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METABOLISM OF CHLOROETHANOL IN THE RAT

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Abstract—When chloroethanol is given orally to rats, liver GSH is rapidly depleted and S-carboxymethylglutathione is formed. Release of ^{36}chloride ion from [^{36}Cl]-chloroethanol has been studied *in vitro* and shown to require stoichiometric amounts of GSH (1 mole) and NAD (2 moles). S-carboxymethyl-glutathione has been identified as the product *in vitro*. Ethanol inhibits the depletion of GSH *in vivo* and release of ^{36}chloride *in vitro*. Chloroethanol is readily dehydrogenated *in vitro* by purified yeast and horse liver alcohol dehydrogenases. The route from chloroethanol to S-carboxymethyl-glutathione has been studied *in vitro*. S-carboxymethyl-glutathione is degraded by kidney homogenate to glycine, glutamic acid and S-carboxymethylcysteine. Subsequent metabolism of the latter compound is discussed. It is suggested that the toxicity of chloroethanol is due to its conversion to chloroacetaldehyde *in vivo*.

CHLOROETHANOL itself does not react with GSH *in vitro* under the influence of glutathione S-alkyl transferase,[1] but when chloroethanol is given orally to rats their liver GSH level drops rapidly.[2] This paper will present evidence that S-carboxymethyl-glutathione (VII in Fig. 1) is formed in rat liver *in vivo* after administration of chloroethanol and that *in vitro* this compound is probably formed via chloroacetaldehyde and S-formylmethyl-glutathione (IV and V in Fig. 1) rather than via the alternative possible routes marked with broken arrows. The breakdown and ultimate fate of VII is considered.

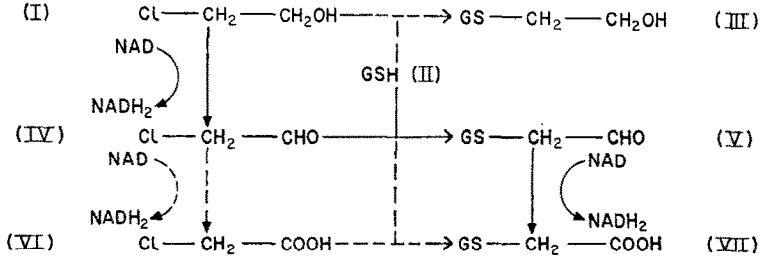

FIG. 1. Metabolic fate of chloroethanol in rat liver.
The full lines indicate route proposed and broken lines indicate possible alternatives.

MATERIALS AND METHODS

Materials

Mature female white rats (Porton strain) of about 200 g weight were used. M.R.C. diet 41B and water were supplied *ad. libitum*. Chloroethanol was administered orally or i.v. dissolved in 0·9% NaCl so that each rat received 1·0 ml saline/kg body weight.

Control rats received saline only or were undosed (no significant difference in effect on GSH levels). All rats were dosed and killed between 10 a.m. and noon.

GSSG, NAD, nicotinamide, and dry crystalline yeast and equine liver alcohol dehydrogenases were obtained from Sigma Chemical Co.; $NADH_2$ from Boehringer Corporation (London) Ltd; GSH from Schwarz Bioresearch Inc; chloroethanol and iodoacetic acid from British Drug Houses; L-cysteine hydrochloride and sodium pyruvate from Roche Products Inc; Tween 80 from Honeywell Atlas Co; L-glutamic acid from L. Light & Co. and ethylene oxide from London Fumigation Co. [^{36}Cl]-chloroethanol (50 μc/m-mole) and Li^{36}Cl were prepared by the Radiochemical Centre, Amersham.

S-carboxymethyl-glutathione was prepared anaerobically by adding a 50% molar excess of solid iodoacetic acid to a small volume of solution of GSH (20 mM) in EDTA (0·2 mM) in an atmosphere of nitrogen and then adding drops of ammonia solution (sp. gr. 0·880) until pH was 9–10. After 20 min thiol could no longer be detected (method below). One tenth volume of triethanolamine hydrochloride buffer (1 M) pH 7·5 was added and the pH reduced to 7–8 with HCl (6 N). S-carboxymethyl-cysteine was prepared in exactly the same way starting with cystine hydrochloride. S-hydroxyethyl-glutathione and S-hydroxyethyl-cysteine were prepared in analogous fashion using a solution of ethylene oxide (4 M) in acetone; three times at 20-min intervals 100 per cent excess of ethylene oxide was added to GSH (20 mM) in EDTA (0·2 mM) made alkaline (pH 9–10) with ammonia and after 40 min the excess of ethylene oxide was removed from the solution by a rapid stream of nitrogen. All the above derivatives were prepared fresh shortly before use. The structure of the prepared S-carboxymethyl- and S-hydroxyethyl-thiols were confirmed by the following reasoning:

In GSH only the amino group of glutamic acid, the carboxyl groups of glutamic acid and glycine and the thiol group are available for attack. That the thiol group had reacted was shown by direct analysis, and that the glycine and glutamic moieties were not attacked was shown by degrading the prepared glutathione derivatives to constituent amino-acids using kidney homogenate as described below. Table 2 shows the chromatographic evidence that the glutathione derivatives were converted to the related cysteine derivatives; compounds indistinguishable from glycine and glutamic acid were produced together with the cysteine derivatives and identified chromatographically at the same time in solvents A–E.

Direct preparation of S-formylmethyl-glutathione by conjugation of chloroacetaldehyde with GSH in alkaline solution proved impossible since chloroacetaldehyde polymerized at pH much above 7. An attempt to prepare the derivative indirectly from the diethyl-acetal of chloroacetaldehyde also failed.

Enzymic preparation of a solution of S-formylmethylglutathione is described in the legend to Fig. 6. Pure yeast alcohol dehydrogenase and NAD were used to convert chloroethanol to chloroacetaldehyde which reacts with GSH immediately. Lactate dehydrogenase and pyruvate were added to regenerate NAD and to prevent accumulation of inhibitory $NADH_2$. Identity of product was deduced from stoichiometry of the reaction which is demonstrated in Fig. 6.

Identification of metabolites

Forty-eight grammes of liver (from eight normal or dosed rats) was deproteinized

by homogenizing in 200 ml of an ice-cold mixture of three volumes ethanol plus one volume KH_2PO_4 (24 mM pH 5·5) containing EDTA (0·1 mM) as described previously.[3] After centrifuging at 20,000 g for 15 min, a sample of the combined clear supernatants (180–190 ml, pale yellow) was assayed for GSH with 5,5′-dithiobis(2-nitrobenzoic acid).[3] The remainder was divided and half was applied to each of two Dowex-carbonate columns (15 × 1·3 cm bed). Basic, neutral and acidic amino-acids were separated by eluting with HCl (0·1 N) according to Gaitonde[4] and each eluant was concentrated to 5 ml in a rotary evaporator at 35°. The acidic fraction concentrate was evaporated to a nearly dry gum with a stream of air overnight. The strongly acidic (HCl) residue was dissolved in water (1 ml) and neutralized to pH 7–8 by addition of 2 N triethanolamine base (about 0·5 ml); the volume was adjusted to 2 ml and samples taken for paper chromatography or for degradation by kidney preparation: GSH and S-carboxymethyl-glutathione when present in the liver extract appeared in this acidic fraction (GSH is oxidized to GSSG during the Dowex treatment). Concentrates of neutral and basic fractions were evaporated in a stream of air to about 2 ml for chromatography.

To identify metabolites produced from chloroethanol *in vitro*, samples (5 ml) from quantitative experiments with 1–2 mM substrates (see below) which had been incubated for 20 min at 37° and pH 7·2 were deproteinized by heating at 100° for 90 sec. Protein was centrifuged off and the supernatant applied to a small Dowex-carbonate column (10 × 0·8 cm) and processed as above.

Degradation of metabolites by kidney homogenate. Glutathione derivatives in triethanolamine hydrochloride buffer (pH 7–8) were hydrolysed to constituent amino-acids by incubation with a cell-free kidney homogenate (25 mg/ml) at 37° for 60 min as described for S-methyl glutathione.[5] Reaction was stopped and the deproteinized extract processed on Dowex-carbonate as above. S-carboxymethyl-cystine and glutamic acid appeared in the acidic fraction, S-hydroxyethylcysteine and glycine in the neutral fraction.

Paper chromatography. Descending chromatography was carried out on Whatman No. 1 Papers for 17–24 hr at 23–25°. The ninhydrin and platinic iodide stains have been described.[5]

Solvents used were: (A) Butan-1-ol:acetic acid:water (11:4:5 vols.—aged 24 hr before use); (B) Phenol:2 N ammonia (80 g:20 ml); (C) Propan-1-ol:pyridine:water (1:1:1 vols.); (D) methyl-ethyl-ketone:pyridine:2N ammonia (1:1:1 vols.); (E) Propan-2-ol:2N ammonia (7:3 vols.); (F) Propan-2-ol:2N HCl:20 mM EDTA (70:30:1 vols.); (G) Propan-2-ol:methyl-ethyl-ketone:water (1:1:1 vols.); (H) Butan-1-ol: formic acid:water (11:4:5 vols.): this solvent separated into two phases within 20 hr and was prepared immediately before use.

Assays in vitro. Unless otherwise specified, the basic reaction medium used in all experiments contained Na-K-PO_4 buffer (66 mM), EDTA (0·2 mM), $MgCl_2$ (5 mM) and nicotinamide (20 mM) pH 7·2. Rat liver was homogenized in ice-cold basic medium using a smooth Perspex pestle with 0·020 in difference in diameters of pestle and tube.[6] Cell debris was removed to give a cell-free homogenate by centrifuging at 600 g for 5 min at 2°. Supernatant [referred to as enzyme preparation (A)] was prepared from the homogenate by centrifuging at 2° in the angle 30 head of the Spinco model L at 60,000 g for 60 min. When a preparation substantially free of cofactors was needed, solid ammonium sulphate was added to (A) (430 g/l. to give 65 per cent saturation);

the cold suspension was allowed to stand in ice for 30 min before the precipitate was centrifuged down, dissolved in basic medium and adjusted to pH 7·2 at 20° to give enzyme preparation (B). For most assays, preparations from 1 g liver were incubated in 8–12 ml solution with added substrates and cofactors at 37° and pH 7·2. Exact details are noted in the Table legends. Except for slight loss of GSH from solutions containing homogenate there was no difference between reactions studied in anaerobic solutions and those shaken in air. Reaction was usually started by addition of chloroethanol but in cases where lactate production was studied it was necessary to start the reaction by addition of NAD/pyruvate since there was considerable production of lactate when tissue was incubated with NAD/pyruvate without any other substrate; this endogenous reaction decayed with time and was observed to be stimulated by magnesium ions: initially it could be as much as 0·17 μmoles/min/g liver. Reaction was stopped by various means according to material being assayed:

(a). GSH. Two-millilitre samples were removed and blown into 4 ml fresh ice-cold sulphosalicylic acid (3% w/v). The assay has been described.[1] GSH in deproteinized extracts of whole liver was assayed by the same method.

(b). Lactate and pyruvate. Four-millilitre samples were removed and blown into 1 ml ice-cold $HClO_4$ (15% w/v). Assays were as described by Bergmeyer.[7]

(c). ^{36}Chloride ion. Five-millilitre samples were removed and blown into 3 ml ice-cold solution containing $HClO_4$ (15% w/v), NaCl (100 mM) and $HgCl_2$ (12·5 mM). The NaCl provided carrier for the isotope and $HgCl_2$ neutralized all thiols present which otherwise exerted a colloidal effect in preventing precipitation of AgCl in the next step. Protein was centrifuged off and 7 ml clear supernatant was mixed with 2 ml $AgNO_3$ (200 mM) containing 0·3% of Tween-80 detergent in a stoppered centrifuge tube. A fine dense precipitate of AgCl quite different from the typical granular precipitate was obtained. The precipitate was collected by centrifuging and, after all supernatant had been removed with suction, was washed by resuspending in a mixture containing $AgNO_3$ (180 mM) and $HClO_4$ (1·5% w/v) and again centrifuged. The supernatant was sucked off and the pellet dissolved in 5 ml KCN (0·5 M). Samples (0·8 ml) were transferred to counting vials and 15 ml of the XDC scintillation medium[8] added. Radioactivity was counted in a Packard series 3000 Tri-Carb counter with 2 per cent gain and window set to 50–1000. Efficiency of counting was 99 per cent and recovery of $^{36}Cl'$ added immediately after deproteinization was 98–100 per cent. No radioactive chloroethanol was found to be converted to inorganic chloride or carried through unchanged during the separation.

(d). Spectrophotometric dehydrogenase assay. Dehydrogenation of chloroethanol at 20° by liver preparation (B) equivalent to 16 mg liver/ml was studied in 2·9 ml basic medium pH 7·5 containing NAD (1 mM); GSH (5 mM); chloroethanol (3 mM). Chloroethanol was added after observing endogenous reaction for 5 min. Reaction was followed by measuring increase in extinction at 340 mμ due to $NADH_2$. Dehydrogenation by purified enzymes was studied similarly but in NH_4Cl buffer (50 mM, pH 9·2); NAD (5 mM); GSH (5 mM); ethanol, chloroethanol or both (50 mM).

RESULTS AND DISCUSSION

Reaction in vivo

Table 1 shows that rat liver GSH levels rapidly drop to less than one third of the controls after oral administration of chloroethanol (52 mg/kg or more). Concomitant

administration of a large dose of ethanol has been stated not to prevent this drop.[2] However, this is now shown to be true only at later periods (2 hr) after dosing while the drop was much less at 30 and 50 min when ethanol was also given. This effect of ethanol is not merely due to inhibition of absorption of chloroethanol from the gut

TABLE 1. INFLUENCE OF ORAL DOSAGE OF CHLOROETHANOL ON GSH CONTENT OF RAT LIVER

Dose (mg/kg body wt.)	Time after dosage (min)	Liver GSH content (% mean control)	
		Group A	*Group B*
52	30	49, 35	89
	50	33	84
	60	27	—
	120	11–22 (6 rats)	12, 24
100	30	30, 36	—
	40	21	—
	45	4	—
	50	32	—
	55	4	—
	120	17, 15	—

In group A, rats received chloroethanol in 0·9 per cent NaCl; group B rats also received ethanol (500 mg/kg) in the dose.
Extraction of tissue and assay have been described.[3] Mean control value for GSH content was 5·3 μmoles/g with range 4·2–6·5 (eleven rats). Each value represents results with one rat.

since the effect was seen at 30 mins if chloroethanol (52 mg/kg) was given intravenously; without ethanol the GSH level dropped to 24 and 26 per cent respectively in two experiments and with ethanol the level was 77 and 94 per cent of the controls. Administration of ethanol alone does not influence rat liver GSH levels.[2]

A derivative of GSH was found in deproteinized extracts of livers taken 45 min after dosing rats with chloroethanol (100 mg/kg). When compared with controls, no substantial new spots were detected in the neutral and basic amino-acid fractions of the Dowex-carbonate effluent. Table 2 shows that the acidic fraction of the effluent contained material indistinguishable from S-carboxymethylglutathione (VII in Fig. 1) according to R_f in five solvents and the platinic iodide and ninhydrin tests. This compound was the only new spot seen on the chromatograms when compared with the acidic fraction of extracts of normal liver. When the dried concentrate of the acidic fraction from chloroethanol-dosed rats was dissolved and treated with kidney homogenate for 60 min at 37° and pH 7–8 and reprocessed on a Dowex-carbonate column (see Methods), an acidic amino acid positive to ninhydrin and platinic iodide was obtained as well as glutamic acid and glycine. This acidic amino acid could not be resolved from S-carboxymethyl-cysteine in four solvents (Table 2) and was only slightly resolved from glutamic acid which, however, does not respond to the platinic reagent. The poor resolution of S-carboxymethyl-cysteine and glutamic acid is hardly surprising when one considers that the molecules only differ by the presence or absence of a single sulphur atom in the middle of the sidechain.

From control rats GSSG was the only ninhydrin and platinic-positive spot found after the first Dowex separation. This was not checked further by the kidney homogenate treatment.

The chromatographic evidence given shows clearly that rat liver GSH is converted *in vivo* to S-carboxymethyl-glutathione after administration of chloroethanol.

TABLE 2. PAPER CHROMATOGRAPHY OF ACIDIC AND NEUTRAL AMINO-ACIDS

	Distance travelled (% of glycine) in solvents							
	A	B	C	D	E	F	G	H
I. Acidic fraction								
A. Reference compounds								
Glutamic acid	120	57	73	70	89	128	93	—
GSH	112	} 23	} 28	} 29	} 49	128	} 61	112
GSSG	35					53		86
SH-ydroxyethyl-glutathione	93	106	101	101	—	—	97	—
*S-Formylmethyl-glutathione	—	<10	<10	—	12	—	29	42
S-Carboxymethyl-glutathione	82	66	57	53	76	86	90	86
S-Carboxymethyl-cysteine	115	85	82	—	86	—	—	—
B. Rat-liver extract								
Chloroethanol-dosed	83	66	56	53	76	—	—	—
Saline-dosed	35	24	27	29	—	53	—	—
C. Kidney-degraded products from:								
S-Hydroxyethyl-glutathione	← No Pt +ve spot →							
S-Carboxymethyl-glutathione	115	85	82	—	86	—	—	—
IB: Chloroethanol-dosed	114	85	82	—	86	—	—	—
II. *Neutral fraction*								
A. Reference compounds								
Glycine	← 100 →							
S-Hydroxyethyl-cysteine	124	159	126	141	114	—	—	—
C. Kidney-degraded products from:								
S-Hydroxyethyl-glutathione	124	159	126	141	114	—	—	—
S-Carboxymethyl-glutathione	← No Pt +ve spot →							
I B: Chloroethanol-dosed	← No Pt +ve spot →							

Separation of acidic and neutral amino-acids by Dowex-carbonate and paper chromatography as described in Methods. (A) Reference compounds; (B) liver extracts; (C) derivatives obtained from (A) or (B) by incubating with kidney homogenate *in vitro*. With the exception of glycine and glutamic acid which do not respond to the platinic iodide reagent, only compounds responding positively to both ninhydrin and platinic reagents are noted. Values reported are per cent of distance travelled by glycine and are measured from centre of spot: they represent mean of two to four values differing by not more than 5 per cent.

* Identity presumed on evidence presented in Results and Fig. 6.

Reaction in vitro

Utilization of glutathione and release of chloride. Table 3 shows that GSH is lost from an incubation medium containing chloroethanol and liver enzymes provided that NAD and pyruvate are also present. The table also shows that the same factors were necessary for chloride ion release from chloroethanol. GSH was also required in the latter reaction but cysteine was partly effective as an alternative.

Figure 2 shows an experiment in which rate of loss of GSH and of production of ^{36}chloride were determined simultaneously in the same reaction. Calculation from

TABLE 3. FACTORS INFLUENCING THE LOSS OF GLUTATHIONE AND THE LIBERATION OF CHLORIDE FROM CHLOROETHANOL *IN VITRO*

Alteration to medium	A Rate of loss of GSH	B Rate of liberation of ^{36}Cl ion
None	100%	100%
Omit NAD	12	32
Omit Pyruvate	25*	10
Omit NAD/Pyruvate	—	15
Omit enzymes	Nil	Nil
Omit chloroethanol	Nil	—
Omit GSH	—	12
Omit GSH but include CySH (5 mM)	—	58

Separate experiments each in basic medium at pH 7·2 and 37° as described in Methods. In (A), medium contained NAD (2 mM); GSH (1 mM); sodium pyruvate (2 mM); chloroethanol (2 mM) and enzyme preparation (B) equivalent to 84 mg liver/ml. Samples taken for assay at 3, 8 and 13 min after start. 100% rate = 0·13 μmoles GSH/min/g liver. In (B), NAD (5 mM); GSH (5 mM); sodium pyruvate (3 mM); [^{36}Cl]-chloroethanol (1 mM, 0·02 μc/ml) and enzyme preparation (B) equivalent to 55 mg liver/ml. Samples taken for assay at 7 and 14 min after start. 100% rate = 0·13 μmoles ^{36}chloride/min/g liver.

* Mean value. Rate almost nil after 8 min.

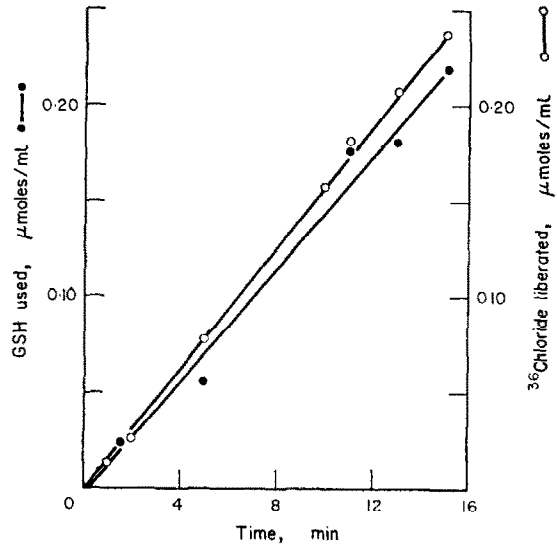

FIG. 2. Correlation of disappearance of GSH and liberation of chloride ion *in vitro*. Reaction at 37° in basic medium pH 7·2 for 16 min as described in Methods. NAD (1mM); GSH (2 mM); [^{36}Cl]-chloroethanol (2 mM, 0·04μc/ml); sodium pyruvate (3mM) and enzyme preparation (B) equivalent to 150 mg liver/ml.

the best straight line through the values shows rates of reaction per min per g liver of 0·092 μmoles GSH and 0·095 μmoles ^{36}chloride respectively. A similar close molar correlation was found in a second experiment in which GSH and chloroethanol concentrations were reduced to 0·5 mM each.

Dehydrogenation. The requirement for NAD and pyruvate shown in Table 3 suggested that dehydrogenation was occurring during a conversion of chloroethanol to a compound which reacted with GSH liberating chloride ion. Direct spectrophotometric determination (Methods) showed that chloroethanol was dehydrogenated by liver supernatant at a slow rate (0·04 μmoles $NADH_2$ produced/min/g liver) at 20° and pH 7·5 and that the rate declined steadily with time. Figure 3 shows that when

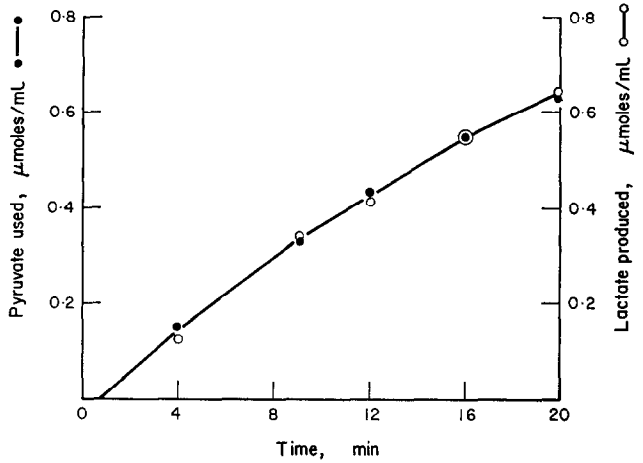

FIG. 3. Correlation of rate of disappearance of pyruvate and appearance of lactate *in vitro*. Reaction at 37° in basic medium pH 7·2 for 20 min as described in Methods. NAD(1mM); GSH (2mM); chloroethanol (2mM); sodium pyruvate (1mM) and enzyme preparation (A) equivalent to 125 mg liver/ml. No distinction is made between reaction occurring in presence and absence of chloroethanol.

chloroethanol was incubated with enzyme preparation (A) in the presence of GSH NAD and pyruvate then pyruvate was lost from the medium and an equimolar quantity of lactate was formed. It may be deduced that chloroethanol was being dehydrogenated and that lactate dehydrogenase (present in liver supernatant[9]) acted with pyruvate to remove $NADH_2$ from the reaction as it was formed. At neutral pH the lactate dehydrogenase equilibrium is much in favour of lactate formation[10] so that lactate produced is a measure of $NADH_2$ produced initially and therefore of NAD utilized in the dehydrogenation of chloroethanol. The stimulation of chloride release and GSH utilization by addition of pyruvate may best be explained by the removal of inhibitory $NADH_2$ from the medium. Stimulation by pyruvate is not explained by the fact that it maintains the NAD concentration constant throughout the reaction since in Experiment (B) (Table 3) NAD concentration would only fall from 5 to about 4·6 mM during the 14 min reaction in absence of pyruvate. If $NADH_2$ inhibits chloride release it seems probable that dehydrogenation is the limiting factor in the overall reaction under study.

Figure 4 shows the rate of lactate production and of GSH utilization in the same reaction. In two such experiments the ratio of these rates was 2·15:1 and 1·84:1 respectively when the lactate production rate had been corrected for endogenous reaction (about 40% total) in the absence of chloroethanol. These values approximating to 2:1 indicate that two dehydrogenation steps were associated with utilization of one glutathione molecule and release of one chloride ion. The experiments do not indicate whether both dehydrogenations occur before the conjugation, but the expected product would be S-carboxymethyl glutathione as was found *in vivo*.

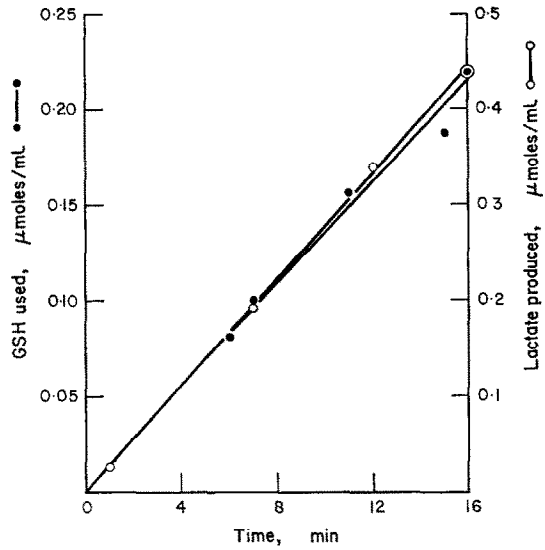

FIG. 4. Correlation of rate of utilization of GSH and appearance of lactate *in vitro*. Reaction at 37° in basic medium pH 7·2 for 16 min as described in Methods. NAD (1mM); GSH (1mM); chloroethanol (2mM); sodium pyruvate (3mM) and enzyme preparation (B) equivalent to 120 mg liver/ml. Lactate results are corrected for control rate in absence of chloroethanol.

Identification of product in vitro. When high concentrations of chloroethanol NAD, pyruvate and GSH (10–20 mM) were incubated with enzyme preparation (A) for 2–3 hr at 37° and initial pH 7·5 very little of the expected S-carboxymethyl-glutathione could be found by paper chromatography. However, if reaction with 1–2 mM substrates was stopped after only 20 min (comparable with quantitative experiments) and the deproteinized solution was then processed on Dowex-carbonate, concentrated and chromatographed in solvents A, B, C and G (Methods) then S-carboxymethyl glutathione was identified as the only platinic positive compound not present in controls incubated for a similar time either with ethanol or with no added substrate. This confirms the quantitative results, but it seems that prolonged incubation of S-carboxymethyl glutathione with liver supernatant causes it to be degraded in a manner which has not been elucidated. It is unlikely that simple hydrolysis to constituent amino acids was occurring since S-carboxymethyl cysteine could have been detected and was not found.

Sub-cellular distribution of chloroethanol-metabolizing enzymes

A homogenate of liver in 0·3 M sucrose was centrifuged at 60,000 *g* for 1 hr and the capacity of the unwashed particles and the supernatant to release ^{36}chloride from [^{36}Cl]-chloroethanol was determined in conditions as in Table 3. Seventy-eight per cent only of the original homogenate activity was recovered and of this 85 per cent was in the supernatant fraction (64 per cent of the activity of the original homogenate). In numerous experiments in which conditions were not entirely standardized, enzyme preparations (A) were found to use GSH or release chloride at about 0·13 μmoles/min/g compared with cell-free homogenates about 0·20 μmoles/min/g. It seems therefore probable that the majority of the rate-limiting enzyme is present in the supernatant fraction but no conclusion concerning distribution of the remaining enzymes is possible without specific assays.

Possible metabolic routes for chloroethanol

The chromatographic studies, the cofactor requirements and the stoichiometric ratios established for GSH and NAD consumption and for chloride release from chloroethanol are fully consistent with the formation of S-carboxymethyl glutathione (VII in Fig. 1) from chloroethanol by two dehydrogenation steps and a conjugation with the thiol group of GSH by any of the routes depicted in Fig. 1. The route itself is considered below.

1. *Direct conjugation of chloroethanol* (I → III). This is excluded by the failure to obtain significant GSH consumption or release of chloride in absence of pyruvate and NAD (Table 3).

2. *Dehydrogenation of chloroethanol* (I → IV). This has been observed spectrophotometrically (see above) and indirectly by lactate production. Further evidence for

TABLE 4. INHIBITION OF RELEASE OF ^{36}CHLORIDE FROM CHLOROETHANOL *IN VITRO*

Alteration to medium	Rate (%)
None	100
Include ethanol (1 mM)	42
Include ethanol (1 mM) and increase pyruvate (10 mM)	43
Increase pyruvate only (10 mM)	93
Include iodoethane (3 mM)	87
Include S-methyl-glutathione (7 mM)	90
Include monochloroacetate (0·8 mM)	27

Reaction at 37° in basic medium pH 7·2 for 14 min as described in Methods. NAD (1 mM); GSH (1·5 mM); [^{36}Cl]-chloroethanol (1 mM, 0·02 μc/ml); sodium pyruvate (3 mM) and enzyme preparation (B) equivalent to 55 mg liver/ml. Additions as noted. 100 per cent rate = 0·13 μmoles ^{36}chloride released/min/g liver.

dehydrogenation comes from studies with ethanol which inhibits the release of ^{36}chloride (Table 4). If this were due to lowering of NAD concentration in the solution by the combined action of ethanol and alcohol dehydrogenase then increase in pyruvate concentration might be expected to relieve the inhibition by speeding the regeneration of NAD. In fact the inhibition is unchanged when pyruvate concentration

is trebled (Table 4) and it seems probable therefore that the inhibition is due to ethanol competing for an enzyme dehydrogenating chloroethanol.

3. *Conjugation with* GSH (IV → V *or* VI → VII). Table 4 shows that neither iodoethane nor S-methyl-glutathione greatly inhibited the release of ^{36}chloride from chloroethanol. At the concentrations used both these compounds would effectively inhibit reactions catalysed by glutathione S-alkyl transferase.[1] Moreover cysteine is not a substrate for that enzyme[1] while here cysteine is a partial substitute for GSH (Table 3). It may be deduced therefore that either the conjugation step is non-enzymic or is catalysed by a glutathione transferase other than that previously described. In the non-enzymic situation chloroacetaldehyde (IV) has been shown to react far faster with GSH than does chloroacetate (VI).[1]

The rate of release of ^{36}chloride ion is considerably reduced in the presence of unlabelled monochloroacetate (Table 4). This could be due to a true inhibition of one of the steps leading to conjugation or be due to an isotope dilution effect if monochloroacetate were an intermediate in the reaction. In the latter case, each molecule of labelled monochloroacetate produced would mix with a pool of the same compound unlabelled: the chance of the labelled molecules reacting with GSH would be much less than if there had been no isotope dilution and initially the observed rate of $^{36}Cl'$ release would be vanishingly small. As more label entered the pool the chance of a labelled molecule reacting with GSH would increase continually so that the observed rate of release of ^{36}chloride should increase steadily from zero. Figure 5 shows that

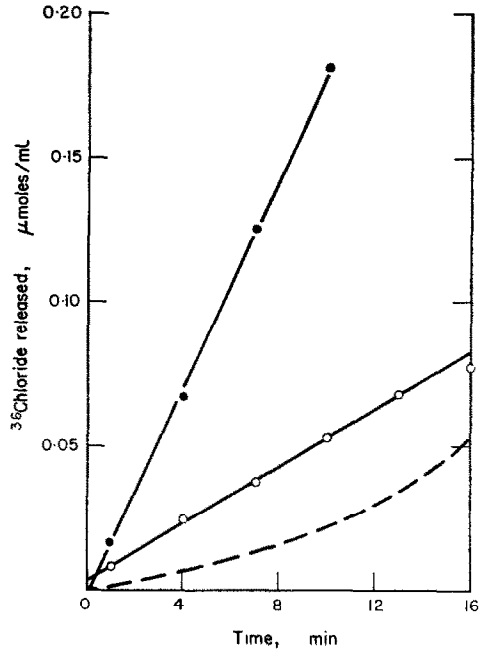

FIG. 5. Influence of presence of unlabelled monochloroacetate on rate of release of ^{36}Chloride from [^{36}Cl]-chloro-ethanol *in vitro*.

Conditions were as in Table 4. ● Control; ○ monochloroacetate (0·8mM) added. Broken line shows the calculated rate in case of isotope dilution of radioactive intermediate monochloroacetate.

the observed rate did not alter with time in an experiment using [^{36}Cl]-chloroethanol (1 mM) in the presence of monochloracetate (0·8 mM). The theoretical curved line for rate of release of ^{36}chloride if isotope dilution occurred has been calculated and is plotted on Fig. 5. Clearly the experimental results are incompatible with the occurrence of significant isotope dilution and since it is difficult to envisage reaction

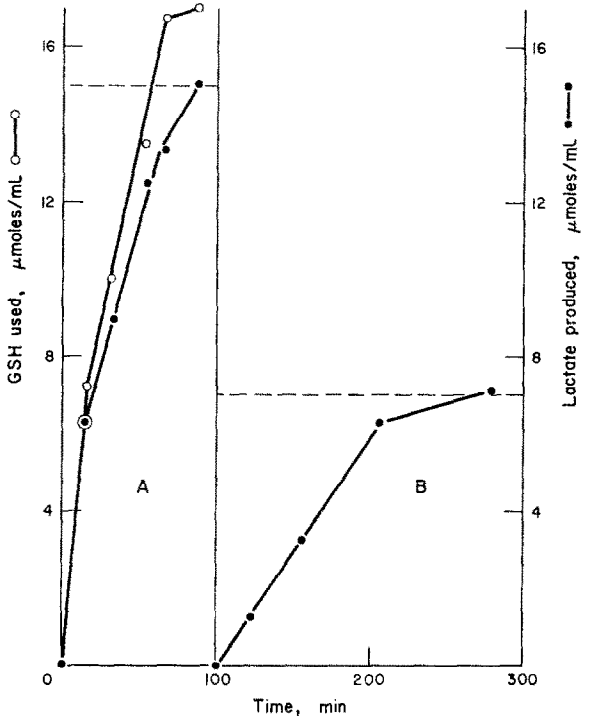

FIG. 6 (A). Enzymic preparation of S-formylmethyl-glutathione.

Reaction at 37° in basic medium initially pH 7·8. NAD (1·8 mM); GSH (22 mM); chloroethanol (15 mM); sodium pyruvate (35 mM) with yeast alcohol dehydrogenase (77 mg dried preparation) and lactic dehydrogenase (0·1 ml suspension in 6M ammonium sulphate) in final volume 6·9 ml. Portions (10μl) were sampled at intervals for direct estimation of lactate and GSH without deproteinization.

(B). Dehydrogenation of S-formylmethyl-glutathione.

After 100 min reaction above, there was added an equal volume of liver enzyme preparation (A) equivalent to 140 mg liver/ml in final 12 ml. Concentrations now were (approx): NAD (0·9 mM); sodium pyruvate (10 mM); chloroethanol (Nil); GSH (4 mM); S-formylmethyl-glutathione (7 mM). 6 ml of mixture was immediately deproteinized at 100°/2 min and chromatographed on paper. Remainder was incubated for 3 hr at 37° with sampling for lactate assay as in (A). Reaction was stopped and a sample taken for chromatography as at start. Broken lines represent initial concentrations of chloroethanol in (A) and S-formyl-methyl glutathione in (B).

occurring via monochloracetate without the radioactive intermediate being free to mix with added monochloracetate in solution this pathway may be excluded. The fact that monochloracetate does substantially reduce the rate of release of ^{36}chloride from [^{36}Cl]-chlorethanol may possibly be explained as being due to competition of added monochloracetate with [^{36}Cl]-chloroacetaldehyde for an enzyme effecting

conjugation with GSH. If this is so it has already been stated that the enzyme must be distinct from the previously described glutathione S-alkyltransferase.

4. *Dehydrogenation of* S-*formylmethyl-glutathione* (V → VII). Attempts to prepare and isolate S-formylmethyl-glutathione chemically were not successful (see Methods). However an enzymic preparation seems to have succeeded. Figure 6A shows that when chloroethanol was incubated with purified yeast alcohol dehydrogenase, purified lactic dehydrogenase, NAD and an excess of GSH and pyruvate then the latter two were only used in quantity equivalent to the chloroethanol present and Table 2 shows that a compound reacting positively to the ninhydrin and platinic reagents was formed which was distinct from GSH, GSSG, S-hydroethyl- and S-carboxymethyl-glutathione. It seems very probable that this compound was S-formylmethyl-glutathione. The fact that the compound hardly moved from the origin of most chromatograms suggests that it may be easily polymerized. Figure 6B shows that when liver supernatant was added to the solution of S-formylmethyl-glutathione with cofactors and incubation was continued, then another molar equivalent of lactate was produced indicating that a second dehydrogenation had occurred. The reaction took three hours to complete and as in the attempted direct preparation from chloroethanol (above) no S-carboxymethyl-glutathione was detected chromatographically after the long period of incubation with liver supernatant. However, it is likely that S-formylmethyl-glutathione (initially about 7 mM) was being dehydrogenated by liver supernatant at an average rate of 0·4–0·5 μmoles NAD/min/g liver during the first hour, and it can be deduced that such reaction is feasible as the last step in the overall conversion of chloroethanol to S-carboxymethyl-glutathione by liver supernatant *in vitro*.

Studies with pure alcohol dehydrogenases

Table 5 shows that chloroethanol is dehydrogenated by pure alcohol dehydrogenases from liver and yeast and that it inhibits the dehydrogenation of ethanol by these enzymes.

TABLE 5. DEHYDROGENATION OF CHLOROETHANOL BY PURIFIED ALCOHOL DEHYDROGENASES

Substrate	Rate (extinction units/min) Yeast		Liver	
	Y_1	Y_2	L_1	L_2
(1) Ethanol	—	0·066	—	0·040
(2) Add chloroethanol to (1)	—	0·007	—	0·023
(3) Chloroethanol	0·046	Nil	0·075	0·005
(4) Add ethanol to (3)	very fast	—	very fast	0·025

Reaction in 2·9 ml as described in Methods at pH 9·2 at 20°. Ethanol and chloroethanol concentrations: 50 mM. Solutions contained either yeast alcohol dehydrogenase (Y_1 = 50 μg; Y_2 = 0·08 μg protein) or equine liver enzyme (L_1 = 400 μg; L_2 = 27 μg protein).

GENERAL DISCUSSION

Metabolism of chloroethanol

The chromatographic and quantitative results presented indicate that chloroethanol which is itself relatively inert is converted to S-carboxymethyl-glutathione by rat

liver *in vivo* and *in vitro*. Although the route of reaction has not been unequivocally proven, such evidence as is available favours the route via S-formylmethyl-glutathione indicated on Fig. 1. It is interesting that liver enzymes will dehydrogenate this complex aldehyde.

It has been shown that S-carboxymethyl-glutathione can be degraded by kidney tissue to S-carboxymethyl-cysteine, a close analogue of glutamic acid. Little is known of its further metabolism. It may follow or interfere with some of the metabolic reactions of glutamic acid and may also follow one or more degradative pathways open to S-alkyl-substituted cysteines which include thioether cleavage and N-acetylation to form mercapturic acids. The factors influencing entry of a compound into these pathways have been discussed.[5] Enzymic cleavage of the thioether bond of S-carboxymethyl cysteine has been demonstrated *in vitro*,[11] although the rate was only one third to one half that for S-methyl and S-ethyl cysteine.

Rat liver alcohol dehydrogenases

Treble[12] has shown that fluoroethanol is dehydrogenated by rat liver and has given some evidence that in horse liver there may be an enzyme capable of dehydrogenating fluoroethanol and which is distinct from the well-known alcohol dehydrogenase. However, Table 5 shows that chloroethanol is a substrate for the latter enzyme. The present studies with chloroethanol in the rat suggest that it is dehydrogenated by an enzyme which also dehydrogenates ethanol since *in vivo* ethanol inhibits early effects of chloroethanol on liver GSH (Table 1) and reduces its toxicity[2, 13] and *in vitro* ethanol inhibits the release of chloride from chloroethanol (Table 4). There appears therefore to be no reason at present to propose the existence of more than one alcohol dehydrogenase in rat liver. It has been suggested[14] that chloroethanol inhibits yeast alcohol dehydrogenase at pH 8·7 by producing inhibitory ethylene oxide in an alkaline hydrolysis reaction. However, since it is now shown that chloroethanol is a substrate for the enzyme it is more probable that inhibition is due to simple competition.

Toxic action of chloroethanol

The toxicity of chloroethanol appears to be related to the possibility of its being metabolized since concomitant administration of ethanol markedly reduced the toxicity.[2, 13] The toxic agent may be the very reactive chloroacetaldehyde produced intracellularly in a target organ. GSH in such a target organ may protect it until sufficient chloroacetaldehyde has been produced to conjugate all or most of the GSH. It thus becomes necessary to give an overwhelming dose of chloroethanol and the lack of effect of life-long daily intake by rats of about 12 per cent the LD_{50} dose (Johnson, unpublished) may be simply explained by the rapid replenishment of tissue GSH as has been seen after repeated doses of iodomethane.[5]

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