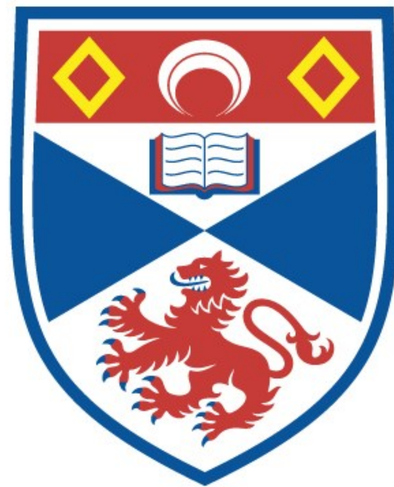
University of St Andrews



Full metadata for this thesis is available in
St Andrews Research Repository
at:
http://research-repository.st-andrews.ac.uk/

This thesis is protected by original copyright

BIOCHEMICAL METHYLATION

WITH

METHYL METHANESULPHONATE

Th 5544

A Thesis submitted by

David. J. Pillinger

for the Degree of Doctor of Philosophy

of the University of St Andrews.

July 1967.

This work was carried out in the Department of Biochemistry, University of St Andrews, ST ANDREWS, and in the Department of Experimental Chemotherapy, Paterson Laboratories, Christie Hospital and Holt Radium Institute, MANCHESTER.

D E C L A R A T I O N

I hereby declare that the following Thesis is based upon 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 under the direct supervision of Dr.G.A.J.Goodlad, B.Sc., Ph.D., of the Department of Biochemistry, University of St Andrews.

C E R T I F I C A T E

I certify that David.J.Pillinger has spent nine terms at research work under my direct supervision, 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 Doctor of Philosophy.

STATEMENT OF RESEARCH

Oct. 1962	Appointed Research Biochemist in Department of Experimental Chemotherapy, Paterson Laboratories, Christie Hospital and Holt Radium Inst., Manchester. Admitted as Research Student (M.Sc.) Ord No 16 (St A).
June 1964	Awarded M.Sc. degree for a Thesis entitled - The Metabolism of Methyl Methanesulphonate.
Oct. 1964	Admitted as Research Student (Ph.D.) Ord No 16 (St A).
Oct. 1965	Leave of absence from Paterson Laboratories for three terms residence in St Andrews, Department of Biochemistry, University of St Andrews.
July 1966	Research continued at Paterson Laboratories, Christie Hospital and Holt Radium Inst., Manchester.

S U M M A R Y

Methyl methanesulphonate has been prepared, labelled with a C^{14} atom in the methyl group, and its metabolism studied in the rat. Subcellular fractionation of liver tissue established the ubiquitous distribution of the labelled methyl group; the majority of the radioactivity being associated with mitochondrial and cell supernatant fractions. Acid extraction of the whole tissue confirmed incorporation of the labelled carbon atom into macromolecules. DNA and RNA, including ribosomal and soluble RNA, were isolated and their specific activities determined at various time intervals after injection. Preliminary studies after repeated daily administration indicated a cumulative build up of radioactivity in the macromolecules with successive doses. The principal sites for reaction at a molecular level were the sulphydryl group present in protein and, in particular, methylation of the cysteine moiety of glutathione, and the N7 atom of guanine in the nucleic acid macromolecule. 7-Methylguanine was also isolated from urine. The mechanism for reaction appears to be a direct methylation although the isolation of labelled choline, formaldehyde and S-adenosylmethionine provide evidence for the entry of the labelled methyl group into normal methylation reactions in the body. The possible biological implications of these results are discussed.

C O N T E N T S

INTRODUCTION Page 1.

Alkylating agents 2.

Alkanesulphonates 4.

Metabolism of methanesulphonates 5.

Methyl methanesulphonate 8.

The concept of biological methylation 11.

The transmethylation reaction 13.

Origin of methyl groups 16.

Regulation of the level of methyl groups _in vivo_ 21.

Methylation of nucleic acids 24.

Biological role of methylation 27.

EXPERIMENTAL 30.

Chemical preparations 30.

Biochemical techniques 36.

Chromatographic and counting techniques 60.

RESULTS 71.

Incorporation of radioactivity into protein 78.

Incorporation of radioactivity into DNA 85.

Incorporation of radioactivity into RNA 89.

Separation of urinary purines 94.

Incorporation of radioactivity into choline Page 97.

Incorporation of radioactivity into formaldehyde 98.

Effect on liver glycogen levels 99.

Effect on the level of ATP 101.

Incorporation into acid soluble liver extract 102.

DISCUSSION 105.

Incorporation into protein 115.

Incorporation into DNA 119.

Incorporation into RNA 122.

The methylation reaction 123.

POSSIBLE BIOLOGICAL IMPLICATIONS OF RESULTS

Methylation of protein 137.

Methylation of DNA 139.

Excretion of 7-methylguanine 141.

Methylation of RNA 142.

Biological role of methylation 143.

BIBLIOGRAPHY 147.

ACKNOWLEDGEMENTS 160.

I N T R O D U C T I O N

The ultimate aim in cancer chemotherapy is the destruction of neoplastic cells. Past experience in chemotherapy has shown that although quantitative differences in cell biochemistry have been utilised initially, the successful application of chemical agents to control disease has ultimately relied on qualitative variations in the susceptibilities of the cells of the host and the invading organism. A pertinent example of this was the treatment of spirochaete infection in the 16th. century with mercury. The sensitivity of the spirochaete was only slightly greater than that of human cells and the amount of mercuric ion required to exert significant control of the disease could only be administered at the cost of appreciable toxicity. Over 400 years later, Ehrlich found that the sensitivity of certain host cells to the noxious effects of arsenical compounds was still the limiting factor in obtaining cures. The search for truly selective agents culminated in the discovery of penicillins, agents that take advantage, not of minor quantitative differences between cells of the host and the parasite, but of a qualitative difference. It is upon such qualitative differences between various types of cells that the most rational approach to chemotherapy can be based.

Knowledge of cellular function and

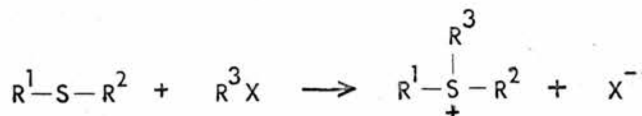
metabolism have rapidly increased with the advent of modern molecular biology. Although a great deal has been learnt of the biochemistry of neoplastic cells, the absence of demonstrable qualitative differences between them and their normal counterparts has emphasised that the problem inherent in a rational approach to the chemotherapy of cancer is enormous.

The work presented in this thesis is designed to extend preliminary studies on the mode of action of the simplest member of a series of alkylating agents used in the chemotherapy of cancer in an attempt to correlate, at a molecular level, the metabolism of the drug with observed biological and physiological effects.

ALKYLATING AGENTS

The term alkylating agent in its widest sense denotes those compounds which are capable of replacing a hydrogen atom in another molecule by an alkyl group. As this involves attack by the alkylating agent at nucleophilic centres, the definition should be extended to include those reactions which involve the addition of a radical to a molecule containing an atom in a lower valency state, for example, the formation of

sulphonium compounds :-



$$R^1-S-R^2 + R^3X \longrightarrow R^1-\overset{\displaystyle R^3}{\underset{+}{S}}-R^2 + X^-$$

Although the biological alkylating agents are a diverse group of organic compounds which include nitrogen and sulphur mustards, ethyleneimines, alkyl halides, nitrosamines, alkyl sulphates, epoxides, alkyl phosphates and alkyl alkanesulphonates, they all possess a common factor in that they are capable of alkylating, under physiological conditions, a variety of sites in biological material. Pharmacologically, they exhibit a diversity of effects which include the capacity to interfere with mitosis, cause mutations and some to initiate and promote malignant tumours. Others are powerful vesicants and lachrymators. They are, furthermore, characterised by a high order of selective action against certain proliferating tissues, namely, haemopoietic cells in bone marrow and lymphoid organs, germinal epithelium, intestinal mucosa and some neoplastic tumours ; attributes which have led to the application of some of them as palliatives in the therapy of cancer.

When attempting to find a parallelism between the pharmacological effects elicited by the alkylating

agents and their chemical reactions at particular cellular sites, it is important to consider, among other variables, the particular mechanism of alkylation involved, since this may well determine the chemical groups which will be attacked inside the cell. The mechanism of action of the alkylating agents has been discussed by Ross (1).

ALKANESULPHONATES

Interest in the mode of action of the simplest members of a series of methanesulphonic acid esters originated from the observation that it was possible to produce selective effects on spermatogenesis with variation in the alkyl group (2)(3). In the rat, the methyl ester produced transient sterility during the second and third weeks after treatment due to interference with spermatids and spermatozoa. The isopropyl ester, however, caused sterility referable to pre-meiotic stages. This antifertility action was associated with damage to genetic factors (4). Although these esters were somewhat less effective by mouth, the remarkable feature was their cumulative action in small divided doses, producing both predictable and fully reversible periods of sterility. It has proved possible to maintain animals on a daily dose of methyl methanesulphonate at

one tenth the LD_{50} for a period of two years without external signs of damage ; the fertility returning to normal when the drug was discontinued.

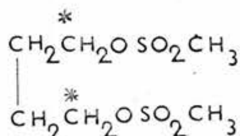
Studies of the effect on the haemopoietic system with the difunctional methanesulphonate of 1,4-butanediol (Myleran, Busulphan), showed that it had a specific depressant action on neutrophils and this has now become the drug of choice in the treatment of chronic myeloid leukaemia. Among the monofunctional alkylating agents, methyl methanesulphonate produced a marked lymphopenia in several species studied only a few hours after administration. In contrast, for the isopropyl ester, the lymphocyte count remained unaffected but a pronounced neutropenia developed ten days after treatment (5).

METABOLISM OF METHANESULPHONATES

Because of the enhanced pharmacological properties, much of the initial metabolic studies on the esters of methanesulphonic acid were performed on the difunctional agent Myleran. One of the earliest of these was made by Peng, who showed that 95 % of the dose of S^{35}-labelled Myleran (4 mg/ kg/propylene glycol) injected into rats, was excreted in the

urine in 32 hours as methanesulphonic acid (6). A selective uptake in the spleen and bone marrow was also claimed. Subsequent studies by Trams et al., indicated that after injection, rapid clearance of the S^{35} label took place from the blood and the highest levels of radioactivity were in those organs concerned with excretion, namely liver, kidney and intestine (7) (8) (9) (10). A comparative study of the urinary metabolites in the rat, mouse and rabbit after injection of S^{35}-Myleran (10 mg/kg) showed that in the rabbit only methanesulphonic acid was excreted, but rat and mouse urine, in addition, contained a little unchanged Myleran, together with two unidentified components(11).

According to Trams, injection of Myleran labelled with C^{14} in the 1 and 4 positions (marked with an asterisk) suggested higher specific activities in kidneys, lungs and liver than in other tissues examined.

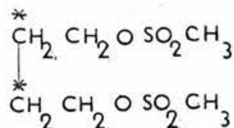


1,4-C^{14}-butane-1,4-dimethanesulphonate

In the liver, only small amounts were associated with fats, proteins and sodium nucleates. The urine contained three major products, also numerous lesser ones, together with unchanged drug.

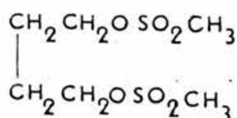
They inferred that the drug had been metabolised to smaller molecules, but did not elaborate this further.

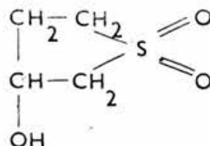
The administration to rats of Myleran labelled with C^{14} in the 2 and 3 positions revealed that only 4 °/o of the radioactivity was exhaled as C^{14}-carbon dioxide in 24 hours.

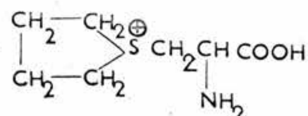


2,3-C^{14}-butane-1,4-dimethanesulphonate

After injection of 2,3-C^{14}-butane-1,4-diol, essentially all the activity was exhaled as C^{14}-carbon dioxide in the same period (12) (13). This suggested that the agent was not converted to the diol *in vivo*. Roberts and Warwick were also able to show that the injection of S-β-alanyltetrahydrothiophenium cation yielded the same urinary product as Myleran (12). This product was identified as 3-hydroxytetrahydrothiophene-1,1-dioxide.





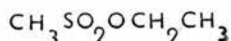


3-hydroxytetrahydrothiophene-1,1-dioxide

Myleran

S-β-alanyltetrahydrothiophenium cation

These authors reasoning that a monofunctional agent might present a simpler picture for analysis than the more complex difunctional agents, studied the metabolic fate of ethyl methanesulphonate (300 mg/kg). Results of this work using C^{14}-labelled material in the 2 position of the ethyl group led to the suggestion that the drug was metabolised by two different routes.



Ethyl methanesulphonate

One involved hydrolysis to ethanol with subsequent exhalation of C^{14}-carbon dioxide, the other resulted in excretion of conjugates of S-ethylcysteine in the urine (14). No attempts were made by these authors to correlate observed metabolic products with the distribution and metabolism of the drug within the animal body.

METHYL METHANESULPHONATE

Preliminary studies on the metabolism and tissue distribution of C^{14}-methyl methanesulphonate have already been published (15) (16) (17). Evidence was presented showing that in the rat the drug was rapidly metabolised after injection. Of the injected radioactivity, 20 % was

accounted for in the urine within the first three days, and, of this, almost 18 % appeared within the first 24 hours. Chromatographic analysis and subsequent autoradiography of the labelled urinary metabolites presented a complex picture for identification. At least 8 radioactive metabolites were present. Although the major metabolite was not identified, up to 60 % of the urinary radioactivity was probably associated with sulphur-containing compounds and of these, conjugated derivatives of S-methylcysteine accounted for at least half this total.

In addition to the urinary excretion of metqbolites, a further 5 % of the administered drug was exhaled as C^{14}-carbon dioxide during the initáal 24 hours. The rate of this excretion increased rapidly at first, reaching a maximum after 7 hours and thereafter tended to fall away only slowly. This was attributed to a relatively slow rate of oxidation of methanol, formed on hydrolysis of the ester, via formaldehyde and formic acid, to carbon dioxide.

Since the work of Baumann and Preusse, conjugation with cysteine has been recognised as a route for detoxication of certain aromatic compounds. (18). The identification of methyl mercapturic acid in the urine of rats

after methyl methanesulphonate confirmed the report by Roberts and Warwick that the excretion in this form represented a general metabolic pathway for this series of compounds (19).

Chromatographic analysis of the bile samples collected by cannulation of the bile duct in the rat showed that after injection of methyl methanesulphonate, the principal metabolite present was S-methylglutathione. The absence of radioactivity in the urine of rats in which the biliary duct had been cannulated implied that the major urinary metabolites were derived from material contained in the bile. Iodate titration of the acid soluble liver extracts revealed a substantial decrease in the level of free glutathione in the liver subsequent to the injection of the drug and the correlation in time between these observations and the maximum excretion of radioactivity through the bile duct is regarded as further evidence in favour of liver glutathione being the major source of cysteine conjugates found in the urine.

The results outlined above indicated that only 25 % of the radioactive methyl groups injected had been metabolised and excreted in 24 hours, by which time, the excretion of C^{14} activity from the animal had dropped to negligible

amounts. Assay of the radioactivity remaining in the major organs and body fluids including liver, kidney, spleen, testis, intestine and whole blood, amounted to no more than a further 20 - 25 % of the injected dose. The remainder of the administered methyl group, 50 - 55 %, was retained in the body other than in these organs, suggesting that widespread methylation had taken place.

THE CONCEPT OF BIOLOGICAL METHYLATION

Prominent among the biosynthetic reactions carried out in vivo is the introduction of an alkyl group into a molecule. The ubiquitous distribution of the methyl group in the body emphasises its importance for the maintenance of normal metabolic reactions. It is of considerable interest in connection with the metabolism of foreign compounds and as such, methylation is a potential reaction of compounds containing hydroxyl, sulphydryl, amino and substituted amino groups. The occurrence of the 8-methyl ether of xanthurenic acid in normal human urine suggests also that isolated phenolic hydroxyl groups can be methylated in vivo (20). The excretion of 2-methylthiouracil after injection of small amounts of thiouracil represents an authentic example of the S-methylation

of a foreign compound (21).

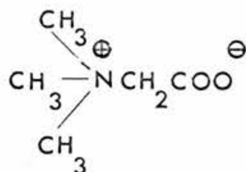
The term biological methylation implies that either an intact methyl group is transferred under physiological conditions from one compound (the donor), to another, as in the case of a transmethylation reaction, or it may refer to the fission of a compound which itself does not contain a methyl group but which gives rise to a molecule of formaldehyde which can be transferred and subsequently converted to a methyl group by reduction.

As early as 1894, Hofmeister suggested that the exhalation of a strong garlic-like odour after the administration of potassium tellurite to animals, noted by Gmelin in 1824, was due to dimethyl telluride formed by transfer of the methyl group already present in the tissues which possess the capacity for methylation (22) (23). The source of the methyl group was not defined. In 1913, Reisser concluded that the methyl groups were probably furnished by choline or betaine (24). This conclusion was unequivocally established by the work of du Vigneaud et al., who showed that homocysteine can replace methionine in the diet of the rat only in the presence of choline or betaine (25) (26). The implication was that the methyl group was

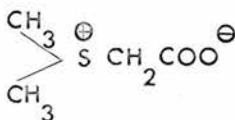
transferred as a whole from the nitrogen of choline or betaine to the sulphur of homocysteine to give methionine. This concept of the biological transfer of the methyl group _in toto_ from one compound to another was shown to be correct when an assay of the deuterium content of the methyl groups of creatine and choline after feeding deuteromethionine to methyl deficient rats did not indicate the intermediate formation of deuteroformaldehyde (25).

THE TRANSMETHYLATION REACTION

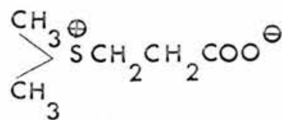
The major pathway for the formation of biologically important methylated compounds is by transmethylation. Reaction involving methyl group transfer can be divided into two types depending upon the nature of the methyl compound involved and the requirement for ATP (27). For those reactions in which the methyl transfer was shown to occur in the absence of ATP, the methyl group was supplied by compounds like betaine, dimethylthetin and dimethylpropiothetin.



Betaine



Dimethylthetin

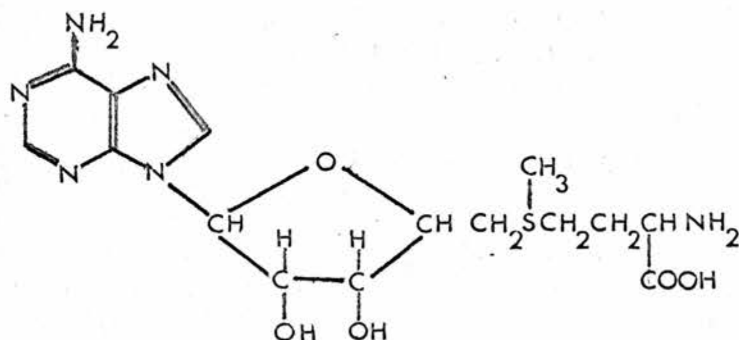


Dimethylpropiothetin

In all cases the methyl radicals are contained in a sulphonium compound. In almost all cases where the methyl group is labile,

it is attached directly to an -onium pole (the exception being arsenocholine). The sulphonium centre places a partial positive charge on the carbon atoms adjacent to it making these susceptible to nucleophilic attack. The acceptor molecules contain nucleophilic sites in which nitrogen, oxygen, sulphur and carbon atoms are the foremost positions for attack.

The main source of methyl groups for the methylation of various compounds *in vivo* is methionine. For this reaction, it was found that ATP and Mg^{++} ions were essential (28). Cantoni showed that ATP does not act as a phosphorylating agent under these conditions but that it takes part in the conversion of methionine to a biochemically active form before the methyl group can be transferred (27) (29). This 'active methionine' was shown not to contain phosphorus and from a study of its properties, Cantoni suggested that it was a sulphonium compound, S-adenosylmethionine, with the following structure (30) :-



S—Adenosylmethionine

His conclusions were shown to be correct when its structure was established conclusively by both degradation and synthesis (31) (32). The finding that this active form of the amino acid is a methylsulphonium compound has greatly clarified the concept of the mechanism for transmethylation.

The biosynthesis of S-adenosylmethionine constituted a new type of enzymic process. A feature of special interest is the complete dephosphorylation of ATP which in all other functions undergoes only partial dephosphorylation. The ease with which the methyl group is severed from the sulphur atom of the sulphonium compound by enzymes is in contrast to the resistance of the C-S bond to chemical cleavage. Unfortunately, no equilibrium measurements have been obtained for any transmethylation reaction. However, an experimental approach has been made by calorimetric determination of the enthalpy changes in several transmethylations (33) (34). It has been shown that methyl transfer reactions from sulphonium poles are exothermic with release of a proton. If this takes place at physiological pH it will make a major contribution to the favourable free energy changes. The free energy of hydrolysis of a sulphonium compound at physiological pH is of the order of the free energy of hydrolysis of the terminal phosphate of ATP.

This leaves little doubt that the sulphonium poles supply the driving force for transmethylations.

ORIGIN OF THE METHYL GROUPS

From the results of the nutritional experiments, it became accepted that the animal organism was incapable of synthesising labile methyl groups and that a supply of methionine, or alternatively choline and homocysteine, was essential in the diet. This concept of the obligatory exogenous origin of a preformed methyl group was at variance with the observations of du Vigneaud, who found that occasionally an animal on a methyl deficient diet, after initially losing weight, started to grow on a homocysteine diet without added choline (35). This suggested that under certain conditions sufficient methyl groups were being synthesised either in the tissues of the animal, or in the intestinal tract, to allow growth to take place. The latter alternative was ruled out when experiments with germ free animals showed synthesis to take place in the absence of intestinal flora (36) (37).

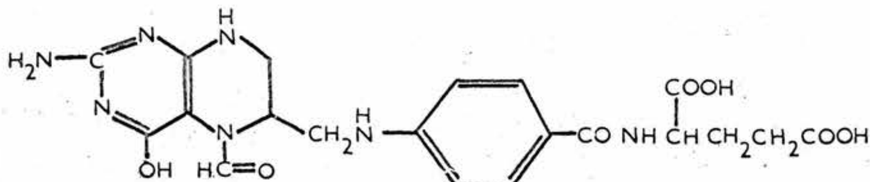
It is now recognised that de novo synthesis of methyl groups does take place and although details

are still obscure, there is evidence that more than one pathway is utilised by different organisms and even by a single organism (38) (39) (40). It is significant that even in the presence of an adequate supply, neogenesis of methyl groups still occurs. It is, however, comparatively small and utilisation of a preformed group is preponderant for transmethylation. No satisfactory evaluation of the relative importance of transmethylation versus methyl group synthesis has been published.

DE NOVO SYNTHESIS

a). Synthesis of the methyl group of methionine.

When tetrahydrofolic acid is incubated with rat liver slices from folic acid deficient rats, N^5-formyltetrahydrofolic acid is produced.

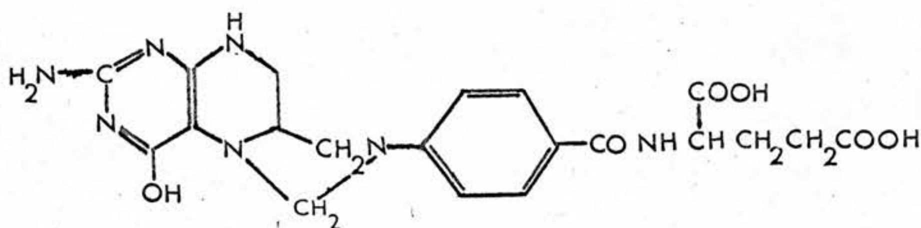


N^5-formyltetrahydrofolic acid

This suggests that a one carbon fragment has been supplied through

the agency of an enzyme. Sakami and Welch showed that in the above system, the addition of folic acid increased the synthesis of methyl groups from added formate (41) (42) (43). This was also true in the intact animal and provided the first incontrovertible evidence for the biosynthesis of methyl groups from formate and formaldehyde.

The first stage in the synthesis involves the transfer of the β-carbon atom of serine to tetrahydrofolic acid to form N^5,N^{10}-methylenetetrahydrofolic acid (Anhydroleucovorin).



N^5, N^{10}– methylenetetrahydrofolic acid .

This reaction is catalysed by serine hydroxymethyl-transferase (EC 2.1.2.1.), and requires the participation of pyridoxal phosphate as a co-factor. The N^5,N^{10}-methylenetetrahydrofolic acid is then reduced to N^5-methyltetrahydrofolic acid. The participation of this methylated derivative of folic acid was suggested by Wilmanns et al., who found that incubation of

N^5,N^{10}-methylenetetrahydrofolic acid with methionine synthetase of pig liver in the absence of a methyl acceptor led to the formation of a folate derivative active as a methyl donor in the formation of methionine (44). This active intermediate was identified eventually as N^5-methyltetrahydrofolic acid (45) (46).

The final step in methionine biosynthesis is the transfer of the methyl group of N^5-methyltetrahydrofolic acid to the acceptor, homocysteine. The requirements for this stage are complex and not yet fully understood, but in addition to homocysteine and N^5-methyltetrahydrofolic acid, ATP, Mg^{++}, NADH, FAD and a vitamin B_{12} containing enzyme are necessary. Recently, it has been observed that the requirement for ATP and Mg^{++} can be replaced by a catalytic amount of S-adenosylmethionine (47). The requirement for vitamin B_{12} was postulated some years ago when it was found that it allowed growth of rats maintained on diets deficient in choline and methionine (48). The exact mechanism has not been deduced, but of interest in this connection is the work of Guest et al. (49). Working with a synthetic analogue of dimethylbenzimidazolylcobamide coenzyme in which a methyl group had replaced the 5'-deoxyadenosine group linked to cobalt (methylcobalamin), they showed that this compound catalysed the formation of methionine when incubated with

homocysteine. This reaction was specific in that the ethylcobalamin homologue was not effective and when cysteine replaced homocysteine, no S-methylcysteine was formed. Final interpretation must await the results of further experimental findings, but it would appear that this methyl transfer step is in contrast to all other methyl transfer reactions in that the apparent methyl donor is not a quaternary compound.

b). Synthesis of the methyl group of choline.

From the rigorous investigations of the biosynthesis of choline by du Vigneaud et al., the concept has arisen that choline is formed by the stepwise transfer of methyl groups from methionine to ethanolamine forming successively methylaminoethanol, dimethylaminoethanol and choline (35) (50). More recent work supports the original idea that there is a single source for all the methyl groups of choline, namely, S-adenosylmethionine (51) (52) (53). There are five possible stages at which methylation could take place, namely, ethanolamine, phosphoethanolamine, glycerylphosphorylethanolamine, CDP-ethanolamine or phospholipidethanolamine. Evidence to date suggests methylation occurs at the phosphatide level.

c). Synthesis of the methyl group of thymine

In the biosynthesis of the methyl group of thymine, it has been conclusively shown that a tetrahydrofolic acid derivative is the sole source of the one carbon unit. Deoxyuridylic acid is the methyl acceptor in thymine systems (53) (54). It would appear that the thymidylate synthetase reaction has a unique reaction mechanism of its own. The major difference from methionine methyl synthesis is the fact that the methylating agent in the formation of thymine methyl is probably N^5,N^{10}-methylenetetrahydrofolic acid itself. Evidence which supports this is the fact that thymidylic acid never exceeds the amount of tetrahydrofolate derivatives in the system and that when folate is supplied as N^5,N^{10}-methylenetetrahydrofolic acid, tritiated in the folate portion, the tritium is located subsequently in the methyl groups of thymidylic acid (55) (56) (57). It would appear that the reaction proceeds in two stages, one in which a one carbon unit is added to deoxyuridylate and a second in which the hydroxymethyl is reduced to methyl.

REGULATION OF THE LEVEL OF METHYL GROUPS <u>IN VIVO</u>

Normally by transmethylation and remethylation, the level of methionine in the body is kept constant. When the level of methyl groups is decreased, as for example when

the animal is maintained on a methyl deficient diet, growth ceases and histological examination of the tissues shows evidence of fatty infiltration of the liver, leading ultimately to centrolobular necrosis. In addition to this impairment of the functional ability of the liver cells, haemorrhagic degeneration of the kidneys can also be recognised. Administration of methionine or compounds containing labile methyl groups rapidly decreases the accumulation of hepatic fat. This lipotropic action of methionine is associated with the donation of methyl groups to choline which promotes the formation of choline-containing phospholipids,eg. lecithins, which are more readily transferred from the liver to the blood. The urinary excretion of methionine continues unchanged during the period the animals are on a methyl deficient diet (58). However, when methionine is restored to the diet, the excretion rises to an abnormally high figure which indicates that in the first few days after the deficiency period nearly all the methionine supplement is lost in the urine. This would suggest a transient loss of ability to metabolise the methionine had occurred during deprivation.

The effect of methyl groups in promoting growth and preventing pathological changes in the liver and kidney does not imply that the animal will tolerate an excess

of these groups. Addition of 4·8 % methionine to a 12 % casein diet caused marked loss of weight which was interpreted as being due to increased demands on the body to cope with excess of methyl groups, either by oxidation or elimination as creatinine (59). Excess choline does not produce the same éffect however, although an absence of choline aggravates it. Glycine and serine are known to counteract the toxicity of methionine. Catabolism of methionine requires simultaneous breakdown of fat which may account for an increased acetone and β-hydroxybutyric acid excretion in these circumstances.

Almost all the dietary excess of methyl groups taken in as methionine is metabolised to carbon dioxide. Studies by Mackenzie et al., showed that when rats were fed a synthetic diet containing 20 amino acids including methionine for six to ten days and then given an identical meal containing 0·6 % (optimal growth conditions) of C^{14}-methyl labelled methionine, the rate of appearance of C^{14} as carbon dioxide rapidly settled down to a steady value (60). When the amount in the diet was doubled, the initial rateof appearance of C^{14} was enormously increased, but this too settled down after a period of digestion and absorption lasting 6-7 hours to that of the 0·6 % diet. This was shown not to be due to adaptive enzymes for a high or low level of methionine supplied.

In cases of severe liver damage, in which transmethylation is prevented, or where the main pathways of metabolism are overloaded, methionine accumulates. The organism may then resort to C-S fission to reduce methionine to methanethiol,- a reaction shown to occur in rat liver mitochondrial preparations (61). The complete oxidation of methyl groups supplied in the diet serves as a regulatory mechanism in the maintenance of the methyl balance within the animal, since the higher the methionine content of the diet the greater the proportion of S-methyl carbon converted to respiratory carbon dioxide.

METHYLATION OF NUCLEIC ACIDS

In addition to the four main bases present in the primary structure of both DNA and RNA, nucleic acids also contain several minor components, many of which are methylated analogues of the four normal bases. Systematic analysis of DNA from various sources has shown that apart from thymine (5-methyluracil), there also exists in plants and animals 5-methylcytosine as a minor constituent and in bacterial DNA, 6-methylaminopurine (62) (63). In RNA, the majority of the minor bases, between 2-3 % of the total RNA, have been found in

soluble RNA and although those present in ribosomal RNA have not yet been characterised, at least ten methylated bases have been demonstrated in transfer RNA, including 2-methyladenine (64), 1-methylguanine (65), 6-methylaminopurine (66), 6-dimethylaminopurine (67), two methylated guanines in yeast RNA (68), together with the pyrimidines thymine (66) and 5-methylcytosine (67).

The existence of methylated bases in nucleic acids has presented somewhat of a paradox, principally because it was difficult to conceive how transfer RNA with its ten methylated bases could be derived by complementary alignment from DNA, which contains only one methylated base. Furthermore, no precursors of methylated bases have been found in any tissues examined. The Watson-Crick hypothesis for the replication of DNA itself or its transcription to transfer RNA offers no mechanism for the determination of the sequence of methylated bases in the nucleotide chain.

The discovery by Borek et al., that a particular mutant of E. coli $K_{12}W_6$, auxotrophic for methionine, was able to synthesise RNA after transfer to a medium lacking its required amino acid provided the clue for resolving these paradoxes. On examination of the properties of the RNA made

during methyl group starvation, they found that although the newly synthesised RNA appeared to be normal, the proportion of methylated bases, including thymine, in the soluble RNA fell as more RNA accumulated in the absence of methionine (70) (71). Since many methylation reactions were known to involve the transfer of the methyl group from methionine, the observation suggested that a similar transmethylation was involved in the formation of the methylated bases. Using C^{14}-methyl labelled methionine, it was found that all the methylated bases in RNA possessed identical specific activity, indicating that they were all derived from the same methyl pool, and that even the methyl group of thymine in RNA was derived from this source.(72).

This latter observation was in antithesis to the established evidence that in DNA the methyl group of thymine was derived from an entirely different source, namely, the transfer of the methyl group from N^5,N^{10}-methylene-tetrahydrofolic acid to deoxyribouridylic acid and pointed to a dichotomy in the pathway for the synthesis of thymine for RNA and DNA. It was later confirmed that as in many other methylating reactions involving methionine, the active agent was S-adenosylmethionine (5).

The mechanism for the insertion of the methylated bases into DNA and RNA was resolved when enzymes were isolated which activate transmethylation at the polymer level of preformed transfer RNA. In E. coli, at least six enzymes are involved in the methylation of RNA, one for each methylated base formed and two for the formation of 1-methylguanine (74). This list may be incomplete because it is now known that ribosomes appear to contain methylases that are probably specific for ribosomal RNA (75). In DNA, it would appear that one enzyme, again using S-adenosylmethionine, gives rise to 5-methylcytosine and 6-methylaminopurine (76).

BIOLOGICAL ROLE OF METHYLATION

The presence of as many as 10 distinct enzymes for methylation of specific nucleic acids would suggest a highly functional role in the metabolism of the cell. From the available experimental data, it is possible only to speculate on the part played by the methyl group in controlling cell reactions. The unique specificity of the methylases for a particular species would suggest that methylation may confer individuality on the respective nucleic acids, thereby protecting the structure against the action of nucleases or

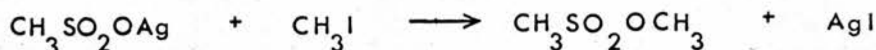
alternatively prevent the integration of a foreign DNA from an infective organism (77). The absence of methylated bases in viral RNA and messenger RNA makes it unlikely that methylation plays a role in coding for amino acids (77). Methylation as a means of punctuation in the transcription of DNA has also been put forward but the variability of the number of bases in DNA of different organisms would argue against this (78). The presence of methyl groups in ribosomal RNA has been taken to indicate a role in the aggregational function of ribosomal precursors (79). A further suggestion is that methylation may play an important part in determining the specific secondary structure of transfer RNA by maintaining it in a single-stranded configuration over a certain region by blocking the hydrogen bonding groups. This however, must be reconciled with the fact that polyribothymidylic acid has a highly ordered structure (80). The methylation of DNA does not interfere with the primary information of the code but it may confer structural form (78). The modifications of the RNA methylases in different biological systems, eg. metamorphosis of insects and tumour tissues, involving phenomena associated with certain metabolic processes suggests the enzymes may be involved in regulatory functions (81) (82). It is evident that although much information has been obtained concerning the site and extent of methylation,

its ultimate biological function remains obscure.

E X P E R I M E N T A L

A. CHEMICAL PREPARATIONS

PREPARATION OF METHYL METHANESULPHONATE



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

Commercial methyl iodide was redistilled before use and the fraction distilling between 42 - 43° was retained as a colourless distillate. Silver methanesulphonate (203 g), prepared as a white crystalline solid by the addition of a slight excess of dry silver oxide to a solution of methanesulphonic acid in acetonitrile, was dissolved in the minimum volume of acetonitrile (1000 mls), with stirring. Methyl iodide (142 g) was dissolved in a further quantity of acetonitrile (100 mls) and added. Precipitation of silver iodide began immediately and was complete after stirring the reaction mixture for 5 hours, during which time, the contents of the flask were shielded from light. Silver iodide was filtered off and the solvent removed from the filtrate under reduced pressure. Thr remaining liquid was distilled and the fraction distilling between 86 - 90° @ 12 mm was retained as a colourless liquid.

Yield 91·0 g (85 % of theoretical).

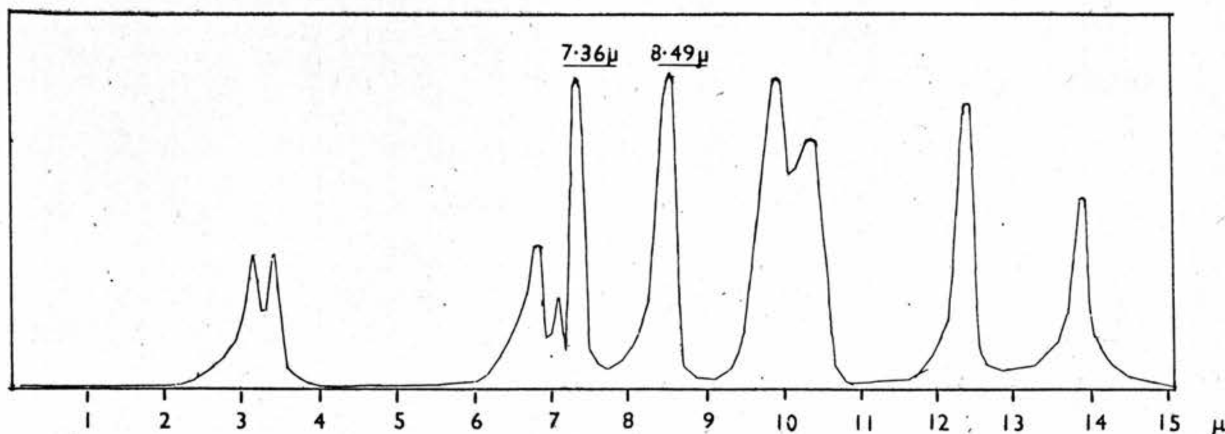
After washing with potassium bicarbonate to remove any free acid and drying over sodium sulphate, the ester was finally redistilled to give a colourless, odourless liquid.

Boiling point 88 - 90° @ 12 mm.

Immediately after preparation, the ester was sealed in 5 g quantities into sterile glass ampoules and stored at -20°. Evidence for the stability of the drug under these conditions was obtained at intervals, chemically by elemental analysis and biologically by evaluation of toxicity data.

Elemental Analysis	C = 21·83 %	H = 5·36 %	S = 29·07 %
Required for $C_2H_6O_3S$	C = 21·82 %	H = 5·46 %	S = 29·10 %

Infra red analysis as a liquid film between sodium chloride plates showed absorption at 7·36 μ and 8·49 μ characteristic of the $R \cdot SO_2 \cdot O \cdot R'$ group.



I R spectrum Methyl methanesulphonate

Confirmatory evidence for the structure of methyl methanesulphonate was provided from the nuclear magnetic resonance spectrum which showed two resonance signals having shifts of 3·04 and 3·94 with respect to the internal standard. These were assigned to the protons present in CH_3S- and CH_3O- environments respectively.

PREPARATION OF C^{14}-METHYL METHANESULPHONATE

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

The radioactive ester, containing a C^{14}-methyl group, was prepared by the method described above for the unlabelled drug. Silver methanesulphonate (6·0 g) was dissolved in acetonitrile (30 mls) in a 100 ml flask fitted with a magnetic stirrer. C^{14}-methyl iodide (2 mc), diluted to 4·1 g with inactive halide, was supplied by the Radiochemical Centre, Amersham. The methyl iodide was transferred to the reaction flask using a dropping pipette and the ampoule washed out several times with small quantities of solvent. After stirring for five hours, protected from light, silver iodide was removed by filtration and the filtrate transferred to a distillation flask. Acetonitrile was removed under reduced

pressure and the product subsequently isolated as the fraction distilling between 84 - 85° @ 11 mm. The drug was stored at -20°.

ABSOLUTE SPECIFIC ACTIVITY

C^{14}-methyl iodide contained 2 mc in 4·10g. This reacted with 5·86g silver mothanesulphonate to give a theoretical yield of 3·175g methyl methanesulphonate. Actual yield 2·182g. Percentage yield 69 %

2·182g labelled product contain 1·374 mc

Absolute specific activity = 630 μc/g.

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

PREPARATION OF 7-METHYLGUANINE

Triacetylguanosine was prepared by the method of Bredereck (83). Guanosine (5·0g), dried in vacuo, was warmed gently with acetic anhydride (50 mls) in pyridine (60 mls) as solvent. On cooling, crystals of the triacetyl compound separated out. The product was recrystallised from

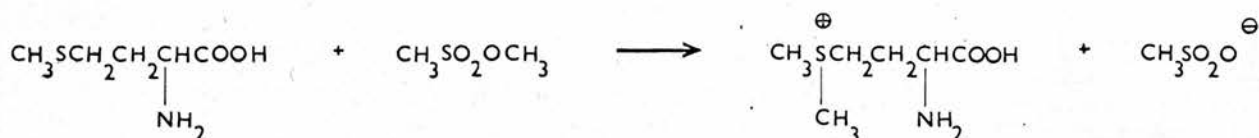
absolute alcohol.

Triacetyl guanosine (1·8 g) was dissolved in methanol (60 mls) and acetone (10 mls) and cooled to 0° in an ice-bath. A solution of diazomethane in ether (20 mg/ml) was added until the presence of a permanent yellow colour suggested excess reagent. On standing overnight at 0°, a precipitate formed which crystallised from methanol as prisms. These were filtered off and dried _in vacuo_ at 100°.

Melting point 164 - 166°. Yield 0·3 g.

The methylated base was isolated from the nucleoside after treatment with N HCl at 100° for one hour in a sealed ampoule (84). The hydrolysate was evaporated to dryness several times to remove residual acid and the white residue purified by fractionation on an ion exchange column of Dowex-1-formate. Fractions containing the methylated base were located, combined and evaporated to dryness. Residual ammonium salts were removed by sublimation at 40°. The identity of the product was confirmed by chromatography, ionophoretic mobility and by comparing the UV absorption characteristics with those of an authentic sample obtained commercially.

REACTION OF METHYL METHANESULPHONATE WITH METHIONINE



Methionine (5·96 g), 98 % formic acid (60 mls), acetic acid (20 mls) and methyl methanesulphonate (17·6 g) were reacted together at room temperature in the dark for three days. The solution was distilled in vacuo to give a syrup which was soluble in methanol. The methanol was removed and the clear sticky residue left for eight weeks in a desiccator. The syrup became more viscous but did not crystallise.

The sulphonium salt was isolated as the reineckate salt. The reaction product was dissolved in a small quantity of water to which was added a freshly prepared solution of ammonium reineckate (15·6 g) in water (100 mls). Reaction took place immediately to yield a pink precipitate which was isolated by centrifugation, washed with ammonium reineckate solution and dried.

The reineckate ion was removed by dissolving the precipitate in the minimum volume of methyl

ethyl ketone and shaking the pink solution with 0·1 N sulphuric acid in a separating funnel. Dissolved methyl ethyl ketone was removed from the aqueous layer and extracted twice with ether. The ether remaining in the aqueous layer was removed on a water-bath and the solution concentrated to small volume. The UV spectra of the product was recorded. Chromatography on paper showed a single ninhydrin positive area with an identical Rf value to the material isolated after treatment of methionine with methyl iodide under identical reaction conditions.

B. BIOCHEMICAL TECHNIQUES

ANIMALS; FEEDING AND DOSING.

Rats of the American Wistar strain, bred in the laboratory, were fed on a basic diet provided by the Scottish N.E.Agricultural Society (Diet 41B). They were allowed food and water ad libitum.

Animals were injected with methyl methanesulphonate, dissolved in physiological saline, by an intraperitoneal route at a dose of 100 mg per kilogram body weight. This represented the maximum tolerated dose of the

drug.

ISOLATION OF PROTEIN

Total protein was isolated from rat liver essentially according to the procedure of Schneider (85). Livers were removed from rats at intervals after injection with C^{14}-labelled methyl methanesulphonate, frozen immediately in liquid nitrogen and stored at -80° until required.

For the extraction, the tissue was weighed out (500 mg) and homogenised in an ice-cold glass homogeniser with cold 6 % sodium p-aminosalicylate (PAS), (6 mls). The volume was then made up to exactly 10 mls with more PAS. To 3 ml aliquots of the homogenate, in triplicate for each sample, was added cold 100 % trichloroacetic acid (TCA) (0·15 ml), and the contents vigorously shaken. A further amount of ice-cold 5 % TCA (3 mls) was added and the tubes centrifuged at 0°. The supernate was removed by pipette and retained for radioactive assay. The residue was washed with a further 5 mls of cold 5 % TCA and the washings retained. This was repeated with three more 5 ml aliquots of TCA and the supernate retained on each occasion and combined as cold TCA soluble extract.

The residue remaining from this extraction, containing nucleic acids, protein and lipids was washed successively with 5 ml portions of water, ethanol, chloroform - methanol (2:1 $^{v}/_{v}$), ethanol - ether (3:1 $^{v}/_{v}$), ether twice and then dried. The washings were combined and retained. The dry powder was suspended in 10 % sodium chloride (5 mls) and heated on a boiling water-bath for 1 hour to extract the nucleic acids (86). This procedure was repeated twice using 5 ml aliquots and heating for 30 minute periods; the three extracts being pooled for radioactive assay.

The protein residue was extracted three times with 5 % TCA (5 mls) at 90° for 30 minutes to remove any remaining nucleic acids (85). Finally, the protein was washed with ethanol, ethanol - ether (3:1 $^{v}/_{v}$), ether and dried. This represented the total protein fraction of rat liver.

ESTIMATION OF PROTEIN

Protein was estimated using the Folin-Ciocalteu reagent (87). The protein sample (0·2 ml), containing approximately 300µg of protein was mixed well with a freshly prepared alkaline copper solution (1 ml), taken from a

mixture of 2 % sodium carbonate in 0·1 N sodium hydroxide (50 mls) and 0·5 % copper sulphate in 1 % potassium tartrate (1 ml), and allowed to stand for exactly 10 minutes at room temperature. The Folin-Ciocalteu reagent (0·1 ml), diluted to 1 N with acid, was added quickly. After 30 minutes, the optical density of the sample was obtained at 750 mμ. The actual protein concentration was obtained by comparison of the optical density with that on a standard curve, obtained each day using a freshly prepared solution of bovine serum albumin, containing exactly 500 μg per ml.

HYDROLYSIS OF PROTEIN

The protein (200 mg) was hydrolysed in a sealed glass ampoule with 6 N HCl (20 mls) at 110° for 16 hours. Before use, analar hydrochloric acid was redistilled three times, the fraction distilling 109 - 111° being retained. Air was displaced from the hydrolysis tubes before sealing by displacement with argon gas. This reduced to some extent, the formation of black humin during hydrolysis (88).

When hydrolysis was complete, the HCl was removed by diluting the contents with water and evaporating

to dryness several times on a rotary evaporator. Finally, the residue was taken into solution in 0·5 N acetic acid (2·0 mls).

ANALYSIS OF PROTEIN

Samples of the hydrolysed protein in acetic acid solution were diluted to contain the equivalent of 2 mg original protein per ml solution. Aliquots (0·5 ml) were analysed automatically for amino acid content.

Isolation of the individual amino acids present in the hydrolysate was carried out on ion exchange columns according to the technique of Hirs, Moore and Stein (89). The acidic amino acids, aspartic acid, glutamic acid and tyrosine were isolated first by passing the hydrolysate from 15 mgs of dry protein down a column of Dowex-1-acetate, 1 x 30 cms. The basic and neutral amino acids were not absorbed on the column and were collected immediately. Elution was carried out using 0·5 N acetic acid, 3 ml fractions being collected. An estimation of the amino acid content of each of the fractions was obtained by the method of Cocking and Yemm (90). Identification of the individual amino acids was made by comparison of the Rf values with those of authentic samples using thin layer

chromatography on silica gel.

The fractions containing neutral and basic amino acids were combined, evaporated to dryness and taken up in 1 N HCl (2 ml) for fractionation on Dowex-50-H^+, previously equilibrated with 1 N HCl. The resin in the hydrogen form was suspended in 1 N HCl and poured into a water-jacketed column, 1 x 130 cms, maintained at 25°. Fractions (3 mls) were again collected and analysed as described above. After the emergence of glycine, the temperature was increased to 50° to improve the separation of methionine from iso-leucine, and gradient elution begun by running HCl (300 mls, 4N) into a reservoir containing HCl (300 mls, 1N). The pooled fractions containing the amino acid hydrochlorides were evaporated to dryness on a rotary evaporator, recrystallised and the specific activity determined.

For the estimation of the amino acid content of the fractions, 0·1 ml of each was added to 0·2 N citrate buffer (0·5 ml pH 5) in a colorimeter tube. A 2 % solution of ninhydrin in methylcellosolve (1 ml) was added, the solutions mixed and heated for 15 minutes at 100°. When cool, the solution was made up to a convenient volume and the intensity

of developed colour read on an EEL colorimeter using a 626 filter.

ISOLATION OF PARTICULATE CELL COMPONENTS

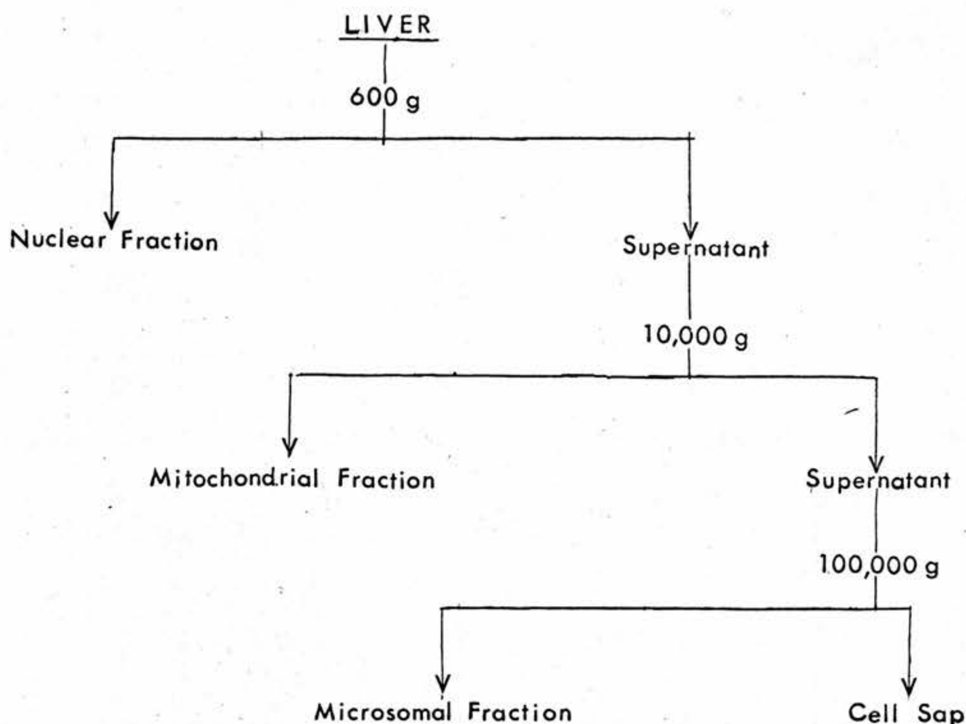
Cell components were isolated using the isotonic sucrose technique of Schneider (91) (92). Rats injected with C^{14}-methyl methanesulphonate were killed 24 hours after injection by exsanguination under ether anaesthesia. Livers were quickly removed and cooled immediately in ice. Tissue samples (2•0 g) were weighed out on an ice-cold watchglass, cut into small pieces and homogenised with cold 0•25 M sucrose solution (12 mls), in a Potter-Elvehjem homogeniser fitted with a teflon pestle. The volume of the homogenate was made up to 25 mls with further sucrose solution and filtered through nylon gauze to remove connective tissue. Aliquots of the filtrate were taken for determination of the DNA and RNA content and for radioactive assay.

The homogenate was centrifuged at 600 x g for 15 minutes at 0°. The residue, consisting of sedimented nuclei, unbroken liver cells and some erythrocytes, was washed twice with 10 ml portions of 0•25 M sucrose and re-homogenised to break up any intact cells before centrifuging

again at 600 x g. The washings were combined and added to the original supernate containing mitochondria, microsomes and cell sap. Aliquots of the supernate and residue were taken for analysis and assay.

Mitochondria were separated from the supernate by centrifuging at 10,000 x g for 20 minutes at 0°. The yellowish-brown residue of mitochondria was re-suspended in 0·25 M sucrose and re-centrifuged. The washings from this were again combined with the previous supernate and aliquots of this, together with the isolated mitochondria, analysed for purity and assayed.

When the above clear yellow supernate was centrifuged at 100,000 x g for 3 hours, a reddish-brown pellet of microsomes was obtained. These were washed with a further 2 mls of 0·25 M sucrose, the washings being combined with the supernate and representing the non-particulate cell sap fraction. Aliquots of microsomes and cell sap were analysed for nucleic acid content and assayed for radioactivity.



ISOLATION OF NUCLEI

Isolation was carried out by the procedure of Hogeboom et al. (93). Samples of liver (2·0 g) were cut into small pieces on an ice-cold watchglass and homogenised in cold 0·25 M sucrose containing 0·0018 M calcium chloride. The volume of the homogenate was adjusted to 20 mls and filtered through nylon gauze to remove intact liver cells. An aliquot (10 mls) of the filtrate was carefully layered onto each of two 10 ml samples of ice-cold 2·2 M sucrose containing

0·0018 M calcium chloride in polypropylene centrifuge tubes. These were centrifuged for 2 hours at 40,000 x g. The cloudy supernate from each tube, containing mitochondria and intact liver cells at the interface, was removed to leave a pale yellow gelatinous pellet at the bottom of the tube. The nuclei were re-suspended in 0.25 M sucrose and a further 5 mls of the 2·2 M sucrose added to the bottom of the tube, underneath the 0·25 M solution. This was re-centrifuged for 1 hour at 40,000 x g. The supernate was discarded, the núclei re-suspended in 0·25 M sucrose and an aliquot taken for estimation of nucleic acids and for radioactive assay.

ISOLATION OF DNA

Deoxyribonucleic acid (DNA) was isolated by the phenol method of Kirby (94). After ether anaesthesia and exsanguination, livers were removed quickly and blotted lightly on filter paper before being dropped into liquid nitrogen.

Rat liver (1 g) was transferred to a Griffiths tube which had previously been cooled in ice, and homogenised in an ice-cold 6 % solution of sodium p-amino-

salicylate (10 mls). The volume of the homogenate was measured and an equal volume of 90% phenol in water added quickly with stirring. The mixture was shaken mechanically for 1 hour. At the end of this time, the straw-coloured emulsion was centrifuged at 3000 rpm for 1 hour. The pale yellow supernate was removed by suction. The dark brown phenolic layer was washed once with a further 10 mls PAS. To the combined aqueous layers after centrifuging was added an equal volume of 2-ethoxy-ethanol. The fibrous strands of high polymer DNA were removed using a glass rod and transferred to 0·2 % sodium acetate (3 mls) to dissolve. The solution was made up to 4 % with respect to sodium acetate and DNA precipitated by the addition of an equal volume of 2-ethoxyethanol. The DNA was removed as before and redissolved in 0·2 % sodium acetate. To the solution was added a further 114 mgs sodium acetate, and a solution of ribonuclease (1.5 mg in 1 ml water) added. The mixture was allowed to remain overnight at 2°. The DNA was subsequently precipitated by the addition of an equal volume of 2-ethoxyethanol and the purification and precipitation repeated three more times. Finally, the purified DNA was taken into solution in 0·2 % sodium acetate (1 ml). Aliquots were taken for DNA analysis and for radioactive assay.

ESTIMATION OF DNA

DNA was estimated using the diphenylamine method of Burton (95). The diphenylamine reagent was prepared by the addition of steam distilled diphenylamine (1·5 g) to a mixture of redistilled glacial acetic acid (100 mls) and conc H_2SO_4 (1·5 mls). Before use, 0·1 ml of aqueous acetaldehyde, containing 16 mg per ml, was added to each 20 mls of reagent. For each analysis, the DNA solution, containing approximately 100 µg in 0·1 ml, was added to a mixture of diphenylamine reagent (2·0 mls), perchloric acid (0·1 ml) and sufficient water to give a final volume of 3·0 mls. Samples in duplicate were incubated overnight at 37°. The optical densities of the solutions were then read at 600 mµ and the values obtained compared with the readings of standard DNA solutions containing 0 - 150 µg DNA.

ANALYSIS OF DNA

DNA was hydrolysed by the method of Vischer and Chargaff (84). The DNA was heated with 1 N HCl in a sealed tube for 1 hour at 100° to yield pyrimidine nucleotides and purine bases. The acid was removed from the

hydrolysate by repeated evaporation and solution, before chromatography on ion exchange columns, according to the method of Weissman (96). Alternatively, the dry nucleic acid preparation was mixed with 70 % perchloric acid (5 mg DNA per 0·1 ml acid) and heated at 100° for 1 hour. The mixture of bases was diluted with water and ground with a glass rod to produce an homogeneous suspension. Centrifuging this removed a black particulate residue and the supernate was used directly for chromatographic analysis. Samples were spotted for paper chromatography and autoradiography.

ISOLATION OF RNA

Rat liver (1·0 g) was homogenised with ice-cold water (10 mls) in a glass homogeniser, which had been cooled in ice. To the homogenate was added an equal volume of 90 % phenol and the mixture shaken mechanically for 1 hour (97). The emulsion was centrifuged at 2000 rpm for 1 hour, the cloudy aqueous layer containing RNA and polysaccharide was removed and the phenol layer washed twice with water. On each occasion, the top layers were removed after centrifuging (2000 rpm, 30 min). The combined extracts were made up to 2 % with respect to potassium acetate and the RNA and carbohydrate precipitated by

the addition of 95 % ethanol (2 vols). Precipitation was allowed to continue overnight at 0°. The resulting precipitate was separated by centrifugation at 2000 rpm for 30 minutes, washed with ethanol - water (3:1), and finally redissolved in water (2o mls). Remaining ethanol was removed in a rotary evaporator at 25°. The aqueous solution was mixed with 2.5 M dipotassium hydrogen phosphate (1 vol), 33•3 % phosphoric acid (0•05 vol) and 2-methoxyethanol (1 vol). After centrifuging, two layers formed in the approximate ratio 5:1 by volume. The upper layer was removed and the lower one washed once with 10 mls of the top layer from a mixture of 2-methoxyethanol - water - 2•5 M dipotassium hydrogen phosphate - 33•3 % phosphoric acid (1:1:1:0•05 by vol). The combined top layers contained all the RNA. To the clear supernate was added two drops toluene as preservative and the solution dialysed against distilled water overnight. The contents of the dialysis bag were then centrifuged and made up to 2 % with respect to potassium acetate and the RNA precipitated by the addition of 95 % ethanol (2 vols). The precipitate was collected by centrifugation, washed twice with ethanol - water (3:1), and dried in a desiccator. The product was a hard, friable, resin-like mass which was soluble in water

ESTIMATION OF RNA

The orcinol procedure, modified by Ceriotti (98), was used for the estimation of pentose. The RNA sample containing approximately 100 µg RNA was mixed well with freshly prepared orcinol reagent (3 mls), made by dissolving pure orcinol in 0·1 % ferric chloride in conc HCl to give a concentration of 10 mg orcinol per ml, was heated for 45 minutes in a boiling water-bath. When the tubes had cooled, the optical densities were read at 660 mµ. A standard curve was obtained using a similar procedure with pure RNA (0 - 150 µg).

ANALYSIS OF RNA

In addition to the acid hydrolysis methods described for DNA, RNA was also subjected to alkaline hydrolysis to liberate nucleotides. The RNA sample was incubated at 37° with 0·3 N potassium hydroxide in a sealed ampoule for exactly 18 hours. The volume of alkali used was in the ratio of 1 ml per 5 mgs nucleic acid. The digest was cooled immediately in ice and brought to pH 1 by the addition of ice-cold 60 % perchloric acid. The insoluble precipitate of potassium perchlorate was removed by centrifugation at 0°.

The residue was washed once, the washings being combined with the supernatant containing the ribonucleotides and adjusted to pH 4 with N KOH with cooling in ice to minimise further hydrolysis of the nucleotides. An aliquot was then submitted to ionophoresis.

ISOLATION OF SOLUBLE RNA

Preparation of the soluble RNA of rat liver was carried out essentially by the method of Brunngraber (99). Rat liver (1·0 g) was homogenised with 1 M sodium chloride (1·5 mls) containing 0·005 M ethylenediaminetetraacetic acid (EDTA) in 0·1 M tris-chloride buffer pH 7·5 in a glass homogeniser cooled in ice. 88 % Phenol (1·5 mls) was added and the mixture stirred thoroughly. The homogenate was centrifuged at 10,000 rpm for 30 minutes at 0°. The aqueous layer was pipetted off and the RNA precipitated by the addition of 95 % ethanol (3 vols) at 0°. After centrifuging and discarding the supernate, the residue was re-suspended in 0·1 M tris-chloride buffer at pH 7·5. The solution was layered on to a column (1 x 10 cms) of DEAE cellulose (2·0 g) which had previously been equilibrated with cold 0·1 M tris-chloride buffer. The column was washed with buffer (20 mls) at pH 7·5

and elution of the RNA commenced using 1·0 M sodium chloride in 0·1 M tris buffer.

UV absorbing material eluted from the column with buffer only was discarded and those fractions containing RNA were combined and freeze dried. The solid residue was taken into solution in distilled water (5 mls) and placed on a column of Sephadex G 25. The column was developed with water. The fractions containing RNA were combined and freeze dried as soluble RNA. Aliquots of the solution were taken for analysis of the RNA content and for radioactive assay.

ISOLATION OF RIBOSOMAL RNA

Ribosomal RNA was prepared by the phenol-m-cresol method of Kirby (100). Liver (1·0 g) was homogenised with 6 % PAS (15 mls) in an ice-cold homogeniser. The phenol m-cresol mixture (15 mls), prepared by the addition of purified phenol (500 g) to redistilled m-cresol (70 mls), water(55 mls) and 8-hydroxyquinoline (0·5 g), was added and stirred for 20 minutes at 20°. The emulsion was centrifuged at 6000 x g for 30 minutes at 5°. To each 100 mls of upper layer was added

sodium chloride (3·0 g) and the mixture re-extracted with 0·5 vol of phenol- m-cresol for 10 minutes at 20° and then centrifuged at 8000 x g for 10 minutes at 5°. The aqueous phase was then removed and mixed with m-cresol - ethanol (1:9, 2 vols) and the mixture allowed to stand for 45 minutes at 2°. The precipitate was centrifuged off and extracted twice with cold 3 M sodium acetate pH 6·0 (5·0 mls). The ribosomal RNA, free from DNA, s-RNA and glycogen, was washed once with a cold mixture containing water, sodium chloride and ethanol in the proportions (25 mls : 1·0 g : 75 mls), then once with 75 % ethanol, twice with pure ethanol and finally dried in a desiccator. An aliquot was taken for radioactive assay and for analysis of the RNA content.

ISOLATION OF URINARY PURINES

For a period of 24 hours after the injection of C^{14}-labelled methyl methanesulphonate, urine was collected over chloroform from two male rats. The animals were maintained with food and water ad libitum in a metabolic cage which allowed collection of the urine free from faecal contamination. The volume of urine excreted was approximately 12 mls.

After separation from the chloroform, the urine was diluted with water and acidified to pH 2 with nitric acid. On standing overnight at 0°, insoluble material separated out. This was filtered off and the clear urine passed through a column (2 x 30 cms) of Dowex-50-H^+ to absorb the purine bases. The column was thoroughly washed with water (1000 mls), and the bases eluted with ammonium hydroxide (500 mls). The ammoniacal eluate was concentrated to 20 mls and acidified to pH 2. M Silver nitrate (7 mls) was added and the mixture stored at room temperature for 24 hours and for a further two days at 5° to complete precipitation. The silver purinate was washed three times with water, collected by centrifugation and decomposed by heating for five minutes with 0·05 N HCl (100 mls) on a boiling water-bath. The silver halide was removed and the purines concentrated to small volume (2 mls) for analysis.

ISOLATION OF CHOLINE

Rat liver (500 mg), together with a saturated solution of barium hydroxide (20 mls), was refluxed on a water-bath for four hours. Fibrous tissue was removed from the hydrolysate by filtration through a Buchner funnel and the clear supernate chilled in an ice-bath. A 3 % methanolic solution of ammonium reineckate was added and the solution

stored at 0° for two hours. The microcrystalline precipitate was filtered off, washed with portions of 0·1 N sodium hydroxide saturated with choline reineckate and finally with n-propanol (2 mls) and dried. The choline reineckate was removed from other contaminating reineckates by solution in acetone. Aliquots of the acetone solution were taken for estimation at 327 mμ and the choline content assessed by reference to a standard curve prepared from samples of known choline content. Aliquots were taken for radioactive assay.

The choline was finally digested with 20 % sodium hydroxide in a flask with an excess of potassium permanganate. The flask was aerated and the evolved gas passed into 0·02 N H_2SO_4. The trimethylamine formed was precipitated as the chloroplatinate.

ISOLATION OF FORMALDEHYDE

Isolation was carried out essentially by the method of Mitoma and Greenberg (101). The cold acid soluble extract of rat liver was made 25 % with respect to trichloroacetic acid and distilled to dryness _in vacuo_, the distillate being trapped in 10 mls of an aqueous solution

containing 0·4 % dimedone (5,5-dimethylcyclohexane-1,3-dione) buffered to pH 7·4. The white crystalline formaldehyde-dimedone complex, methylene bis-(5,5-dimethylcyclohexane-1,3-dione) was filtered off and purified by repeated solution in alkali and precipitation in acid. The distillation was repeated three times until there was no further evidence for the formation of the complex. The dimedone compound was assayed for radioactivity.

ISOLATION OF THE β-CARBON ATOM OF SERINE

The residue from the isolation of the formaldehyde complex was oxidised with periodic acid and the distillation into dimedone repeated. As before, the complex was isolated and assayed.

ESTIMATION OF GLYCOGEN

Glycogen was estimated by the method of Tarnoky et al. (102). The frozen liver (300 mg) was thawed in ice-cold 10 % trichloroacetic acid and after homogenising, was made up to 30 mls with acid, to give a final concentration of 10 mg/ml. This was centrifuged and an aliquot (0·5 ml) of the clear supernate added to 95 % ethanol

(1 ml) for 30 minutes to precipitate glycogen. After centrifuging, the supernate was discarded and the residue digested in 2 N H_2SO_4 (0·2 ml) on a boiling water-bath for 1 hour. the digest was cooled and a 6 % solution of redistilled o-toluidine (8 mls) in glacial acetic acid containing 0·15 % thiourea was added. The colour was developed by heating on a water-bath for 8 minutes. The optical density was determined at 635 mμ against a blank of o-toluidine reagent (8 mls) with 2 N H_2SO_4 (0·2 ml). The values obtained were compared with those from solutions containing known amounts of sugar and using a factor of 0·925 to convert these to glycogen equivalents.

ESTIMATION OF ADENOSINE TRIPHOSPHATE

a) Whole blood.

Blood (1 ml), taken from the vena cava of a rat after ether anaesthesia, was deproteinised with 4 % perchloric acid (1 ml). The precipitated protein was removed by centrifugation at 3000 rpm for 10 minutes at 4°. To an aliquot of the clear supernatant (0·2 ml) was added 0·1 M tris-ethanolamine buffer (2·5 mls, pH 7·6), prepared by dissolving tris-ethanolamine hydrochloride (1·86 g) in distilled water (50 mls), adding magnesium sulphate (125 mg), disodium-

-3-phosphoglycerate (210 mg) and EDTA-disodium salt (50 mg), adjusting to pH 7·6 with 1 N sodium hydroxide and making the volume up to 100 mls. DPNH solution (0·05 ml), containing DPNH-disodium salt (10 mg) in distilled water (1 ml), was also added. The optical density was determined at 340 mμ in a silica-glass cell measured against air. An enzyme suspension (0·05 ml), containing glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9.) (4 mg/ml) and phosphoglycerate kinase (E.C.2.7.2.3.) (1 mg/ml) was added to the cell. After 5 minutes, the optical density was recorded. From the difference between the two values, the concentration of ATP was calculated by reference to the results obtained from a similar assay on a standard ATP solution containing 1 mg/ml. Each assay was performed in duplicate.

b) Liver.

The assay was repeated on cold acid soluble extracts of liver tissue, prepared by homogenising liver (1·0 g) in ice-cold perchloric acid (3 ml) and making the volume up to 5 mls. An aliquot (0·4 mls) was taken for assay and the optical densities determined as before.

FRACTIONATION OF ACID SOLUBLE LIVER EXTRACT

Rats which had received a single injection of methyl methanesulphonate, were anaesthetised with ether, exsanguinated and the livers rapidly dissected out. Samples of tissue (4 g) were weighed out and homogenised in ice-cold 10 % TCA (16·0 mls). The homogenates were allowed to stand in an ice-bath for 30 minutes and were then centrifuged at 0°. The clear supernate was removed and the residue washed with cold 5 % TCA (10 mls), the washings being added to the original extract. The TCA was removed by extracting the combined supernatant solutions with five portions of ether (50 mls), the ether being subsequently removed from the aqueous phase by aeration. The extract (pH 4-5) was fractionated on a column of Dowex-1-formate (1 x 30 cms). After absorption of the extract, the column was washed with water until the elution of UV absorbing material ceased. These UV absorbing fractions which were not retained on the column were pooled. The solution was freeze dried to 10 mls, then cooled in ice and cold 95 % ethanol (10 mls) added. The supernatant from this was freeze dried and chromatographed. The UV absorbing area corresponding to S-adenosylmethionine was eluted off with 0·1 N HCl and aliquots taken for UV analysis and for radioactive assay.

Nucleotides were eluted from the column using a gradient of formic acid - ammonium formate (103). Analysis and assay of the eluted material was carried out by paper chromatography on Whatman No 17 paper, followed by elution from the paper and characterisation from the UV absorption spectra.

C. CHROMATOGRAPHIC AND COUNTING TECHNIQUES

INFRA RED SPECTROSCOPY

The technique of spectroscopy in the infra red region was applied to confirm the identity of methyl methanesulphonate. The sample was prepared as a liquid film between sodium chloride plates and examined over the range 2·5-15·0 µ using a Perkin Elmer model 137, sodium chloride prism instrument, or the model 237 grating instrument.

ULTRA VIOLET SPECTROSCOPY

Nucleic acid degradation products, isolated by chromatography, were identified by comparison of their UV absorption characteristics at pH 1 and pH 9, over the range 200 - 300 mµ, with spectra of authentic samples, using a Unicam SP 800 recording spectrophotometer. Other UV data was

recorded from the Unicam SP 500 spectrophotometer.

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

To obtain confirmatory evidence for the structure of methyl methanesulphonate from the nuclear magnetic resonance spectrum, the ester was dissolved in deuterated chloroform to yield an approximately 30 % solution and placed in the magnetic field. Trimethylsilane was used as an internal standard.

ION EXCHANGE CHROMATOGRAPHY

In all the procedures utilising ion exchange techniques, Dowex Analytical Grade resins were used. The high quality, consistent mesh size and freedom from organic and inorganic impurities permitted use without backwashing or other preliminary treatment.

Anion exchange.

Dowex AG 1 - X8, 200 - 400 mesh was used in the chloride form as a strongly basic anion exchange resin. For fractionation and isolation of nucleic acid components, the resin was converted to the formate form, through an intermediate hydroxy form. The Dowex-1-Cl^- was slurried

into a column (2 x 60 cms) and washed with 1 N sodium hydroxide solution (1750 mls = 9 bed vols), by which time the presence of chloride ion could no longer be detected in the effluent. Excess sodium hydroxide was removed with 4 bed volumes of glass distilled water until the pH was less than 9. The eluting solution was changed to 1 N formic acid (360 mls = 2 bed vols) until the pH was greater than 2. The column was washed with water until pH 4•8 , showing that the remaining formic acid had successfully been washed off the column. The acetate form was prepared likewise, using a 2 M sodium acetate solution in place of formic acid.

Cation exchange.

Dowex AG 50W - X8, 200 -400 mesh in the hydrogen form was used as a strongly acidic cation exchange resin. Until required for use, the resins were stored in a moist condition in dark bottles, as the anion exchange resin in particular was shown to be light sensitive.

PAPER CHROMATOGRAPHY

Single dimension - ascending

Two samples of approximately 10 µl

were applied 4 cms apart on each strip of Whatman No 1 paper 8 x 50 cms, using a fine capillary tube, and dried in warm air. Two solvent systems were found to give adequate separation of components for most purposes.

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 treated with locating agents. Duplicate chromatograms were marked off in 1 cm lengths and the strips assayed for radioactivity by immersing the paper in phosphor and counting in a liquid scintillation counter.

Single dimension - descending

For nucleic acid derivatives, the descending procedure was also used. Samples were spotted 9 cms from one end of a strip of Whatman No 1 paper. At the end of 24 hours, the paper was removed from the tank, dried and spots located by the usual techniques. An iso-propanol/HCl system was found to give adequate separation of the bases contained in hydrolysates.

Peroxides were removed from commercial iso-propanol by shaking the solvent with ferrous

sulphate until acidification of the alcohol and addition of a crystal of potassium iodide failed to yield a brown colour in the solution. The iso-propanol was fractionally distilled and the fraction boiling between 82-84° @ 750 mm collected and stored in the dark. The solvent was prepared by adding the peroxide-free isopropanol (65 mls) to 12 N HCl (16·7 mls) and making the volume up to 100 mls with water. Fresh solvent was made up for each separation. After locating the bases under UV light, the areas were cut out and the UV spectrum characteristics determined, using an equivalent area of paper not containing UV absorbing material as blank.

Two dimension - ascending

To effect a positive identification of the various samples, two dimensional chromatograms were prepared by the ascending technique. The sample 20 µl was spotted on sheets of Whatman No 1 paper (20 x 20 cms) and placed in airtight glass tanks 12" x 12" x 12" containing the solvent. After developing in one direction, the paper was turned through 90° before being placed in the second solvent for development. The solvents used were BA as first solvent followed by phenol - ammonia. The latter was prepared by adding 125 mls of water to 500 g phenol in a dark bottle and leaving overnight to

produce a homogeneous solution. Before use, 0·88 ammonia (1 ml) was added to each 200 mls of liquid phenol. It was found that the presence of a small quantity of solid sodium cyanide in a beaker inside the tank increased the resolution of the system.

THIN LAYER CHROMATOGRAPHY

For identifying the individual amino acids present in the eluates from ion exchange separation of protein hydrolysates, thin layer chromatography produced adequate resolution of components in two to three hours. Plates (20 x 20 cms) were prepared using a suspension of Silica gel G (60 g) in water (30 mls). The slurry was poured into a Shandon spreading device to produce a layer 250 µ thick, and the plates dried in air. Samples (5 µl) were spotted, dried and the chromatogram developed in BA. Amino acids were located with a 2 % solution of ninhydrin in acetone and the position of the spots compared with authentic samples of the amino acids.

AUTORADIOGRAPHY

A permanent record of the exact position of areas of radioactivity after paper chromatography

and ionophoresis was made by exposing the paper to X-ray film for an appropriate length of time. The film used was conventional Ilford Red Seal X-ray film (25·4 x 30·5 cms).

Before exposure, the developed chromatograms and ionophoretograms were located on a piece of stiff cardboard (25 x 30 cms) so that the surface in contact with the film should remain flat. The film and cardboard, protected from light, were left in a darkroom for 4 - 6 weeks. The exposed film was then developed and fixed in the usual manner.

AMINO ACID ANALYSIS

Analyses were performed on the amino acids present in aliquots of protein hydrolysates equivalent to 1·5 mg of original protein, using the Technicon Auto Analyser. The sample was placed on the column of Chromobed resin, previously equilibrated with buffer at pH 2·87, under nitrogen pressure. Elution of amino acids was carried out with a combination of citrate buffers pH 2·87, pH 3·8 and pH 5·0, using an Autograd 9-chamber mixer. The column was maintained at 60° during chromatography. Analysis of the colour developed when the eluate reacted with ninhydrin at a temperature

of 90-95° was obtained using three colorimeters, two set at 570 mμ and having 16 mm and 8 mm cells, and the third using a 16 mm cell at 440 mμ. The absorptions were recorded automatically as the log of the optical densities. For quantitative estimation comparison was made with the optical densities produced by a standard solution of the amino acids present at a concentration of 1 μM per ml, except for cysteine, 0•5 μM per ml, and proline 2•0 μM per ml. At the end of each analysis, the column was regenerated using 0•2 N sodium hydroxide solution.

IONOPHORESIS

An aliquot of the nucleotide solution was spotted 9 cms from one end of a strip of Whatman 3MM paper (72 x 5 cms). Additional material was added where necessary after drying the spots in a stream of warm air - excessive heat, which causes spontaneous formation of isomeric nucleotides, was avoided. The strip was then moistened with 0•02 M citric acid - trisodium citrate buffer pH 3•5, prepared by a 50 fold dilution of a mixture of 3 vols M citric acid and 1 vol trisodium citrate. The paper was then suspended over a glass rod above an electrophoresis tank so that the two ends were immersed to a depth of 2 cms in two separate glass dishes

containing 500 mls buffer. Electrical contact was made by two platinum electrodes dipping in the dishes and connected to a source of DC current, so that the anode was in the dish furthest away from the spot on the paper. The DC supply consisted of an EEL power pack delivering up to 600 volts. The ionophoresis was carried out for 18 hours at a potential gradient of 8 volts per cm. The apparatus was covered during the run with a glass case to minimise evaporation. At the completion of the run, the strips were removed, dried and the nucleotides located using a Hanovia UV lamp. Identification of the spots was achieved by cutting out the UV absorbing areas, together with a blank area, and eluting with 0·1 N HCl (3 mls). The UV absorption characteristics of each sample were determined at pH 1 and pH 9.

COUNTING TECHNIQUES

Chromatogram scanning.

Where sufficient radioactivity was present, paper chromatograms, after development, were scanned using an instrument designed by Gilbert and Keene (104). This consisted 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° in an ice-bath to ensure constant composition of the gas. Counting was continued for 30 minutes and the accumulated charge read from a meter as total counts per 30 minutes. After subtracting a background, the distribution of activity along the paper was recorded.

Solid counting.

The assay of radioactivity in biological samples was made by plating an aliquot of the liquid on to a 2 cm filter paper disc contained in an aluminium planchette. The contents were then dried to the solid state under an infra red lamp. In this way, a uniform distribution of material was obtained over the surface of the disc. Assay was made using either a thin mica end-window geiger tube, or a windowless gas-flow counter. For the latter, the quenching gas used was pure argon saturated with ethyl alcohol vapour at 0° and the efficiency was just less than 1 %. In all measurements, an absorption factor for each sample was determined using a C^{14} source of known specific activity.

Liquid scintillation counting.

Liquid samples were counted using an IDL Tritomat, a Nuclear Enterprises α/β spectrometer or a Beckman spectrometer. The phosphor used in each case was a solution of naphthalene (80·0 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 (105).

An aliquot of the sample (0·1 ml), dissolved in 4 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 recorded count. The efficiency for carbon using a standard sample of known specific activity was approximately 50 %.

R E S U L T S

A prominent feature of whole body autoradiographs prepared from frozen sections of animals injected with C^{14}-methyl methanesulphonate was the high level of radioactivity present in the liver (15). Preliminary studies on the metabolism and tissue distribution of the C^{14}-labelled ester had shown that approximately 8 - 10 % of the injected dose was retained in the liver and that this value decreased only slowly over a period of days. Radioactivity was still detectable after 10 - 14 days.

Results from the present series of experiments, based upon the radioactive assay of livers from 12 animals, have shown that the average value for the percentage of the initial dose of 100 mg/kg remaining in the liver at 24 hours was 7·90 %. This was equivalent to a specific activity of 0·94 % of the injected dose per gram wet weight of liver.

In order to assess the degree of incorporation of the radioactivity into the various subcellular components, nuclear, mitochondrial and microsomal fractions were isolated by differential centrifugation in isotonic sucrose from liver samples taken 24 hours after injection. In addition to

microscopic examination, estimation of the DNA and RNA content of the various fractions was employed as a measure of the success of the fractionation procedure. Results are expressed as a percentage of the initial dose remaining in the liver after 24 hours and are the mean of values obtained from duplicate determinations on four animals (Table 1).

TABLE 1

	% of dose injected			DNA	RNA
	4hrs	16hrs	24hrs	mg/g	mg/g
Nuclear (crude)	1·91	1·12	1·00	2·63	0·41
Nuclear (purified)	–	–	0·47	1·90	0·08
Mitochondrial	2·70	2·41	2·30	–	0·63
Microsomal	0·96	2·03	1·84	–	6·05
Cell supernate	11·03	3·64	2·76	–	1·09
Whole liver	–	–	–	2·90	8·40
% Dose in liver	16·60	9·20	7·90	–	–

For comparative purposes, similar fractionation was carried out on livers taken from animals 4 and 16 hours after injection. These values are the mean of duplicate determinations on tissues from two animals in each case.

Results showed that after 24 hours, 1 % of the initial dose was associated with the nuclear fraction.

Microscopic examination showed however, that in this preparation the individual nuclei were agglutinated in large clumps and that it was far from an homogeneous nuclei preparation, containing in addition some free mitochondria, intact liver cells and red blood cells. Analysis of the nucleic acid content of this fraction showed that almost all the DNA present in the whole homogenate was present in this fraction, 2•63 mg/g wet weight of liver, but that 5 % of the total RNA content of the liver was also present, confirming contamination with intact liver cells. Preparation of nuclei in sucrose medium containing calcium chloride reduced this contamination and the nuclei exhibited the morphological appearance characteristic of those in intact cells. The number of cells was less than 1 % of those present initially in the homogenate, but there was evidence for the presence of a structure resembling cell membrane. An estimate of the DNA content suggested that 60 - 70 % of the nuclei were recovered. In this purified preparation, the radioactive content decreased to 0•47 % of the dose injected.

Centrifugation of the supernatant from the nuclear separation in sucrose at 10,000 x g yielded a fraction containing mitochondria on microscopic examination and having low RNA content. 2•30 % of the dose was present in

this particular fraction. On standing at 4° in a refrigerator, rapid decomposition of this fraction took place with the liberation of a sulphurous odour.

A submicroscopic fraction containing microsomes was obtained by subjecting the supernatant from the mitochondrial preparation to centrifugation at 100,000 x g. This fraction contained approximately 75 % of the total RNA present in the liver but only 1·84 % of the injected dose.

The majority of the injected radioactivity, 2·76 % was not associated with any of the cellular particles and remained in the non-particulate cell sap fraction.

An assessment of the distribution of radioactivity in liver tissue was obtained on a chemical rather than a morphological basis using the acid extraction technique of Schneider (85). Preliminary studies were also made on the effect of repeated doses of methyl methanesulphonate on incorporation into nucleic acid and protein.

Four hours after injection of a single dose of C^{14}-methyl methanesulphonate, the majority of the isotope

in the liver, representing 13 % of the initial dose, was extracted with cold 5 % trichloroacetic acid (TCA). A further 0·49 % was soluble in lipid solvents. The residue from this extraction, containing acid soluble, non-lipid phosphorus material was extracted with hot sodium chloride solution which separated off the nucleic acids as soluble sodium nucleates. This fraction contained only 0·34 % of the injected dose. The protein residue contained 1·45 % of the injected dose.

Similar extractions on liver tissue taken 24 hours after a single dose showed that the decrease in the amount of injected radioactivity present at this time, compared with the shorter interval, was reflected in the cold acid soluble fractions. Only 2·68 % of the dose was associated with this extract. There was little change in the lipid extract, 0·38 % and only a marginal increase in the amount present in the protein residue. The sodium nucleates however, accounted for four times the activity detected after 4 hours. The results, which are summarised in Table 2, are based upon an average of duplicate determinations on the livers from two rats for each time interval. At both time intervals, the fractionation failed to account for all the radioactivity thought to be present in the whole homogenate. At 4 hours, 8 % of the radioactivity

present in the liver could not be located, whilst at 24 hours, despite repeated attempts, 10 % was lost during the fractionation procedure.

TABLE 2

	% of dose injected	
	4 hrs	24 hrs
Cold acid soluble extract	12·95	2·68
Lipid extract	0·49	0·38
Hot NaCl extract	0·34	1·45
Protein residue	1·45	1·88
% Dose in liver	16·60	7·90

The fractionation was repeated on samples of liver tissue taken at 48 and 72 hours after a single dose of the C^{14}-methyl methanesulphonate in order to study the persistence of the carbon atom derived from the methyl group. Assay of the radioactivity in the hot TCA soluble fraction remained remarkably constant, suggesting a relatively permanent incorporation of the C^{14} into the nucleic acid components. The labelling of the protein entity also remained consistent over this period. The slight decrease in total radioactivity of

the whole tissue with time could be ascribed almost completely to loss of activity from the cold acid soluble fraction, and, to a lesser extent, the lipid fraction. The results, summarised in Table 3, are based upon duplicate determinations on livers from two animals killed at 48 and 72 hours after injection.

TABLE 3

	% of dose injected		
	24 hrs	48 hrs	72 hrs
Cold acid soluble extract	2·68	2·10	1·87
Lipid extract	0·38	0·27	0·13
Hot NaCl extract	1·45	1·43	1·38
Protein Residue	1·88	1·74	1·58
% Dose in liver	7·90	6·90	6·20

In order to investigate the cumulative nature of the drug when given on a repeated dose regime, the extraction procedures were repeated on the livers from a pair of animals injected with a second and third dose of methyl methanesulphonate (100 mg/kg), given at 24 hour intervals. Three successive doses at this level was the maximum tolerated by the animals.

Results showed that with successive doses, greatly increased amounts of radioactivity could be extracted in the ice-cold TCA soluble fraction. Three times as much was extracted from the liver of the animal given two doses compared with a single dose and six times this amount after three doses. Similar increases in the nucleic acid fraction were noted although only one and a half times the activity was found after two doses and three times as much after three. Table 4 summarises these results which are expressed as total counts extracted per aliquot of liver.

TABLE 4

	1 x 100 mg/kg	2 x 100 mg/kg	3 x 100 mg/kg
Cold acid soluble extract	4950	15320	31790
Hot NaCl extract	3050	4530	8990
Protein residue	3650	7460	12070

INCORPORATION OF RADIOACTIVITY INTO PROTEIN

The protein fraction obtained as a residue from the Schneider procedure was freeze dried and samples taken for accurate radioactive assay and protein estimation. Approximately 100 mgs of each of the dry protein powders was

dissolved in 0·3 ml sodium hydroxide by incubating overnight at 37°. An aliquot (0·1 ml) was then taken for estimation of the protein content and an equivalent aliquot counted immediately in a liquid scintillation counter (IDL Tritomat). The results obtained from this latter assay were far in excess of the values to be expected from the preliminary data evident from assaying aliquots of tissue homogenate. Furthermore, the initial result was much decreased when the same samples were recounted 20 minutes later. This spurious count was eventually attributed to a chemiluminescent reaction between the alkali and a component of the phosphor mixture. An accurate count was obtained by digesting the weighed protein powder (20 mgs) in a solution of pronase (1·0 ml, 1 mg/ml) inphosphate buffer pH 6·4. An aliquot of the digest was taken for counting. From the results obtained for the protein samples, absolute value for the specific activity of the protein after successive doses was calculated. These are expressed in Table 5.

TABLE 5

Specific activity of Protein

Dose	Specific activity
1 × 100 mg/kg	7·40 c/sec/mg
2 × 100 mg/kg	15·00 c/sec/mg
3 × 100 mg/kg	22·00 c/sec/mg

SITE OF INCORPORATION OF RADIOACTIVITY

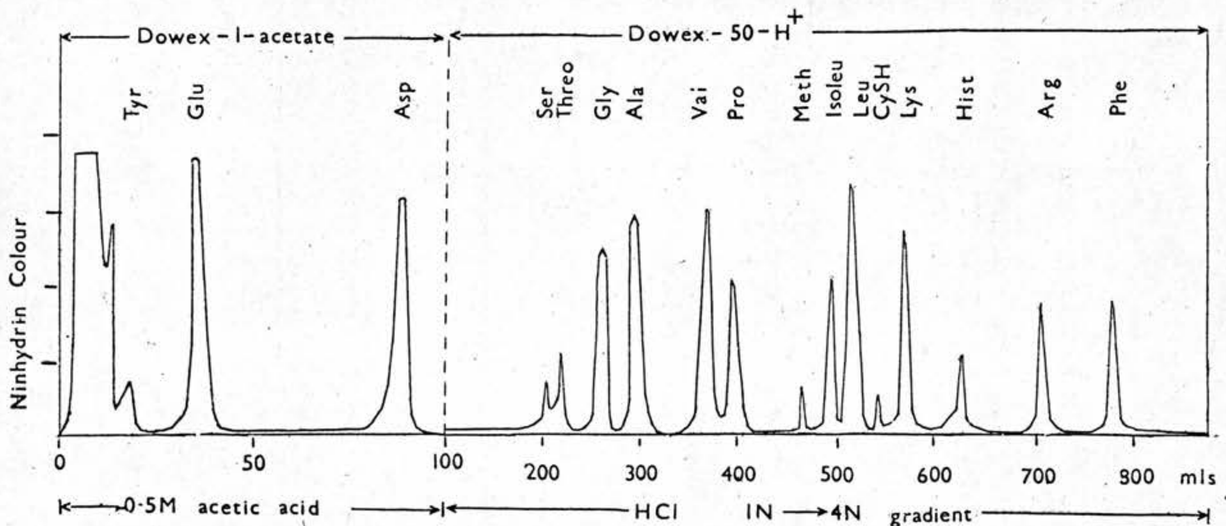
Knowing the extent to which incorporation of radioactivity had taken place, the location of the isotope in individual amino acids was determined. Protein samples (10 mgs), obtained from the livers of animals treated with one, two and three doses of the ester, were hydrolysed in acid and their amino acid content quantitatively compared with a similar hydrolysate prepared from a liver of an untreated rat.

TABLE 6

	Control µM/mg	Treated µM/mg
Aspartic acid	0·327	0·317
Threonine	0·147	0·138
Serine	0·077	0·086
Glutamic acid	0·390	0·417
Proline	0·211	0·188
Glycine	0·264	0·316
Alanine	0·291	0·284
Valine	0·300	0·295
Cysteine	0·019	0·017
Methionine	0·065	0·059
Isoleucine	0·216	0·247
Leucine	0·380	0·380
Tyrosine	0·101	0·118
Phenylalanine	0·179	0·194
Lysine	0·279	0·291
Histidine	0·105	0·100
Arginine	0·190	0·210

There was no evidence for any quantitative variation in the amount of amino acids present in the total liver protein when compared with protein from an untreated animal. There was, however, some evidence for the appearance in trace amounts of a ninhydrin positive substance immediately after proline in the protein sample prepared from the animal which received three successive doses. This was tentitatively identified as S-methylcysteine by comparison with the position of an authentic specimen of the amino acid.

Fractionation of the protein hydrolysate on ion exchange columns enabled isolation of larger amounts of the individual amino acids.



Ion exchange separation of Liver Protein Hydrolysate

Aspartic acid, glutamic acid and tyrosine were isolated and identified in crystalline form after fractionation of the hydrolysate on Dowex-1-acetate. The neutral and basic amino acids were collected within the first 25 mls of eluate and these were followed by tyrosine, glutamic acid and aspartic acid in that order as discrete peaks. All the radioactivity present in the original hydrolysate was present in the basic and neutral fractions. Further fractionation on Dowex-50-H^+ with gradient elution up to 4 N HCl revealed a radioactive material eluting after proline. From the amount obtained however it was not possible to obtain a positive identification nor to estimate the specific activity. For an accurate assessment of the individual labelled amino acids present at such a low level, methyl methanesulphonate of higher specific activity would be necessary.

After hydrolysis of the protein residue (500 mgs) in conc HCl, and subsequent chromatography on Whatman No 17 paper, two major radioactive areas were located. Elution of the paper corresponding to these and successive chromatography in three different solvent systems resulted in the isolation of two chromatographically homogeneous compounds. One of these was positively identified as S-methylcysteine by

comparison of its chromatographic properties with those of an authentic sample of the amino acid. The other had identical Rf values in each of the three solvent systems to a specimen of S-methylcysteine sulphoxide. Furthermore, oxidation of the former material gave a radioactive product identical chromatographically with the second. It is therefore confirmed that the principal product from the action of methyl methanesulphonate on protein is S-methylcysteine.

There was no evidence on these chromatograms for any radioactivity associated with either 1- or 3-methylhistidines. Trace amounts were, however, seen on autoradiographs in areas corresponding to glycine, serine and methionine, particularly in 24 hour liver samples. The amount present was not sufficient to allow estimations of the individual specific activities. There was no loss of radioactivity from the protein sample after acid hydrolysis suggesting that alkylation of free carboxyl and amino groups was not significant. Substantial loss did take place on hydrolysis under alkaline conditions.

After hydrolysis of the freeze dried mitochondrial fraction obtained from the subcellular fractionation

of a 24 hour liver sample, the amino acids were isolated by passing the hydrolysate through a column of Dowex-1-Cl. Radioactive scanning of the paper chromatogram of the material not absorbed by the column showed a similar distribution of the C^{14} atom to that produced from hydrolysis of the whole liver protein residue from the Schneider procedure. The two principal areas of radioactivity were eluted from the paper and subsequently identified as S-methylcysteine and its sulphoxide. The former accounted for 55 % of the radioactivity present and the sulphoxide, a further 25 %. S-methylcysteine was isolated in a chromatographically homogeneous form and a quantitative estimate of the amount present in an aliquot showed that the specific activity of the isolated material was approximately 480 µc/grm.

The distribution of radiocarbon in the hot sodium chloride extract and the particulate fractions of rat liver suggested that an assessment of the relative specific activities of DNA, RNA and their constituent bases after injection of C^{14}-methyl methanesulphonate might provide an indication of the biological importance of alkylation of nucleic acids.

INCORPORATION OF RADIOACTIVITY INTO DNA

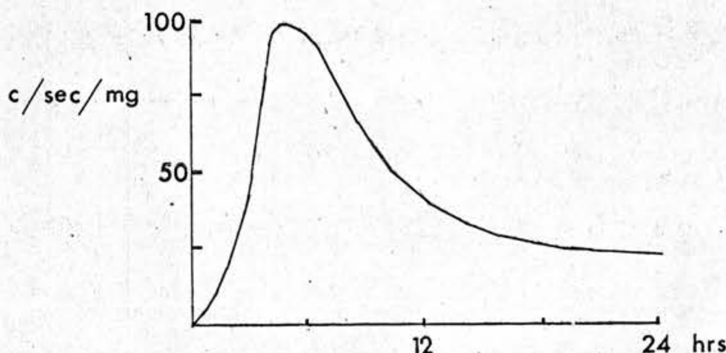
Deoxyribonucleic acid was isolated from the livers of rats using the Kirby phenol procedure (94). Samples were obtained at various intervals after injection in order to study the time course of labelling. Salts were removed from the precipitated DNA by repeated solution in sodium acetate and precipitation with ethanol followed by incubation with ribonuclease to remove RNA. Aliquots were taken for radioactive assay and for analysis of the DNA content using the diphenylamine colour reaction of Burton (95). Contamination with RNA and protein was shown to be minimal.

During the isolation, it was observed that the solution of DNA in acetate was markedly less viscous in treated animals than in untreated controls. In all cases, the amount of high polymer DNA extracted from treated animals was less than that obtained from controls, which suggested in the absence of any demonstrable quantitative loss in DNA content of the liver that the DNA had become fragmented and was not being extracted by this procedure. It was also noted during the preparation that the aqueous phase from the phenol extraction remained clear after centrifugation in comparison with the cloudy

phase obtained from untreated animals. This was shown to be due to polysaccharide contamination present in normal livers.

The sequence of labelling showed a fairly rapid incorporation of radioactivity over the first few hours, reaching a maximum around five hours after injection. Subsequently, the specific activity tended to decrease slowly until, by 24 hours, the level had dropped to 25 % of its maximum value

Incorporation of Radioactivity into DNA



The specific activity of DNA after repeated dosage was also determined to investigate any cumulative action on the nucleic acid. Results showed that the first dose produced the maximum degree of alkylation when the DNA was shown to have a specific activity of 25 c/sec/mg. It would appear from this that each succeeding dose is responsible for

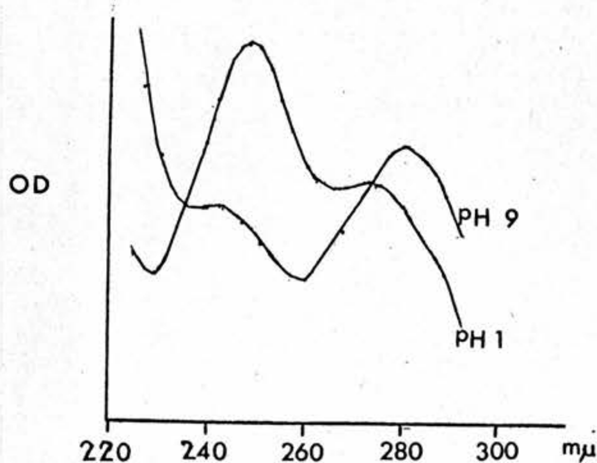
contributing less C^{14} to the isolated DNA (Table 7).

TABLE 7

Specific Activity of DNA

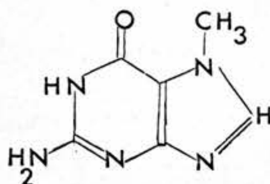
1 x 100 mg/kg	25·1 c/sec/mg
2 x 100 mg/kg	35·6 c/sec/mg
3 x 100 mg/kg	40·6 c/sec/mg

The site of the alkylation in the nucleic acid was determined by isolation of the individual bases from DNA after acid hydrolysis. Hydrolysates were subjected to paper chromatography and ionophoretic analysis. In each case, a single radioactive component was detected distinct from the four normal bases present. The UV spectra of this material eluted from the paper showed a λ_{max} at 250 mμ, with a point of inflection around 275 mμ at pH 1 and at 280 mμ and 245 mμ at pH 9.



UV absorption spectra 7-Methylguanine

Deamination with nitrous acid gave a radioactive material having an Rf value in the solvent systems used identical with 7-methylxanthine. Spectral data showed a shift at pH 1 for λ_{max} from 250 mμ to 266 mμ. This evidence indicated that the labelled material was 7-methylguanine, and the spectral data was consistent with this reasoning when compared with an authentic specimen of this methylated base.



7-Methylguanine

This was confirmed when aliquots of the hydrolysate were fractionated on Dowex -50-H^+ ion exchange resin. A single radioactive peak occurring between adenine and guanine was isolated. This, when refractionated on Dowex-1-formate, gave a single radioactive peak distinguishable from the guanine peak. The UV spectra of the isolated material was not sufficient to allow an accurate assessment of the specific activities of the methylated base to be made after repeated doses.

There was no evidence for alkylation at other sites in the macromolecule. It would seem therefore that the increase in the specific activity of the isolated DNA under these circumstances may be due to an increased methylation of the guanine moiety.

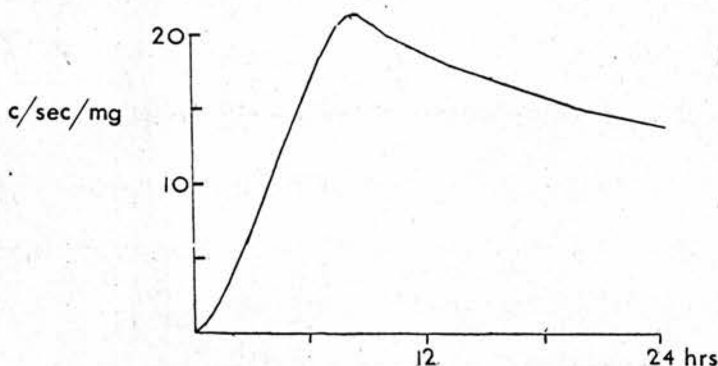
INCORPORATION OF RADIOACTIVITY INTO RNA

Total RNA was isolated from rat liver using the phenol procedure of Kirby (97). After centrifugation of the emulsion obtained by adding the phenol to the homogenate of the liver sample in water, RNA and polysaccharides were present in the aqueous layer and were precipitated out with ethanol. The RNA was then separated from polysaccharides by extraction with 2-methoxyethanol in phosphate buffer. The latter was removed on dialysis and the RNA precipitated as a white flocculent material with ethanol. The precipitate was freeze dried to yield a hard friable mass, easily soluble in water.

The presence of radioactivity from C^{14}-methyl methanesulphonate was detected within the first two hours of injection. From this time, the specific activity built up in a broad peak to reach a maximum around 8 hours after injection. By 24 hours, the specific activity had dropped to

60 % of the peak value.

Incorporation of Radioactivity into RNA



After a single dose of the drug, the isolated RNA had a specific activity of 14·0 c/sec/mg at 24 hours. RNA isolated at a similar interval of time after a second dose showed an increase to 35 c/sec/mg. This was further increased to 43 c/sec/mg after a third. From this it can be seen that the greatest increase in incorporation of C^{14} into RNA occurred after the second dose (Table 8).

TABLE 8

Specific Activity of RNA

1 x 100 mg/kg	14·0 c/sec/mg
2 x 100 mg/kg	35·0 c/sec/mg
3 x 100 mg/kg	43·0 c/sec/mg

Similar chromatographic and ionophoretic

techniques to those used for the analysis of DNA were carried out. Acid hydrolysis of the isolated RNA, followed by fractionation of the purine bases on Dowex-50-H^+ using HCl elution, produced a similar result to the DNA analysis. A single radioactive peak between guanine and adenine was found. Fractions within this peak were combined, freeze dried and re-fractionated on Dowex-1-formate. A white residue was isolated, identical with the product from the DNA hydrolysis and which, on the basis of chromatographic and spectral data, was identified as 7-methylguanine. No other radioactive material could be detected in the hydrolysate.

The demonstration that the RNA had been alkylated after methyl methanesulphonate led to the possibility that the alkylation might be specific to a particular RNA species present in the cell. The microsomal fraction prepared from rat liver had been shown to contain around 20 % of the remaining activity in this organ at 24 hours, and, as 80 % of the cell RNA is associated with the microsomes in the form of ribosome particles, the preparation of ribosomal RNA was undertaken.

(i) Ribosomal RNA

The method used was based upon that of

Kirby (100). A two stage extraction was necessary for the preparation of a stable product. In the first stage, all the nucleic acids were released into the aqueous phase with PAS and m-cresol - phenol, but some protein was also solubilised into this phase. The sodium chloride extraction effectively removed protein. After precipitation with ethanol, DNA, sRNA and glycogen were separated by extraction with sodium acetate. Estimation of the specific activity of the ribosomal RNA showed that after one dose the isolated product had a similar specific activity to the total RNA isolated previously. This was doubled after a second dose to 26 c/sec/mg. A third dose only added a further 2 c/sec/mg (Table 9).

TABLE 9

Specific Activity of Ribosomal RNA

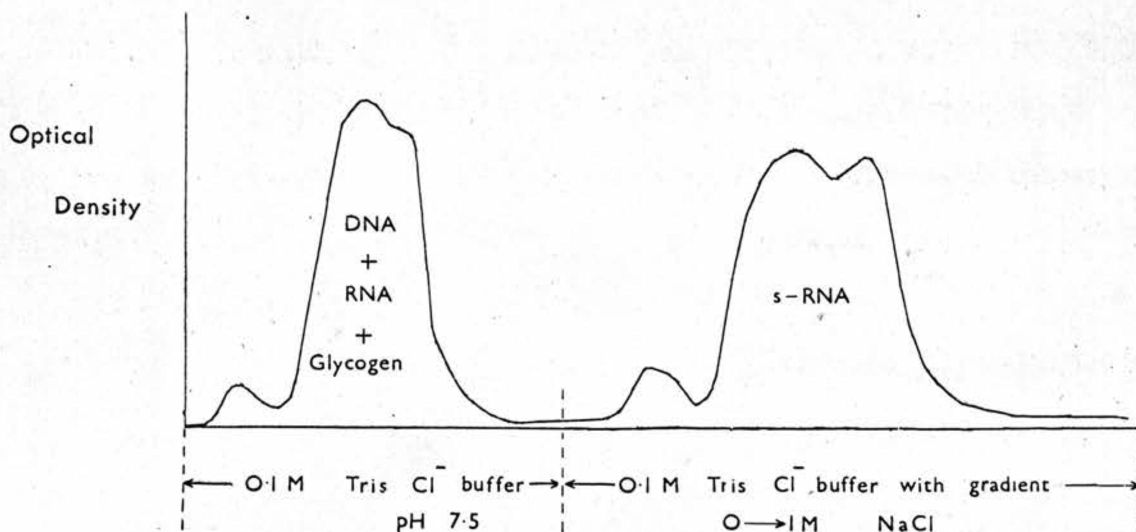
Dose	Specific Activity
1 × 100 mg/kg	12·0 c/sec/mg
2 × 100 mg/kg	26·0 c/sec/mg
3 × 100 mg/kg	28·0 c/sec/mg

Although this preparation of RNA undoubtedly results in a selective separation of the ribosomal component, some soluble RNA may still be associated with the product.

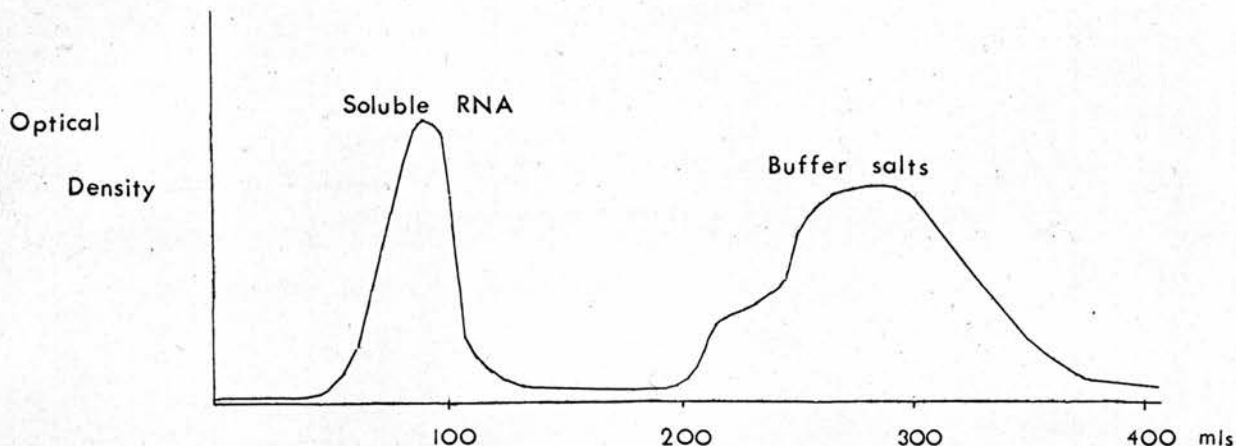
(ii) Soluble RNA

Isolation of soluble RNA was achieved

by the method of Brunngraber (99). The mixture of DNA, glycogen and RNA containing sRNA was fractionated on a column of DEAE cellulose. Only soluble RNA was retained on the column when washed with tris Cl^- buffer pH 7·5 containing EDTA, and this, in turn, was eluted with a sodium chloride gradient O - 1 M.



Fractions containing the sRNA were combined and freeze dried. The sodium chloride was removed by passing the product down a column of Sephadex G 25 and washing the column with water. The material contained in the sRNA peak was isolated and assayed for radioactivity.



The results are summarised below (Table 10).

TABLE 10

Specific Activity of soluble RNA

1 x 100 mg/kg	20·5 c/sec/mg
2 x 100 mg/kg	26·7 c/sec/mg
3 x 100 mg/kg	22·4 c/sec/mg

SEPARATION OF URINARY PURINES

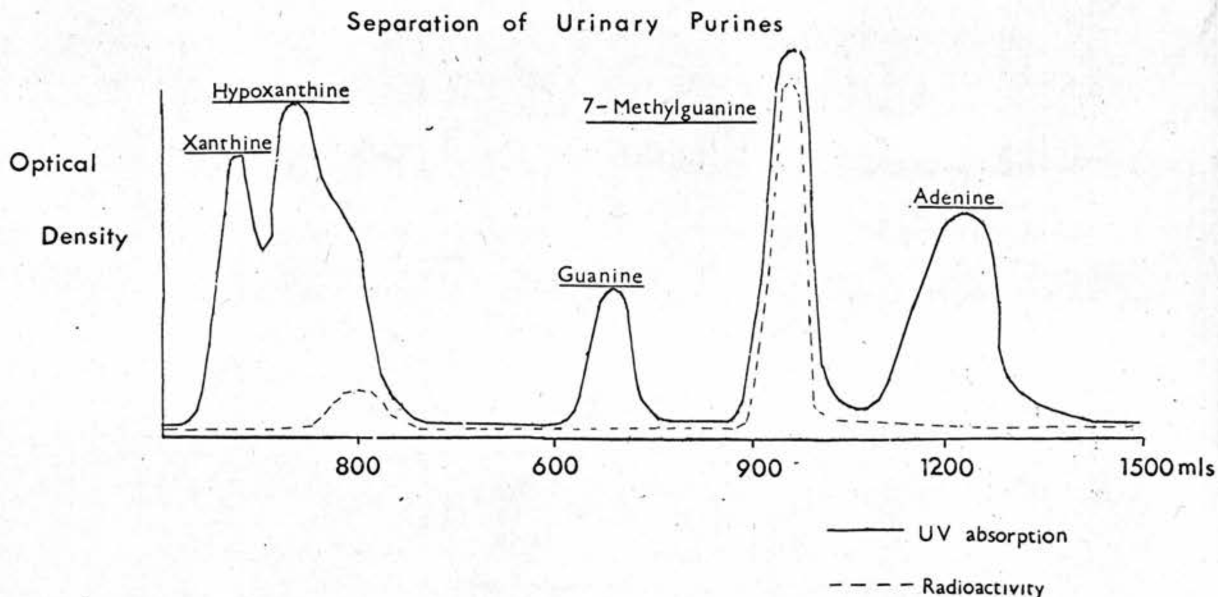
Chromatography of the purine concentrate prepared from the urine of rats treated with C^{14}-methyl methanesulphonate showed that it contained a large number of components and the presence of strongly fluorescing substances having similar Rf values made interpretation of the

results difficult. Autoradiography indicated the presence of one component having a markedly higher content of C^{14} than the other spots. Slightly improved resolution was achieved after chromatography in a second dimension. Areas of strong UV absorption were cut out and the paper eluted with 0·1 N HCl. An absorption curve was obtained from 200 - 300 mμ for each spot.

Isolation of the purine bases was carried out by the method of Weissmann(96). The purine concentrate, representing a 5 ml aliquot of the 24 hour sample was placed on a column 1 x 40 cms of Dowex-50-H^{+} and washed in with 0·05 M HCl (25 mls). The column was developed with an HCl gradient 1 - 4 N, and 10 ml fractions collected. An absorption curve was plotted for each of the fractions and an aliquot taken for radioactive assay.

Five major peaks of UV absorption were obtained and characterised by comparison of their spectra with authentic materials. Only one of these peaks contained radioactivity. The fractions within this peak were combined, freeze dried and the white crystalline solid remaining refractionated on a column of Dowex-1-formate 1 x 30 cms, using a gradient 0 - 0·05 M

ammonium formate. The single radioactive peak obtained corresponded exactly with the peak of UV absorption. The contents of the tubes containing the material were pooled, evaporated to dryness and the ammonium formate removed by sublimation at 40°. The UV spectra of the isolated material was almost identical with that obtained from an authentic sample of 7-methylguanine and with that reported in the literature (106). Furthermore, the positions of the spots obtained on paper chromatography and on ionophoretic analysis corresponded with the synthetic material.



The content of radioactivity in the major bases such as adenine and guanine was practically negligible.

The widespread distribution in the animal of the labelled methyl group from C^{14}-methyl methanesulphonate as well as the excretion of the isotope as carbon dioxide, suggested that the alkyl group had entered the metabolic reactions involving 'one-carbon' transfer. Evidence to confirm this was obtained from the isolation of labelled choline and formaldehyde.

INCORPORATION OF RADIOACTIVITY INTO CHOLINE

Choline was extracted from rat livers taken from animals which had received one, two and three doses of the drug at 24 hour intervals, by refluxing the acid soluble extract with saturated baryta. The choline was isolated as the reineckate. After a single dose of the ester, the choline isolated had a specific activity of 20•6 c/sec/mg, rising to 33•3 c/sec/mg after a second and 60•8 c/sec/mg after a third dose respectively. These are summarised in Table 11.

TABLE 11

Specific Activity of Choline

Dose	Specific activity
1 x 100 mg/kg	20·60 c/sec/mg
2 x 100 mg/kg	33·30 c/sec/mg
3 x 100 mg/kg	60·80 c/sec/mg

That the radioactivity was present in the methyl groups was shown when, after digestion of the choline reineckate in alkali, at least 60 % of the activity was liberated as trimethylamine and precipitated as the chloroplatinate.

These results would further suggest that there is an appreciable amount of the radioactive carbon atom entering the normal methylation reactions in the body.

INCORPORATION OF RADIOACTIVITY INTO FORMALDEHYDE

Formaldehyde was isolated from the acid soluble fraction of rat liver according to the method of Mitoma et al. (101). Assay of the formaldehyde-dimedone complex showed that after repeated purification by solution in alkali and reprecipitation with acid, it still contained radioactivity. It was not possible to assess the specific activity in this experiment however, due to the small quantities of material present.

Similarly, serine in the acid extract was shown to contain a radioactive carbon atom in the β-position.

EFFECT ON LIVER GLYCOGEN LEVELS

During the isolation procedure for DNA, it was observed that the aqueous phenol layer after centrifugation remained clear in comparison with the cloudy samples obtained from control animals which had not received an injection of methyl methanesulphonate. It was suspected that this cloudiness was due to polysaccharide contamination. Further investigation showed that this was in fact due to glycogen present in normal livers. It appeared that injection of methyl methanesulphonate caused depletion of hepatic glycogen reserves almost completely.

Estimation of the glycogen content of rat liver at intervals after a single injection of methyl methanesulphonate confirmed that the level of polysaccharide decreased. One hour after injection, the glycogen contené had dropped to 65 % of the untreated controls, and by 24 hours, only 10 % remained in the liver. It was reasoned that this decrease could be due to loss of appetite induced by the general toxic effect of the drug. The untreated controls and animals receiving the drug were therefore fasted from the time of injection. After 1 hour, the level was 62 % of the control

and this decreased to 25 % at 3 hours. By 5 hours, only 10 % of the glycogen content of the fasted animals remained. These results showed that at short time intervals, the glycogen content in the livers of fasted-treated animals decreased very rapidly compared with livers from fasted-untreated rats, showing that the drop was drug induced and not related to the carbohydrate intake. When feeding was resumed after 24 hours, the level in the control animals increased to more than twice the initial level, but returned to normal after a further 24 hours. This was not the case in the treated livers. At 48 hours, the glycogen content was still 50 % lower than the original control level and by 72 hours, this had only risen to 75 % of control. Even after 4 days, there was still a difference of 15 - 20 % but this discrepancy was no longer evident at the end of one week. Results are tabulated in Table 12.

TABLE 12

Liver Glycogen Levels (mg/grm wet wt)

	1	3	5	18	24	48	72	96	168 hrs
Untreated Control	25·4	23·6	24·9	—	26·1	27·6	—	—	—
Fasted Control	24·5	22·2	21·8	6·0	1·7	52·6	28·1	27.3	27.8
Fasted–MMS–Treated	14·6	5·5	2·1	—	0·6	13·6	21·1	22·7	26·4

EFFECT ON THE LEVEL OF ATP

The effect of administration of methyl methanesulphonate on the level of ATP present in the acid soluble extracts of rat liver and whole blood was studied using an enzymatic technique. The change in optical density of the DPNH solution when oxidised to DPN^+ was utilised as a measure of the ATP concentration. Control animals received an injection of saline only and in each case, estimations were performed in duplicate and results expressed as an average of the duplicate determinations on two animals.

In whole blood taken from treated animals $^1/_2$, 1, 2, 4 and 24 hours after injection with methyl methanesulphonate, there was no significant difference in the level of ATP when compared with control animals. The level in the latter averaged 18·4 µM/100 mls blood and in the treated samples varied between 15·9 and 18·0 µM/100 mls, without any correlation with time.

In liver extracts, the control level of ATP ranged from 1·60 to 1·97 µM/grm tissue with an average value of 1·78 µM/grm tissue. The ATP concentration in

treated livers was only slightly lower than this, 1·69 µM/grm, ranging from 1·48 to 1·76 µM/grm. These results suggested that, under these experimental conditions, there was no significant effect on the level of ATP in animals which had received the drug.

FRACTIONATION OF COLD-ACID SOLUBLE EXTRACT OF LIVER

The UV absorbing fractions of the cold acid soluble extract, prepared from the livers of rats killed 4 and 24 hours after injection of C^{14}-methyl methanesulphonate, which were not retained on Dowex-1-formate during water elution of the column, were combined. Assay of aliquots from the 4 hour sample showed that 16 % of the radioactivity in the total cold acid soluble extract was present in this fraction. This corresponded to 2·07 % of the injected dose. Paper chromatography and subsequent autoradiography revealed that the majority of the radioactivity (70 %) was associated with a single radioactive material having an Rf value identical to S-methylcysteine in three different solvent systems. A further 20 % of the activity was attributed to S-methylcysteine sulphoxide. A third area of radioactivity, having an Rf 23 in BA solvent, was unidentified. Elution of the area

corresponding to S-adenosylmethionine (Rf 10 in BA) produced a material which gave a UV spectrum with λ_{max} at 260 mμ (pH 7) and at 256 mμ (pH 1). Only trace amounts of radioactivity were associated with this product.

Formic acid elution of the material absorbed on the column gave one component equivalent to 4·4 % of the injected radioactivity which was identified from chromatographic and spectral data as 7-methylguanine. Two other unidentified metabolites were present as minor components.

For the corresponding extract prepared from the 24 hour liver sample, 29 % of the radioactivity was not absorbed on Dowex-1-formate, equivalent to 0·78 % of the initial dose. Only one third of this was recognised as S-methylcysteine whilst 52 % coincided with the material previously identified as S-methylcysteine sulphoxide. In addition, radioactivity was detected on autoradiographs in the area corresponding to S-adenosylmethionine. This was estimated to be equivalent to approximately 0·15 % of the dose.

In the eluate from the formic acid elution, there were two radioactive UV absorbing materials

present. One of these was identical with the component isolated from the 4 hour sample and identified as 7-methylguanine. The other was not identified, but eluted from the column with a concentration of formic acid at which nucleotide monophosphates would be expected. 0·80 % of the dose was present in the total eluates.

D I S C U S S I O N

The biological activity of the alkylating agents is undoubtedly due to the one property they have in common, that of reaction with nucleophilic groups. In a reaction as complex as alkylation, however, where several pharmacological events are evident, it is particularly difficult to establish which biochemical reactions are primarily responsible for the observed effects. Considering also the large number of nucleophilic sites in the body which are accessible to the drug, it is evident that much of it will be wasted in reactions which are not biologically significant. For methyl methanesulphonate, these difficulties are enhanced as the alkyl group in question could enter the normal pool of methyl groups in the body and take part in essential methylation reactions. Before an attempt can be made to relate pharmacological effects of this drug to its reaction with particular cell components or constituents, it is essential to be able to distinguish between those which are normal reactions of the methyl group and those which are abnormal methylations.

Earlier studies on the excretion and metabolism of the methyl group after injection of C^{14}-labelled methyl methanesulphonate into the rat had indicated that, of the administered dose, at least 70 % of the radioactivity remained

within the animal body for a period greater than 24 hours (15). The initial detoxication reactions were judged to be complete by this time and it was reasoned that this retained activity represented widespread incorporation of the labelled carbon atom into cell components as tissue bound activity. This tolerated methylation was of particular interest in view of the studies on the quantitative relationship between the total dose and the biological effect produced, which as in the treatment of chronic myeloid leukaemia with the difunctional agent, Myleran, indicated a cumulative action when given in small divided doses (107).

Differential centrifugation of the liver homogenate in sucrose medium allowed the separation of a limited number of subcellular fractions, each designated by the name of the preponderant component, nuclear, mitochondrial, microsomal and cell supernatant. Limitations on the technique, including the time taken for fractionation with the consequent possibility of alteration, resulted in each fraction containing several components and a certain degree of cross contamination could not be avoided. The heterogeneous nature of the fractions precluded meaningful estimates of the specific activity of the individual components. Assay of the radioactivity present in

the subcellular fractions did indicate, however, the intracellular distribution of the C^{14} atom. Radioactivity was detected in each of the fractions examined, demonstrating the widespread distribution of the labelled carbon atom.

Cell fractionation of liver tissue taken from rats 24 hours after a single injection of C^{14}-methyl methanesulphonate showed that the majority of the radioactivity present was in the cell supernatant. This non-particulate fraction contained in addition to soluble cell constituents, those compounds which had been solubilised in the course of fractionation, together with any particulate elements such as microsomal vesicles, ribosomes and cellular debris which may have escaped centrifugation. The results suggest, therefore, that the radioactivity in the liver may be associated with low molecular weight material including free amino acids, nucleic acid constituents and the important metabolic enzymes normally present in the cytoplasm. The RNA present is principally low molecular weight (15,000 to 30,000) ; RNA which is of fundamental importance in the biosynthesis of proteins. At shorter time intervals after injection, the products of the detoxication reactions contribute to the greater percentage of radioactivity present in this soluble fraction.

The microsomal fraction, composed essentially of structures derived from fragmentation of the endoplasmic reticulum, contains the majority of the RNA present in the cell (108). The radioactivity in this fraction appears to increase with time, possibly resulting from increased utilisation of radioactive precursors for normal metabolic processes. No attempt was made to subfractionate the isolated material into ribosomes and microsomal membranes.

The fraction isolated by centrifugation at 10,000 g, referred to as the mitochondrial fraction, contains a number of diverse enzyme systems which play an extremely important role in cell metabolism. Mitochondria themselves are derived particularly from parenchymatous cells and are the main energy donors in the cell through oxidative phosphorylation reactions (109). They contain a considerable quantity of protein bound phosphoric esters and are rich in lipids (110)(111). At each of the time intervals studied, the mitochondrial fraction accounted for almost half the radioactivity in the particulate fractions. Assay of the RNA present showed that this was relatively low and that contamination with microsomal material was not significant. Little is known regarding permeability of mitochondrial membranes to substrates although mitochondria

are known to be particularly effected during ingestion of carcinogenic azo dyes (112). Liver mitochondria are able to incorporate amino acids into their protein _in vivo_. The distribution of enzymatic activities in the mitochondrial fraction has led to the concept of the existence of lysosomal entities (113). Their sedimentation characteristics put them in an intermediate class between mitochondria and microsomes, whilst biochemical and cytochemical studies classify them as dense pericanalicular bodies. As organelles preferentially located along the bile canaliculi and associated with the destruction of foreign substances, they may play an important role in the metabolism of methyl methanesulphonate.(114).

In this connection, the detection of radioactivity in this particular fraction may be of significance in view of earlier experiments, in which the bile duct of animals which had received an injection of C^{14}-methyl methanesulphonate was cannulated, in which it was shown conclusively that the principal detoxication reaction appeared to involve methylation of the sulphydryl group of glutathione (15). Studies of the intracellular localisation of glutathione indicated that negligible amounts occurred in nuclear and microsomal preparations but that the tripeptide was present, not only in the soluble

extract, but was also associated with the mitochondrial fraction (115). Based upon studies of the effect of stress conditions on the level of glutathione in rat liver, it has been postulated that it is a labile fraction of the tripeptide which exists in the cell supernate, but that which is associated with the mitochondrial fraction is relatively stable (116).

The nuclear fraction as isolated by centrifugation in 0·25 M sucrose contained approximately 10 % of the dose present in liver after 24 hours. Estimation of the RNA content showed that it was contaminated with other cell components. A purified preparation in sucrose containing a calcium salt produced a purer sample with 6 % of the liver radioactivity associated with it. Hypertonic solutions of sucrose are known however to extract soluble constituents from rat liver nuclei (117). Almost all the DNA of the cell was located in this fraction together with some RNA present even in the purified preparation. This was probably nuclear RNA of the heterochromatin or that located in the nucleolus (117).

The presence of radioactivity in each of the fractions examined illustrated the widespread distribution of the C^{14}-labelled atom at a subcellular level. It would

appear that incorporation has taken place into several cell components including macromolecules. For this to have occurred, either the drug is metabolised extracellularly and a metabolic product then enters the cell and becomes incorporated into the various structural elements of the cell, or the cell membrane is permeable to the drug and a direct alkylation occurs *in situ*. The hydrophilic nature of the ester would favour the passage across the cell membrane into the cytoplasm where it would find numerous nucleophilic sites available for reaction. If, however, the alkyl group enters the normal metabolic reactions of one-carbon compounds, the widespread distribution of the group would be no less surprising. The ubiquitous distribution of the methyl group and its importance for the maintainance of normal metabolism was discussed earlier.

Considering, in general, the small amount of drug required to produce a biological effect, reaction must occur at a very specific site *in vivo*. For the alkylating agents, however, there is no agreement as to the site of alkylation which may lead to the destruction of neoplastic cells. From their studies, Roberts and Warwick have suggested that the significant reactions are with thiol groups probably derived from protein (118)(119)(120). More recently, Wheeler

et al. implied that inhibition of plasma cell tumours in hamsters with nitrogen mustards and cyclophosphoramide was the result of interference with purine biosynthesis and inhibition of nucleotide incorporation into DNA (121).

An assessment of the distribution of radioactivity in liver tissue on a molecular basis was obtained using the modified techniques of Schneider (85). Results showed that the majority of the radioactivity was present in the cold acid soluble extract, thus confirming that the low molecular weight compounds were principally involved. The five-fold decrease in the amount of C^{14} activity present in this fraction at 24 hours compared with the shorter time interval accounted almost completely for the lower specific activity of the whole tissue at this later time. Previous work had shown that the liver was the principal organ concerned with detoxication of the drug and that, 4 hours after injection, the passage of radioactivity in the form of S-methylglutathione and its derivatives, down the biliary duct was at its peak. The material present in the acid soluble fraction presumably represents metabolites immediately prior to detoxication or the products of detoxication before entry into the bile duct.

By 4 hours, ,it was evident that the incorporation of the C^{14} atom into macromolecules had taken place. The sodium nucleates extracted after 4 hours contributed to the tissue radioactivity only to the extent of 0•34 % of the dose, but by 24 hours, this had increased to almost three times this amount. This increased incorporation cannot solely be due to direct action of the drug on the macromolecule as it is unlikely such a reactive ester with a half-life of 13 minutes at 37° would remain in the free state. It would seem more likely that the increased radioactivity was either due to increased utilisation of the labelled precursors for synthesis of macromolecules, or to increased utilisation of the C^{14}-methyl group for the methylation reactions which take place normally. Similarly, the increased radioactivity of the insoluble protein residue at 24 hours would be due to increased use of the carbon atom for synthesis or transmethylation reactions. At both the time intervals studied, it was not possible to prepare a complete balance sheet of the radioactivity in each of the fractions. At 4 hours, 8 % of the activity present in the whole tissue was unaccounted for in the four fractions examined. At 24 hours, as much as 19 % was lost during the fractionation procedure. This deficit may be attributed to loss of volatile material e.g. methanol, methyl sulphides, during the isolation procedure.

The persistence of the radioactive label in similar fractions prepared from liver tissue taken 48 and 72 hours after injection established that the incorporation of the carbon atom from the methyl group was relatively permanent and not a feature of the initial detoxication reactions. Although the percentage of the dose in the liver decreased by 20 % over the three days, the relative amounts of C^{14} activity associated with protein and nucleic acid fractions remained surprisingly constant. The decrease in the specific activity of the whole tissue was principally due to loss from the lipid and cold acid soluble fractions. This suggested that it might be possible to demonstrate a cumulative build up of radioactivity in macromolecules after repeated doses of the ester. This deduction was confirmed by assay of the radioactivity present after successive doses of methyl methanesulphonate given at 24 hour intervals. Three times as much radioactivity was extracted in the cold acid soluble fraction after a second dose compared with a single dose, and six times as much after the third. Similarly, the sodium nucleates contained 1·5 times and 3 times and the protein residue twice and three times the amount compared with a single injection. In calculating a hypothetical level of methylation in the liver after successive doses, based upon figures of 7·9 %, 6·9 %, and 6·2 %, for retained

activity at 24, 48 and 72 hours after a single dose, the rat which had two injections should have 6·9 % of the first dose remaining and 7·9 % of the second. Similarly, the animal which had three doses will have 6·2 % of the first, 6·9 % of the second and 7·9 % of the third dose remaining. If this were in fact the case, the ratio of the amount of C^{14} activity present, expressed as a percentage of the initial dose would be 7·9 %, 14·8 % and 21·0 % respectively. Aliquots of the whole tissue homogenates were assayed for radioactivity and the ratios of the amounts present were 8 : 19:37. This would imply that a significantly greater amount of the third dose is retained in the liver, thus substantiating to a great extent the highly cumulative property of this drug.

Having obtained an indication of the distribution of radioactivity in the cellular components and constituents of the liver and shown the extent to which incorporation of C^{14} atom occurs into protein and nucleic acids, more detailed studies were made to assess the exact sites of chemical action at a macromolecular level.

INCORPORATION INTO PROTEIN

Under physiological conditions, the

reactive centres susceptible to alkylation in a protein molecule include free carboxyl, amino, mercapto and imidazole groups and reaction has been demonstrated in vitro in all these cases (122). For reaction at most of these sites, however, the amount of agent used was greatly in excess of that likely to be encountered in vivo.

Amino acid analysis of protein isolated after treatment with methyl methanosulphonate showed conclusively that the principal radioactive component was S-methylcysteine. Quantitative studies of the amino acids present in the hydrolysed liver protein after the drug showed that there was no appreciable variation in the amount of other amino acids normally present. Only trace amounts of a material corresponding to S-methylcysteine could be detected in the chromatograms from treated livers suggesting that this methylated amino acid was not a normal protein constituent. A comparison of the specific activity of the isolated compound with the activity of the original drug confirmed within experimental error that the isolated material had not been diluted by naturally occurring unlabelled material. Considering the high pK_a value (10·8) of the sulphydryl group of cysteine when present in protein molecules and its existence largely in an unreactive unionised

form, alkylation at this site presents an apparent anomaly (1). The character of each sulphydryl group within a protein structure is, however, unique and its reactions are dependant upon the particular environment within that structure. The trace amounts of radioactivity detected on autoradiographs prepared from the hydrolysates of liver tissue taken 24 hours after injection were tentitatively attributed to methionine, serine and glycine. The extent of dilution of this activity by unlabelled amino acids prevented accurate assessment of the specific activities. Methyl methanesulphonate of higher specific activity would be necessary to obtain this information. From studies of the methylation of protein with the carcinogenic dimethylnitrosamine, Magee showed that after incubating slices of the rat liver with the C^{14}-methyl labelled compound, the radioactivity was associated with 1-methyl and 3-methyl histidines (123). No evidence was obtained in the present studies for methylation at this site after methyl methanesulphonate.

S-methylcysteine was recognised as one of the principal components present after hydrolysis of the mitochondrial fraction isolated from the 24 hour liver sample. Although the mitochondrial fraction is known to contain around 35 % of the total protein in the liver, it seems possible that

the majority of this labelled amino acid is derived from hydrolysis of methylated glutathione present in the mitochondria (124).

Hydrolysis of the total liver protein obtained from the Schneider procedure showed on chromatography that up to 55 % of the radioactivity bound to protein was associated with S-methylcysteine. On the basis of a figure of 1·88 % of the injected dose remaining in the trichloroacetic acid insoluble fraction, it is estimated that approximately 1 % of the dose exists as S-methylcysteine. As the majority of the remaining activity in the protein hydrolysate was identified as the sulphoxide of this amino acid, almost all the radioactivity present is due to reaction with the sulphydryl group. No alkylation of free amino or carboxyl groups was apparent. In terms of the cumulative build up of radioactivity with repeated doses of methyl methanesulphonate, it would appear that with successive doses the drug is able to seek out more sulphydryl groups for reaction, almost on a stochiometric basis. The significance of this reaction in terms of cell metabolism will be discussed later.

INCORPORATION INTO DNA

Since the importance of nucleic acids

in cellular metabolism was established, the idea that the biological properties of the alkylating agents could be explained on the basis of reaction with nucleic acids, particularly DNA, has frequently been propounded. At present, perhaps the most significant evidence bearing on this question is that from studies of the effects of alkylating agents on DNA-containing viruses. The work of Herriott, comparing the inactivation of enzymes, cells and viruses by a variety of alkylating agents, gave a clear indication that DNA was the most sensitive component of biological entities ; DNA-containing viruses being the most readily inactivated of the systems studied (125). From the evidence available, it appeared likely that both the cytotoxic and mutagenic effects resulted from an action on genetic material, and hence DNA has attracted attention as a possible site of alkylation in biological systems.

Radioactive assay of the DNA isolated from the livers of rats given a single injection of C^{14}-labelled methyl methanesulphonate confirmed that incorporation into the macromolecule had taken place. A rapid uptake of the labelled carbon atom occurred during the first few hours and this reached a maximum around five hours after injection. The specific activity of the nucleic acid then decreased slowly until by 24

hours it had dropped to 40 % of its maximum value. The metabolic stability of DNA and the relative absence of mitosis in normal liver cells make it unlikely that this was due to dilution of the radioactivity by newly formed DNA during normal synthesis. If the drug had induced synthesis of new DNA or if repair processes had been stimulated, a decrease in the specific activity would have been observed. A cumulative increase in the specific activity was shown with repeated doses although the maximum uptake occurred at the initial exposure. This could be attributed to there being fewer reaction sites available or to an increased efficiency for removing the labelled carbon atom.

Considering the nucleic acid structure as a whole, no reaction would be expected, nor has any been shown, with the hydroxyl groups of the deoxyribose moieties. Reaction with phosphoryl groups is theoretically possible on purely chemical reasoning for agents of the mustard type and evidence for this has been published, although the criterion which these authors used, namely reduction in acidity on reaction, would not distinguish between esterification of an acid group and quaternisation of a tertiary nitrogen atom (122). Esterification of phosphate groups would be possible although due to lability

of the products formed, it would prove difficult to demonstrate. Other centres where reaction would theoretically be possible include the purine and pyrimidine bases. From studies of the distribution of electrons in base pairs of DNA, based upon the Watson-Crick model for DNA, Pullman et al. showed that the N7 atom of guanine was the most nucleophilic centre not involved in hydrogen bonding (126). Hydrolysis of the DNA isolated from rat liver after methyl methanesulphonate yielded a radioactive component which was distinct from the four normal bases present. Identification of the isolated material as 7-methylguanine confirmed these predictions and supported the *in vitro* findings of Brookes and Lawley that this was the principal site of methylation (127). Although reaction with cytosine and adenine bases was theoretically possible, in undenatured DNA the N1 positions are involved in hydrogen bonding with guanine and thymine respectively, but the N3 atom is sterically available. No reaction was detected at these sites in the present studies but this may have been due to the low specific activity of the drug and the low degree of alkylation at these sites rather than lack of evidence for reaction. There was no evidence for the incorporation of radioactivity into the purine ring.

INCORPORATION INTO RNA

The isolation of radioactively labelled 7-methylguanine from the hydrolysate of rat liver RNA after injection of C^{14}-methyl methanesulphonate confirmed that methylation of guanine base had taken place. As with DNA, radioactivity was rapidly associated with the macromolecule and the specific activity was greatest around 8 hours after injection. It was found possible to increase the specific activity of the RNA almost stochiometrically after repeated doses. Fractionation of the RNA into ribosomal and soluble forms showed that the alkylation was not specific to a particular RNA species since the specific activities were similar in both cases. Cell fractionation studies had shown that almost 25 % of the radioactivity in the liver was present in the microsomal fraction. Alkylation of ribosomal RNA would explain this result as 80 % of the cellular RNA exists in association with microsomes in the form of ribosomal particles.

Evidence has been obtained from the present studies that the predominant reaction of methyl methanesulphonate *in vivo* is methylation. At a macromolecular level, the methyl group is incorporated into nucleic acids by

reaction at the N7 position of guanine and into cell protein by reaction with the thiol group. Before implicating methylation at these sites as being directly responsible for the biological effects, it is necessary to enquire into the mechanism by which alkylation has taken place and to assess any similarity with the normal methylation reaction.

THE METHYLATION REACTION

The presence of 7-methylguanine in mammalian urine as a normal excretion product has been recognised since 1898 (128)(129). Its biological origin has, however, remained obscure. Ingested 7-methylguanine is rapidly degraded to the xanthine stage prior to excretion so the urinary component is unlikely to be of dietary origin. Methylation at this locus must, therefore, represent a normal metabolic reaction and this suggests that a mechanism exists normally for the transfer of the methyl group *in vivo*. The isolation of 7-(C^{14})-methylguanine from the urine of rats after a single injection of C^{14}-methyl methanesulphonate would suggest that transfer of the labelled carbon atom may have taken place by a similar mechanism.

Current concepts for the biological transfer of the methyl group within the body have already been discussed. The principal source of methyl groups for transmethylation reactions was shown to be dietary methionine. Synthesis *de novo* and incorporation from the pool of one-carbon compounds were also shown to occur (38)(39)(40).

The isolation of choline containing radioactivity after injection of C^{14}-methyl methanesulphonate provided clear evidence for the entry of the methyl carbon atom into the one-carbon pool. Alkaline digestion in the presence of an oxidising agent proved conclusively that the labelled atom was located in the methyl group of the choline molecule. The mechanism for the formation of choline has been clarified by the findings of Artom with respect to methylation at the phosphatide level and it is now recognised that under normal conditions S-adenosylmethionine is the sole source of the methyl groups (81)(130). From the work of du Vigneaud, it would be reasonable to assume that the methyl group may be transferred to homocysteine in the course of time, resulting in the formation of labelled methionine. An alternative possibility would be that a C^{14}-methyl group of methionine had been synthesised *de novo* from formate via the tetrahydrofolate pathway and

subsequently transferred in toto from methionine to choline by transmethylation. The classical work of du Vigneaud, using Deuterium-labelled methionine has shown that in this reverse reaction, the transfer of methyl to choline does not take place through a formate or formaldehyde intermediate (25). The labelled methyl group of choline detected after methyl methanesulphonate must therefore have arisen by transmethylation from methionine or by direct action of the ester at the phosphatide level.

During a study of the distribution and fate of various alkylating agents, Trams et al. considered the possibility that the radioactivity assayed in biological samples after administration of C^{14}-labelled nitrogen mustard to rats, might be present in metabolites rather than in intact drug (131) (132). They isolated the radiocarbon from choline present in liver phospholipid and found that it amounted to less than 0·5 % of the dose. From their calculations of the total body choline content, they estimated that it did not exceed 4 % of the total injected dose and concluded that transmethylation did not represent a major metabolic pathway for the drug. A feature of special interest in connection with the present studies was the increase in specific activity of choline which they noted

when smaller doses of the mustard were given over a period of 2-4 days. This implied the possibility of a cumulative build up of methyl groups derived from the N-methyl groups of the mustard with fractionated doses.

The mechanism by which the level of methyl groups in the body is maintained was discussed earlier when it was shown that almost all the dietary excess of methyl groups given as methionine was metabolised to carbon dioxide. The majority of the excretion took place around six hours after administration of the methionine. The formation of C^{14}-carbon dioxide after injection of ethyl methanesulphonate into rats, representing 34 % of the dose, was presented by Roberts and Warwick as evidence for the hydrolysis of this ester to ethanol being a major pathway for metabolism in the rat.(19). Comparison with the exhalation of carbon dioxide after injection of 1-C^{14}-ethanol showed that for the alcohol, metabolism was extremely rapid and within 5 hours, 75 % of the administered radioactivity had been accounted for by this route. The rate of excrétion of carbon dioxide after ethyl methanesulphonate was therefore taken by these authors as representing the rate of hydrolysás of the drug _in vivo_. The maximum levels of excretion did not occur until 3 hours after injection. This

would imply that this biochemically reactive drug had remained unreacted in the body for this length of time. In relating these results to the methyl ester, it should be remembered that the dose given in these studies with the ethyl ester was 2·42 mM/kg compared with 0·91 mM/kg in the ppesent studées. This could account for the lower excretion of radioactivity as carbon dioxide after methyl methanesulphonate - only 5 % in 24 hours. When ethyl methanesulphonate was given at a dose of 0·81 mM/kg, a similar level of carbon dioxide excretion was found to the methyl ester at this dose.

These results could equally well be interpreted in relation to the methylation reaction, namely that the metabolism to carbon dioxide be regarded as disposal of excess alkyl groups over and above those required for the maintenance of normal methylation reactions, rather than due to chemical hydrolysis of the esters _in vivo_. This obviates the need to postulate the existence of the intact drug for a period of six hours or so after injection.

From more recent studies, it has been suggested that the percentage of the injected drug excreted as carbon dioxide after methyl methanesulphonate should be much

higher. (133). In this particular study, in which 25 % was metabolised to carbon dioxide, the drug was administered intravenously at a toxic dose level (1·09 mM/kg). It is therefore not surprising that if metabolism is taking place by normal metabolic pathways, a significantly higher proportion would be exhaled as carbon dioxide.

In this connection, it is interesting to note a curious sex specificity which has been reported for metabolism of the methyl groups in rats (134). The oxidation of administered methyl groups to carbon dioxide is significantly greater in the female animal that in the male and as the incorporation into tissue protein is inversely related to the extent of oxidation, it might be expected that protein isolated from male animals would have higher specific activity than that from females. This phenomenon may, in part, account for the LD_{50} of methyl methanesulphonate in the female rat being 120 mg/kg compared with 110 mg/kg in the male (135). A similar sex difference in the extent of demethylation of morphine has been shown to occur in the female rat where 8 % of the radioactivity administered as N-methyl-C^{14}-morphine was eliminated as respiratory carbon dioxide compared with 0·6 % in the female (136).

The isolation of formaldehyde containing a radioactive carbon atom from the liver provided evidence that dealkylation and oxidation of the methyl group had taken place. This metabolic formation of formaldehyde and its subsequent entry into the C_1 metabolic pool must inevitably be followed by labelling of all the cell constituents on the normal metabolic pathways of this compound. This would explain the incorporation of radioactivity into the amino acids serine, glycine and methionine which utilise the formate pathway for normal synthetic reactions. The low specific activity of these compounds would be due to significant dilution with unlabelled amino acids.

Although methionine is the principal source for the methyl group for transmethylation in vivo, it requires conversion to a biochemically active form, S-adenosylmethionine, before transfer can take place. Direct evidence for the formation of S-adenosylmethionine containing a labelled methyl group derived from methyl methanesulphonate was obtained by isolation of this sulphonium salt from rat liver. The low level of S-adenosylmethionine in this organ (0·07 µM/grm wet wt.) together with the inevitable dilution of radioactivity by unlabelled material did not allow accurate measurement of its specific activity. As the methyl groups derived from the methyl ester

have entered the normal metabolic pool of one-carbon compounds in the body, it is reasonable to assume that some of the reactions have taken place under the auspices of the normal methylating system. Studies on the radioactive distribution of the drug therefore do not necessarily refer to abnormal reactions.

The discovery of methylated bases present normally in nucleic acids stimulated a study of their methylation at enzymic and cellular level and the occurrence and distribution of methylated purines in the subcellular fractions of RNA has been reported in detail (137). The meticulous investigations of Dunn et al. on RNA from various sources have led to the isolation of numerous methylated bases (65)(66)(138). Furthermore, it is now recognised that the methyl groups in methylated purines and pyrimidines of RNA originate from the methyl group of methionine (72). If the labelled carbon atom from methyl methanesulphonate has become incorporated into the S-adenosylmethionine intermediate, labelling of the RNA would be expected at the normally methylated bases. According to Dunn, there is a substantially higher proportion of methylated purines in the soluble fraction of RNA isolated from rat liver than in the ribosomal fraction (67). After methyl methanesulphonate, the specific activity of the soluble RNA was

almost twice that of the ribosomal fraction which is in agreement with the above work. For soluble RNA, six different methylase enzymes have been isolated from bacterial cells which are responsible for methylating specific sites (74). They include three enzyme fractions for the methylation of guañine. Two of these achieve methylation at the 1 position and the third is responsible for methylating the 7 position. After injection of C^{14}-methyl labelled methionine into rats, 7-methylguanine has been detected in soluble RNA and it is degradation of these macromolecules which is thought to yield the methylated purines found in urine (139). The extent to which 7-methylguanine occurs naturally in nucleic acids has not been determined quantitatively and it is therefore not possible to deduce how much of this base, isolated after injection of methyl methanesulphonate is a result of normal methylation reactions at these macromolecules. The presence of 7-methylguanine as the predominant methylated base in the urine of normal animals is surprising as it occurs as such a relatively minor component in RNA.

DNA from mammalian sources has been shown to contain only 5-methylcytosine as a minor methylated base (62). The isolation of 7-methylguanine from the DNA of rats injected with the C^{14}- methionine provided evidence that

methylation at this site can take place. The detection of this methylated base after administration of methyl methanesulphonate may not, therefore, represent an abnormal methylation.

The present studies have conclusively shown that after a single dose of methyl methanesulphonate, the methyl group has entered the one carbon pool. Subsequent incorporation of the labelled atom into various molecules by normal metabolic processes under the control of appropriate enzyme systems would be anticipated. It seems likely that the biological effects observed after methyl methanesulphonate would not be due to such reactions unless quantitative control of the number of methylation reactions was bypassed. There was no observable decrease in the level of hepatic adenosine triphosphate to indicate increased synthesis of S-adenosylmethionine as has been observed after the administration of ethionine and the corresponding formation of S-adenosylethionine (140).

Whilst the results obtained on the site of in vivo alkylation of various molecules after giving methyl methanesulphonate could conceivably be explained by reference to the entry of the methyl group into normal reactions, the extent to which this reaction has been shown to occur makes it most

unlikely that it is primarily responsible for the biological effects. It does not preclude the possibility that the observed reactions could be the result of the direct action of the drug on cell components. As was discussed earlier, in the absence of ATP, the methyl group may be transferred when it is contained in a sulphonium compound. The formation of such a compound was demonstrated _in vitro_ by the reaction of the methyl ester with methionine. The product, identified as S-methyl methionine, has been suggested as a methyl donor as it occurs naturally in plants (141). No evidence was obtained from the present _in vivo_ studies to suggest that this represented an intermediate for these methylation reactions.

In favour of a theory of direct action of the drug _in vivo_ is the reaction with protein. Although S-methylcysteine occurs naturally in the plant kingdom, there is no evidence for its normal occurrence in animals (142). Methylation of protein at this site after methyl methanesulphonate is therefore unlikely to occur via the normal methylation reaction utilising S-adenosylmethionine as donor. Reaction would appear to be the result of the direct action of the drug with the protein _in situ_. Similarly for DNA, where there is no evidence for the existence of a methylase enzyme capable of

introducing a methyl substitueht into the N7 position of the guanine mölecule in a mammalian system.

Direct action on cellular components in situ would seem to imply either passage of the intact drug through the cell and nuclear membranes into direct contact with the constituents, or the extracellular formation of an alkylating entity which then passed across the cell membrane by ionic diffusion. The small size of the molecule of methyl mëthanesulphonate might favour passage into the cell but its water solubility would argue against diffusion across a lipoidal membrane. A more plausible hypothesis would involve reaction of the intact molecule with the cell surface, resulting in the liberation of a positively charged carbonium ion after reaction with the cell membrane, and passage into the cell as an alkylating entity.

In summary, these studies have shown that after a single dose of methyl methanesulphonate, methylation of nucleic acids occurs at the N7 position of guanine and at the sulphydryl group present in peptide and protein molecules. The mechanism by which the alkylation has taken place would appear to be by direct reaction within the cells. The entry of the

administered carbon atom into the pool of methyl groups would suggest that methylation through the normally occurring S-adenosylmethionine must be taken into account when assessing the significance of the site of action _in vivo_ with the biological effects produced.

Before discussing in detail the possible biological relevance of the demonstrated metabolic reactions, attention should be drawn to the dose used in these studies compared with that necessary to produce a biological effect.

In order to obtain cellular material of sufficiently high specific activity to perform these studies with the labelled drug available, it was necessary to administer it at a dose level of 100 mg/kg, which was just less than the lethal dose. This level was approximately four times greater than that required to produce an observable biological effect. As a result of this, some of the metabolic reactions detailed may be related to overall toxic effects of the drug on the animal, rather than those pertaining to the subtle biological effects which prompted the study of this particular alkylating agent. It has been reported from preliminary clinical studies in humans

that the type of changes to be expected in liver and which were visible 8 days after a total cumulative dose of 30 mg/kg, involved destruction of the limiting plate which normally demarcates the portal structures from the liver parenchyma. In addition, cholangioles were irregularly shaped and some had been destroyed. Histological examination of liver, kidney, spleen and intestine did not indicate any gross damage, including any fatty infiltration or centrilobular necrosis, within the first 24 hours of treatment.

POSSIBLE BIOLOGICAL IMPLICATIONS OF METHYLATION

The diversity of the pharmacological effects produced by the alkylating agents in general, together with the large number of cellular sites with which they can interact, precludes facile explanation of their mode of action. For this methyl ester, attempts to correlate methylation with biological results are frustrated by a lack of precise knowledge of the role methylation plays in the normal metabolism of the animal. It is possible, however, to assess the likely biological implications of the reaction by reference to the methylation reactions known to occur naturally and to those resulting from chemical administration.

The present studies have implicated both the sulphydryl group of protein and the guanine base of nucleic acids as central sites for reaction at a molecular level.

METHYLATION OF PROTEIN

The reactivity of methyl methanesulphonate with the cysteine moiety present in glutathione, which was shown in earlier work to represent the principal detoxication reaction, suggests that the role of this sulphydryl group may be to render inocuous compounds which otherwise damage essential sites in the cell (16). It does not follow that reaction with the tripeptide at other specific cell sites, for example, mitochondria, does not play an important part in subsequent biological effects. If this protective role of the acid soluble sulphydryl fraction is mediated by competition with protein bound sulphydryl sites, a decrease in the level of exogenous sulphydryl will lead to increased methylation of protein bound sulphydryl groups. The role of the sulphydryl group in maintaining normal cellular metabolism has been emphasised by the work of Rapkine, who observed the variation in concentration of free sulphydryl groups throughout the mitotic cycle (143)(144). The mitotic apparatus was shown to be composed of proteins rich in sulphydryl groups.

Attack at these nucleophilic sites could lead to inhibition of cell division(145). It was subsequently shown that only cells treated with alkylating agents during the earliest part of the resting stage showed chromosome aberrations in the next mitosis (146). One possible consequence of reaction with protein is an inactivation of essential sulphur containing enzymes. The fact that not all such enzymes are inactivated by sulphydryl reactants does not invalidate this possibility as the properties of the group are dependant upon a particular environment. It would seem therefore that the general cytotoxicity of these agents may be due to an inability to detoxicate the ester quickly enough to prevent reaction at other cell sites. Preliminary evidence to support this has been obtained from experiments in which a second dose of the methyl ester was given 5 hours after the first, at a time when the level of liver glutathione was known to be at a minimum. Much increased levels of tissue activity were noted after a second dose compared with the first or with a similar injection given when the level of glutathione in the liver had returned to control level. The role of the sulphydryl group in maintaining the configuration of certain proteins is well recognised. Alkylation of these fibrous proteins could conceivably modify the architecture of the cell or alter the structure of the cell membrane in such a way that

its subsequent metabolism would be affected.

Where reaction with exogenous thiols was not sufficiently fast to reduce the amount of alkylating agent available, alkylation of other molecular sites e.g. nucleic acids, may occur freely.

METHYLATION OF DNA

The decreased viscosity of the DNA prepared from liver after treatment with methyl methanesulphonate, noted during the isolation procedure, suggested that alteration in the secondary structure of DNA may have taken place. A similar reaction was noted by Strauss, who showed that the biological properties of alkylated DNA were altered by methylation with respect to loss of transforming activity, due to insertion of single strand breaks into DNA (147). The introduction of the bulky, hydrophobic methyl group into macromolecules must produce profound alterations in the structure of nucleic acids due to steric and electronic perturbations. The introduction of the methyl group into the N7 position of guanine has been shown to render the deoxyguanosine structure sufficiently unstable for the glycosidic bond to be split under neutral pH conditions (148).

It also enhances ionisation of the proton from the N1 position at physiological pH. This would affect the hydrogen bonding capacity of the 7-methylguanine with cytosine and it has been suggested that this base may pair aberrantly with thymine instead of cytosine (149). Although depurination may cause inactivation of the DNA, these effects could conceivably result in a mutation by causing base-pair deletion.

Based upon this reasoning, alkylation of DNA by methyl methanesulphonate could lead to depurination of the guanine entity producing fission of the macromolecule and loss of secondary structure. This would be evidenced by a lowered viscosity of the DNA solution. The results of more recent work have confirmed that fragmentation of DNA does occur (150). The ultimate effect on biological systems of the depurination will depend to some extent on the efficiency of the repair mechanisms. The persistence of alkylation in DNA and a cumulative build up of methyl groups would imply that alkylated purines are incompletely released <u>in vivo</u>. A consequence of the depurination process is that estimation of the number of guanine residues methylated, based upon radioactivity measurements on the macromolecule, will be inaccurate if much of the methylated base has already been removed from the macromolecule. The

presence of 7-methylguanine in the cold acid soluble extract of the whole tissue indicated that this may have occurred.

The demonstration that methylation leads to alterations in the structure and function of DNA implies interference with cellular integrity. The gross mitotic abnormalities and gene mutations could satisfactorily be explained on this basis. Such reaction does not rule out the possibility of alterations mediated as a result of an indirect effect at a more distant site.

EXCRETION OF 7-METHYLGUANINE

The origin of the 7-methylguanine excreted in urine has not been located. Transfer RNA is known to contain a variety of methylated purines and degradation of this macromolecule would result in the release of these components. They are present, however, only in very small amounts in RNA so that it is surprising to find 7-methylguanine as the predominant methylated base if this were the principal source. The depurination of DNA may provide a hitherto unrecognised contribution to this metabolite. This urinary excretion, together with the relatively high level of the methylated base in the cold acid

soluble fraction of rat liver at a time when detoxication is taking place, leads to the suggestion that this molecule may feature primarily as a repository for excess methyl groups. The biological significance of this excretion remains unknown. The output is, however, increased after X-ray treatment, ingestion of glandular material and after increased destruction of leukocytes (151). Elevated levels of excretion of methylated purines in general have been reported from animals bearing mammary carcinoma and thymic lymphoma (152). This excretion is important in view of the relatively small size of the tumours in comparison with the whole animal, and suggests that the metabolism leading to the excretion of greater amounts of methylated products must be much higher in tumour tissue. Whether the excretion of increased amounts of methylated guanine in leukaemic patients represents a greater methylation capacity or a failure to demethylate compounds present in abnormal amounts is not known.

METHYLATION OF RNA

The biological consequences of methylating RNA are not known. One of the earliest suggestions for the role of methylation of transfer RNA was that it protects

the RNA strand against the action of nucleases (78). Studies of the site of action of these enzymes on methylated and non-methylated RNA's have failed to confirm this (82). It has also been reported that methyl deficient RNA accepts amino acids normally, ruling out involvement in the amino acid specificity of RNA (153).

Although the RNA methylase enzymes are widely distributed over tissues, studies in several laboratories simultaneously reported that heterologous enzymes are capable od adding methyl groups in vitro to fully methylated transfer RNA (154)(155)(156). No correlation has been made so far on the possible biological consequence of this. If overmethylation is possible through heterologous enzymes, then, by analogy, it may be possible to overmethylate transfer RNA by chemical means, for example, by direct action of methyl methanesulphonate on RNA.

BIOLOGICAL ROLE OF METHYLATION

The characteristic species specificity of enzymes for forming methylated bases, and therefore the distribution of such bases would suggest a vital and unique function to that particular species. The biological function

which exhibits maximum species specificity is differentiation. It is tempting to suggest that methylation may act as a control mechanism responsible for switching on pre-existing enzyme capabilities manifesting later as differentiated cells. The studies of Mudd on the biosynthesis of hordenine (N,N-dimethyl-tyramine) in germinating barley may be relevant in this connection. The correlation in the time course for the accumulation of hordenine by successive methylation of tyramine suggests that this is a metabolic function related to tissue maturation or differentiation (157)(158)(159). Whether direct methylation by methyl methanesulphonate of cells at a post meiotic phase in spermatogenesis is responsible for sterility due to cells being 'downgraded' from mature to immature stages is open to conjecture.

The present studies have not ruled out the possibility that the critical reaction giving rise to the observed biological effects may be due reaction at a site other than on nucleic acid or protein. The general reactivity and reported mutagenicity of formaldehyde, for example, have invited speculation as to the role this metabolite might play in producing certain pharmacological effects (160)(161). From the evidence available it is not possible to implicate the

presence of formaldehyde in the liver after methyl methanesulphonate as being responsible for the characteristic biological effects.

In view of the central role played by S-adenosylmethionine during cell development, it is tempting to suggest that interference with this mechanism might lead to some of the biological effects observed after administration of alkylating agents. The detection of 3-hydroxytetrahydrothiophene-1,1-dioxide as a urinary metabolite after injection of myleran into rats, was attributed to reaction of this difunctional agent with the sulphydryl group present in glutathione (19). The same product could be observed if reaction had taken place with S-adenosylmethionine or S-adenosylhomocysteine.

While advances have been made in the therapy of cancer, the morbidity and mortality of the disease have not seriously been affected. It has, therefore, become increasingly apparent that fundamental knowledge about the nature of the cancer cell and its distinguishing properties must be obtained before approaches can be made to therapy on truly rational grounds. From studies on the radioactive distribution of labelled alkylating agents *in vivo*, Mandel concluded that they

had provided little information as to the nature of the effect of the drugs on tissues and that clear cut evidence for alkylation of protein and nucleic acids _in vivo_ was lacking (162). The present studies on the metabolism of methyl methanesulphonate have confirmed that methylation of nucleic acids has occurred _in vivo_, predominantly at the N7 position of guanine and at the sulphydryl group of the protein macromolecule.

In the absence of demonstrable qualitative differences between neoplastic cells and their normal counterparts, it is, perhaps, not surprising that the chemotherapy of neoplastic diseases has not yet emerged from an era corresponding to the treatment of spirochaete infection with mercury. The cures which have been obtained as a result of the treatment of choriocarcinoma with Methotrexate, suggests we may be entering a period analogous to the use of arsenicals (163). What cannot be predicted is whether future developments in chemotherapy will be analogous to the treatment with penicillin.

\- - - - - - - - - - - -

BIBLIOGRAPHY

1. Ross, W.C.J. in Biological Alkylating Agents, p3 1962.
Butterworths.

2. Jackson, H., Craig, A.W. & Fox, B.W.
Brit.J.Pharmacol. 14 149 1959.

3. Jackson, H., Fox, B.W. & Craig, A.W.
J.Reprod.Fertil. 2 447 1961.

4. Partington, M. & Jackson, H.
Genet.Res. 4 333 1963.

5. Fox, B.W. Nature. 212 1058 1966.

6. Peng, C-T. J.Pharmacol. 120 229 1957.

7. Nadkarni, M.V., Trams, E.G. & Smith, P.K.
Cancer Res. 19 713 1959.

8. Trams, E.G., Salvador, R.A., Maengwyn-Davies, E.D., de Quattro, V. Proc.Amer.Assoc.Cancer Res. 2 256 1957.

9. Trams, E.G. & Smith, P.K.
7th Intern.Canc.Cong.Abstr. 168 1958.

10. Trams, E.G., Nadkarni, M.V., de Quattro, V., Maengwyn-Davies, G.D. & Smith, P.K.
Biochem.Pharmacol. 2 7 1957.

11. Fox, B.W., Craig, A.W. & Jackson, H.
Biochem.Pharmacol 5 27 1960.

12. Roberts, J.J. & Warwick, G.P.
Nature 183 1509 1959.

13. Roberts, J.J. & Warwick, G.P.

Biochem.J. 72 3P 1959

14. Roberts, J.J. & Warwick, G.P.

Nature 179 1181 1957.

15. Pillinger, D.J., Fox, B.W. & Craig, A.W. in Isotopes in Experimental Pharmacology, ed. Roth. Chicago Press.

Chap 32 415 1965.

16. Pillinger, D.J., Craig, A.W. & Jackson, H.

Biochem.J. 90 43P 1964.

17. Craig, A.W. & Pillinger, D.J.

BECC Report. 504 1964.

18. Baumann, E. & Preusse, C.

Ber.dtsch.chem.Ges. 12 806 1879.

19. Roberts, J.J. & Warwick, G.P.

Biochem.Pharmacol. 1 60 1958.

20. Pråce, J.M. & Dodge, L.W.

J.Biol.Chem. 223 699 1956.

21. Sarcione, E.J. & Sokal, J.E.

J.Biol.Chem. 231 605 1958.

22. Hofmeister, F.

Arch.exp.Path.Pharm 33 198 1894.

23. Gmelin, C. Versuche über die Wirkungen Beryts, Strontians, Tellurs etc. auf den thierishen Organisms

Lampp, Tuebingen 96 1824.

24. Reisser, M. Z.physiol.Chem. 86 440 1913.

25. du Vigneaud, V., Cohn, M., Chandler, J.P. & Simmonds, S.
J.Biol.Chem. 140 625 1941.

26. Simmonds, S., Cohn, M., Chandler, J.P. & du Vigneaud, V.
J.Biol.Chem. 149 519 1943.

27. Cantoni, G.L. J.Biol.Chem 189 203 1951.

28. Borsook, H. & Dubnoff, J.W.
J.Biol.Chem. 171 363 1947.

29. Cantoni, G.L. J.Biol.Chem. 189 745 1951.

30. Cantoni, G.L. J.Biol.Chem. 204 403 1953.

31. Baddiley, J., Cantoni, G.L. & Jamieson, G.A.
J.Chem.Soc. 2662 1953.

32. Baddiley, J. & Jamieson, G.A.
J.Chem.Soc. 1085 1955.

33. Durell, J., Rawitscher, M. & Sturtevant, J.M.
Biochim.Biophys Acta. 56 552 1962.

34. Durell, J. & Sturtevant, J.M.
Biochim.Biophys.Acta. 26 282 1957.

35. du Vigneaud, V., Chandler, J.P., Moyer, A.W. & Keppel, D.M.
J.Biol.Chem. 128 cviii 1939.

36. du Vigneaud, V., Ressler, C. & Rachele, J.R.
Science. 112 267 1950.

37. du Vigneaud, V., Ressler, C., Rachele, J.R., Reyniers, J.A. & Luckey, T.D. J.Nutrit. 45 361 1951.

38. Toennies, G., Bennett, M.A. & Medes. G. Growth. 7 251 1943.

39. Bennett, M.A., & Toennies, G. J.Biol.Chem. 163 235 1946.

40. Stekol,,J.A., Bennett, M.A., Weiss, K., Halpern, P. & Weiss, S. Fed.Proc. 9 234. 1950.

41. Sakami, W. Fed.Proc. 9 222 1950.

42. Welch, A.D. & Sakami, W. Fed.Proc. 9 245 1950.

43. Sakami, W. & Welch, A.D. J.Biol.Chem. 187 379 1950.

44. Willmans, W., Rucker, B. & Jaenicke, L. Z.physiol.Chem. 322 283 1960.

45. Larrabee, A.R., Rosenthal, S., Cathon, R.E. & Buchanan, J.M. J.Amer.Chem.Soc. 83 4094 1961.

46. Jaenicke, L. Z.physiol.Chem. 326 168 1961.

47. Mangrum, J.H. & Scrimgeour, K.G. Fed.Proc. 21 242 1962.

48. Bennett, M.A. Science, 110 589 1949.

49. Guest, J.R., Friedman, S., Woods, D.D. & Smith, E.L. Nature. 195 340 1962.

50. du Vigneaud, V., Chandler, J.P., Moyer, A.W. & Keppel, D.M.
J.Biol.Chem. 131 57 1939.

51. Bremer, J. & Greenberg, D.M.
Biochim.Biophys.Acta. 37 173 1960.

52. Bremer, J., Figard, P.H. & Greenberg, D.M.
Biochim.Biophys.Acta. 43 477 1960.

53. Phear, E.A. & Greenberg, D.M.
J.Amer.Chem.Soc. 79 3737 1957.

54. Greenberg, D.M., Nath, R. & Humphreys, G.K.
J.Biol.Chem. 236 2271 1961.

55. Friedkin, M. Fed.Proc. 18 230 1959.

56. Friedkin, M. Fed.Proc. 19 312 1960.

57. Pastore, E.J. & Friedkin, M.
J.Biol.Chem. 237 3802 1962.

58. Albanese, A.A. Adv.in Prot.Chem. 3 227 1947.

59. Roth, J.S. & Allison, J.B.
J.Biol.Chem. 183 173 1950.

60. Mackenzie, C.G., Rachele, J.R., Cross, N. & du Vigneaud, V.
J.Biol.Chem. 183 617 1950.

61. Canellakis, E.S., Arch.Biochem.Biophys. 42 387 1953.

62. Wyatt, G.R. Biochem.J. 48 581 1951.

63. Dunn, D.B. & Smith, J.D.
Biochem.J. 68 627 1958.

64. Markham, R. & Smith, J.D.
Biochem.J. 52 558 1952.

65. Smith, J.D. & Dunn, D.B.
Biochem.J. 72 294 1959.

66. Littlefield, J.W. & Dunn, D.B.
Biochem.J. 70 642 1958.

67. Dunn, D.B. Biochem.Biophys.Acta. 34 286 1959.

68. Adler, M., Weissman, B. & Gutman, A.B.
J.Biol.Chem. 230 717 1958.

69. Borek, E., Ryan, A. & Rochenbach, J.
Fed.Proc. 13 184 1954.

70. Mandel, L.R. & Borek, E.
Biochem.Biophys.Res.Comm. 6 138 1961.

71. Mandel, L.R. & Borek, E.
Biochemistry. 2 560 1963.

72. Mandel, L.R. & Borek, E.
Biochemistry. 2 555 1963.

73. Fleissner, E. & Borek, E.
Fed.Proc. 22 229 1963.

74. Hurwitz, J., Gold, M. & Anders, M.
J.Biol.Chem. 239 3462 1964.

75. Gordon, J. & Bowman, H.G.
J.Molec.Biol. 9 638 1964.

76. Gold, M. & Hurwitz, J.
J.Biol.Chem. 239 3866 1964.

77. Srinivasan, P.R. & Borek, E.
Science. 145 548 1964.

78. Borek, E. & Srinivasan, P.R.
Ann.Rev.Biochem. 35 291 1966.

79. Dagley, S., Turnock, G. & Wild, D.G.
Biochem.J. 88 555 1963.

80. Szer, W. & Ochoa, S.
J.Molec.Biol. 8 823 1964.

81. Baliga, B.S., Srinivasan, P.R. & Borek, E.
Nature, 208 555 1965.

82. Tsutsui, E., Srinivasan, P.R. & Borek, E.
Proc.Natl.Acad.Sci. 56 1003 1966.

83. Bredereck, H. Chem.Ber. 80 401 1947.

84. Vischer, E. & Chargaff, E.
J.Biol.Chem. 176 715 1948.

85. Schneider, W.C. J.Biol.Chem. 161 293 1946.

86. Davidson, J.N. & Smellie, R.M.S.
Biochem.J. 52 594 1952.

87. Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J.
J.Biol.Chem. 193 265 1951.

88. Tristram, G.R. Adv.in Prot.Chem. 5 83 1949.

89. Hirs, C.H.W., Moore, S. & Stein, W.H.
J.Amer.Chem.Soc. 76 6063 1954.

90. Cocking, E.C. & Yemm, E.W.
Biochem.J. 58 xii 1954.

91. Schneider, W.C. & Hogeboom, G.H.
J.Biol.Chem. 183 123 1950.

92. Schneider, W.C. J.Biol.Chem. 176 259 1948.

93.- Hogeboom, G.H. & Schneider, W.C.
J.Biol.Chem. 197 611 1952.

94. Kirby, K.S. Biochem.J. 66 495 1957.

95. Burton, K. Biochem.J. 62- 315 1956.

96. Weissman, B., Bromberg, P.A. & Gutman, A.B.
J.Biol.Chem. 224 407 1957.

97. Kirby, K.S. Biochem.J. 64 405 1956.

98. Ceriotti, G. J.Biol.Chem. 214 59 1955.

99. Brungraber, E.F. Biochem.Biophys.Res.Comm. 8 1 1962.

100. Kirby, K.S. Biochem.J. 96 266 1965.

101. Mitoma, C. & Greenberg, D.M.
J.Biol.Chem. 196 599 1952.

102. Tarnoky, K. & Nagy, S.
Clin.Chêm.Acta. 8 627 1963.

103. Hurlbert, R.B., Schmitz, H., Brumm, A. & Potter, V.R.
J.Biol.Chem. 209 23 1954.

104. Gilbert, C.W. & Keene, J.P. in Radioisotopes in Scientific Research, Pergamon Press 1 698 1958.

105. Moutschen, J. & Ezell, H.K. Rad.Res. 13 898 1960.

106. Magee, P.N. & Lee, K.Y. Biochem.J. 91 35 1964.

107. Jackson, H. BECC Rep. 503 1964.

108. Schneider, W.C., Hogeboom, G.H. & Ross, H.E. J.Natl.Canc.Inst. 10 977 1950.

109. Moule, Y. & Chauveau, J. in The Liver, Academic Press. 1 410 1963.

110. Moule, Y. Bull.Soc.Chim.Biol. 38 175 1956.

111. Spiro, M.J., & MacKibbin, J.M. J.Biol.Chem. 219 643 1956.

112. Allard, C., Lamirande, G. & Cantero, A. Cancer Res. 17 862 1957.

113. de Duve, C. Exptl.Cell.Res.Suppl. 7 169 1959.

114. Novikoff, A.B. in The Cell, ed Brachet & Mirsky, Academic Press. 2 423 1961.

115. Edwards, S. & Westerfield, W.W. Proc.Soc.Exp.Biol.Med. 79 37 1952.

116. Stout, D.A., McCartney, R.L., Lewis, B.M., Smith, H.M. & Register, U.D. Proc.Soc.Exp.Biol.Med. 112 334 1963.

117. Mirsky, A.E. & Osawa, S. in The Cell, ed. Brachet & Mirsky. Academic Press. 2 677 1961.

118. Roberts, J.J. & Warwick, G.P. Biochem.Pharmacol. 6 205 1961.

119. Roberts, J.J. & Warwick, G.P. Biochem.Pharmacol. 12 1329 1963.

120. Roberts, J.J. & Warwick, G.P. Biochem.Pharmacol. 12 1321 1963.

121. Wheeler, G.P. & Alexander, J.A. Cancer Res. 24 1338 1964.

122. Alexander, P., Cousens, S.F. & Stacey, R.A. in Ciba Symposium Drug resistence in microorganisms. 294 1957.

123. Magee, P.N. & Hultin, T. Biochem.J. 83 106 1962.

124. Schneider, W.C. & Hogeboom, G.H. Cancer Res. 11 1 1951.

125. Herriott, R.M. J.Gen.Physiol. 32 221 1948.

126. Pullman, B. & Pullman, A. Biochim.Biophys.Acta. 36 343 1959.

127. Brookes, P.D. & Lawley, P. Biochem.J. 80 496 1961.

128. Kruger, M. & Salomon, G. Z.physiol.Chem 24 364 1898.

129. Kruger, M. & Salomon, G.

Z.physiol.Chem 26 330 1898.

130. Artom, C. Biochem.Biophys.Res.Comm. 15 201 1964.

131. Smith, P.K., Nadkarni, M.V., Trams, E.G. & Davison, C.

Annal.N.Y.Acad.Sci. 68 834 1958.

132. Trams, E.G. & Klopp, C.T.

Cancer Res. 15 617 1955.

133. Swann, P.F. Nature. 214 919 1967.

134. Natori, Y. in Transmethylation & Methionine Biosynthesis,

Univ. Chicago Press. p69 1965.

135. Craig, A.W. Personal Communication.

136. March, C.H. & Elliott, H.W.

Proc.Soc.Exp Biol.Med. 86 494 1954.

137. Bergquist, P.L. & Matthews, R.E.F.

Biochem.J. 85 305 1962.

138. Dunn, D.B., Smith, J.D. & Spahr, P.F.

J.Mol.Biol. 2 113 1960.

139. Stekol, J.A. in Transmethylation & Methionine Biosynthesis,

Univ. Chicago Press. p250 1965.

140. Smith, R.C. & Salmon, W.D.

Arch.Biochem.Biophys. 111 191 1965.

141. Sato, C.S., Byerrum, R.U., Albersheim, P. & Bonner, J.

J.Biol.Chem. 233 128 1958.

142. Synge, R.L.M. & Wood, J.C.

Biochem.J. 64 252 1956.

143. Rapkine, L. Ann.physiol.physicochem.biol. 9 383 1931.

144. Mazia, D. in Glutathione; Symposium ed. Colowick, S.P.,

Academic Press. p209 1954.

145. Mazia, D. in The Cell, ed. Brachet & Mirsky,

Academic Press. 3 77 1961.

146. Revell, S.H. Heredity. 6 107 1953.

147. Strauss, B.S. J.Gen.Physiol. 30 89 1963.

148. Lawley, P. & Brookes, P.

Biochem.J. 89 127 1963.

149. Lawley, P.& Brookes, P.

Nature. 192 1081 1961.

150. Fox, B.W. Personal Communication.

151. Jackson, K. & Entenman, C.

Amer.J.Physiol. 189 315 1957.

152. Park, R.W., Holland, J.F. & Jenkins, A.

Cancer Res. 22 469 1962.

153. Starr, J.L. Biochem.Biophys.Res.Comm. 10 181 1963.

154. Srinivasan, P.R. & Borek, E.

Proc.Nat Acad.Sci. 49 529 1963.

155. Gold, M., Hurwitz, J. & Anders, M.

Proc.Nat.Acad.Sci. 50 164 1963.

156. Svensson, I., Boman, H.G., Eriksson, K.G. & Kjellin, K.
J.Mol.Biol. 7 254 1963.

157. Mudd, S.H. Biochim.Biophys.Acta, 37 164 1960.

158. Mudd, S.H. Biochim.Biophys.Acta 38 354 1960.

159. Rabitzsch, G. Plants.Med. 7 268 1959.

160. Rapoport, I.A. Dokl.Akad.Nauk.SSSR 59 1183 1948.

161. Boyland. E. Pharmacol.Rev. 6 345 1954.

162. Mandel. H.G. Pharmacol.Rev. 11 743 1959.

163. Hertz. R., Ross, G.T. & Lipsett, M.B.
Amer.J.Obstet.Gyn. 86 808 1963.

ACKNOWLEDGEMENTS

I wish to express my thanks to Dr.G.A.J.Goodlad, B.Sc., Ph.D., of the Department of Biochemistry, University of St Andrews, for supervising the work of this Thesis. I am grateful to Dr.L.G.Lajtha, M.D., D.Phil., Director of Research, for allowing the work to be conducted in the Paterson Laboratories, Professor.G.R.Tristram, Ph.D., for the facilities provided in St Andrews, and Dr.H. Jackson, M.B., Ch.B., Ph.D., D.Sc., for suggesting the problem and providing facilities in the Department of Experimental Chemotherapy.

I am particularly indebted to Dr.A.W. Craig, M.Sc., Ph.D., and Dr.B.W.Fox, B.Sc., Ph.D., for their patience, guidance, encouragement and helpful discussion at all stages of the work.