates that they are bound to the enzyme and that the reaction is not merely between 'activated GSH' and any alkyl compound in solution. That the alkyl substrates are actually bound to an active site is confirmed by

Fig. 3. Influence of pH on the Lineweaver-Burk plot for GSH. The reaction of GSH (0.4–1.5 mM) with iodomethane (1 mM, 200 μ C/m-mole) was assayed for 3 min. at 25° at either pH 7.0 (●) or pH 6.2 (○) by method e. The rates are corrected for non-enzymic reaction (negligible at pH 6.2).

Fig. 4. Inhibition of the reaction by GSSG. Lineweaver-Burk plots are shown for the reaction of GSH (0.4–1.5 mM) with iodomethane (1 mM; 200 μ C/m-mole) in the absence of GSSG (●), in the presence of 0.8 mM-GSSG (○) and in the presence of 3.2 mM-GSSG (▲). The reaction was assayed for 3 min. at 25° and pH 7.0 by method e. The rates are corrected for non-enzymic reaction.

Fig. 5. Inhibition of the reaction by *S*-methylglutathione. Lineweaver-Burk plots are shown for the reaction of GSH (0.4–1.5 mm) with iodomethane (1 mm; 200 μ C/m-mole) in the absence (●) and presence (○) of *S*-methylglutathione (1 mm). The reaction was assayed for 3 min. at 25° and pH 7.0 by method *e*. The rates are corrected for non-enzymic reaction.

the kinetic and inhibition experiments described below.

(ii) Kinetics. Fig. 6 shows that the rate of enzymic reaction of iodomethane and bromomethane approaches saturation in the range 0.5–3 mm, whereas the rates of the ethyl and *n*-propyl substrates increased nearly linearly with respect to concentration in this range, indicating that they had low affinity for the enzyme. A Lineweaver-Burk plot over a range of iodomethane concentrations (0.5–3 mm) shows a linear relationship (Fig. 7) with K'_m 0.37 at a GSH concentration of 2.1 mm and at pH 7.0. No determination was made to see if either the concentration of GSH or the pH influenced the result. The determination of K'_m of bromomethane was impracticable because of losses of the very volatile substrate during the assay, but these were not so great as to vitiate the observation of saturation of the enzyme by this substrate. No attempt was made to determine K'_m for the ethyl or propyl halides; values calculated from Lineweaver-Burk plots for substrates of low affinity are very inaccurate since the intercept on the abscissa is small and errors are magnified. That these substrates are bound to an active site on the enzyme is shown by the competition between them (see below).

(iii) Inhibitions. The last column of Table 4 shows the extent of inhibition that occurred when

Table 4. Comparison of alkyl substrates and inhibitors

The substrate survey used methods *b* and *e* at 25° and pH 7.0 with GSH (2 mm) and alkyl substrate (1.4 mm). The inhibition studies used method *e* with GSH (2 mm), inhibitor (2 mm) and iodo[14 C]ethane (1.8 mm). 100 rate units are equivalent to the conversion of 0.4 μ mole of substrate/min. All enzymic rates refer to an arbitrarily fixed quantity of enzyme and were corrected for non-enzymic reaction.

Compound	Reaction rate (arbitrary units)		Ratio A/B	Inhibition of iodoethane reaction (%)
	Enzymic (A)	Non-enzymic (B)		
Iodomethane	100	2	50	90
Bromomethane	40	< 1	—	—
Iodoethane	20	0.4*	50	—
Bromoethane	13	< 1	—	approx. 20
1-Iodopropane	13	0.16*	80	—
1-Bromopropane	6	< 1	> 6	5–10
2-Bromopropane	< 5	< 1	—	—
Bromochloromethane	< 5	< 1	—	—
2-Chloroethanol	< 5	< 1	—	None
2-Bromoethanol	< 5	< 1	—	None
Chloroacetaldehyde	55	55	1	20
Chloroacetic acid	< 5	< 1	—	None
Chloroacetamide	< 5	< 1	—	None
Chloroacetonitrile	20	4	5	50
Iodoacetic acid	< 5	12	< 0.4	—
Iodoacetamide	100	32	3	10
2-Bromopropionic acid	< 5	—	—	—
3-Bromopropionic acid	< 5	—	—	—
β -Propiolactone	18	3	6	50

* Extrapolated from Ingold (1953).

Fig. 6. Relationship of rate of enzymic reaction to alkyl halide concentration. The reaction of GSH (2–3 mM) with alkyl halide (0.5–4 mM; variety of specific activities) was assayed for 4–16 min. at 25° and pH 7.0 by method *b* (for bromomethane) or method *e*. Rates (corrected for non-enzymic reaction) are calculated in arbitrary units and are not comparable between substrates. Comparative rates are given in Table 4. ●, Iodomethane; ○, bromoethane; ▲, iodoethane; △, bromomethane; ■, 1-bromopropane.

the listed substrates (2 mM) were tested as inhibitors of the reaction of iodoethane (1.8 mM) with GSH (2 mM). All the compounds found to inhibit the reaction were also substrates for the enzyme, and those that did not were not substrates. Characteristically the most effective inhibitors (iodomethane, propiolactone and chloroacetonitrile) were those compounds most influenced by the enzyme when they were offered as substrates (i.e. the *A/B* ratio in Table 4 was comparatively high for these compounds). The converse was not universally true,

Fig. 7. Lineweaver-Burk plot for iodomethane. The reaction of GSH (2 mM) with iodomethane (0.5–3 mM; 10–60 μ C/m-mole) was assayed for 3 min. at 25° and pH 7.0 by method *e*. The rates are corrected for non-enzymic reaction.

Table 5. *Inhibitions of enzymic reaction of alkyl halides*

Radioactive substrates were assayed by method *e* in the presence of non-radioactive inhibitor with GSH (2 mM) at pH 7 and 25°. Inhibitor and substrate were added simultaneously. The reaction times were 8–16 min.

Substrate	Inhibitor	Inhibition (%)
Bromoethane (2 mM)	Iodoethane (3 mM)	37
	Iodomethane (1.9 mM)	75
Iodoethane (5 mM)	Bromoethane (6 mM)	40
	1-Bromopropane (8 mM)	20
Iodoethane (1.8 mM)	Chloroacetonitrile (3 mM)	67
	β -Propiolactone (3 mM)	66
	Iodoacetamide (2 mM)	10
	Chloroacetaldehyde (2 mM)	23
Iodoethane (1.3 mM)	2-Chloroethanol (10 mM)	None
	2-Bromoethanol (10 mM)	None
	Chloroacetic acid (10 mM)	16
	Chloroacetamide (10 mM)	16
1-Bromopropane (8 mM)	Iodomethane (1.9 mM)	82
	Bromoethane (5.5 mM)	30
	Iodoethane (5.5 mM)	37

for the A/B ratio was high for the higher alkyl halides, which competed only poorly.

Table 5 shows the results of various inhibition experiments with pairs of alkyl halides in which the substrate was radioactive and inhibitor not. It was not possible to plot inhibition curves with iodomethane or other reactive compounds as inhibitors of more slowly reacting substrates since a considerable quantity of the inhibitor would be lost by enzymic reaction during the assay period. However, when unlabelled iodoethane was used as inhibitor for the reaction with bromo[^{14}C]ethane a linear plot was obtained of $1/v$ against inhibitor concentration according to the method of Dixon (1953) over a limited range of inhibitor concentration. Unfortunately, insufficient labelled bromoethane was available for several determinations to be made of inhibition at different substrate concentration, so that the nature of the inhibition could not be clearly demonstrated.

All the above observations suggest that the inhibitions studied were competitive. High competitive-inhibitory capacity of a compound implies that it has high affinity for the enzyme and this was found only in the smaller comparatively hydrophobic molecules: the significance of this fact in relation to the role of the enzyme is considered in the Discussion section.

Effect of sundry inhibitors and activators. *S*-Methylglutathione has been shown to inhibit the reaction (Fig. 5). It is not clear whether this is by direct competition for the GSH-binding site or by repressing the dissociation of *S*-methylglutathione from an enzyme-product complex. However, I^- ion (2mM), which is another product, also inhibits the reaction (34% with iodomethane and GSH, each 2mM). When I^- ion and *S*-methylglutathione (both 2mM), which separately inhibited the reaction 34 and 19% respectively, were both present the inhibition was 42%. Inhibition by I^- ion has not

Table 6. *Aromatic nitro compounds as inhibitors of glutathione S-alkyltransferase*

The reaction of iodomethane (2mM) with GSH (2mM) catalysed by the purified enzyme was assayed by the radioactive method *c*. Nitro compounds were present at 1mM in both substrate and inhibition studies. The test of reaction of nitro compounds with GSH used assay method *b*.

Compound	Inhibition (%)	Enzymic reaction with	
		Iodomethane	Glutathione
(I)	77	*	*
(II)	28	*	*
(III)	96	None†	None
(IV)	58	*	*
(V)	31	*	approx. 3%‡

* Not tested.

† Non-enzymic action also nil during the assay period.

‡ The 3% value refers to the rate (= 100%) with iodomethane as substrate instead of (V).

been investigated over a range of substrate and inhibitor concentrations. The effect of other halide ions has not been examined except that no difference in rate was found when comparing reaction media with buffers (50mM) consisting of alkali phosphates and triethanolamine hydrochloride respectively. The back reaction of *S*-alkylglutathiones with halide ions has not been studied. Table 6 shows that a variety of aromatic nitro compounds inhibit the reaction, including 3,4-dichloronitrobenzene (V), which is a good substrate for glutathione *S*-aryltransferase (Booth *et al.* 1961). Traces of this enzyme are still present in the purified alkyltransferase (see Table 4) and this probably accounts for the observed slight reaction with GSH. However, the remoteness of the structure of these inhibitors from those of GSH and iodomethane, together with the fact that 2-amino-4-nitrophenol (compound III), which is the most effective inhibitor, will not replace either substrate in the reaction, suggests that these are probably not reversible competitors for the active site. It seems more likely that these inhibitors are bound near the active site in some way similar to the well-known binding of nitrophenols to serum albumin (Teresi & Luck, 1948).

The addition of SO_3^{2-} ion (2–8 mM) to the complete reaction medium (GSH, 2 mM; iodomethane, 1.2 mM; pH 7.0) stimulated the enzymic reaction rate by 27%, although negligible reaction of sulphite and iodomethane occurred in the presence or absence

of enzyme when GSH was omitted. Whether the reaction product was still purely *S*-methylglutathione could not be decided with the assay procedure (e) used, but chromatographic investigation should be informative. Since this work was completed an enzyme-catalysed reaction of sulphite with cysteine to form cysteic acid and hydrogen sulphide has been reported (Fromageot & Sentenac, 1964). The enzyme was extracted from the vitelline sac of chick embryos. It is possible that something analogous was happening in my experiments. Non-enzymic reaction was also stimulated by sulphite, possibly by increasing the degree of ionization of the thiol group; no product other than *S*-methylglutathione appears to be formed in this case.

Activity in various species. The distribution among species (Table 7) distinguishes this enzyme from the aryltransferase, the activity of which is higher in rat liver than in all other species tested (Grover & Sims, 1964): livers of the larger species had less than one-quarter of the rat-liver aryltransferase activity. The high liver alkyltransferase activities in lamb, ox, pig and monkey are not matched by high activity in the kidney of these animals.

Some observations on glutathione S-aryltransferase of rat liver. This enzyme has been examined in considerable detail (Booth *et al.* 1961; Al-Kassab *et al.* 1963; Grover & Sims, 1964). I have briefly examined the specificity and compared some inhibitory effects with those seen with the alkyltransferase.

Table 7. *Activity of glutathione S-alkyltransferase in various species*

The 2% NaCl extracts of liver and kidney were prepared as described in the text. Activity was assayed by methods *b* and *d* with GSH (1.4 mM) and iodomethane (2 mM) at pH 7.0 and 25°.

Species	No. tested	Activity ($\mu\text{moles/min./g.}$ of tissue)	
		Liver	Kidney
Rat (Porton strain) (♀)	>20	3–5	1.8, 2.3
Rat (Porton strain) (♂)	2	1.5, 2.1	1.5, 2.7
Mouse (A.2.G. strain) (♂)	8*	1.5	0.7
Mouse (c.52.L. strain) (♂)	8*	1.6	0.7
Guinea pig (♂)	3	1.2, 0.5, 1.8	0.2, 0.3, 0.6
Rabbit (♂)	1	5.0	0.4
Lamb	1	15.4, 14.1, 12.1	1.1, 1.5
Ox	2	3.3, 3.8	0.6 (cortex) 0.6 (medulla)
Monkey (Rhesus)	1	7.9	0.5
Pig	1	4.0	<0.1
Cat	1	3.8	0.9
Chicken	1	0.7	0.5
Toad	5*	2.2	—
Goldfish	5*	1.5	—
Human (biopsy)	1	0.9	—

* Pooled tissues

(1) Thyroxine and its precursors are the only known physiological aromatic halogen compounds in mammals, and it was therefore considered as a possible substrate. However, thyroxine (0.5mM) did not react measurably with GSH (0.5mM) when incubated for 10min. with an extract from 50mg. of liver in 12ml. at pH 8 and 37° (assay *b*). Activity as little as 6% of that towards 3,4-dichloronitrobenzene would have been detected. It seems from this experiment that the enzyme is not concerned in thyroxine metabolism.

(2) Various aromatic halogen compounds (1mM) were screened in an attempt to find a freely soluble substrate. When compared with 3,4-dichloronitrobenzene at 37° and pH 8 the total rates of reaction were: 4-iodobenzoic acid, less than 3%; 3,5-diiodoanthranilic acid, less than 3%; 2,6-dichlorophenol, 15%; 4-chloro-3,5-dimethylphenol, 15%; 4-amino-2,6-dichlorophenol, 25%; 2-amino-4-nitrophenol, less than 3%. No measure was made of non-enzymic rates. The failure of the last compound to react is significant in view of its inhibitory power towards both the alkyltransferase and aryltransferase [see (3) below].

(3) The reaction of GSH (6mM) with 3,4-dichloronitrobenzene (0.6mM) at pH 8 and 20° assayed spectrophotometrically (Booth *et al.* 1961) was inhibited by 31% by *S*-methylglutathione (6mM), by 42% by GSSG (6mM) and by 58% by 2-amino-4-nitrophenol (compound III in Table 6) (1mM). These effects are qualitatively similar to the effects on the alkyltransferase.

DISCUSSION

Identity. The ten mammalian enzymes listed in Table 8 have all been described as specifically requiring GSH as substrate. Glutathione *S*-alkyltransferase (no. 10) is clearly distinguished from three of the others (nos. 2, 7 and 8) by differences in distribution. It is distinguished from all the rest except nos. 3 and 4 (which may be identical) by its instability when dialysed against water. It is also distinguished from several by being inactivated when attempts are made to precipitate it from aqueous solution by ethanol or acetone. The only enzyme from which it cannot be clearly distinguished by these criteria is the glutathione-homo-

Table 8. *Mammalian enzymes using glutathione as substrate*

References: 1, Rall & Lehninger (1952); 2, Mills (1957); 3, Mills (1960); 4, Neubert, Rose & Lehninger (1962); 5, Neubert, Wojtczak & Lehninger (1962); 6, Racker (1955); 7, Narahara & Williams (1959); 8, Tomizawa & Halsey (1959); 9, Heppel & Hilmo (1950); 10, Binkley & Nakamura (1948); 11, Revell & Ball (1959); 12, Goldberg *et al.* (1960); 13, Fodor, Miller & Waelsch (1953); 14, Booth *et al.* (1961); 15, Grover & Sims (1964); 16, Boyland & Williams (1965); 17, this paper.

Enzyme	Organs of high activity	Characteristics	References
1. GSSG reductase	Rat liver and kidney (some activity in spleen and brain, little in blood)	(a) Active enzyme precipitated by acetone; (b) stable to water dialysis	1
2. GSH peroxidase	Liver and erythrocytes of many species		2-5
3. GSH-homocystine transhydrogenase	Ox liver		6
4. GSH-oxidized CoA transhydrogenase	Ox liver	? Identical with no. 3	6
5. GSH-insulin reductase	Rat liver	Stable to water dialysis and to 40% (v/v) ethanol	7, 8
6. Nitroglycerine reductase	Rabbit liver, kidney stomach and uterus, and 'several organs of rat'	Pig-liver enzyme: (a) stable to water dialysis; (b) is precipitated in active form by ethanol (54-65%, v/v) at pH 5; (c) has optimum pH 7-8	9
7. γ -Glutamyltransferase (glutathionase)	Predominantly kidney and pancreas of many species (also sheep brain and calf liver)		10-13
8. GSH <i>S</i> -aryltransferase	Liver of many species Little in rat kidney	(a) Stable to water dialysis; (b) optimum pH 8	14, 15
9. GSH <i>S</i> -epoxidettransferase	Rat liver	(a) Stable to water dialysis; (b) optimum pH 6.5	16
10. GSH <i>S</i> -alkyltransferase	Liver and kidney of several species	(a) Inactivated by water dialysis; (b) inactivated by 30% (v/v) acetone or ethanol at 0°; (c) optimum pH 8-9	17

cystine transhydrogenase (no. 3), for which only limited data are available, but which is not reported to occur in kidney. It seems probable therefore that glutathione S-alkyltransferase is an enzyme distinct from any previously characterized as being specific for GSH.

Besides those listed above, an enzyme catalysing the production of H^+ and Br^- ions from bromochloromethane is present chiefly in liver and kidney of the rat and is precipitated from liver supernatant by ammonium sulphate between 45 and 55% saturation (Heppel & Porterfield, 1949). Although I have not been able to show bromochloromethane to be a substrate for the transferase, these characteristics match those of S-alkylglutathione transferase. However, the authors state that their enzyme required either GSH or cysteine and has a pH optimum of 7.1–7.4. Their Table III shows that up to 9 μ moles of Br^- ion were produced in an experiment with unfortified liver extract, which would have contained only 2 μ moles of GSH. It is obvious therefore that the major portion of the reaction was not catalysed by an enzyme utilizing GSH as substrate in stoichiometric amount.

Function. The normal function of all the three glutathione transferases present in rat liver (Table 8) is obscure since in no case has a physiological second substrate been found. Thyroxine is almost the only organic halogen compound that has a definite place in mammalian metabolism, and this has been shown not to be a substrate for either the alkyltransferase or the aryltransferase: it is known to be excreted conjugated with glucuronic acid rather than as a mercapturic acid (Taurog, Briggs & Chaikoff, 1952). The substrates most effectively converted by the alkyltransferase are small in size and tend to be hydrophobic in character, although neither criterion is absolute (Table 4). Brodie (1956) has pointed out that hydrophobic molecules are often preferred to hydrophilic analogues as substrates for the so-called drug-metabolizing enzymes located on liver microsomes, although this is not universally true (Mazel & Henderson, 1965), and he has suggested that they exist specifically to solubilize and aid in the excretion of hydrophobic foreign compounds accidentally ingested. Whether or not a hypothesis similar to Brodie's (1956) is reasonable for the glutathione transferases, it is now becoming apparent that the production of a urinary mercapturic acid from an administered compound may depend on the existence of an appropriate glutathione transferase. However, it seems strange that a general detoxicating capacity should exist whereby aryl substrates may all be dealt with by an enzyme present only in liver, whereas alkyl substrates require one enzyme in liver and also one in kidney.

An alternative explanation of function is that a

true physiological second substrate exists for glutathione S-alkyltransferase, although it reacts slowly compared with iodomethane. Less reactivity would be no hindrance *in vivo* where much larger amounts of enzyme are present in the liver than were used *in vitro*. Screening tests *in vivo* or with very large amounts of purified enzyme *in vitro* might reveal some hitherto unsuspected substrates that react slowly.

Problem of 2-chloroethanol. 2-Chloroethanol and 2-bromoethanol have been shown to influence the GSH concentration in the liver of rats after oral administration (Johnson, 1965), yet appeared to be unreactive in the screening tests (Table 4). It may be that these compounds are metabolized *in vivo* to compounds such as chloroacetaldehyde or ethylene oxide, which would react rapidly even without enzymic influence. A possibility that has not been excluded is that an enzyme exists in liver extracts that can catalyse the reaction of 2-chloroethanol at a rate undetected in the presence of non-specific blanks and that this enzyme was lost during the purification of the enzyme utilizing iodomethane.

I thank Dr W. N. Aldridge for much stimulating discussion and encouragement and Mrs M. A. Thomas for skilled technical assistance. Most of the work described here has been included in a Thesis for the Ph.D. award of the University of London.

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