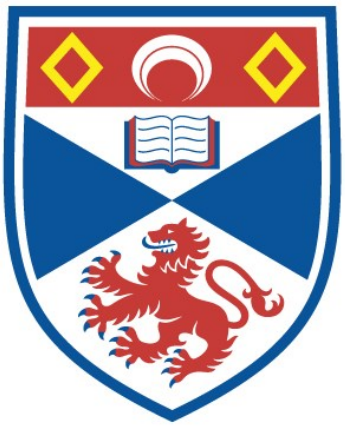
# THE METABOLISM OF METHYL METHANESULPHONATE

David J. Pillinger

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1964

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A Thesis submitted by

David J. Pillinger

for the Degree of Master of Science

of the University of St. Andrews.

July 1964.


This work was carried out in the Department of Experimental Chemotherapy, Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester.

# C E R T I F I C A T E

I certify that David. J. Pillinger has spent six terms at research work under the direct supervision of Dr. H. Jackson, Ph.D., M.B., Ch.B., D.Sc., that he has fulfilled the conditions of Ordinance No. 16 (St. Andrews) and that he is qualified to submit the accompanying Thesis in application for the degree of Master of Science.

# D E C L A R A T I O N

I hereby declare that the following Thesis is based on the results of experiments carried out by me, that the Thesis is my own composition and that it has not previously been presented for a Higher Degree.

The Research was carried out in the Paterson Laboratories of the Christie Hospital and Holt Radium Institute, Manchester, under the direct supervision of Dr Harold Jackson, Ph.D., M.B., Ch.B., D.Sc.

C O N T E N T S

# S U M M A R Y

Methyl methanesulphonate has been prepared labelled with radioactive carbon and its metabolism studied in rat, mouse and rabbit. The distribution of the drug after injection has been determined in the rat. Quantitative studies in this species have shown that less than 05 % of the injected drug is excreted or exhaled in 24 hours. Assay of tissue levels of radioactivity after injection has confirmed that the majority of the drug remains bound within the animal body. In the rat, the principal metabolic reaction has been shown to take place in the liver with glutathione. The S-methylglutathione formed is excreted in bile and this intermediate was the source of the urinary metabolites. In the urine, conjugates of S-methylcysteine and S-methylthioglycollic acid, together with small quantities of radioactive urea, have been recognised. The main metabolite has not been identified but appears to be a substituted guanidine compound. The possible significance of these results has been discussed in terms of cell biochemistry.

# INTRODUCTION

All efforts made so far in the field of chemotherapy of human cancer have been in vain. At the present time there is not one chemotherapeutic agent which has a lasting beneficial effect.

Even a cursory glance at the literature reveals that in many cases the rationale behind the selection of a particular compound for testing is obscure and, on occasion, non-existent. In the past, the demonstration of an apparent beneficial effect of one drug prompted the indescriminate screening of many hundreds of related compounds, incorporating minor chemical modifications in an attempt to improve the efficacy. The singular lack of success of this approach in general emphasises the inadequacy of observing the effects of routine screening in biological systems without at the same time enquiring why such modifications are more or less effective. Recently, a new phase of experimental chemotherapy has developed concerned, not only with the immediate, if limited, practical aspect but with a more theoretical and fundamental approach to the problem.

The work presented in this thesis represents the initial stage of a larger project to correlate observed differences in biological activity with changes in chemical structure.

An analysis of the relation of structure to activity serves a twofold purpose in that it may be regarded not only as an attempt to find rules which may guide the chemist in the synthesis of new drugs, but also as an opportunity to seek clues regarding the various factors governing biological response. A given change in the structure may have one effect on the uptake of the drug by the host, a different effect on the distribution within the host, and still another effect on the metabolic fate of the drug or on its toxicity to the animal. Change in chemical structure within a series of related compounds may be accompanied by extensive alteration in physiological distribution. It is important to bear in mind when considering these points that, owing to species differences in distribution or metabolism, results obtained with one animal may not be applicable to another, or to man. Knowledge of the metabolic fate of a drug may suggest advantageous modifications, as in the studies on the fate of quinine which has had useful application in guiding synthesis of compounds with greater chemotherapeutic activity.

## RADIOMIMETIC COMPOUNDS

The usefulness of radiomimetic substances to cellular pharmacology was first predicted in 1947 by Peters (1)

and has been subsequently confirmed by their application to cancer research and therapy. The term 'radiomimetic' was first applied by Dustin (2) to chemical agents which gave rise to the same effects in cells as ionising radiation. The observed effects listed by Boyland (3) include inducement of chromosome abnormalities, mutagenic action, vesication, characteristic damage to bone marrow and delayed lethal effects, for example, leucopenia. Although there is a general similarity, more detailed comparison reveals characteristic differences in the effects produced suggesting that the mode of action is not necessarily similar.

## ALKYLATING AGENTS

The most active of the radiomimetic compounds contain two or more groups which are capable of reacting with electrophilic centres under physiological conditions. This reaction is more specifically defined as an alkylation as it frequently involves the replacement of a hydrogen atom in a molecule by an alkyl group. The term is also applied to the addition of a radical to a molecule containing an atom in a lower valency state, as in the formation of sulphonium ions (Fig 1). The radical may be a simple one eg. $-CH_3$ , or it may be a compound one eg. $-CH_2 \cdot CH_2OH$ , but in all cases the carbon atom through which

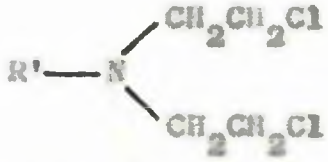
Attachment is made is saturated.

Fig. 1

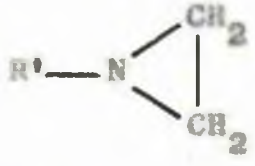
A compound which effects the introduction of an alkyl group into a recipient molecule is referred to as an alkylating agent.

The chief types of cytotoxic alkylating agents are :-

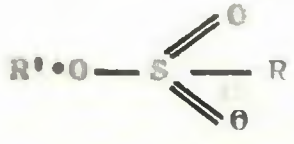
1.) Nitrogen mustards in which one or more β-chloroethyl groups are directly attached to a nitrogen atom. R' may be aliphatic or aromatic.



2.) Ethyleneimines having a 3-membered heterocyclic ring.



3.) Esters of alkanesulphonic acids.



4.) Epoxides.

These substances fall into a variety of chemical categories and vary widely in both chemical reactivity and biological activity, not only from one group to another, but also as individual members of a particular group.

During the past few years, some work has been done on the mechanisms of action of biologically active alkylating agents and the results of this work have made evident the complexity of the problem. Although a number of hypotheses have been proposed, and subsequently discarded, considerable progress has been made in establishing reactive sites for these compounds. Nevertheless, the exact modes of action at molecular level remain unknown.

The types of alkylation which can occur in vivo are numerous and the problem is to determine what reactions do occur and which of these are primarily responsible for the observed biological and physiological effects. Whilst the biochemical mechanisms underlying the effects of the alkylating

agents are not known, biological activity as measured by the inhibition of tumour growth or the induction of chromosome abnormalities has been shown, by *in vivo* studies, to correlate both quantitatively and qualitatively with alkylating ability.

## BIOLOGICAL EFFECTS OF ALKYLATION

The subtle nature of the biological vicissitudes shown by members of the sulphonic acid ester series is best examined by considering briefly the individual systems in which effects have been recorded.

### 1.) Effects on germinal tissue. (Diag 1).

Perhaps the most significant evidence which points to a selectivity of action within this type of compound came as an indirect result of the work of Jackson (4) on the development of tumour resistance using triethylenemelamine, a trifunctional member of the ethyleneimine series of alkylating agents. During these experiments, it was noticed that male rats receiving the drug became sterile during the 4th week after administration (0·2 mg/kg/ip). Subsequent application of serial mating techniques to studies of these antifertility effects in a range of compounds produced a wide spectrum of results.

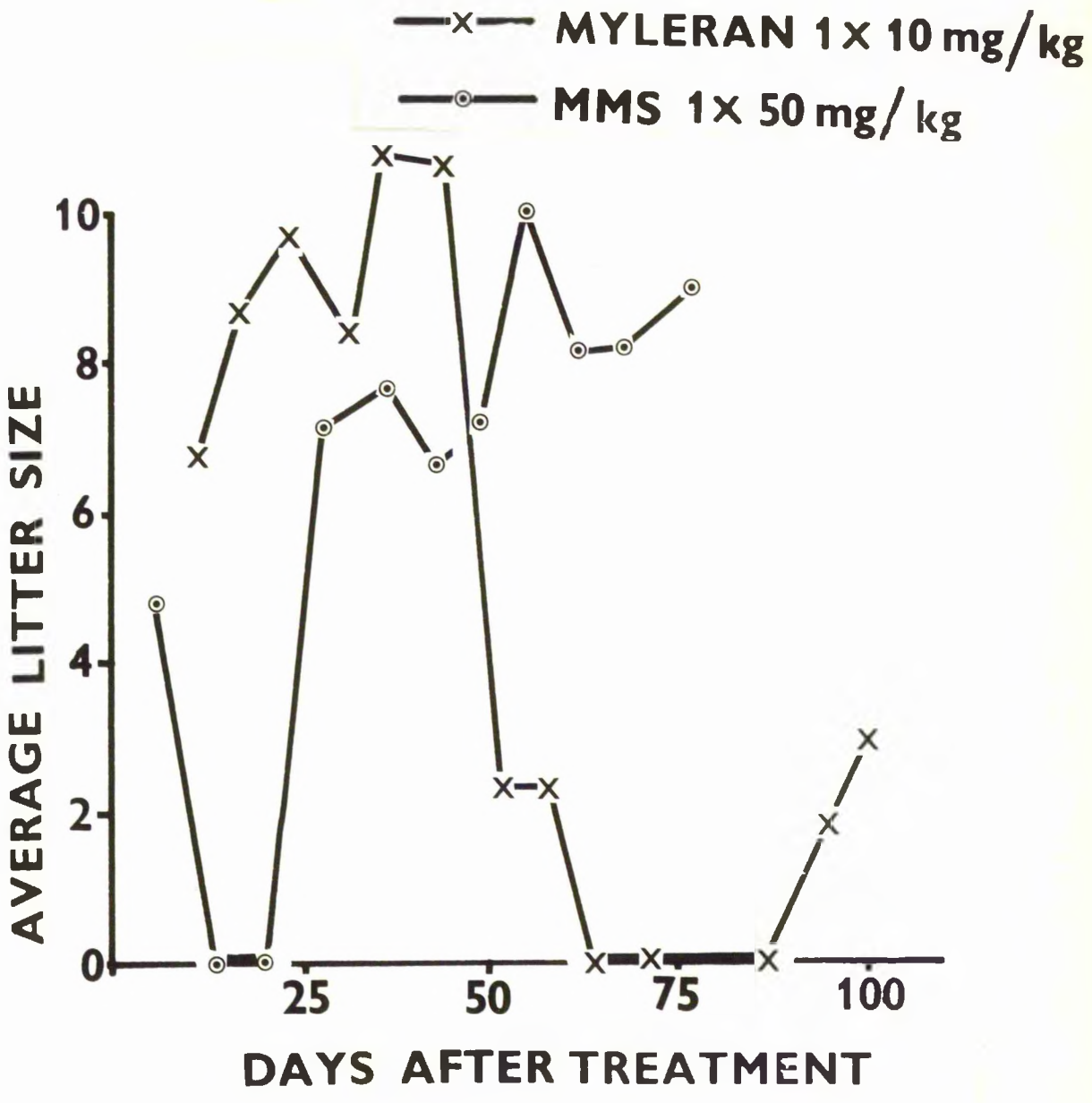



DIAGRAM 1. Comparative effects of a monofunctional and a difunctional alkanesulphonate on rat fertility.

With the difunctional agent Myleran (Fig 2) at a dose of 10 mg/kg, fertility remained normal until the 8th week when sterility associated with oligospermia or aspermia developed: normal potency only being restored after a further 45 days.

$$CH_3SO_2O\cdot CH_2CH_2CH_2CH_2\cdot OSO_2CH_3$$

Fig 2

Taking into account modern views on the duration of spermatogenic events in the rat (5), this pattern of response would be consistent with an effect on the early stages of spermatogenesis. If, for example, Myleran affected cell division in the spermatogonial phase but had little effect on the spermatocytes, spermatids and mature sperm, then fertility would be normal until spermatogonia were due to reach maturity after about 8 weeks. Sterility would become apparent and would be maintained until spermatogonia resumed mitosis and normal spermatogenesis returned.

Reference was made earlier to the radiomimetic nature of these alkylating agents and it is pertinent at this stage to compare these effects with those following radiation. Craig et al. (6,7) showed that there was no striking effect on the fertility of male rats after a whole-body dose of 200 r. After

300 r, litter size was below normal during the 2nd and 4th weeks but a period of sterility occurred associated with oligospermia in the 9th and 10th weeks. Prior to this there was a decline in fertility from the 7th week onwards. The duration of the sterile phase varied from 2 to 8 weeks, but all were fully fertile 100 days after treatment. Animals exposed to a dose of 500 r were subfertile immediately after treatment but did not become sterile until 40 - 45 days after irradiation. They remained sterile for a minimum of 3 months after this dose.

The simplest monofunctional sulphonyloxy-alkane is methyl methanesulphonate (Fig 3).:-

$CH_3SO_2OCH_3$

Fig 3

A single intraperitoneal dose of 50 mg/kg produced complete sterility in the 2nd and 3rd weeks after treatment with no apparent change inthe number or appearance of the sperm. This suggested that the susceptible cells in this case are the spermatozoa in the testis and in the epididymis which will emerge as mature sperm after 2 to 3 weeks. The ethyl ester of methanesulphonic acid (Fig 4)

$CH_3SO_2OCH_2CH_3$

Fig 4

required a higher dose but produced a similar effect. A dose of 300 mg/kg produced sterility during the 3rd week after treatment. Normal fertility returned and persisted from the 5th week onwards except for subfertility during the 8th week. Increasing the length of the carbon chain of the alkylating moiety showed a similar action to the other esters but in general required a higher dose, for example, n-propylmethanesulphonate (400 mg/kg) which caused subfertility during the 2nd and 3rd weeks.

The antifertility effect of the iso-propylmethanesulphonate was strikingly different from the above cases. There was no effect on spermatozoa after a single injection (100 mg/kg) as the animals remained fertile during the 2nd and 3rd weeks but a prolonged sterility was observed from the 6th week onwards.

The influence of the acidic part of the molecule was examined by observing the effect of the methyl ester of ethanesulphonic acid. If it is the alkyl group which is responsible for the effects outlined above then it is to be expected that a similar response to that found with methyl methanesulphonate would result as the alkylating entity is still the methyl group. This reasoning was supported as sterility was induced during the 2nd and 3rd weeks. Similarly, iso-propylethanesulphonate gave an

essentially similar antifertility pattern to iso-propylmethane-sulphonate.

2.) Mutagenesis

Somewhat related studies to those described above have been carried out by Fahmy and Fahmy (8) by observing the action of different alkylating agents on the stages of cell development in the germinal tissue of male Drosophila. After injecting male flies with the agent, fractionation of the germ line by sequential mating gave rise to a mutation rate (induced by sex-linked recessive lethals) which fluctuated in successive broods. As the sperm used in these matings must have come from progressively younger germ cells at the time of treatment, the variation in brood mutation response probably reflects the relative susceptibility of the various cell stages to the action of these agents.

They found that while triethylenemelamine (TEM) produced a maximum effect on spermatids, the mutagenic activity of monofunctional and difunctional sulphonyloxyalkanes was greatest for mature sperm.(9). It would appear that the cell stage response in fertility and mutagenicity is to some extent complementary, that is, with respect to fertility, spermatocytes are most sensitive to TEM and spermatogonia to Myleran, but for sex-

linked recessive lethals, spermatocytes are not particularly sensitive to TEM and spermatogonia are least sensitive to Myleran.

More recently they have shown that although methyl methanesulphonate gave a higher yield of mutants than the ethyl ester at the same dosage(by a factor of 4), the lower toxicity of ethyl methanesulphonate permitted higher doses to be given with consequent higher maximum mutational yield (9). In addition, these authors report that methyl methanesulphonate was ineffective in inducing chromosome breaks or translocations but did induce small deficiencies and deletions.

Loveless (10), in comparing the effects of a number of alkylating agents on T2 bacteriophage <u>in vitro</u> found that T2 wild-type phage was completely inactivated by treatment with 0·02M methyl methanesulphonate at 37° for 24 hours. Of several alkyl methanesulphonates tested at doses comparable with respect to percentage of phage inactivated, the ethyl ester was unique in being highly mutagenic. Methyl methanesulphonate was also non-mutagenic for T4 wild-type in which high mutation rates were effected by ethyl methanesulphonate. The relationship between mutagenicity and the carcinogenicity of alkylating agents has been discussed by Boyland (11).

3.) Effects on neoplastic tissue.

The tumour growth inhibitory activity of the alkylating agents is well established and has recently been reviewed (12) Clinically they are useful in the management of malignant lymphomas and in chronic myeloid and lymphatic leukaemias. They have also been shown to have a therapeutic effect against carcinoma of the ovary. Experimentally, the tumour inhibitory action of the nitrogen mustards has been extensively evaluated against the Walker rat carcinosm 256 since the early studies by Haddow (13) and Burchenal (1 One feature of interest arising from this work is that, with only a few exceptions, tumour growth inhibition is dependant upon the presence of two or more alkylating moieties in the molecule.

On the basis that the biological action of a series of dimethanesulphonyloxy alkanes (Fig 5) might resemble that of the nitrogen mustards, since both types of compound can act as difunctional alkylating agents, members of this series were examined by Haddow et al. (13).

$$CH_3SO_2O\cdot(CH_2)_n\cdot OSO_2CH_3$$

Fig 5

They found that these compounds actively inhibited the growth of

various experimental tumours in animals. As a result of the intense inhibitory effect on the growth of the Walker rat carcinoma 256 and its depressive action on the myeloid series in the rat at a lower dose than other members, the compound with n = 4, dimethane-sulphonyloxy butane, was selected for clinical trial in cases of advanced malignant disease. Under the trade name of Myleran (Busulphan) this has now become an established drug for the treatment of chronic myeloid leukaemia.

Among the monofunctional alkylating agents, ethyl methanesulphonate has been shown to possess some carcinostatic activity against the transplanted Walker rat carcinoma (16). However , in contrast, methyl methanesulphonate has been shown to have no action on either the Walker 256 or against the Dunning ascites tumour. It is particularly effective in the rat against both recently implanted and established Lymphoma 8 (17).

4.) Effects on the haemopoietic system.

Reference has already been made to the specific depressant action of Myleran on neutrophils. In contrast to the difunctional agents (Diag 2), a single injection of methyl methanesulphonate (75 mg/kg/ip) to rats produced a marked lymphopenia within 24 hours. A transient neutropenia occurred

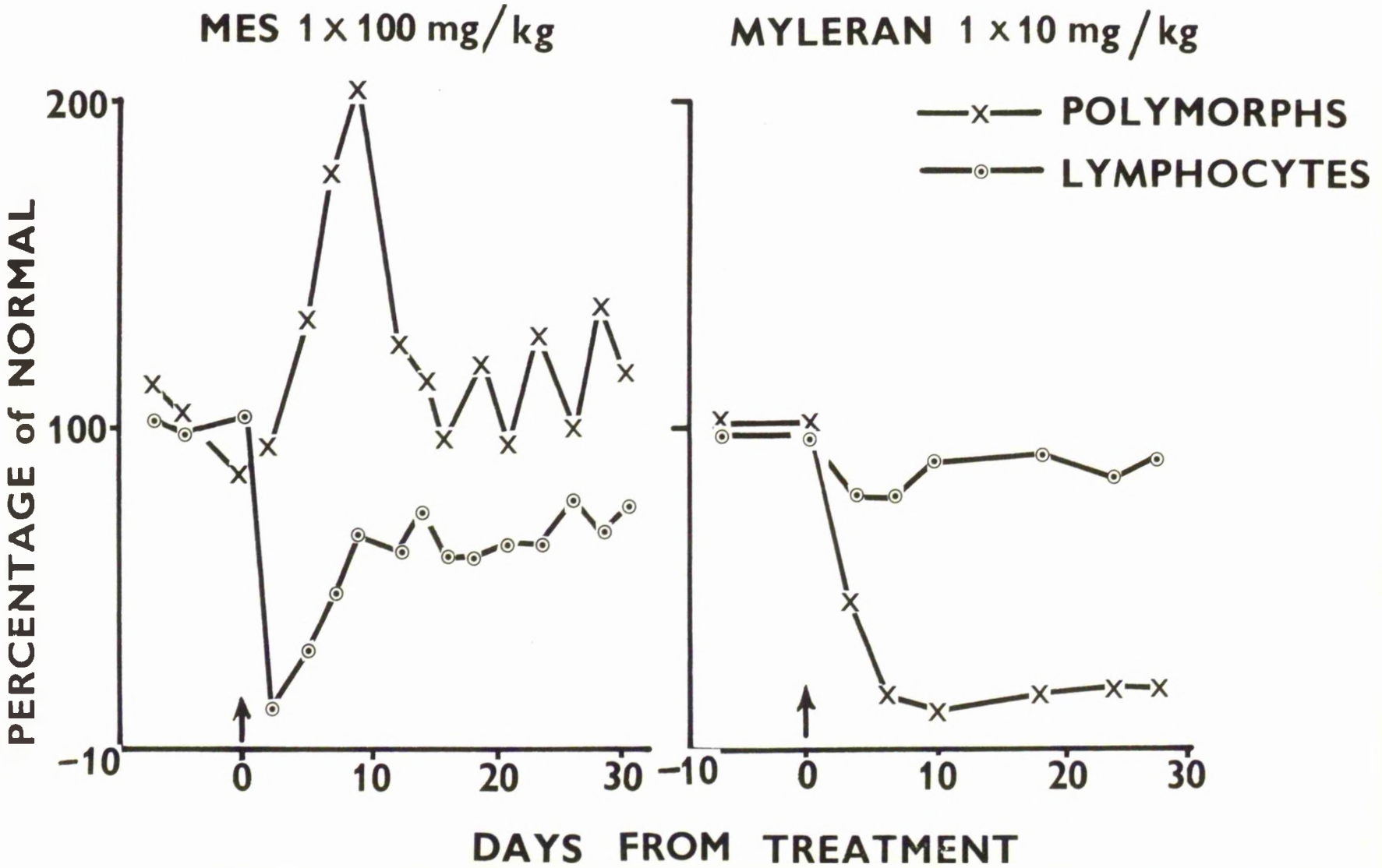



DIAGRAM 2. Comparative effects of a monofunctional and a difunctional alkanesulphonate on leukocytes.

2 - 3 days after treatment compared with a prolonged effect with Myleran. In the rabbit, the lymphocyte count was substantially reduced only 7 hours after a single dose (30 mg/kg/ip). A similar picture was produced in monkey (40 mg/kg/iv), furthermore in this case repeated doses at 7 - 10 day intervals were capable of maintaining the low lymphocyte levels (18). It was reported by Elson (19) that ethyl methanesulphonate had no observable effect on the white cell count. This was confirmed by Fox (18) who found however, that after a higher dose (300 mg/kg) a rapid leukopenia developed in which lymphocytes were more effected than neutrophils. In this the compound resembled the nitrogen mustards.

As in the spermatogenic studies, iso-propyl methanesulphonate (100 mg/kg) showed a quite different effect. This time the lymphocyte count was relatively unaffected but a marked neutropenia developed 10 days after treatment,; this returned to normal by the 15th day. A similar effect was found in rabbit (100 mg/kg) and in monkey (20 mg/kg). With the latter species 5 successive daily doses (10 mg/kg/iv) rapidly depleted neutrophils 16 days after the first dose. Platelet levels were also reduced.

Variations in the sulphonic acid part of the molecule eg. methyl ethanesulphonate and ethyl ethanesulphonate

produced comparable results in peripheral count to the corresponding methanesulphonic acid derivatives but, in general, required a higher dose to produce the same effect.

METABOLISM OF THE SULPHONYLOXY ALKANES

One of the earliest studies concerning the metabolic fate of a member of the sulphonic acid ester series of alkylating agents was made by Peng (21), who showed that after the injection into rats of $S^{35}$-Myleran (4 mg/kg/propylene glycol), 95 % of the dose was excreted in the urine within 32 hours, mainly as methanesulphonic acid. Selective uptake of $S^{35}$ was observed in the spleen and bone marrow. In the same year, Trams et al. (22, 23, 24) reported rapid disappearance of $S^{35}$-Myleran from the blood after injection into rats (2 mg/kg/ip). Soon after injection, the activity was concentrated in the liver, kidney and small intestine. After 24 hours, 85 % of the dose had appeared in the urine. A comparative study of urinary metabolites in the rat, mouse and rabbit after injection of $S^{35}$-Myleran (10 mg/kg) by Fox, Craig and Jackson (25) showed that in rabbit only methanesulphonic acid was excreted; rat and mouse urine, in addition, contained a little unchanged Myleran together with two other unidentified components. Nadkarni et al. (26, 20) reported that

administration of this agent to humans produced a comparable pattern in that the $S^{35}$ was rapidly cleared from the blood, 45-60 °/o of the injected activity appearing in the urine within 48 hours.

According to Trams (22, 24), after the injection into rats of Myleran labelled with $C^{14}$ in the 1 and 4 positions (marked with an asterisk * in Fig 6), the specific

$$CH_3SO_2O\cdot\overset{*}{C}H_2CH_2CH_2\overset{*}{C}H_2\cdot OSO_2CH_3$$

Fig 6

activities of the kidneys, lungs and liver were higher than the other tissues examined. In the liver only, small amounts of activity were associated with fats, proteins and sodium nucleates. The urine contained three major products, also numerous lesser products, together with unchanged drug. There was some evidence that the drug had been metabolised to smaller molecules which were incorporated into various compounds by normal biosynthetic pathways.

The injection of Myleran labelled with $C^{14}$ in the 2 and 3 positions into rats revealed that only 4 °/o was exhaled in 24 hours as $C^{14}O_2$. After injecting 1,4-butanediol-2,3-$C^{14}$, essentially all the $C^{14}$ was exhaled as $C^{14}O_2$ in the same

period (26, 27). This suggested that the agent is not converted to the diol *in vivo*. Roberts and Warwick were also able to show that the injection of the S-β-alanyltetrahydrothiophenium cation (Fig 7) yielded the same urinary product as Myleran.

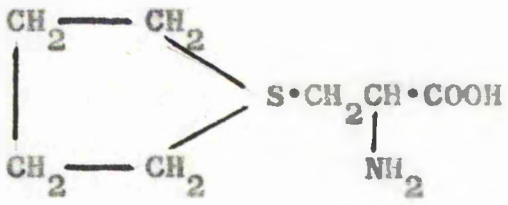


Fig 7

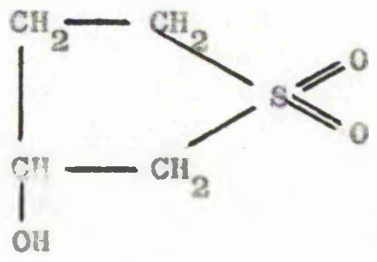


Fig 8

This product was identified as 3-hydroxytetrahydrothiophene-1, 1-dioxide(Fig 8).

The above authors, reasoning that a monofunctional alkylating agent might present a simpler picture for analysis than the more complex difunctional agents, studied the metabolic fate of ethyl methanesulphonate or 'half Myleran' (Fig 9).

$$CH_3SO_2O{\cdot}CH_2CH_3$$

Fig 9

Results of this work using $C^{14}$ labelled material in the 2 position

of the ethyl group, led them to suggest that the drug was metabolised by two distinct routes. One involved hydrolysis to ethanol with subsequent exhalation of $C^{14}$ labelled carbon dioxide, the other resulted in the excretion of conjugates of S-ethylcysteine in the urine (28). More detailed reference to this work will be made in the discussion. No attempts were made by these authors to correlate the observed metabolic products with the distribution and metabolism of the drug within the animal body.

Since the work referred to above was undertaken, a conspicuous diversity of biological effects produced by minor chemical modifications within the sulphonylexy alkane series has become apparent. Before an explanation of these phenomena can be attempted, a more detailed knowledge of their metabolic fate is essential.

# EXPERIMENTAL

## A. CHEMICAL PREPARATIONS

### 1.) SYNTHESIS OF ALKANESULPHONATES.

A general method for the preparation of the alkanesulphonates by the base catalysed reaction of a sulphonyl chloride with an alcohol has been described by Suter (29). This method, is dependant upon the availability of the appropriate alcohol and is limited to those esters which are not sensitive to base. Attempts to prepare methyl methanesulphonate by this method gave a relatively poor yield (30). In view of the high degree of purity also imperative for biological experiments an alternative method was sought.

The reaction between an alkyl halide and the silver salt of a sulphonic acid received little attention owing to the high temperature necessary to complete the heterogeneous reaction until the method was improved by the use of acetonitrile as solvent (31). Preparation by this route gave yields of sufficiently high order for the method to be subsequently applied on a small scale to the synthesis of isotopically labelled material. Furthermore, the ester could be isolated free from toxic impurities.

2.) PREPARATION OF METHYL METHANESULPHONATE

(a). Preparation of silver oxide.

$$2AgNO_3 + 2NaOH \longrightarrow Ag_2O + 2NaNO_3 + H_2O$$

The stochiometric quantity of sodium hydroxide solution was added to a stirred solution of silver nitrate. Heat was evolved during the formation of the dark brown precipitate. The solid oxide was filtered off, washed twice with ether before drying under vacuum in a desiccator.

(b). Preparation of silver methanesulphonate.

$$Ag_2O + 2CH_3SO_2OH \longrightarrow 2CH_3SO_2OAg + H_2O$$

Commercial methanesulphonic acid was redistilled before use, the fraction distilling between 112-113° @ 0·01 mm was collected. The silver salt was prepared by adding a slight excess of silver oxide (30·0 g) to a stirred solution of methanesulphonic acid (40·0 g) in acetonitrile (150 mls). As a precaution against decomposition, the reaction flask was covered with a black cloth whilst stirring continued for 4 hours. Unreacted silver oxide was filtered off. The solvent was

removed from the filtrate by distillation under reduced pressure from a water pump to yield greyish-white crystals of the silver salt. this product was redissolved in the minimum volume of warm acetonitrile, washed with charcoal and the solid reprecipitated by the addition of dry ether (100 mls). This mixture was filtered and the white crystalline product washed with ether before drying under vacuum in a dessicator protected from light. Yield 70·4 g (83 % of theoretical).

(c). Preparation of methyl methanesulphonate.

$$CH_3SO_2OAg + CH_3I \longrightarrow CH_3SO_2OCH_3 + AgI$$

Methyl iodide was redistilled before use, the fraction distilling 42-43° being collected as a colourless distillate. A solution of silver methanesulphonate (20·3 g) in the minimum volume of acetonitrile (100 mls) was stirred during the addition of methyl iodide (14·2 g), dissolved in a further 10 mls of acetonitrile. Immediate precipitation of silver iodide was observed. The reaction mixture was stood overnight in the dark. This solution was filtered and the solvent removed under reduced pressure. The remaining liquid was distilled, the fraction 84-87° @ 12 mm was collected as a

colourless liquid. Yield 9•4 g (85 % of theoretical).

As prepared, this product possessed a faint odour and gave a slight positive reaction for halogen when an acidified solution was tested with silver nitrate. After washing with potassium bicarbonate and drying over sodium sulphate, the ester was finally redistilled to give a colourless odourless liquid. Boiling point 84-85° @ 11 mm.

Elemental analysis gave:- C= 21•78 % H= 5•34 % S= 29•07 %

Required for $C_2H_6O_3S$ :- C= 21•80 % H= 5•46 % S= 29•04 %

(d). Preparation of $C^{14}$-methyl methanesulphonate

$$CH_3SO_2OAg + C^{14}H_3I \longrightarrow CH_3SO_2OC^{14}H_3 + AgI$$

The radioactive compound containing a $C^{14}$-methyl group was prepared by the method described above for the unlabelled drug. 2 mc of $C^{14}$-methyl iodide, diluted to 4•10 g with inactive methyl iodide was supplied by the Radiochemical Centre, Amersham, in a sealed ampoule. Silver methanesulphonate (5•10 g) was dissolved in acetonitrile (25 mls) in a 100 ml flask containing a magnetic stirrer. The labelled methyl iodide was transferred to the flask using a dropping pipette and the ampoule washed out several times with small quantities of acetonitrile. After

stirring for 6 hours, protected from direct light, precipitated silver iodide was removed by filtration and the filtrate transferred to a distilling flask. Acetonitrile was distilled off under reduced pressure and the product subsequently isolated as the fraction distilling between 84-85° @ 12 mm. The drug was stored at -20°.

CALCULATION OF ABSOLUTE SPECIFIC ACTIVITY

$C^{14}$-methyl iodide contained 2 mc in 4·10 g.

3·55 g of this reacted with 5·10 g silver methanesulphonate to give a theoretical yield of 2·75 g methyl methanesulphonate.

Actual yield = 2·10 g

Percentage yield = 76·4 .

Activity in 2·10 g labelled product = $1·74 \times \frac{2·10}{2·75}$

= 1·328 mc.

Absolute specific activity = 633 μc per grm.

During the work, appropriate precautions were taken when handling and disposing of radioactive material.

4.) PREPARATION OF S-METHYLGLUTATHIONE

Reduced glutathione (1·0 g) was dissolved

in N. sodium hydroxide solution (25 mls) and a further 2·5 mls of water. Freshly distilled methyl iodide (0.45 g) was added together with ethanol (30 mls) and the whole shaken mechanically for 3 hours. At the end of this time, the solution was filtered and the ethanol removed to give a colourless viscous liquid. Addition of excess ethanol (300 mls) produced a milky solution and a gumsy precipitate. This was removed with a glass rod and transferred to a mortar, where on triturating with ethanol, a white precipitate was produced. The solution was filtered and the solid dried in a desiccator. The product was chromatographically homogeneous. Yield 0·64 g.(61 % of theoretical).

Nitrogen 16·25 % $C_{10}H_{17}O_6N_3S$ requires 16·40 %.

5.) PREPARATION OF S-METHYLCYSTEINE

Alkylation of cysteine hydrochloride at the sulphur atom, using methyl iodide in a comparable reaction to that used for the preparation of the corresponding ethyl homologue (30), was carried out. Sufficient product was obtained to provide a reference spot for chromatography, but the yield did not allow isolation of the material in crystalline form.

The reaction was successfully carried out by adding dimethyl sulphate (5·10 g) to cysteine hydrochloride (5·0 g)

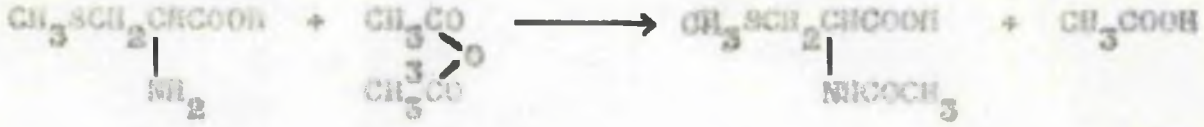
dissolved in 0·4 N barium hydroxide (200 mls). Excess barium hydroxide was removed as the sulphate and the filtrate concentrated in vacuum to approximately 40 mls. After the addition of ammonium hydroxide until the solution was only slightly acid, ethanol (150 mls) precipitated out the S-methylcysteine. The product was recrystallised from aqueous ethanol and dried in a desiccator.

Yield 3·24 g (65 % of theoretical).

Elemental analysis gave:- C=35·82 % H=6·69 % N=10·04 % S=23·30 %

Required for $C_3H_7NO_2S$ :- C=35·81 % H=6·66 % N=10·38 % S=23·70 %

6.) PREPARATION OF N-ACETYL-S-METHYLCYSTEINE (Mercapturic acid).



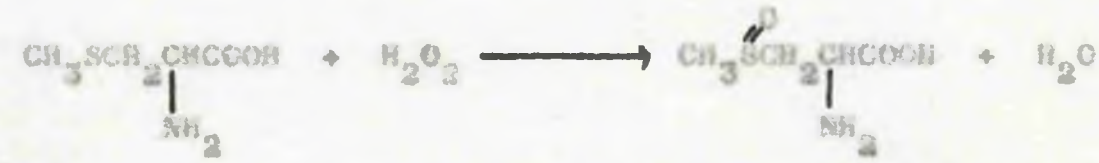
S-methylcysteine (2·0 g) was treated with acetic anhydride (10 mls) in a stoppered flask and the mixture vigorously shaken until solution was attained. After standing overnight at room temperature, the mixture was diluted with an equal volume of water and solvent removed by distillation from a water-bath under reduced pressure. The mercapturic acid was recrystallised from chloroform.

Yield 0.86 g (35 % of theoretical).

Elemental analysis gave:- C=40·45 % H=6·21 % N=7·85 % S=18·04 %

$C_6H_{11}NO_3S$ requires :- C=40·60 % H=6·23 % N=7·90 % S=18·10 %

7.) PREPARATION OF S-METHYLCYSTEINE SULPHOXIDE



S-methylcysteine (1·5 g) was dissolved in conc. hydrochloric acid (1·1 mls) and water(7·5 mls). Methanol (12·5 mls) and hydrogen peroxide 30 % (1·5 mls) were added. After shaking for 1 hour, dicyclohexylamine (2·25 mls) was added giving a white acicular precipitate of the hydrochloride. This was removed and the colourless solution neutralised. Addition of acetone (125 mls) produced an immediate white precipitate.

Yield 0.42 g (25 % of theoretical)

Elemental analysis gave:- C=31·57 % H=5·86 % N=9·17 % S=20·50 %

Required for $C_4H_9NO_3S$ :- C=31·70 % H=5·96 % N=9·27 % S=21·30 %

8.) PREPARATION OF N-ACETYL-S-METHYLCYSTEINE SULPHOXIDE

To N-acetyl-S-methylcysteine (0·5 g) in water (1·0 ml) was added hydrogen peroxide (1·0 ml). The solution was shaken for 1 hour after which time the solvent was evaporated from a water-bath. A white chromatographically homogeneous

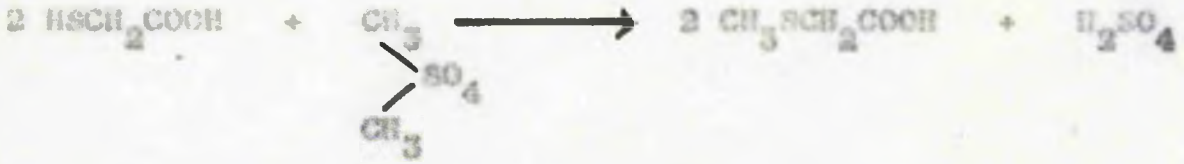
solid remained.

Yield 0·25 g (46 °/o of theoretical).

Elemental analysis gave:- N = 7·26 °/o (Kjeldahl method).

Required for $C_6H_{11}NO_4S$ :- N = 7·26 °/o

9.) PREPARATION OF S-METHYLTHIOACETIC ACID



$$2\ HSCH_2COOH + (CH_3)_2SO_4 \longrightarrow 2\ CH_3SCH_2COOH + H_2SO_4$$

To a solution of sodium hydroxide containing 36 g of solid in 43 mls of water was added thioglycollic acid (46 g). During the addition of dimethyl sulphate (63·0 g), the solution was stirred and heated on a steam-bath until a single phase was produced. After cooling the reaction mixture and acidifying with dil. sulphuric acid, the free acid was extracted with three portions of ether. The extract was dried with anhydrous sodium sulphate, the ether evaporated and the residue distilled under reduced pressure. A fraction distilling as a colourless oil at 110° @ 17 mm. was collected, representing 53 °/o of the theoretical yield.

10.) PREPARATION OF N-(METHYLTHIOACETYL)GYLCINE.

(a). Preparation of S-methylthioacetyl chloride.

$$CH_3SCH_2COOH + SOCl_2 \longrightarrow CH_3SCH_2COCl + HCl + SO_2$$

S-methylthioacetic acid (10·6 g) and thionyl chloride (12·0 g) were heated gently on a steam-bath until evolution of HCl ceased. The residue was distilled to yield a c colourless, fuming liquid. Boiling point 56° @ 18 mm.

(b)· Preparation of N-(methylthioacetyl)glycine ethyl ester.

$$CH_3SCH_2COCl + NH_2CH_2COOEt \longrightarrow CH_3SCH_2CONHCH_2COOEt + HCl$$

Glycine ethyl ester (4·96 g), prepared by treating the amino acid hydrochloride with a 2 % solution of ammonia in chloroform and distilling off the free ester as a colourless oil (B.Pt 50° @ 12 mm), was shaken with S-methyl-thioacetyl chloride (3·0 g) in dry ether (150 mls) for 1 hour. The solution was evaporated and the residue extracted with three 30 ml portions of chloroform. The solvent was removed and the residue crystallised from solution in ether by the addition of light petroleum (40-60); Recrystallisation of the crude product gave /·62 g of material in 79 % yield. Melting point 50-50°.

(c). Preparation of N-(methylthioacetyl)glycine.

$$CH_3SCH_2CONHCH_2COOEt + H_2O \longrightarrow CH_3SCH_2CONHCH_2COOH + EtOH$$

The ethyl ester (0·93 g) was dissolved in methanol (25 mls) and N sodium hydroxide solution (4·9 mls) was added. After 18 hours, the solution was evaporated at 40° and the residue taken up in water. This solution was passed through a column (5 cm x 2 cm diam.) of Amberlite resin IR 120 (H+ form) and the resin washed with water until the pH of the eluate rose from pH 2 to pH 4. The total eluate was evaporated at 45° under reduced pressure and gave a crystalline residue which, on recrystallising from chloroform, gave 0·60 g of white crystalline product. Melting point 99-100°.

## B. BIOLOGICAL TECHNIQUES

### 1. ANIMALS

Rats, of the American Wistar strain, and mice, of an albino strain derived from I.C.I. stock, were fed on a basic diet provided by the Scottish, N. E. Agricultural Society. They were allowed food and water ad libitum.

Rabbits used were of Dutch breed maintained on a standard diet supplemented with fresh carrots or cabbage.

### 2. DRUG TOXICITY

The toxicity of the drug was determined by injecting groups of male animals with a range of dose levels. Results are based upon deaths within 30 days.

| | | | |
|---|---|---|---|
| Rats | 100 mg/kg/ip/saline. | | 0/5 |
| | 110 | " " | 0/5 |
| | 120 | " " | 2/5 |
| | 130 | " " | 4/5 |
| Mice | 100 mg/kg/ip/saline | | 0/10 |
| | 120 | " " | 0/10 |
| | 130 | " " | 2/10 |
| | 200 | " " | 10/10 |

## 3. ADMINISTRATION

Rats and mice were injected with the drug, dissolved in physiological saline, usually by an intraperitoneal route at 100 mg per kilogram of body weight. In the rabbit, the drug was injected intravenously.

Rabbits were injected in an ear vein, initially at 40 mg per Kilogram of body weight in saline. This dose, however, produced signs of distress in the animal and was subsequently lowered to 30 mg per kilogram in later experiments.

## 4. FROZEN SECTION TECHNIQUE (38)

A group of 5 mice, of approximately 30 g body weight , were injected with the radioactive drug. An animal was killed after 16, 30, 71, 103 minutes and 4 hours. Each animal was placed in a metal former and , suspended by its tail, dipped into liquid nitrogen. The frozen animals were then sectioned using a band saw. The cut surface was cleaned with fine emery paper before recording the section by photography.

For the preparation of autoradiographs, the cut sections were covered with a thin layer of Melinex plastic film and placed in contact with rontgen film. After exposure

for 14 days, the film was developed. Superimposing the autoradiograph on the photograph of the cut section revealed the localisation of the drug in the various tissues of the animal.

5. DISTRIBUTION OF RADIOACTIVITY IN TISSUES AND BLOOD

(a). Tissues.

The specific activities of the major organs were determined at various intervals after injection of the labelled drug into rats.

The liver, kidney, spleen and testis were selected for examination. The whole dissected tissue was weighed and approximately 500 mg of each was homogenised in exactly 2·0 ml of physiological saline. 0·1 ml of this homogenate was counted in the solid counter. By preparing standard absorption curves for each tissue it was found that a 0·1 ml sample, containing of the order of 23 mg of tissue, would provide sufficient activity for an accurate count after allowing for absorption.

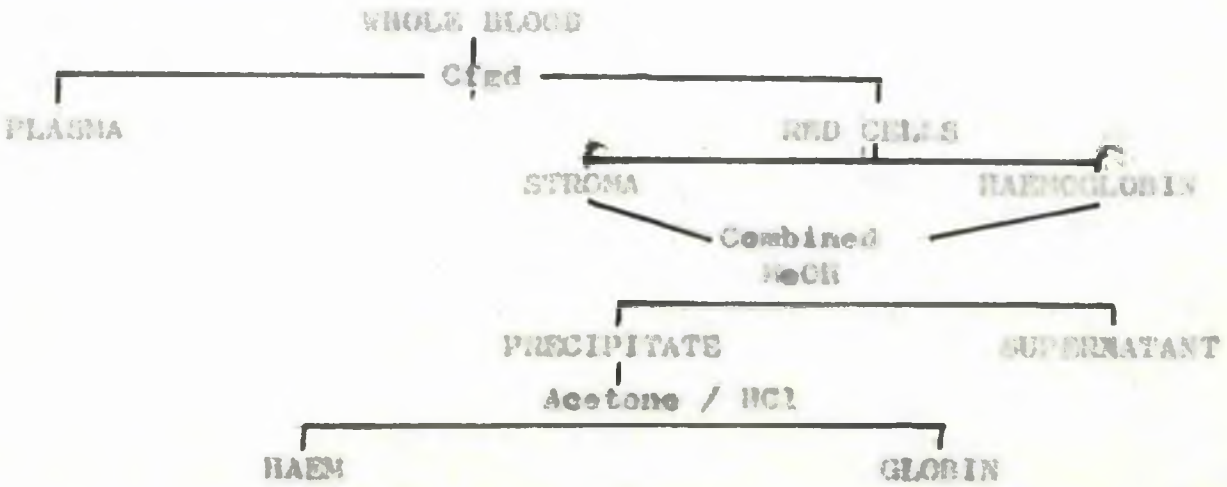
In addition to the organs listed above, homogenates were also prepared from lung, heart, brain, salivary gland, thymus adrenals, seminal vesicles, skin, fat, muscle, stomach and intestine of an animal 24 hours after injection of the labelled

drug to provide information on the specific activities of these tissues.

(b). Blood.

To determine the distribution of the drug within the blood at various intervals after administration of the radioactive compound, animals were anaesthetised and exsanguinated. The blood was collected in a heparinised syringe to prevent clotting. 0·1 ml samples were counted in duplicate in the solid counter. The plasma was separated by centrifuging the remainder of the blood and removing the clear supernatant. 0·1 ml samples were plated for counting. After allowing for absorption, the amount of activity associated with red cells and plasma could then be calculated.

In order to detect further localisation within the constituents, whole blood was fractionated according to the following scheme:-



6. COLLECTION OF CARBON DIOXIDE

Five rats were maintained for 24 hours in a sealed metabolism tank. By applying a slightly negative pressure, dry air could be drawn into the tank. The expired air was led by a tube extending to the bottom of the tank, in turn through 3 wash-bottles each containing 100 mls of 40 % potassium hydroxide solution, a trap containing saturated barium hydroxide, a further potassium hydroxide trap to retain any carbon dioxide which escaped the previous traps and finally through a bottle with barium hydroxide. The animals were given food and water ad libitum. Urine free from faeces was funnelled into a container sealed to the base of the cage. In order to reduce the humidity inside the apparatus, calcium chloride was provided as drying agent in containers constructed from gauze. The apparatus is illustrated in Diagram 3.

Animals were placed in the cage for periods of at least 24 hours before the injection of the drug so that they would become acclimatised to the environment. From these trial runs it was found that after 3 hours there was evidence that carbon dioxide was reaching the first of the barium traps. For the actual experiment, 5 animals were each injected with $C^{14}$-methyl methanesulphonate (100 mg/kg/ip) and placed in the tank which was then sealed. The respiratory carbon dioxide was removed from

COLLECTION of VOLATILE METABOLITES

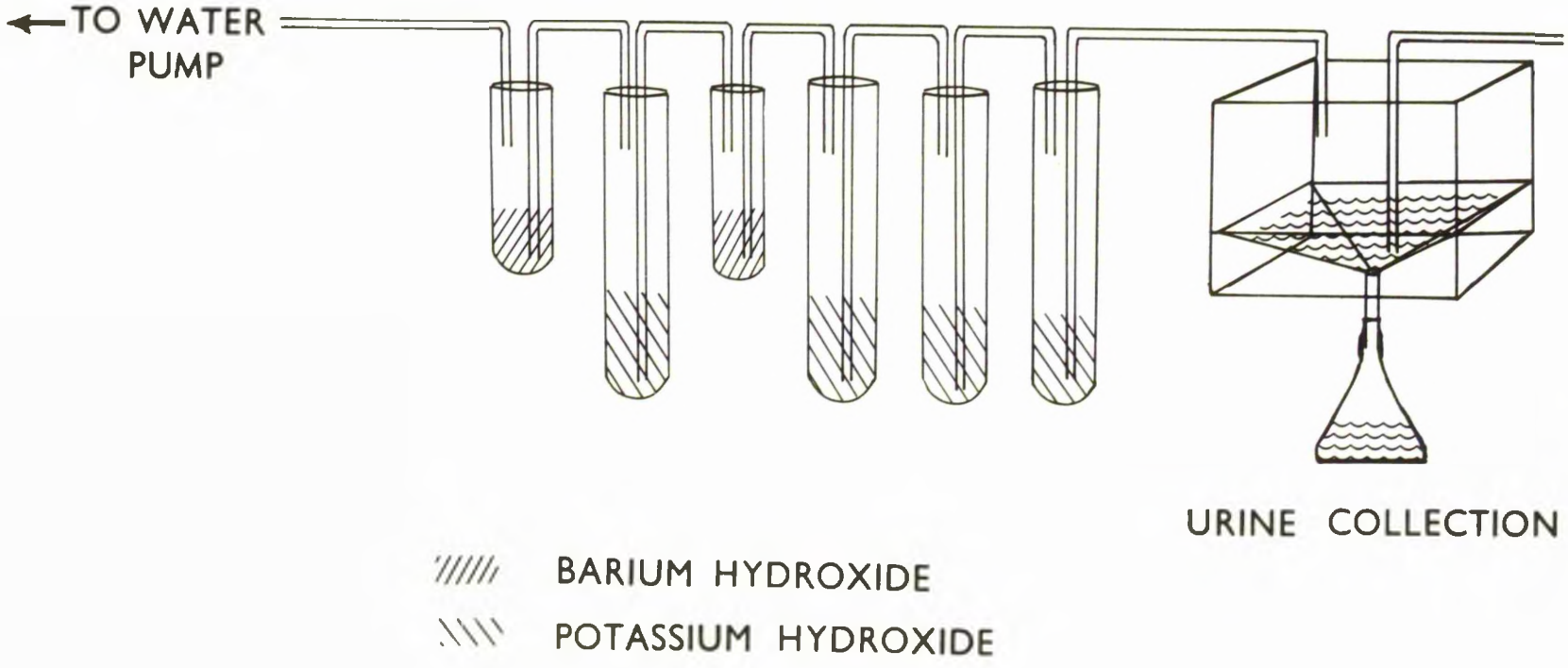



DIAGRAM 3. Collection of volatile metabolites.

the expired air and collected in bottles containing the caustic potash. At intervals of 3 hours, the potassium hydroxide in all 4 traps, and the barium hydroxide in the other traps were renewed.

Aliquots from each 3 hour sample were counted for radioactivity using the liquid scintillation counter. From the results obtained it was possible to calculate the total activity in the form of $C^{14}$-labelled carbon dioxide which had been passed during this period. Knowing the dose given, the percentage exhaled as $C^{14}O_2$ was evaluated. In order to estimate any errors in counting due to the effect of the strong potassium hydroxide solution on the phosphor, absorption was checked using $C^{14}$-adenine standard. The efficiency of the trapping system was also checked by liberating a known amount of radioactive carbon dioxide quantitatively from $C^{14}$-labelled sodium carbonate.

## 7. COLLECTION OF URINE

Groups of 5 rats or 10 mice were maintained in a metabolism cage so constructed to enable collection of urine free from faecal contamination. The animals were placed in the cage at least 48 hours before the injection. Food and water were available to the animals ad libitum. Urine was funnelled into a glass vessel located in a Dewar flask packed with solid $CO_2$ and

remained frozen during the period of collection. Samples were taken at 24 hourly intervals until the excreted activity had dropped to less than 1 % of the administered dose. The total volume excreted in the 24 hour samples was recorded and aliquots plated for counting in duplicate before being chromatographed. An absorption factor was determined for each sample. Knowing this, the total number of counts excreted was calculated and expressed as a percentage of the dose given.

The excretion in the rabbit was determined by a similar technique, but used only one animal in each experiment. As a result of the dose tolerated by the rabbit being approximately 1/3 rd that of rats and mice, urine was of lower activity. This, together with the larger volume excreted by the rabbit, necessitated reduction of the volume by freeze drying before counting and analysing.

8. BLADDER CANNULATION

A male rat, 382 g., was anaesthetised and the bladder cannulated. The animal was placed in a restricted movement cage (Diagram 4) and allowed to recover from the operation overnight. By the following morning, the blood present initially had cleared and urine was flowing freely. The labelled drug was introduced directly into the peritoneum through an external tube.

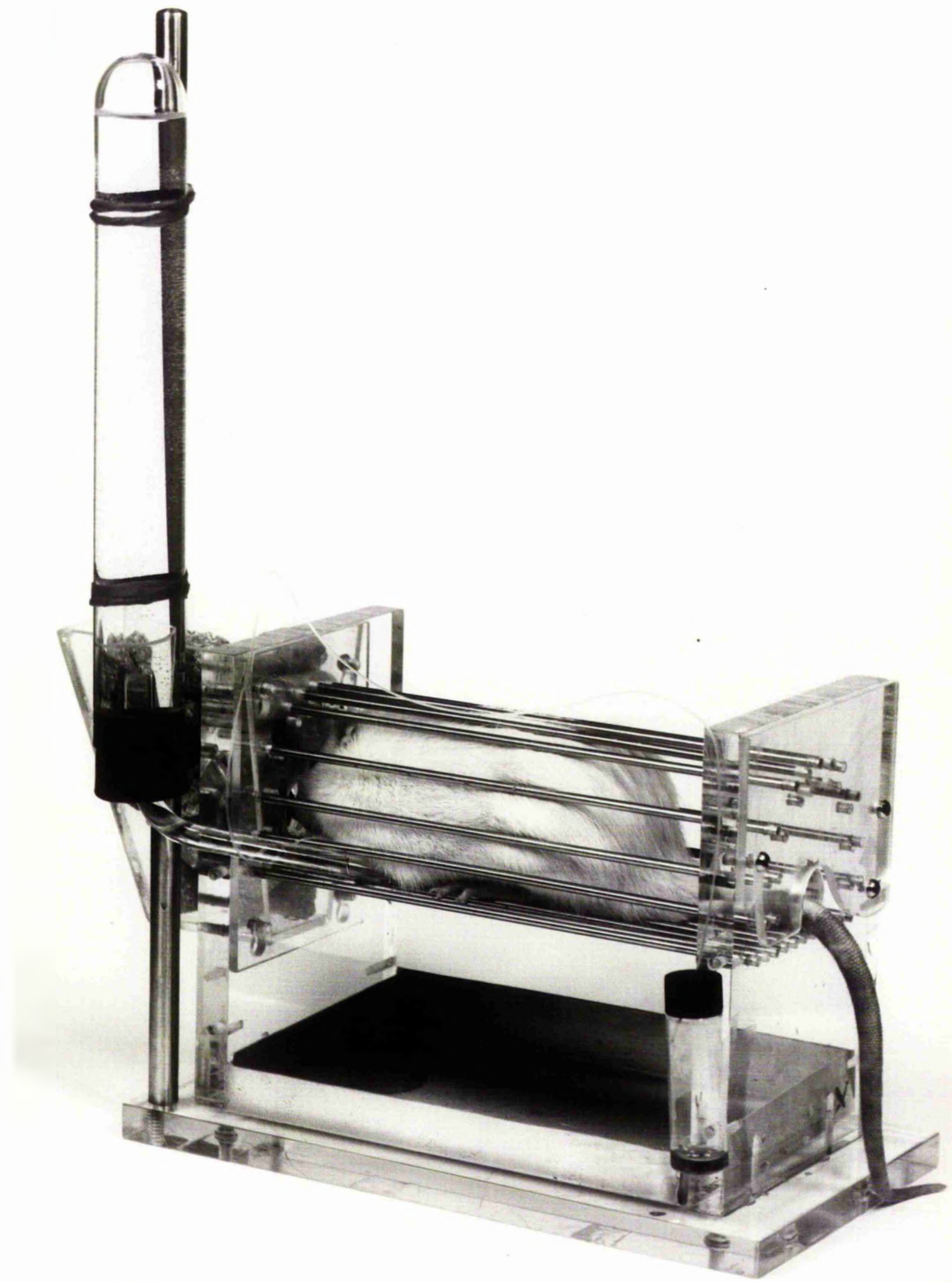


DIAGRAM 4. Restricted movement cage.

Samples were taken every 10 minutes for the first 60 minutes and subsequently at hourly intervals for 24 hours. Aliquots of each sample were counted and the total excreted activity calculated. Alternate samples were chromatographed.

9. HYDROLYSIS OF URINE

Urine collected from the metabolic experiments within 24 hours of the administration of the labelled drug was hydrolysed under acid and alkaline conditions.

Urine (0·3 ml) was pipetted into four 2 ml glass ampoules. To two of these was added hydrochloric acid, one containing 0·3 ml concentrated acid, the other 0·3 ml of 0·1N acid. The other two contained alkali, 0·3 ml of 40 % sodium hydroxide solution, and 0·3 ml of 0·1N solution respectively. Each ampoule was then sealed . Those with strong acid and alkali were heated at 100° for 24 hours; the other two were left at room temperature for the same period. On opening, 0·1 ml samples were counted in the solid counter. The products were analysed chromatographically.

Aliquots of each hydrolysate (0·3 ml) were pipetted into an evaporating dish and gently warmed in an oven. At dryness, 0·3 ml of distilled water was added to reconstitute. This was repeated twice. The final solution was again counted

and chromatographed.

10. INCUBATION OF URINE WITH β-GLUCURONIDASE

To determine whether the drug was excreted as a glucuronide, the 24 hour rat urine (0·5 ml) was incubated with a few milligrams of β-glucuronidase (Bovine) in acetate buffer (0·5 ml) at pH 4·5. After 24 hours at 37° the material was chromatographed.

11. CANNULATION OF THE BILE DUCT

A male rat, 322 g, was anaesthetised with ether. A mid-line incision was made in the upper abdomen to expose the duodenum. The lower part of the bile duct was located and a cannula of polythene tubing inserted. Bile flowed continuously through a 6" length of tubing and was collected in a weighed bottle. The incision was then closed.

When satisfied that cannulation had been successful, the femoral vein was exposed and methyl methanesulphonate solution (0·20 ml), containing 38 mg of $C^{14}$-labelled drug dissolved in physiological saline (0.38 ml) was injected. The animal was placed in a securing cage to limit its movement and the bile collected for 30 minute periods. Each sample was weighed and 20 µl aliquots plated for solid counting and chromatography. The

total counts in each specimen was calculated. After 24 hours, the activity had dropped to less than 2% of the peak value and the animal was killed.

12. INCUBATION OF BILE WITH KIDNEY HOMOGENATE

Kidney homogenate was prepared as described by Booth et al. (32). Rat kidney (1·0 g) taken from a freshly killed animal was homogenised with 3 mls of 0·154M saline. Bile, collected 60 minutes after injection of radioactive drug was used. To a 100µl aliquot was added 100µl of 0·1M pyrophosphate buffer pH 8, together with 100µl of kidney homogenate. The mixture was incubated at 37° for 30 minutes when a sample was taken for chromatography. A similar sample was taken after 60 minutes. As a control experiment, the same bile (50µl) was incubated under similar conditions with 100µl buffer. This was also chromatographed after 30 and 60 minutes.

At the end of this time, slices of rat liver (100 mg) were added to the mixture and incubation resumed for a further 4 hours. Samples were taken for chromatography.

13. INCUBATION OF BILE WITH INTESTINE

Bile (100µl), taken 30 minutes after

administering the drug, was incubated at 37° with the small intestine from a rat (120 mg), containing portions of duodenum, jejunum and pancreas, in the presence of pyrophosphate buffer pH 8. This mixture was chromatographed immediately after the addition of the intestinal material and subsequently after 15, 30, 60 minutes and 5 hours.

The experiment was repeated, incubating approximately 100 mg quantities of duodenum, pancreas and jejunum in three separate incubation bottles, each with radioactive bile (100µl) in buffer for 1 hour at 37°. The products were analysed as before.

14. INCUBATION OF BILE WITH β-GLUCURONIDASE

Bile (100µl), containing labelled metabolites, was incubated with β-glucuronidase in the presence of acetate buffer (100µl) pH 4·8 for 24 hours at 37°. A sample was then chromatographed.

15. QUANTITATIVE ESTIMATION OF GLUTATHIONE

(a) In blood.

The iodate titration method of Woodward and Fry (33) was used for the determination of glutathione levels in

rat blood.

Rats were injected with inactive methyl methanesulphonate (100 mg/kg) and at known times after administration were anaesthetised and exsanguinated. Aliquots (1 ml) of whole blood were laked in distilled water (8 mls) for 10 minutes. A solution of N. sulphosalicylic acid (1 ml) was pipetted into the sample to precipitate out protein. This was filtered to give a colourless solution. The filtrate (5 mls) was placed in a 25 ml flask with 4 % solution of sulphosalicylic acid (1·25 mls), 5 % potassium iodide (1·25 mls) and 3 drops of starch indicator. This was titrated against 0·001M potassium iodate solution until the appearance of a permanent blue colour. The volume run in was recorded. The estimation was repeated in duplicate and the average titre calculated.

A standard solution of glutathione was prepared by dissolving 10 mg in 4 % sulphosalicylic acid and making the volume up to 100 mls. A mixture of 10 mls of this solution, 2.3 mls of potassium iodide, 2·5 mls of 4 % sulphosalicylic acid and 3 drops of 1 % starch solution were similarly titrated against 0·001M potassium iodate solution. This estimation was repeated three times and an accurate value obtained

for the iodate required per mg of glutathione. The total amount present in blood could then be calculated.

(b). In liver.

The liver was removed and weighed. A weighed sample was then homogenised with physiological saline, the protein precipitated with sulphosalicylic acid and an aliquot of filtrate titrated as before.

## C. CHROMATOGRAPHIC AND COUNTING TECHNIQUES

### 1. CHROMATOGRAPHY.

#### (a) Single dimension.

In the first instance, material was analysed on Whatman No 1 paper using the ascending technique. Two 5µl samples were spotted 4 cms apart on each strip of paper, measuring 8 x 30 cms, using an Agla micrometer syringe or a micro-pipette. On occasion, 10µl was applied where the activity of the sample was considered low. Two solvent systems were found to give adequate separation of components and were used throughout.

| | | |
|---|---|---|
| BA | n-butanol - acetic acid - water | (12 : 3 : 5) |
| BDN | n-butanol - dioxan - ammonia | ( 4 : 1 : 5) |

After developing overnight at 20°, the chromatograms were dried in air and scanned for radioactivity before being treated with locating reagents. Comparison of the position of the spots was then made with those produced by compounds of known chemical constitution.

Ninhydrin.

This was prepared as a 2 % solution in

acetone (%v), and containing a few drops of pyridine where it was considered that strong acid might adversely affect the development of the characteristic blue colouration in the presence of α-amino acids.

Iodoplatinate

Chloroplatinic acid, ($H_2PtCl_6 \cdot 6H_2O$)(5 mgs in 5 mls water),potassium iodide( 50 mgs in 0·5 ml water),2N hydrochloric acid (0·5 ml) and acetone (25 mls) were mixed as required. The presence of sulphur containing amino acids, other than oxidised forms, was indicated by the appearance of white spots on a pink background. The reagent is unstable on standing and a freshly prepared solution was used on each occasion.

Iodine - azide

Solution A, containing iodine (1·27 g) in ethanol (100 mls) and solution B, sodium azide (3·25 g) in water (25 mls) and ethanol (75 mls) were mixed in equal volumes when required. White spots on a brown background were produced in the presence of all thiols and disulphides but not with thioethers.

Ehrlich reagent

A 10 % solution of p-dimethylaminobenzaldehyde in conc. HCl was mixed with 4 volumes of acetone just before use. The chromatograms were dipped and laid flat. Immediate yellow spots are produced in the presence of urea, ureides and certain aromatic

amines, red or blue colours are characteristic of certain indolic compounds.

Pentacyanonitrosoferriate

Equal volumes of a 10 % solutions of sodium hydroxide, potassium ferricyanide and sodium nitroprusside were mixed with 3 volumes of water. After adding an equal volume of acetone to the resulting solution, the paper was dipped. Red colours are obtained with NN-di-substituted and mono- guanidines, but NN'-di-substituted compounds do not react.

Jaffe reagent

Solution A, 1 % picric acid in 95 % ethanol and solution B, 5% potassium hydroxide (%v) in 80 % ethanol, were prepared. The chromatograms were first dipped in solution A and dried, then in B and dried. Red spots on a yellow background are obtained with creatinine.

Potassium iodide

A freshly prepared solution of potassium iodide (20·0 mg) in 2N hydrochloric acid (100 mls) gives red spots on a yellow background for certain oxidised compounds containing sulphur. Thiols and disulphides do not react but sulphonic, sulphinic and thiosulphonic acids react positively (34).

Potassium dichromate - silver nitrate

0·1% potassium dichromate and glacial acetic

acid were mixed in equal volumes. Chromatograms were dipped in this solution and dried. After dipping in a solution of 0·1M silver nitrate, mercapturic acids appeared as orange spots on a red-brown background.

(b) Two dimension.

To effect a better separation of components, two dimensional chromatograms were prepared in certain cases by the ascending method . The techniques and apparatus used were based upon those described by Datta, Dent and Harris (33).

The sample (3µl) was spotted on sheets of Whatman No 1 paper (20 x 20 cms) which were fitted five at a time on to a polythene frame and placed in airtight glass tanks 12" x 12" x 12", containing the solvent. After developing in one direction, the frame was removed and dried. It was then turned through a right angle, placed in the second solvent and developed as before.

(c) Electrophoresis.

Separation of plasma proteins was carried out on Whatman No 1 paper using barbitone buffer pH 8·6. For the electrophoretic run, the papers were dipped through the buffer and blotted before being placed in the Shandon Universal tank.

The plasma sample, taken 24 hours after injection of labelled methyl methanesulphonate into the rat, was applied (10µl) in duplicate. After running overnight at constant current (0•3 ma), the papers were removed and dried. One of the strips was stained for 10 minutes in Light Green (0•5 % solution in 25 ml ethanol, 75 ml water plus 5 ml acetic acid). The second strip was cut in sections for counting in an end window counter. In this way the distribution of activity along the strip was obtained and could be directly compared with the individual bands of protein.

2. COUNTING TECHNIQUES

(a) Chromatogram scanning.

The instrument, designed by Gilbert and Keene (36), consists essentially of a counter unit composed of 31 identical geiger counters each connected through an insulated stainless steel probe to a high insulation capacitor. The quenching gas used was highly purified argon with approximately 7 % by weight ethyl acetate vapour, obtained by passing the gas through ethyl acetate held at 0°C. by melting ice to ensure constant composition of the gas.

The advantage of this scanner over apparatus using a single detector is that several chromatograms with

only low activity can be measured in one day. The chromatogram to be counted, either single or two dimension, was taped to a metal slider which was then inserted into the instrument. Counting was continued for 30 minutes in all cases. The accumulated charge was read from the meter as total counts per 30 minutes. After subtracting the background, the distribution of activity along the paper was recorded.

(b) Solid counting.

The assay of radioactivity in biological material after the injection of $C^{14}$-labelled drug was made by plating an aliquot of the liquid onto a 2 cm filter paper disc contained in an aluminium planchette. The planchette was gently heated in an oven to dry the contents to the solid state. In this way a uniform distribution of material was obtained over the surface of the disc.

The aluminium planchette was placed in a holder and mounted directly under the thin mica end window counter. An absorption factor for the particular sample was determined using an aliquot of $C^{14}$-labelled adenine of known specific activity. The counter had an efficiency of just less than 1 % for carbon.

(c) Liquid scintillation counting.

Liquid samples were counted on an IDL counter. The phosphor used throughout was a solution of Naphthalene (80 g), 2,5-diphenyloxazole (0·5 g) and 1,4-bis-2-(phenyloxazol)benzene (0·05 g) in Xylene (385 mls), 1,4-dioxan (385 mls) and Ethanol (230 mls), as described by Moutschen (37).

An aliquot of the sample, dissolved in 6 mls of phosphor solution was counted until at least 10,000 counts were recorded. A background count from the phosphor alone was subtracted from the observed count. The efficiency for carbon, using a standard $C^{14}$-adenine sample of known specific activity, was approximately 60 %.

It was observed that when assaying the radioactivity in the traps used in the experiment to measure excretion of volatile metabolites, the strong alkali affected the properties of the phosphor. The nature of this interference was apparently due to chemiluminescence. Samples were therefore allowed to stand for 1 hour before commencing counting by which time constant values for repeated counts on the same sample were obtained. A standard solution of $C^{14}$-adenine was prepared in 40 % potassium hydroxide for each series of counts.

(d) Autoradiography

The most satisfactory method for recording the exact position of areas of radioactivity on single and two dimensional chromatograms is to expose the paper to X-ray film for a suitable length of time and then to develop the film. The developed chromatograms, having been counted were secured on a piece of stiff cardboard to provide a flat surface. X-ray film was carefully placed in contact with the paper. The film and cardboard, protected from light, were left in a darkroom for 4 - 6 weeks. At the end of this time, the film was developed and fixed in the usual manner.

ANALYSIS OF URINARY METABOLITES

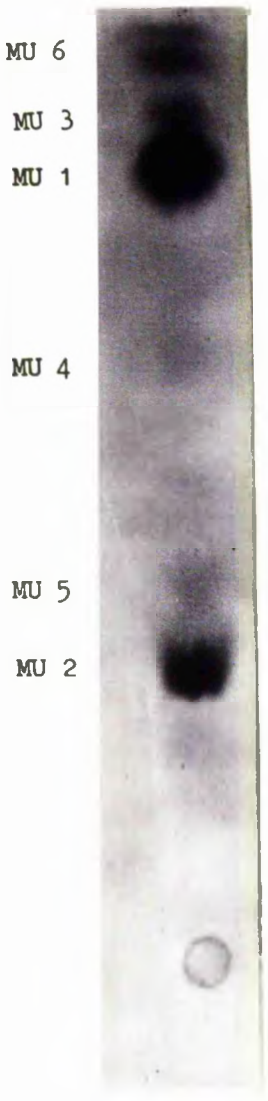
(a) Mouse.

Radioactive assay of urine samples taken at 24 hour intervals after injection of $C^{14}$-methyl methanesulphonate showed the rate at which the drug was being excreted. The results obtained are expressed as a percentage of the injected dose :-

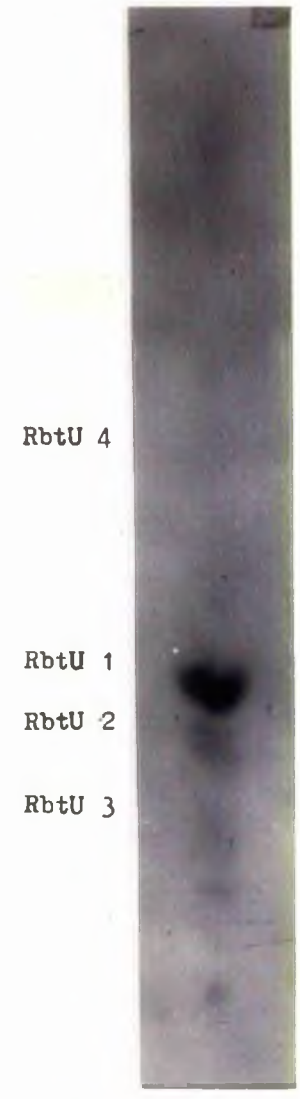
| | % |
|---|---|
| 0 - 24 hours | 10•9 |
| 24 - 48 hours | 3•3 |
| 48 - 72 hours | 2•3 |
| 72 - 96 hours | 1•1 |
| 96 -120 hours | 0•5 |

Approximately 20 % of the injected activity is excreted in the urine in 4 days.

Autoradiographs prepared from chromatograms of urine samples revealed the presence of at least six metabolites (Diag 5). The drug itself was volatile under the conditions employed for chromatography and therefore excretion by the animal of unchanged drug would escape detection by chromatography.




24 hr MOUSE URINE




24 hr RABBIT URINE

DIAGRAM 5.

A comparison of autoradiographs of chromatograms of 24 hr urine samples from mouse and rabbit.

However, the presence of unreacted material in the urine was discounted when an aliquot of fresh urine, counted in the liquid scintillation counter, gave a similar value for activity present to that obtained by evaporating an aliquot to dryness, reconstituting to the original volume and recounting.

The chromatographic patterns from specimens of urine taken 4, 24, 48, and 72 hours after injection were similar suggesting that qualitatively there was no variation in metabolites present with time. Results from three separate experiments were the same. The areas of radioactivity evident from the autoradiographs are referred to numerically in decreasing order of total counts present expressed as a percentage of the total activity on the chromatograms and are summarised thus :-

| | Rf | % | Nin | Ipl | |
|---|---|---|---|---|---|
| MU 1 | 72 | 50 | - | + | N-(methylthioacetyl)glycine |
| MU 2 | 26 | 20 | - | - | |
| MU 3 | 83 | 10 | - | + | N-acetyl-S-methylcysteine |
| MU 4 | 53 | 6 | - | - | Urea |
| MU 5 | 35 | 5 | + | + | S-methylcysteine |
| MU 6 | 87 | 4 | - | + | S-methylthioacetic acid |
| Origin | | 5 | + | + | |

The figures quoted are for BA solvent system, except where specific reference is made, it is to be assumed that corroborative results were obtained from chromatograms developed in BDN.

The major metabolite, MU 1, representing 50 % of the activity on the paper, would appear to be a sulphur containing compound (iodoplatinate +ve). Comparison of the Rf with that of an authentic compound has suggested that this component is N-(methylthioacetyl)glycine. The other predominant metabolite, MU 2, gives a positive yellow reaction with Ehrlich reagent indicating the presence of a ureide. MU 3 reacts positively with the potassium dichromate/silver nitrate reagent and has been identified as N-acetyl-S-methylcysteine. 80 % of the radioactivity excreted in the urine is associated with these three metabolites. The distribution of activity was not altered after incubation of an aliquot of urine with β-glucuronidase.

After mild acid and alkaline hydrolysis, there was no evidence that either the position or percentage distribution of the metabolites originally present had altered. Autoradiographs of urine subjected to hydrolysis with strong acid showed that MU 1, MU 2 and MU 3 had completely disappeared and that

50 % of the activity remaining was concentrated on the origin. This was probably a result of the effect of the concentrated acid on the chromatography paper. There was an increase in the percentage of MU 5 from 8 % to 40 % suggesting that one or more of the metabolites initially present had been broken down to this component and was therefore related to it chemically. The results from strong alkaline hydrolysis were in general similar to those from the hydrolysis with strong acid but were less clearly defined, probably due to salt effects during chromatography. MU 3 has been shown to possess the same Rf value in both solvent systems as S-methylcysteine. Likewise, MU 4 has been recognised as urea and MU 6 as S-methylthioacetic acid.

(b) Rabbit.

Less than 10 % of the injected drug is excreted in the urine within 7 days. The excretion, expressed as a percentage of the dose given is as follows :-

| | % |
|---|---|
| 0 - 24 hours | 2·3 |
| 24 - 48 hours | 0·1 |
| 48 - 72 hours | 4·8 |
| 72 - 96 hours | 1·1 |
| 96 -168 hours | 1·4 |

Autoradiographs provided evidence of 4 areas of radioactivity on chromatograms of the urine samples (Diagg ) :-

| | Rf | % | Nin | Ipl | |
|---|---|---|---|---|---|
| RbtU 1 | 27 | 55 | - | - | |
| RbtU 2 | 23 | 25 | + | - | |
| RbtU 3 | 16 | 12 | - | + | |
| RbtU 4 | 52 | 8 | - | - | Urea |

Hydrolysis of the urine with concentrated HCl produced a sulphur containing - ninhydrin positive area of radioactivity which had the same Rf as an authentic sample of S-methylcysteine. At least one of the original metabolites must therefore be a conjugate of this amino acid.

(c) Rat.

Quantitative collection of urine showed that 20 % of the injected dose was excreted by this route in 4 days:-

| | % |
|---|---|
| 0 - 24 hours | 17·7 |
| 24 - 48 hours | 1·7 |
| 48 - 72 hours | 1·0 |
| 72 - 96 hours | 0·3 |

At least 11 distinct areas of radioactivity are evident on chromatography, presenting a complex picture for analysis (Diag 6). 70 % of the activity is associated with three of these, the remaining 8 spots representing minor metabolites :-

| | Rf | % | Nin | Ipl | |
|---|---|---|---|---|---|
| RU 1 | 37 | 43 | - | + | |
| RU 2 | 30 | 15 | - | - | |
| RU 3 | 40 | 10 | + | + | S-methylcysteine |
| RU 4 | 83 | 7 | - | + | N-acetyl-S-methylcysteine |
| RU 5 | 63 | 6 | - | - | |
| RU 6 | 78 | 5 | - | + | N-(methylthioacetyl)glycine |
| RU 7 | 50 | 5 | - | - | Urea |
| RU 8 | 20 | 3 | - | - | |
| RU 9 | 12 | 2 | - | + | N-acetyl-S-methylcysteine sulphoxide |
| RU 10 | 86 | 2 | - | + | S-methylthioacetic acid |
| RU 11 | 6 | 2 | + | - | |

The major metabolite RU 1 appears to be a sulphur containing compound and in addition gives a positive reaction with the pentacyanoaquoferrinate reagent suggesting the presence of a substituted guanidine residue. RU 2 appears as a yellow spot after Ehrlich reagent. RU 4 gives the characteristic orange spot

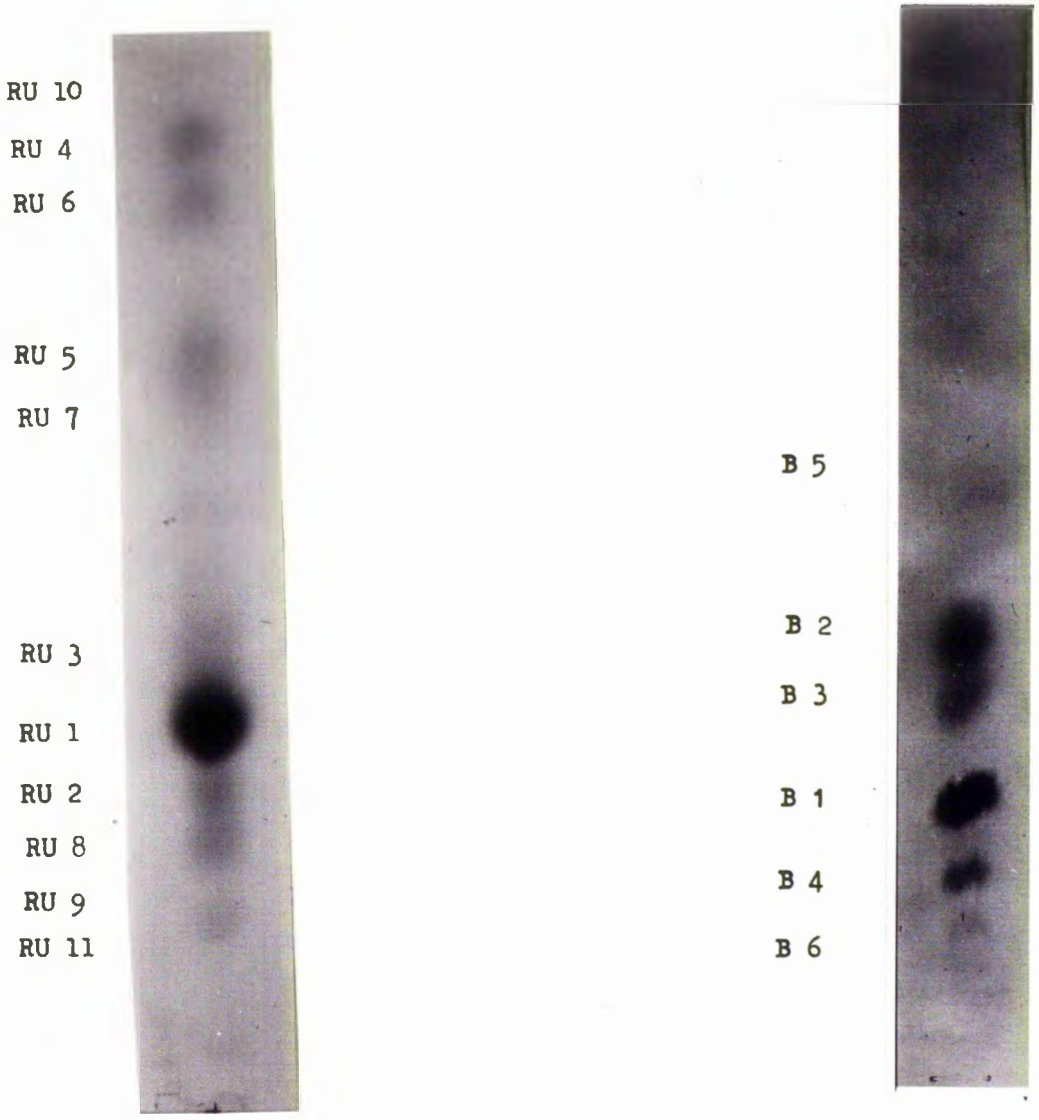



DIAGRAM 6.

Autoradiographs of chromatograms of 24 hr rat urine and of a typical bile sample.

on a red-brown background for mercapturic acid. There was no evidence for the existence of labelled thiols or disulphides in the urine

Incubation of the rat urine with β-glucudenidase followed by chromatographic and autoradiographis analysis did not suggest that the major metabolites were excreted as glucuronides.

Quantitative hydrolysis of the rat urine in sealed ampoules indicated that there was no loss in radioactivity of the samples during this reaction. Elimination of the labelled methyl group as volatile material was thereby excluded. When the urine was hydrolysed under mild acid conditions, the area corresponding to RU 1 becomes less prominent, there being a corresponding increase in RU 4 which now appears as the major metabolite with 55 % of the total activity on the paper. RU 10 is also more prominent, accounting for 15 % of the remaining activity on the paper. There is no evidence for alteration in the other metabolites. The position of the components after mild alkaline hydrolysis is not markedly different from the original distribution but on heating with concentrated alkali RU 2 features as the most conspicuous spot. Strong acid hydrolysis reveals RU 3

as almost the only radioactive material on the paper.

Results from the experiment designed to show the rate of excretion of $C^{14}$-labelled methyl methanesulphonate by assessing the radioactivity present in daily samples of urine from the rat indicated that the majority of the drug metabolised by this route was voided within the first 24 hours after injection. In order to obtain more precise information on the rate of metabolism by the animal, data for shorter intervals of time was required. Collection of urine using the metabolic cage technique was impracticable. By direct cannulation of the bladder of an animal maintained with food and water ad libitum in a restraining cage, urine samples could be collected continuously over a period of at least 24 hours.

Immediately after injection of the drug, output of urine dropped but within 10 minutes had returned to normal. The presence of radioactive material was evident in the collected sample after 20 minutes. The specific activity increased steadily to reach a maximum at $6\frac{1}{2}$ hours, this level being maintained until 17 hours after injection when the specific activity fell away sharply. By 20 hours, the excretion had dropped to 20 % of the peak value and the fall had become more gradual. In the 24 hour period 19 mls of urine were collected and 16·8 %

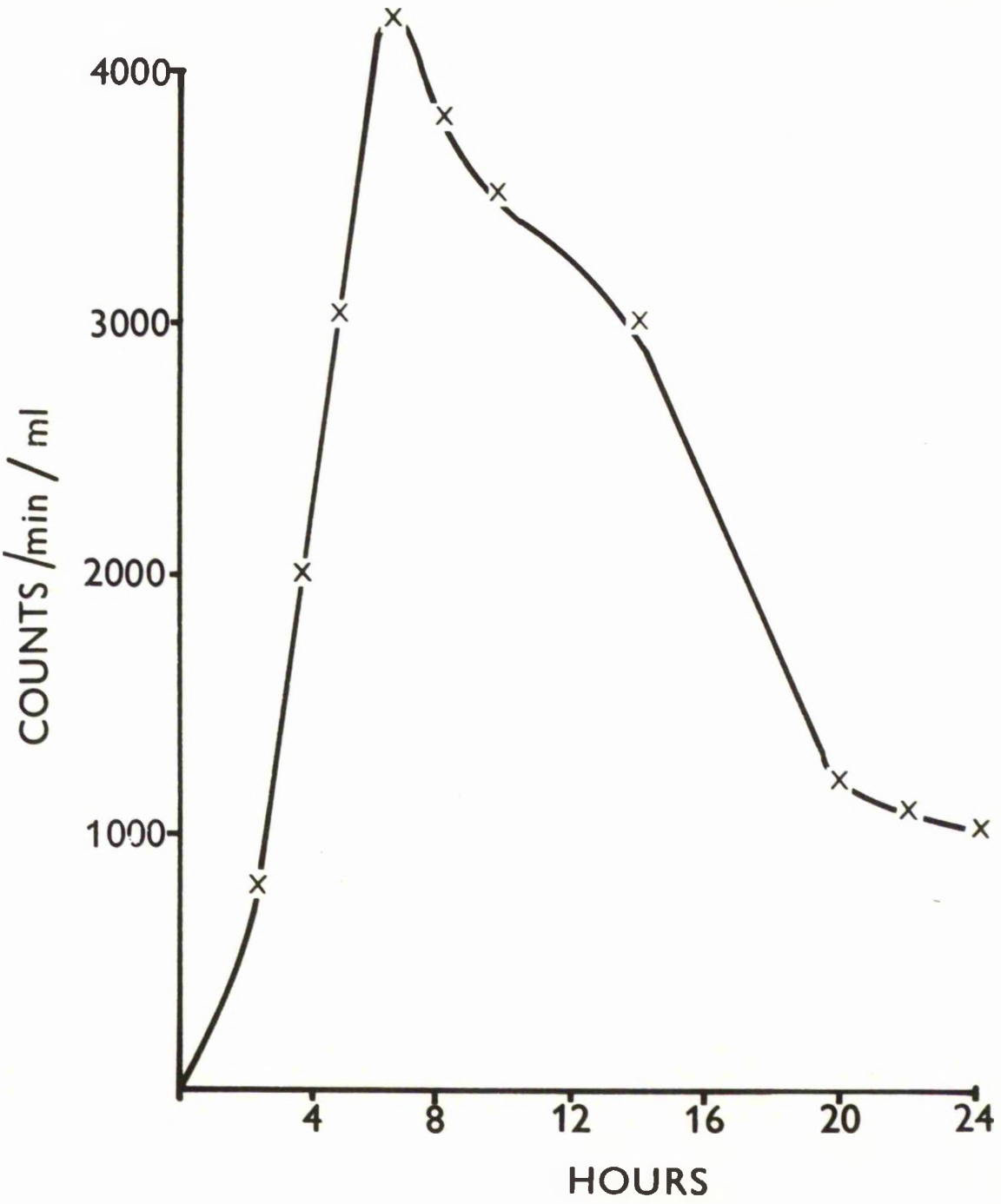



DIAGRAM 7. Excretion of radioactivity in rat urine.

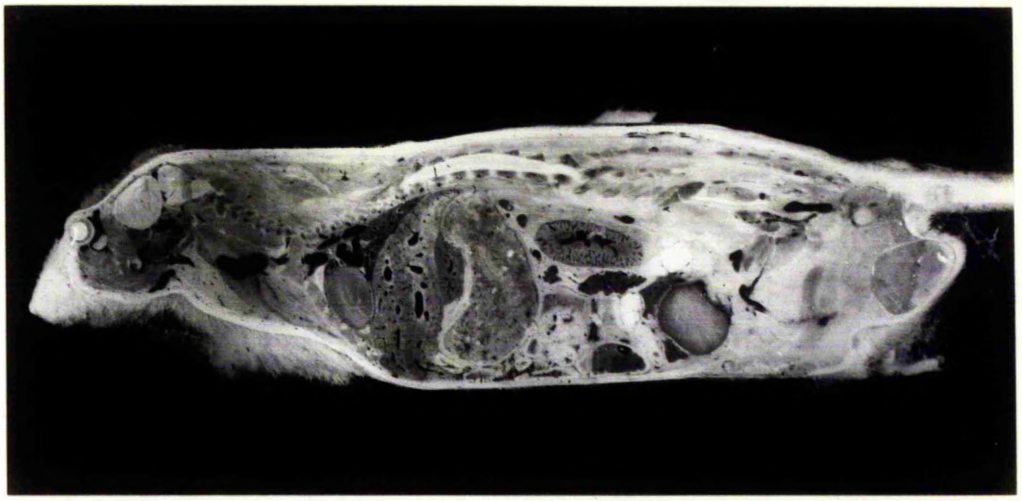
of the injected activity was excreted ; a figure which agreed favourably with that obtained from the metabolic cage over the same period of time. The results are illustrated in Diag 7.

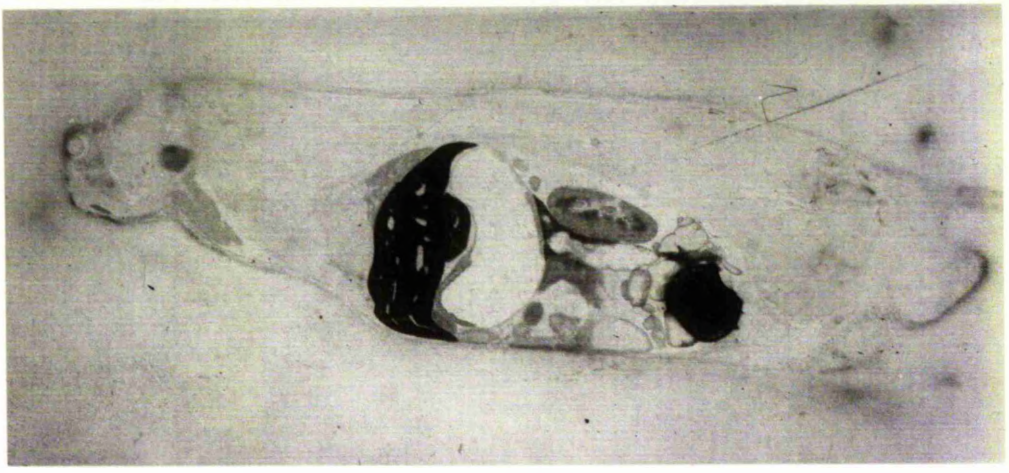
DISTRIBUTION OF THE RADIOACTIVE DRUG AFTER INJECTION

Examination of the autoradiographs prepared from frozen sections of mice at intervals after administration of the $C^{14}$-labelled drug provided preliminary evidence for its overall distribution.(Diag 8).

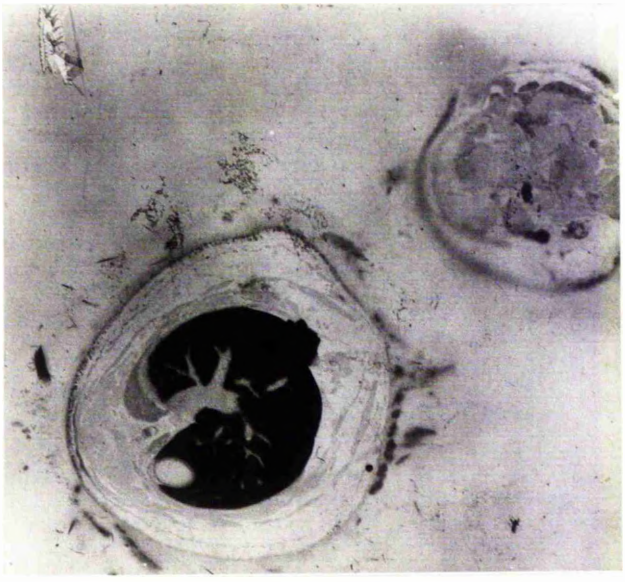
The most prominent feature of a sagittal section 16 minutes after injection was the high activity associated with the liver. Traces of radioactivity in the rectal area were probably due to discharge of urine when the animal was anaesthetised. The background of activity in the animal as a whole would suggest fairly general distribution of the drug. A similar section after 30 minutes shows an increase in intestinal activity. The walls of the rectum are clearly outlined in the autoradiograph. After 70 minutes, the radioactivity is present in liver, intestine, bladder, rectum and salivary glands. These features are also seen after 105 minutes, a kidney being clearly seen in section. There was no obvious localisation in other organs.

Autoradiographs of frozen sections.





Sagittal Section 100 mins.



Transverse Section 4 hrs.

DIAGRAM 8.

The high activity associated with the liver suggested that the organ played a significant part in the metabolism of the drug. Furthermore, a transverse section through the animal 4 hours after dosing chanced to pass through the gall bladder and showed an area of activity of particular intensity. This concentration of radioactivity itself was not surprising as the gall bladder serves to concentrate waste materials. However, it did suggest that this might represent an important route for the metabolism of the drug.

All further studies were carried out in the rat due to practical difficulties encountered in dealing with an animal as small as a mouse.

METABOLITES PRESENT IN BILE

To investigate the relative importance of biliary excretion as a pathway for the metabolism of the drug, quantitative studies were made of the radioactivity present in samples of bile fluid, collected at known intervals of time upto 24 hours after injection of $C^{14}$-labelled drug. Aliquots of bile, collected by the direct cannulation of the bile duct in the rat, were counted on the solid counter. The specific activity

when plotted as a function of time, showed the presence of the labelled material in a sample taken within the first 10 minutes (Diag 9). The level increased sharply, reaching its maximum value in 90 minutes. By 8 hours, the specific activity had dropped to 25 % of the peak and subsequently fell more gradually until by 24 hours, the content of radioactive metabolites was negligible. During the 24 hours, 16 % of the injected activity had passed through the bile duct. There was no activity in the urine when the bile duct was cannulated.

Autoradiographs prepared from bile samples chromatographed immediately after collection showed 6 radioactive metabolites to be present (Diag 6)

| | Rf | % | Nin | Ipl | |
|---|---|---|---|---|---|
| B 1 | 26 | 33 | + | + | S-methylglutathione |
| B 2 | 39 | 18 | + | + | S-methylcysteine |
| B 3 | 34 | 16 | + | + | S-methylcysteinylglycine |
| B 4 | 21 | 5 | + | - | |
| B 5 | 54 | 4 | - | - | |
| B 6 | 12 | 2 | + | - | |

Results from three separate experiments showed that the pattern of excretion did not vary from animal to animal. B 5 was not

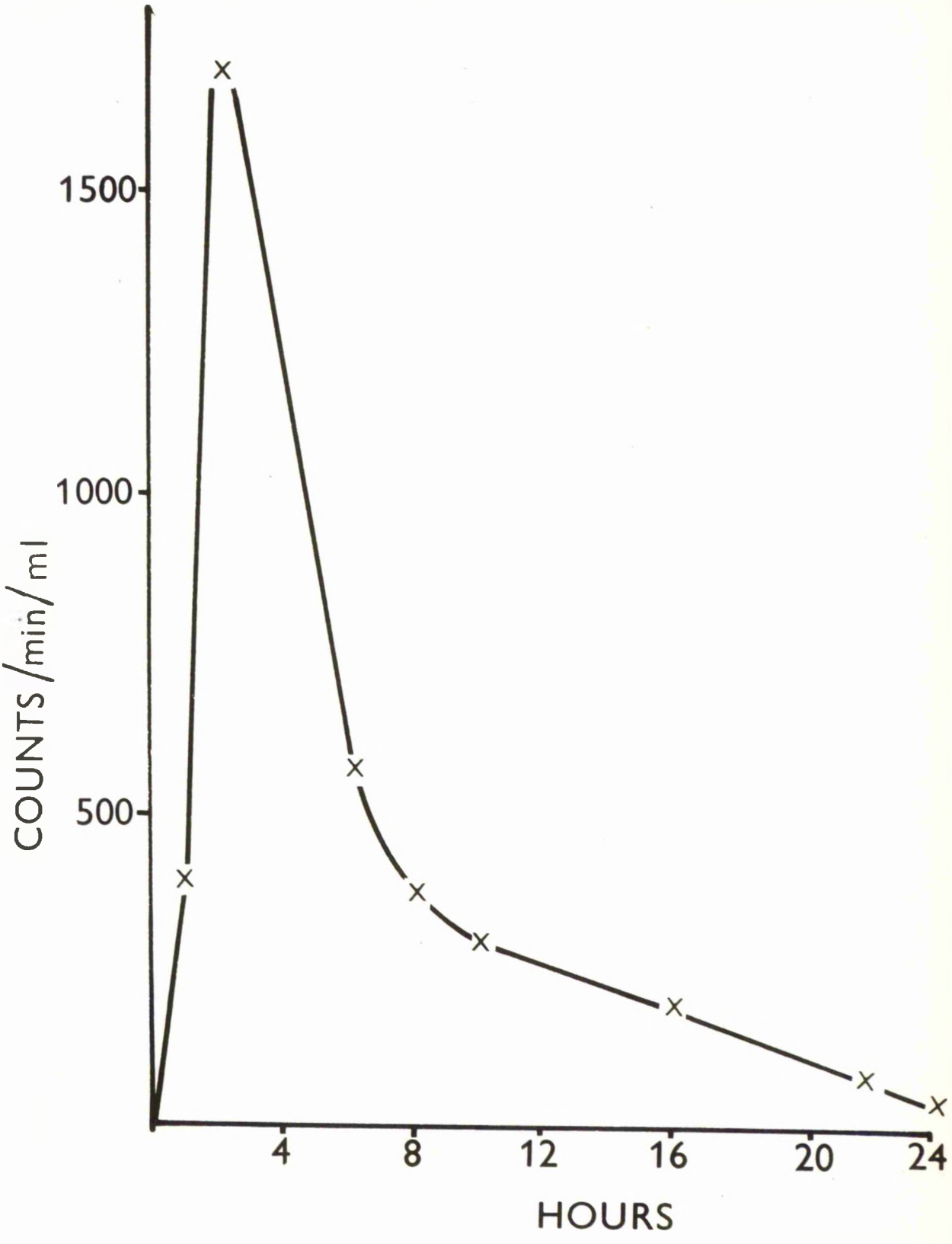



DIAGRAM 9. Excretion of radioactivity in rat bile.

present in the initial samples but reached maximum percentage (less than 10 %) in bile collected 2 hours after the experiment was begun. It was observed that samples chromatographed after storage overnight in a refrigerator showed the presence of only 3 metabolites. It was deduced that B 3 was unstable and, together with an increase in the percentage activity of B 2, breakdown to this latter component was indicated. This implied a chemical relationship between these two compounds. Five of the areas of radioactivity reacted positively with ninhydrin indicating the presence of the α-amino acid structure. The major metabolites, in addition, contained sulphur and by comparison of Rf values with authentic compounds B 1 and B 2 have been identified as S-methyl-glutathione and S-methylcysteine respectively. It was deduced that the unstable sulphur containing amino acid B 3 was probably S-methylcysteinylglycine, the peptide bond of which split on standing to yield the individual amino acids. B 4 and B 5 have not been positively identified but have similar Rf values and comparable reactions to ninhydrin and iodoplatinate as RU 8 and RU 9 respectively, present in rat urine. It is tentatively suggested that these compounds are S-methylcysteine sulphone and N-acetyl-S-methylcysteine sulphoxide.

Chromatography in BDH supported these results.

In general, the separation in the alkaline solvent was not as precise, but the decomposition of B 3 to B 2 on standing was convincing.

IN VITRO EXPERIMENTS

(a) Incubation of bile with kidney homogenate

Information regarding the nature of the metabolites resulted from incubation with a kidney homogenate followed by chromatography. There was evidence of a very rapid breakdown of components B 1 and B 3 within 2 minutes of adding an aliquot of freshly prepared kidney homogenate. After 30 minutes, only three of the components originally present in the bile remained. Estimation of the radioactivity present after incubation suggested that, within experimental limits, the loss in percentage activity from the decomposition of B 1 and B 3 corresponded to the gain in the percentage activity of B 2. After 30 minutes, this area accounted for 95 % of the activity on the paper. This suggested a definite chemical relationship between B 1, B 2 and B 3. A control experiment without the addition of kidney revealed that some breakdown did occur under chemical control, but the slower rate at which this occurred implied that the reaction was essentially

under the influence of an enzyme present in the kidney.

(b) Incubation of normal bile with $C^{14}$-methyl methanesulphonate

*In vitro* incubation of normal bile, collected from a cannulated bile duct before injection of radioactive material, with $C^{14}$-labelled drug gave rise to at least six radioactive products. There was however, very little resemblance to the results obtained *in vivo* and, it was decided that, for the purposes of the present investigation, not to persue this further.

(c) Incubation of bile with intestinal material

Having shown that the major metabolite present in bile after administration of methyl methanesulphonate was S-methylglutathione, and that at least two other urines were related to this compound, an attempt was made to relate these facts to the urinary excretion pattern. As material passing down the bile duct would enter the small intestine, there being exposed to enzymic action, it was reasoned that this might prove to be a site at which further modification of the metabolites might take place. Under the experimental conditions employed, there was no evidence of further metabolism of biliary excretion products when aliquots were

incubated in turn with duodenum, pancreas and jejunum.

(d) Incubation of bile with β-glucuronidase

The distribution of radioactivity on chromatograms of bile was unchanged after incubation with β-glucuronidase

EXCRETION AS VOLATILE MATERIAL

It was found that approximately 5 % of the injected activity was excreted by the rat as volatile material within the first 24 hours (Diag 10). Only trace amounts were detected subsequent to this period. The rate at which excretion takes place is expressed as a percentage of the dose of labelled drug :-

| | % |
|---|---|
| 0 - 3 hours | 0•97 |
| 3 - 6 hours | 1•31 |
| 6 - 9 hours | 1•04 |
| 9 - 12 hours | 0•54 |
| 12 - 15 hours | 0•43 |
| 15 - 18 hours | 0•36 |
| 18 - 21 hours | 0•24 |
| 21 - 24 hours | 0•21 |

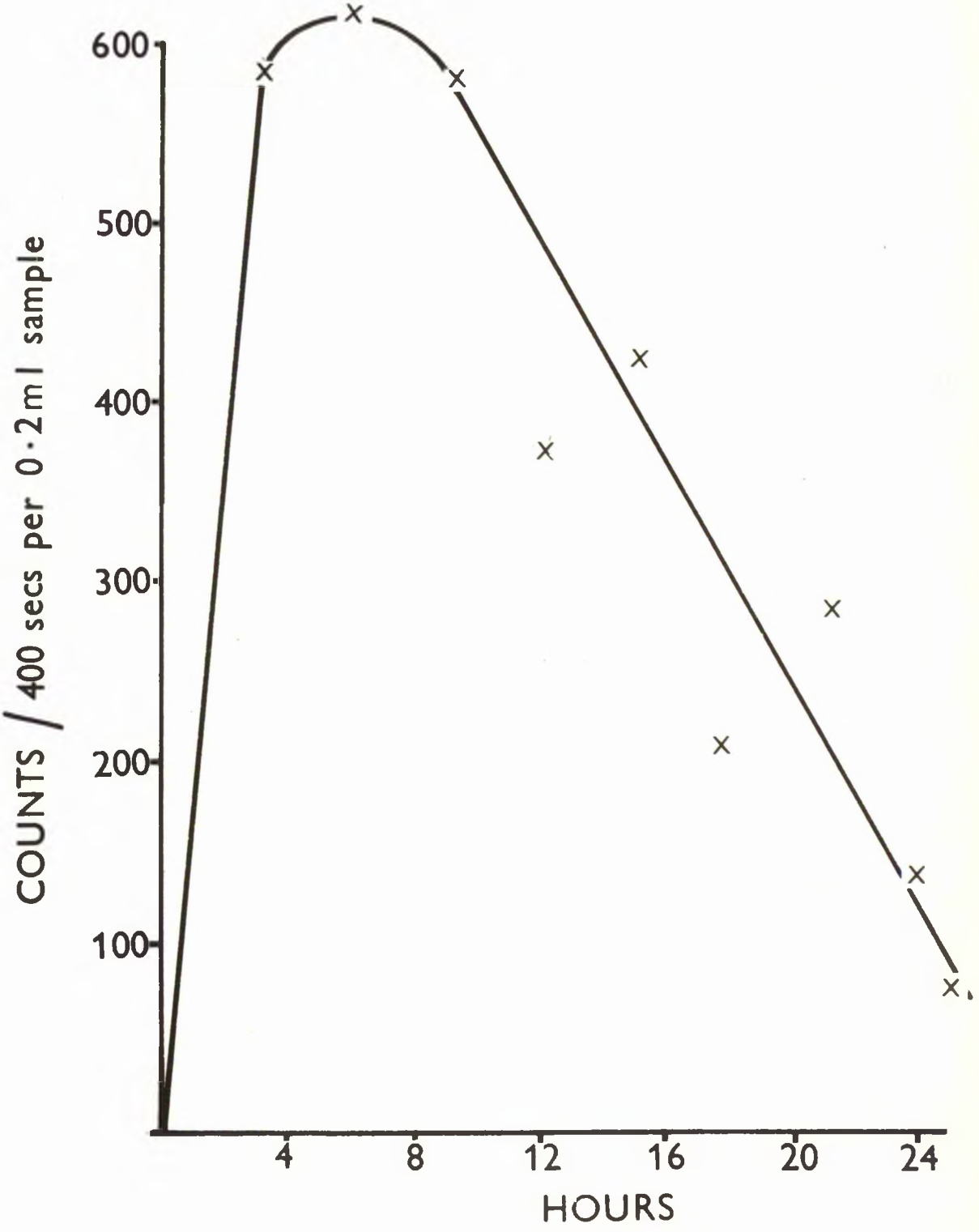



DIAGRAM 10. Excretion of $C^{14}$- labelled carbon dioxide.

The results tabulated above were obtained using a liquid phospher counting system for the potassium hydroxide solutions. Similar figures were obtained by counting the activity present in barium carbonate precipitates on the solid counter, suggesting that the activity present was very probably labelled carbon dioxide exhaled by the animal.

LEVELS OF ACTIVITY IN TISSUES

In an attempt to locate the radioactivity remaining in the animal, assay of certain organs of the rat was undertaken. Results for a series of time intervals are expressed as percentage of the dose present, beneath which in red type is the specific activity of the tissue :-

| | 10 m | 30 m | 1 hr | 2 hr | 4 hr | 7 hr | 12 hr | 24 hr | 48 hr | 72 hr |
|---|---|---|---|---|---|---|---|---|---|---|
| LIVER | 14•3 | 19•2 | 21•5 | 20•0 | 16•2 | 11•2 | 9•2 | 10•4 | 14•5 | 6•3 |
| | 1•69 | 2•05 | 3•82 | 2•16 | 2•00 | 1•69 | 1•06 | 1•24 | 1•51 | 0•70 |
| KIDNEY | 2•2 | 2•1 | 2•5 | 2•3 | 1•1 | 0•6 | 1•3 | 1•3 | 1•2 | 11•0 |
| | 1•16 | 1•06 | 1•69 | 1•20 | 0•90 | 1•00 | 0•67 | 0•82 | 0•49 | 0•55 |
| SPLEEN | 1•3 | 0•8 | 0•7 | 0•7 | 1•0 | 0•4 | 0•3 | 0•2 | 0•2 | 0•3 |
| | 0•77 | 0•48 | 0•79 | 0•73 | 1•46 | 0•70 | 0•39 | 0•52 | 0•40 | 0•35 |
| TESTIS | 1•7 | 0•9 | 1•8 | 1,3 | 1•4 | 1•3 | 0•7 | 1•3 | 0•5 | 0•3 |
| | 0•49 | 0•27 | 0•53 | 0•47 | 0•53 | 0•56 | 0•25 | 0•41 | 0•18 | 0•20 |

In addition to the tissues listed above, the following were also removed from an animal 24 hours after injection and the specific activities per gram of tissue determined :-

| | | | | | |
|---|---|---|---|---|---|
| Lung | 0•40 | Salivary gland | 0•40 | Adrenals | 0•25 |
| Heart | 0•26 | Seminal vesicles | 0•15 | Skin | 0•11 |
| Brain | 0•15 | Stomach | 0•20 | Fat | 0•10 |
| Thymus | 0•22 | Intestine | 0•20 | Muscle | 0•20 |

ESTIMATION OF GLUTATHIONE LEVELS

(a) In liver

Although the principal metabolite present in bile had been recognised as S-methylglutathione, the source of the glutathione had not been located. In view of the high glutathione content of the liver, it seemed likely that this organ might play a significant part in the reaction. Accordingly, estimations of the glutathione content of the liver were made at a series of time intervals after administration of the unlabelled methyl methanesulphonate.

Results showed that within 1 hour of the injection, the level had dropped to 40 % of the control value

obtained from an animal injected with saline. A minimum occurred after 90 minutes when the level reached 13 %. Thereafter, the glutathione content rose slowly until by 7 hours, the original content of the tissue was regained. (Diag 11).

(b) In blood.

As the concentration of glutathione in whole blood is known to be relatively high, it was of interest to determine whether the drug reacted directly with the tripeptide present. Estimation of the reduced glutathione at various times showed that there was a steady decrease to a minimum corresponding to 70 % of the control at 2 hours. By 6 hours, the levels in the treated animals had returned to normal. (Diag 12).

BLOOD FRACTIONATION

Assay of whole blood for radioactivity after injection of the labelled compound into the rat indicated that there was appreciable retention of the labelled methyl group for at least 3 days. Separation of the plasma showed that the level of activity remained constant at approximately 25 % of the total activity found in the whole blood. The remaining 75 % was therefore associated with the red cell. Results obtained for a

Liver glutathione levels after methyl methanesulphonate.

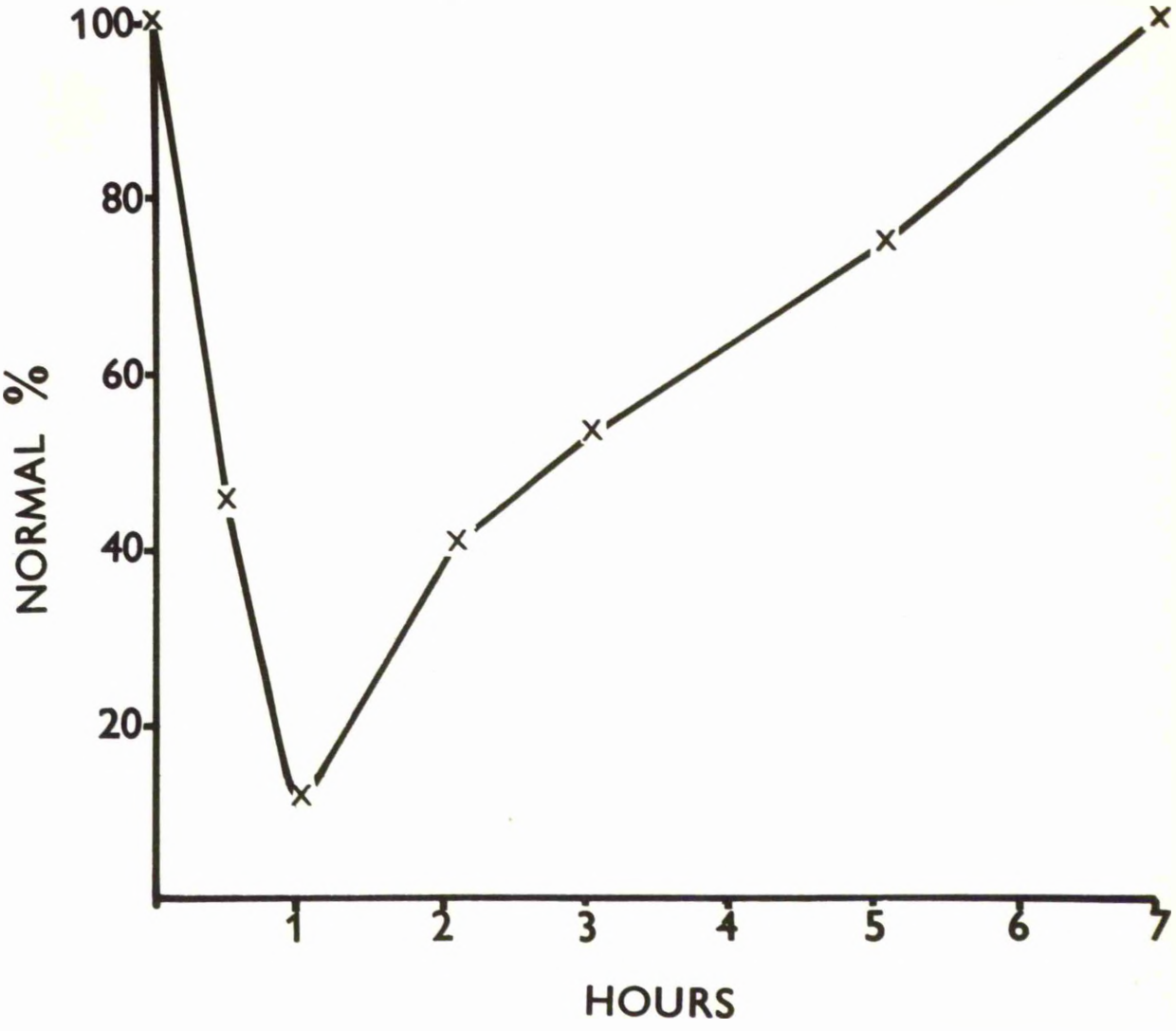



DIAGRAM 11.

Blood glutathione levels after methyl methanesulphonate.

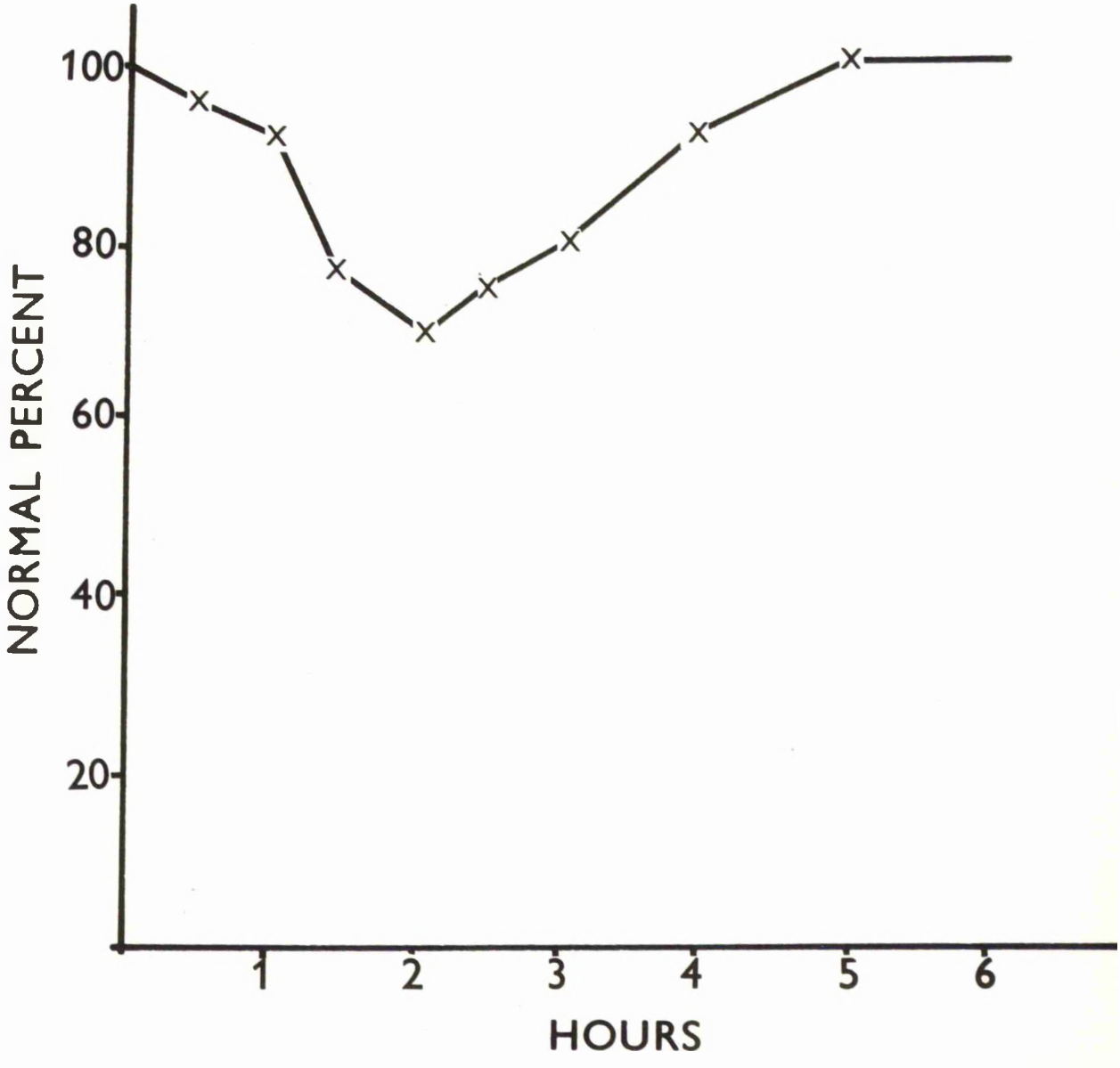



DIAGRAM 12.

series of time intervals are expressed as the percentage of the injected dose in the whole blood. The specific activity of the whole blood per ml is shown in red type. Also shown is percentage of the whole blood activity in the red cells, and the percentage of the injected dose which is therefore associated with the red cells :-

| | 10 m | 30 m | 1 hr | 2 hr | 4 hr | 7 hr | 12 hr | 24 hr | 48 hr | 72 hr |
|---|---|---|---|---|---|---|---|---|---|---|
| % dose in WHOLE BLOOD | 7·2 | 5.3 | 6·7 | 6·4 | 6·7 | 8·7 | 7·7 | 6·0 | 5·9 | 4·4 |
| Sp act. of WHOLE BLOOD | 0·42 | 0·28 | 0·45 | 0·29 | 0·39 | 0·49 | 0·37 | 0·35 | 0·32 | 0·24 |
| % activity RED CELLS | 71 | 71 | 75 | 73 | 63 | 72 | 68 | 66 | 78 | 83 |
| % dose in RED CELLS | 5·3 | 3·9 | 5·0 | 4·7 | 4·2 | 6·3 | 5·2 | 4·1 | 4·6 | 3·6 |

Electrophoresis of the plasma sample showed the activity to be in the albumin fraction. After lysing the red blood cells, there was no activity in the stroma, the haemoglobin being the methylated entity. It was further shown that it was the globin part of the molecule which contained the labelled methyl group.

# DISCUSSION

In any experimental work designed to relate observed biological effects with chemical structure, the purity of the administered material is of paramount importance ; the increased sensitivity of biological systems in general over conventional methods of chemical analysis making this problem particularly acute. The selection of satisfactory criteria upon which to base evidence of purity has not received the prominence in the literature that it deserves.

Soon after detailed studies were begun on methyl methanesulphonate, it became available commercially. This material was however, unexpectedly toxic to rats and mice, killing all 10 animals in the group within 24 hours at a dose which had hitherto been regarded as a tolerated dose (100 mg/kg). The toxic material was effectively removed by washing the commercial sample with sodium carbonate and redistilling the ester. Although the nature of this impurity has not been ascertained, the possibility that it was free methanesulphonic acid, produced by hydrolysis of the methyl ester, was ruled out as the free acid was not toxic in rats at a dose of 200 mg/kg. It is suggested that the commercial sample may be prepared by the reaction of methanesulphonyl chloride with methanol in an ether / pyridine solution :-

$$CH_3SO_2Cl + CH_3OH \longrightarrow CH_3SO_2OCH_3 + HCl$$

The acid chloride may be prepared either by oxidation of the corresponding mercaptan or disulphide with nitric acid, followed by chlorination of the resulting acid :-

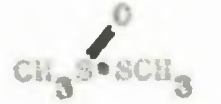
$$CH_3SCH_3 \xrightarrow{HNO_3} CH_3SO_2OH \xrightarrow{SOCl_2} CH_3SO_2Cl$$

or by the direct action of chlorine on the disulphide in the presence of acetic acid :-

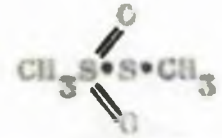
$$CH_3SSCH_3 \xrightarrow{Cl_2} CH_3SCl_3 \xrightarrow{CH_3COOH} CH_3SO_2Cl$$

Both these reactions proceed through a series of intermediates and it is to be expected that isolation of the methanesulphonyl chloride free from other closely related compounds will not be easy to achieve. Subsequent reaction of the impure material with methanol could give rise to numerous contaminants including (39) :-

Methyl methanesulphinate



Methyl methanethiosulphinate



Methyl methanethiosulphonate

Methyl methanesulphonate

By making use of the metathetical reaction between the silver salt of methanesulphonic acid and methyl iodide, the sulphonic acid could not only be redistilled before use but recrystallisation at the silver salt stage further reduced the possibility of contamination of the final product from impurities present initially.

Where long-term biological experiments are envisaged, the conditions under which the drug is stored must also be standardised. Immediately after preparation therefore, the material was sealed in 5 grm quantities into ampoules and stored at -20°. The drug was dispensed for daily use from a glass stoppered bottle contained inside a desiccator stored in the dark at room temperature. These precautions would reduce the risk of moist air being drawn into the bottle which could occur if it was removed from the deep freeze and opened before reaching room temperature. Evidence for the stability of the drug under these conditions was obtained at intervals, chemically by elemental analysis and biologically from evaluation of toxicity data.

## THE METABOLISM OF METHYL METHANESULPHONATE

Evidence that the drug was rapidly

metabolised after injection was obtained when, in both rat and mouse, 30 % of the radioactivity was located in the urine within 96 hours of injection, the majority of this excretion occurring within the first 24 hours. In the rabbit, less than 10 % was excreted in the urine in 7 days, suggesting that either excretion is primarily by another route or that the turnover of labelled material is slower than in the rat. If the latter is true, then the drug, most probably in a bound form since no evidence was obtained for the existence of the free drug after injection, will remain in the system for a greater length of time and this increased exposure time may, in part, explain why the tolerated dose of 30 mg/kg/iv is substantially less than in the other two species studied. As there is no evidence for qualitative variation in the metabolites present with time, the mode of metabolism as reflected in urinary excretion is not dependant upon the concentration of the drug in the system.

After the injection of $C^{14}$-labelled ethyl methanesulphonate into the rat (300 mg/kg), Roberts and Warwick (30) showed that 34 % of the total injected activity was exhaled within 24 hours. This, together with the detection of trace quantities of labelled urea in the urine, led them to suggest that hydrolysis of the drug to ethanol was a major pathway

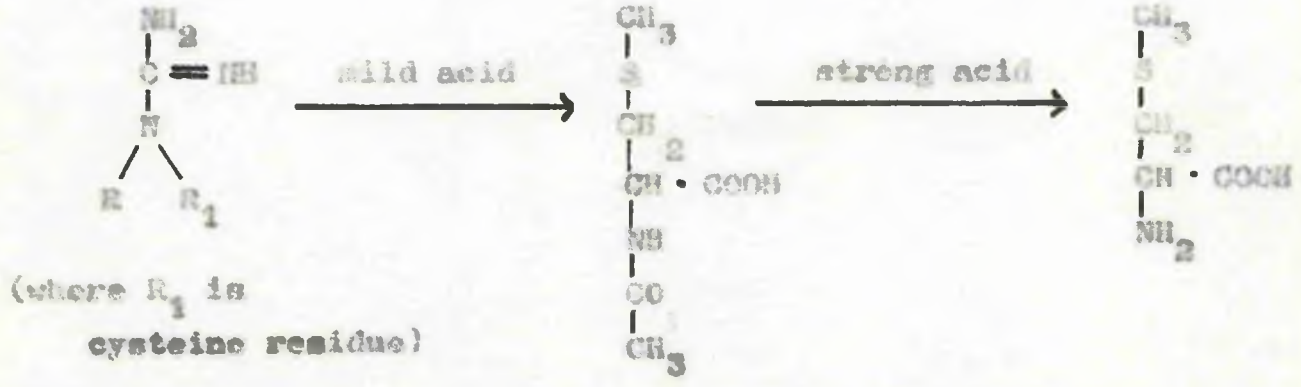
for metabolism. Using $C^{14}$-ethanol injected in an amount equivalent to that which would be formed if all the ethyl methanesulphonate were hydrolysed, they found that the exhalation of $C^{14}O_2$ was extremely rapid and almost quantitative. From this they deduced that the rate of excretion of carbon dioxide after $C^{14}$-ethyl methanesulphonate was a measure of the rate of hydrolysis of the drug in vivo. This infers that the drug remains intact within the animal for several hours. The present studies with methyl methanesulphonate have indicated that only 5 % of the injected drug is excreted as $C^{14}$-carbon dioxide in 24 hours and that although greater during the first 9 hours, excretion is relatively constant during this period. By analogy, it is to be expected that the methyl ester would undergo hydrolysis in vivo to form methyl alcohol. An explanation for the diversity in the rate and quantity of exhaled carbon dioxide is apparent on consideration of the corresponding alcohols. Ethanol is known to be rapidly converted to carbon dioxide by the body (40), acetaldehyde and acetic acid being intermediates in this oxidation (41). The oxidation of methanol however provides the classical example of the conversion by the body of a toxic material to oxidation products which are even more toxic, namely, formaldehyde and formic acid :-

$$CH_3OH \longrightarrow H{\cdot}CHO \longrightarrow H{\cdot}COOH \longrightarrow CO_2$$

Formaldehyde can enter into the metabolism of one-carbon compounds and ultimately give rise to labile methyl groups eg. in choline. It also reacts rapidly with the amino groups of proteins and amino acids. Methanol is also anomalous among the simpler aliphatic alcohols in that it possesses characteristic chronic toxicity by virtue of its much slower rate of oxidation. Using $C^{14}H_3OH$, the rate of oxidation has been shown to be 23 mg/kg/hr compared with 175 mg/kg/hr for ethanol (12). Methanol is, however, the least acutely toxic, the lethal dose by the intravenous route in rabbit being 15•9 grm/kg compared with 9•4 grm/kg for the ethyl homologue (43). It cannot therefore be reasoned that the greater toxicity of a single injection of methyl methanesulphonate compared with that of the ethyl ester is directly due to difference in the metabolism of the corresponding alcohols. However, it seems likely that, by virtue of the greater chronic toxicity of methanol compared with that of ethanol,,repeated or fractionated doses of methyl methanesulphonate would be relatively more toxic than ethyl methanesulphonate.

The figure of 20 % for the urinary excretion of radioactivity after methyl methanesulphonate by the rat agrees very favourably with the results obtained by Roberts and Warwick from their study of the metabolism of ethyl methanesulphonate.

Those authors observed that at least 35 % of the urinary activity was associated with S-ethylcysteine and its derivatives, namely S-ethylcysteine sulphone and N-acetyl-S-ethylcysteine (ethyl mercapturic acid). The present studies with methyl methanesulphonate indicate that approximately 60 % of the urinary activity is associated with sulphur containing compounds but the positive identification in the urine of S-methylcysteine, N-acetyl-S-methylcysteine (mercapturic acid), and N-acetyl-S-methylcysteine sulphoxide accounting for only 23 % suggests that either the remaining sulphur containing compounds are derivatives of compounds other than cysteine or that methylated cysteines are present in a conjugated form. The breakdown of the substituted guanidine component RU 1 on acid hydrolysis to RU 4, N-acetyl-S-methylcysteine, would suggest that one of the substituted groups in the guanidine residue is derived from S-methylcysteine. After strong acid hydrolysis, the mercapturic acid is decomposed to S-methylcysteine:-




(where $R_1$ is cysteine residue)

RU 1 RU 4 RU 3

In agreement with the work of Roberts and Warwick (30), there was no evidence for the presence of the corresponding S-alkylcysteine sulphoxide. They were able to show that after the administration of S-ethylcysteine sulphoxide to rats, none of the material was excreted unchanged. Furthermore, the sulphone was not detected after such an injection ; evidence which led them to suggest that the sulphoxide was not an intermediate in the oxidation of S-ethylcysteine to its sulphone. The presence of N-acetyl-S-methylcysteine sulphoxide after injection of methyl methanesulphonate casts doubt on this reasoning, particularly as Kuchinskas (44) reports the existence of S-methylcysteine sulphoxide in the urine of rats dosed with S-methylcysteine ; an observation confirmed by Barnsley (45). More recently, Barnsley et al. (46), have reported the presence of N-acetyl-S-ethylcysteine sulphoxide in the urine of rats dosed with S-ethylcysteine.

It is of interest to note that the presence of small amounts of S-methylcysteine has recently been reported both in the acid soluble fraction of liver and in the liver protein of rats dosed with the powerful carcinogen and hepatotoxin dimethylnitrosamine (47) (Fig 10).

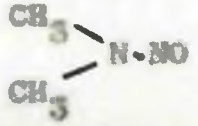


Fig 10

The nitrosamines have been studied recently in connection with their carcinogenic activity and from the limited quantity of data available , it appears that with regard to alkylation reactions, probably through the intermediate diazoalkane, they may give rise to some of the biological effects, e.g. inhibition of cell division, mutation and chromosome abnormalities as the alkylating agents (46).

The presence of trace quantities of radioactivity associated with urea after injection of $C^{14}$-methyl methanesulphonate would infer that the labelled carbon atom has been incorporated into a guanidine derivative at some stage.

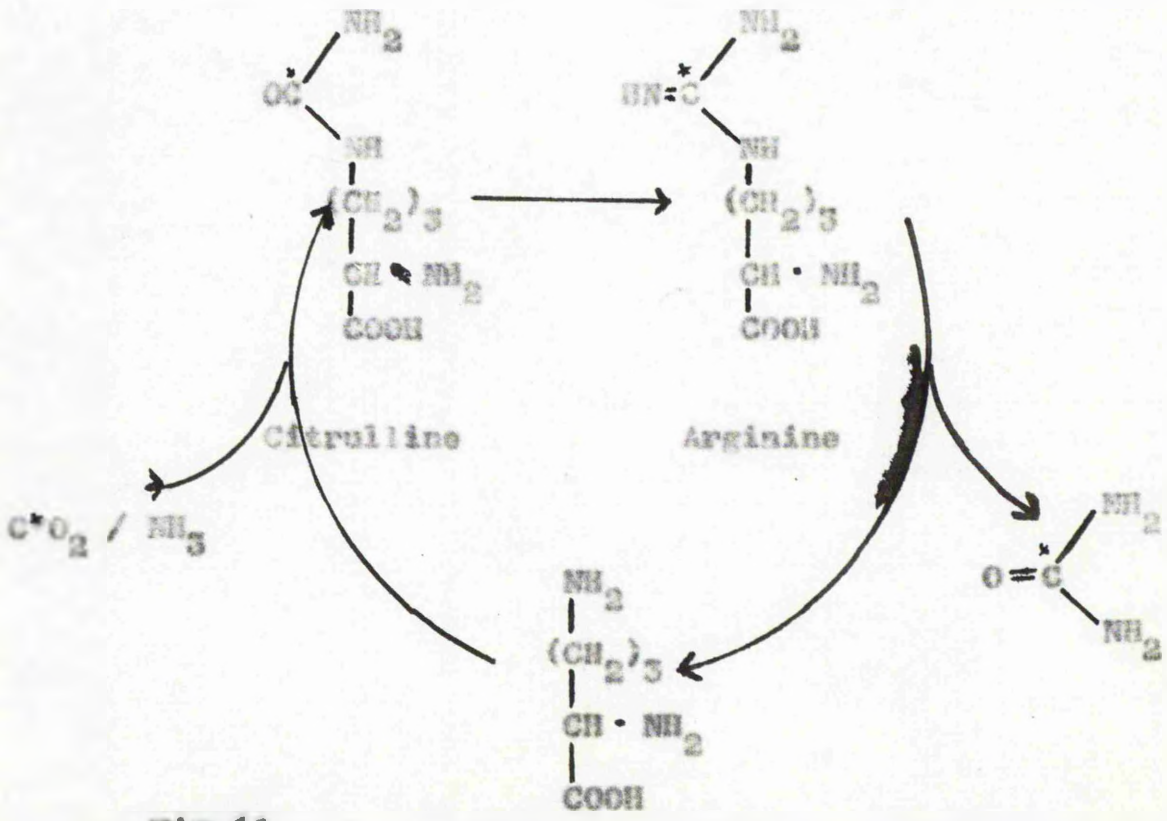



Fig 11

As very little is known about the biochemistry of these compounds other than that they result from protein metabolism, it is not possible to comment constructively on the biological implications of such an observation. It has been suggested that $CO_2$ from methanol oxidation can, in the presence of ammonia, enter the ornithine cycle by reacting with this latter compound to form citrulline. Further addition of ammonia to citrulline produces arginine which under the influence of arginase regenerates ornithine with the simultaneous production of urea. This is illustrated in Fig 11.

Since the work of Baumann and Preusse (49) and Jaffe (30), conjugation with cysteine has been recognised as a route for detoxication of certain aromatic compounds. The detection of ethylmercapturic acid in the urine of rats after ethyl methanesulphonate provided the first instance of mercapturic acid formation from an aliphatic compound (30). The recognition of mercapturic acid in the urine after injecting the methyl ester confirms that the formation of these conjugates represents a general metabolic pathway for this series of compounds. Although Roberts and Warwick do not provide quantitative estimates for the metabolites present, it would appear from a visual comparison of the autoradiographs after ethyl methanesulphonate with those from the present study that the

amount of N-acetyl-S-methylcysteine is considerably less than the corresponding N-acetyl-S-ethylcysteine. Such a discrepancy may be related to the amount of drug given and in making comparisons between the two studies, it should be remembered that the tolerated dose for the ethyl ester is 3 times that of the lower homologue. The presence of N-acetyl-S-ethylcysteine sulphoxide was not noted by Roberts and Warwick.

The immediate source of the cysteine required for conjugation with foreign compounds has been the subject of much speculation. Possible sources are dietary cysteine, cysteine residues of tissue proteins and glutathione. In view of the significance of the thiol group in maintaining the physiological reactions of the cell, the origin of the cysteine for this reaction might provide a lead to explaining the pharmacological effects. In 1937, Barnes and James (51), showed that the administration of dietary cysteine or methionine did not increase the rate of mercapturic acid synthesis in the rabbit. This, together with the observation of Marsden and Young (52), that only a very small proportion of $S^{35}$ given as L-($S^{35}$)-cysteine was excreted as labelled mercapturic acid when rats were dosed with naphthalene, has been taken as evidence that dietary sulphur-containing amino acids are not the immediate

source of more than a trace of the cysteine used for mercapturic acid formation. The larger quantities of mercapturic acid excreted after the administration of some mercapturic acid precursors and the rapid rate at which they are formed makes it unlikely that tissue protein could provide all the cysteine required for theprocess. The possible role of glutathione in furnishing the amino acid was first considered by Waelsch (33). More recently Barnes et al. (34) have reported results which could be explained by assuming that the first stage in mercapturic acid formation in vivo is the formation of S-substituted glutathione.

Chromatographic analysis of bile samples collected by cannulation of the biliary duct in the rat has shown that after the injection of methyl methanesulphonate the principal radioactive metabolite present is S-methylglutathione (B 1) Diag6. The absence of radioactivity in the urine of rats in which the bile duct had been cannulated implies that all the urinary metabolites are derived from material contained in the bile. Metabolites B 4 and B 6 (Diag 6), possess comparable Rf values to MU 8 and MU 9 (Diag 6) which have been recognised in the urine as S-methylcysteine sulphone and N-acetyl-S-methyl-cysteine sulphoxide respectively. Although B 3, a sulphur-

containing amino acid, has not been positively identified it is suggested that this spot represents S-methylcysteinylglycine, an intermediate in the breakdown of S-methylglutathione(B 1) to S-methylcysteine (B 2). The fact that it was unstable on standing and appeared to break down to B 2 is strong evidence in favour of this view. The iodate titration revealed a substantial decrease in the level of free glutathione in the liver subsequent to the injection of the drug, together with the correlation between this result and the maximum excretion of radioactivity through the bile is regarded as further evidence in favour of the liver glutathione being the major source of cysteine conjugates found in the urine. *In vitro* incubation of bile containing radioactive metabolites with intestinal material gave no indication that further metabolism occurred during the passage of bile fluid through the small intestine. Incubation with a kidney homogenate resulted in enzymic degradation of S-methyl-glutathione and S-methylcysteinylglycine to S-methylcysteine.

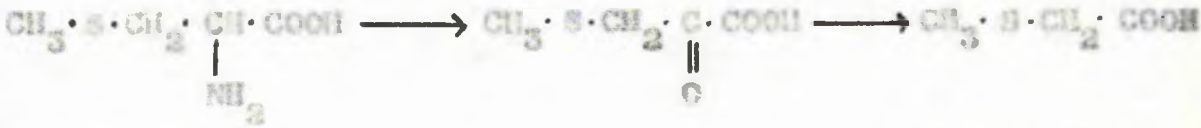
One criticism of the cannulation technique which must be taken into account when interpreting results is that in so far as the animal is being deprived of bile, true physiological conditions are not being maintained. However, preliminary results in which bile is returned to the distal part

of the bile duct by a second cannula, the flow only being interrupted during actual sampling, do not suggest any significant variation in the metabolic picture.

From the results, it appears that the formation of mercapturic acids and other cysteine conjugates involves reaction of the labelled methyl group from methyl methanesulphonate with glutathione of the liver. Although other organs and body fluids contain appreciable amounts of glutathione, for example brain, intestine and peripheral blood, there is no evidence for any appreciable reaction at these sites apart from a slight decrease in the blood level. This would suggest that the reaction in the liver is under the influence of a specific enzyme only present at this site. In support of this theory is the report by Booth et al. (32) that the enzyme responsible for the specific conjugation of organic compounds with glutathione is located in the supernatant fraction of rat liver homogenate. The methylated glutathione then passes down the bile duct where some breakdown takes place, probably under chemical control. The bile metabolites after passing through the small intestine may then be absorbed by the lymphatic system and conveyed via the thoracic duct and the blood to the kidney where degradation is completed before excretion through

the urine. From a study of the metabolites of aromatic compounds, Boyland (55), believes that after passing through the kidney, the metabolites return to the liver for acetylation to form mercapturic acid. As the formation of mercapturic acid does not apparently form a major route for the metabolism of methyl methanesulphonate, it was not possible to confirm this.

The identification of S-methylthioacetic acid and N-(methylthioacetyl)glycine, although representing less than 10 % of the total urinary radioactivity of treated rats is of interest. Barnaley (45), has reported the presence of these two compounds in the urine of rats dosed with S-methyl-cysteine and suggests that they may be formed by oxidative deamination (Fig 12).



S-methylcysteine — S-methylthioacetic acid

Fig 12

Deamination under the influence of the amino acid oxidase found in liver and kidney is one of the general pathways for amino acid

degradation. Decarboxylation may occur in the presence of bacterial enzymes. Whilst such degradative procedure is theoretically possible, there is little evidence for this sequence of reactions taking place *in vivo*. Dubnoff and Borsook (86) were able to show that in the presence of rat liver suspensions, dimethylthetin was able to methylate homocysteine under anaerobic conditions, methylthioacetic acid being formed as a demethylation product.(Fig 13).

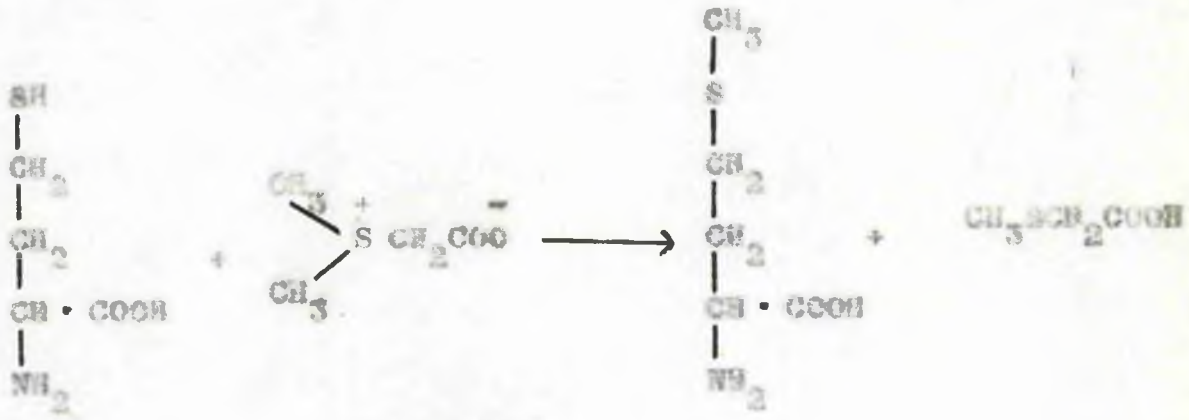


Fig 13

This explanation, concerning the mechanism for biological transmethylation, presents a more plausible pathway for the formation of thioacetic acid derivatives.

In preparing a balance sheet to account for the injected radioactivity, activity retained in the tissues was determined. The 24 hour level of radioactivity in liver, kidney, spleen and testis (13·2 %) , blood (6·0 % ),

together with the activity in urine (17·7 %) and volatile metabolites (3·1 %) account for a total of 42 % of the injected activity. Although excretion of volatile metabolites not retained by the potassium hydroxide traps,.e.g. sulphides and unchanged drug, cannot be ruled out, it appears that the majority of the injected radioactivity is retained within the animal body. Calculation of the specific activities of the body organs including lung, heart, brain, thymus, salivary gland, seminal vesicles, stomach, intestine, adrenals, skin, fat and muscle does not suggestfurther localisation in these tissues other than a general level of approximately 0·2 % of the dose per grm. Assuming that an animal weighed 250 grm after removal of the major organs, an average specific activity of this order would account for 50 % of the radioactivity injected, which makes an almost quantitative account of the drug injected. It would appear from this that widespread methylation throughout the whole animal has taken place.

As far as the relative levels of radioactivity of individual tissues with time is concerned, the results are not particularly significant. The maximum level in the liver between 1 and 2 hours after injection corresponds with the minimum level of glutathione in that organ and this in turn is related to the excretion of radioactive metabolites in

the bile which reaches a maximum value at 90 minutes(Fig 9). Although the excretion of the labelled group was at a peak at this time, it was not until 5 hours later that the corresponding maximum in the urinary excretion occurred. This problem has not been resolved and is still being investigated.

Assay of the radioactivity associated with whole blood at intervals of time after administration of methyl methanesulphonate showed that the initial level of 7 % of the dose dropped only slowly subsequent to injection implying firm binding of the methyl group with a component of whole blood. Having shown that the methyl group reacted with glutathione of the liver, it was reasoned that after an intraperitoneal injection, reaction might occur initially with the glutathione of the whole blood. This view was encouraged when preliminary fractionation of whole blood revealed that 75 % of the activity in the whole blood after injection of $C^{14}$-methyl methanesulphonate was associated with the red cells. The glutathione content of the whole blood is known to be concentrated almost exclusively in the red cells. Furthermore, estimation of the glutathione content of whole blood after injection did show a decrease in the value as compared with a control. Subsequent systematic fractionation showed conclusively that

almost all the radioactivity was bound to the haemoglobin. What effect alkylation of the haemoglobin will have *in vivo* is not known.

## POSSIBLE BIOLOGICAL IMPLICATIONS OF THE RESULTS

The reaction of methyl methanesulphonate with the thiol group of glutathione, which has been shown to take place in the liver, should be discussed further. It is suggested that the reaction is under enzymic control and is therefore not a general reaction between the tripeptide present at other sites, and the drug. The wide distribution of glutathione in living cells as the principal component of soluble thiol groups, concerned with maintaining the regulatory mechanisms of cell metabolism, emphasises its importance biologically. There is abundant evidence that many hydrolytic, oxidising and reducing, and transferring enzymes are dependant on one or more intact thiol groups for their activity (57). Glutathione itself may play an important role *in vivo* in the protection and reactivation of sulphydryl enzymes. The maintainance of the varied physiological reactions of the cell may be severely impaired if the groups are not available, as would be the case after thioether formation with the methyl group

of methyl methanesulphonate. The classical work of Rapkine (38) has underlined the importance of the thiol group during cell division. The higher content of thiol group in tumour tissue is explained on the basis that the tumour has a higher proportion of cells undergoing division than normal tissue. It can be visualised that the blockage of the thiol group at a critical stage during mitosis could prevent this process taking place, or at least lead to a delay in the cell division. The tumour inhibitory property of some alkylating agents could conceivably be related to a localised reduction in thiol groups within the tumour. Perfusion of the tumour with the agent should be more effective for inhibiting growth than the intravenous route if this were true. Although not understood, the protective action of thiol compounds as a means of modifying the effects of ionising radiation is well recognised. Conversely, if the level of thiol groups were reduced by alkylation, the appearance of phenomena associated with radiation damage, is not necessarily surprising. It must be remembered however, that not all thiol-reacting compounds produce radiation-like effects <u>in vivo</u>.

Taking into account the profound nature of the biological effects of the alkane sulphonates, it would appear that their action is immediately upon the cells themselves

or upon a system which in turn controls cell metabolism. Reference has already been made to the possibility of inhibiting mitosis by reducing free thiol groups at a critical stage during cell division. A further possibility concerns the nature of the alkylation reaction itself, particularly as the alkylating group in this instance is the biologically essential methyl group. There are many forms in which the body may obtain its supply of methyl groups, e.g. choline, methionine and betaine. In the process of transmethylation, du Vigneaud et al. (59) have shown that the animal body is able to usethe methyl group of methionine to methylate certain nitrogen and sulphur containing compounds in the body, as for example in the synthesis of creatine in which the methyl group is derived from methionine. Intimately linked with the metabolism of methionine is the biochemistry of sulphonium compounds. Borsook and Dubnoff (60) showed subsequently that the methyl transfer required adenosine triphosphate. Continuation of this work by Cantoni (61) indicated that the actual transfer of methyl groups from such compounds as dimethylacetothetin took place through the intermediate formation of a sulphonium structure of S-adenosylmethionine.(Fig 14). In the biosynthesis of S-adenosylmethionine, the adenosine triphosphate needed for the conversion of methionine into a methyl donor undergoes dephosphorylation. This constitutes

a new type of enzymic process which is of special interest in that complete and irreversible dephospherylation of adenosine-triphosphate occurs whereas in all other functions, the dephospherylation is only partial.(Fig 15).

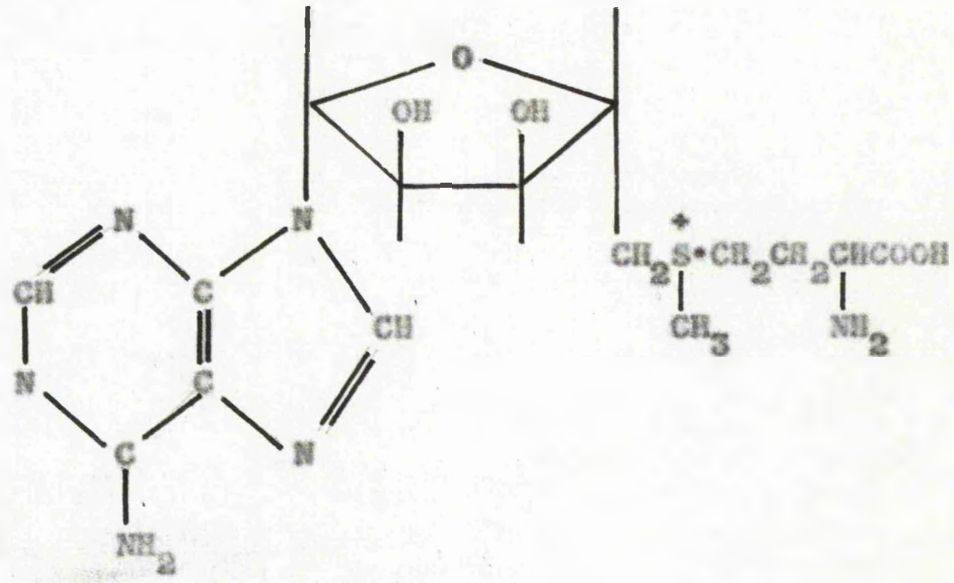


Fig 14.

L-methionine + ATP ⟶ S-adenosyl-L-methionine + Phosphate + Pyrophosphate

Fig 15

The normal breakdown of adenosinetriphosphate with the hydrolysis of the two phospheric acid groups is accompanied by a considerable

decrease in free energy (approx. 10,000 cals). Most of the energy of living matter is derived from oxidative processes of this kind.

It is conceivable that the methyl groups introduced into the animal body by methyl methanesulphonate could take part in the essential methylation reactions through the intermediate formation of a sulphonium compound with adenosine-triphosphate. This unique reaction could be responsible for depriving many cells of the source of energy required to carry out their role efficiently. Obviously more information is required before definite conclusions can be reached. However, from the present study, the identification of labelled S-methyl-thioacetic acid, possibly as a demethylation product from the thetin, and its glycine conjugate, could be accepted as preliminary evidence that the methyl group has entered into the pool of methyl groups concerned with the normal methylation processes of the body. If this is true, then as dimethylthetin itself contains two methyl groups, the amount of methylation which may have taken place could be twice that suggested by the associated radioactivity in the urine. In this connection, it is of interest to note that Stein & Moore (62) have reported that bis-(β-chloroethyl) sulphide (mustard gas) reacts with methionine to form a sulphonium derivative. Similarly, Windmueller (64) observed a reaction

of methionine with ethylene oxide, also resulting in the formation of a sulphonium derivative. Both of these materials are known to alkylate *in vivo*. More recently, Roberts & Warwick (63) have presented evidence for the formation of the sulphonium ion during the *in vitro*. reaction of 2-chloroethyl-arylamines with thiol compounds. The enzyme inhibitor iodoacetic acid has been recognised by Grundlach et al. (65) to react with methionine to form a sulphonium compound.

Any attempt to relate pharmacological effects of a drug with its mode of metabolism is frustrated by a lack of precise knowledge of the metabolic pathways involved. A further complication arises from the ability of the body to devise alternative routes for the synthesis and degradation of the materials it requires to carry on its essential processes should a particular route become impossible. In a reaction as complex as alkylation where several pharmacological effects are evident, it is particularly difficult to establish which biochemical reactions are primarily responsible for the observed effects. For this reason, it is not possible at this stage to assign unequivocally a particular reaction of the alkane sulphonates with their biological activity. It is hoped however,

that by extending these studies to other simple monofunctional and difunctional members of the sulphonic acid series of esters, such an analysis will become possible.

In conclusion, it must be emphasised that, whilst the use of alkylating agents is unlikely to provide the ultimate answer to cancer chemotherapy, experimental studies of their action, particularly at cellular level, should provide valuable information on the control of normal cellular metabolism without which all attempts to regulate the abnormal will be frustrated.

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