METABOLIC STUDIES OF SOME SULPHANILAMIDOPYRIMIDINES

BY

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ABSTRACT

1. The purpose of this study was to investigate species variation in response to some substituted 6-sulphanilamidopyrimidines, which is related to differences in absorption, distribution, tissue binding, rate and route of metabolism and excretion.

2. The fate of the short-acting 2,4-dimethyl-6-sulphanilamidopyrimidine (sulphasomidine), and the medium-long acting 4-methoxy-2-methyl-6-sulphanilamidopyrimidine (sulphamethomidine) in man was studied in the rat, rabbit, monkey and man. Sulphasomidine was excreted mainly as unchanged drug in all the species examined. The major metabolite of sulphamethomidine was the N^4-acetyl derivative in the rat and rabbit, and a glucuronide in monkey and man which was identified as an N^1-glucuronide.

3. The in vitro synthesis of sulphadimethoxine (2,4-dimethoxy-6-sulphanilamidopyrimidine) N^1-glucuronide was studied in the rat, rabbit and monkey. These results confirmed the in vivo findings that the rabbit is unable to form N^1-glucuronides whereas the monkey and rat are able to perform this type of conjugation.

4. The in vitro acetylation rates of a series of methyl and methoxy substituted 6-sulphanilamidopyrimidines were determined in the rabbit and monkey. The measurement of certain physical properties for the same series (i.e. solubilities, partition coefficients and pKa values) helped in correlating the in vivo and in vitro metabolism.

5. Protein binding of sulphasomidine, sulphamethomidine and sulphadimethoxine in human plasma was an important contribution in answering the problem of differences in excretion of these three drugs. The longer acting sulphonamides are more highly bound than the short-acting sulphasomidine.

6. Pretreatment of animals with phenobarbitone often alters not only the species response to a drug as far as stimulation of metabolising enzymes is concerned, but also its duration of action. Pretreatment of rats with phenobarbitone daily for 3 days as well as a single injection (i.p.) just prior to the ingestion of [^{35}S]sulphadimethoxine altered its metabolism and excretion. The problem of interaction of drugs as related to renal and biliary excretion was investigated as it has great importance clinically.

7. The results are summarised and hypotheses proposed concerning the role of N^1-glucuronide formation in nutrient metabolism. Factors affecting long action and the design of sulphonamides, the complexity of predicting species differences and the implications of phenobarbitone in therapy are discussed.

To my wife Maureen

and my parents.

ACKNOWLEDGMENTS

The work described in this thesis was carried out in the Department of Biochemistry, St. Mary's Hospital Medical School. It is an attempt to elucidate the factors that affect the duration of action and the species differences of three closely related 6-sulphanilamidopyrimidines.

I wish to express my sincere thanks to Professor R. T. Williams for his help and supervision of my work over the last three years. In particular I would like to thank Dr. J. W. Bridges for his advice and interest in my project, and for the many hours of stimulating discussion that we have had together. In addition, I am grateful to the other members of the staff and my fellow research workers for the stimulating atmosphere which they have provided.

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S. R. Walker

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King Solomon said:

"Whatsoever thy hand findeth to do, do it with thy might, for there is no work, nor device, nor knowledge, nor wisdom, in the grave, whither thou goest."

Ecclesiastes 9 v. 10.

CHAPTER I

INTRODUCTION

1) THE SULPHONAMIDE STORY.

   a) History

   b) Pharmacological Properties.

2) FACTORS AFFECTING DURATION OF ACTION.

   a) Absorption and Distribution

   b) Metabolism

   c) Excretion

   d) Tissue Binding.

3) SPECIES DIFFERENCES.

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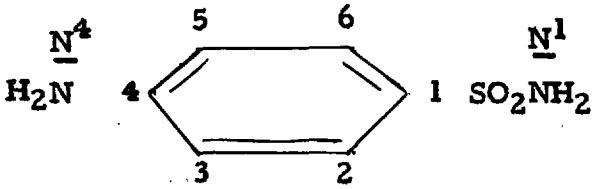
1) THE SULPHONAMIDE STORY

a) HISTORY.

The discovery and widespread use of sulphonamides in chemotherapy is an outstanding example of progress in the field of medicine. Since it was discovered in the latter part of the nineteenth century that bacteria were the cause of many diseases, an intensive effort has been made to find chemicals that will destroy these organisms.

The early interest in sulphonamides was as dyes. In 1909, Hoerlein and his co-workers synthesised the first azo dyes containing sulphonamides and substituted sulphonamides, and noted that they were superior in colour fastness to the existing dyes. As the azo dyes formed a firm complex with proteins of wool and silk it was reasoned that these agents might react with bacterial protoplasm. In 1913, Eisenberg discovered the *in vitro* bactericidal action of chrysoidin (2,4-diaminoazobenzene), another azo dye, and suggested its use in chemotherapy. Subsequently various azo compounds such as pyridium (2,6-diamino-3-phenylazopyridine hydrochloride) and serenium (2,4-diamino-4-ethoxyazobenzene hydrochloride) were introduced into medicine as urinary disinfectants, but the therapeutic results were not very satisfactory.

The major breakthrough came when Domagk (1935) observed that the dye Prontosil (4'-sulphamyl-2,4-diaminoazobenzene hydrochloride) had outstanding antibacterial properties in experimental animals, but lacked any activity in vitro. In France, the Tréfouëls, Nitti and Bovet (1935) discovered that in vivo the azo linkage was split so that prontosil yielded p-aminobenzenesulphonamide, which they thought to be the chemotherapeutic moiety of the molecule. Fourneau et al. (1936) demonstrated that sulphanilamide was as effective as prontosil in curing experimental infections. Fuller (1937) isolated sulphanilamide from the urine of patients treated with prontosil, which confirmed the findings of the Tréfouëls et al. Sulphanilamide became the accepted treatment for all bacterial infections, but at the same time attempts to improve its therapeutic performance were made. It was discovered that substitution of the free amine group (N^4-position) led to a marked reduction or abolition of the antibacterial activity of sulphanilamide. Of the many adaptions of the sulphanilamide molecule that were made, only those which were amido substituted (N^1-position) were of any value as antibacterial agents.



Sulphapyridine (M & B 693) (Whitby, 1938) was reported to be most effective in the treatment of pneumococcic infections in man. This was the first of the N^1-heterocyclic sulphanilamides that became established as a chemotherapeutic agent. Its side effects were nausea and vomiting together with a tendency to produce anuria and oliguria. Continuing the search for new drugs which would be more effective and at the same time less toxic, led to the synthesis of sulphathiazole and sulphamethylthiazole (Fosbinder and Walter, 1939). Sulphadiazine and its derivatives (Roblin et al. 1940) were found to cause less tissue damage and yield higher blood levels than either sulphapyridine or sulphathiazole. The favourable properties of these sulphanilamidopyrimidines together with a high therapeutic activity against pneumococcal, streptococcal and staphylococcal infections led to the synthesis of many related compounds including sulphamerazine and sulphamethazine (Roblin, Winnek and English, 1942).

In 1945, a sulphonamide was discovered with extended duration of action (Van Dyke et al.). However, this compound (4, 6-dimethoxy-

2-sulphanilamidopyrimidine) was found to be poorly absorbed from the intestine in man and to exhibit toxic effects, due to the low solubility of the drug and its principle metabolite (Bevan and Luxton, 1947).

During the decade after the war, the sulphonamides fell into disfavour due to the discovery of penicillin and other antibiotics which were highly active against a broad spectrum of bacteria and well tolerated. The discovery that antibiotics too produced toxic effects such as sensitization, the emergence of resistant bacterial strains and the problem of treatment of secondary infection led to a gradual reappraisal of the value of sulphonamides in therapy.

Various attempts were made to reduce the toxic effects and enhance the antibacterial activity of sulphonamides firstly by the use of combinations of different sulphonamides. It was thought that if the dose of an individual sulphonamide could be reduced by giving a sulphonamide combination, one might be able to improve the urinary excretion of the sulphonamides and to lessen their toxicity, since several sulphonamides should dissolve in body fluids independently of each other (Lehr, 1945). However, the use of these mixtures of sulphonamides did not succeed because in many instances toxic effects were heightened rather than reduced.

Within the last few years experimental research has been directed to such factors as improved water solubility and prolonged duration of therapeutic action. The first of the clinically used long-acting sulphonamides were sulphadimethoxine (Schnitzer et al. 1958) and sulphamethoxypyridazine (Clark et al. 1958). However, in some countries, notably Australia, long-action is viewed unfavourably since it has been reported to enhance the occurrence of a toxic side-effect termed the Stevens-Johnson syndrome.

b) PHARMACOLOGICAL PROPERTIES.

Sulphonamides differ from most antibiotics in that their action is bacteriostatic rather than bactericidal. Many hypotheses have been advanced to explain the mechanism of the antibacterial action of sulphonamides, but the accepted action is that proposed by Woods (1940) and Fildes (1940). Those bacterial organisms which require p-aminobenzoic acid (PABA) for growth utilize it for the synthesis of folic acid. This is prevented by the competitive antagonism of PABA by sulphonamides.

Brown (1962) showed that sulphonamides inhibited the enzyme reaction involved in the formation of dihydropteroic acid from PABA and 2-amino-4, 6-dihydroxymethyldihydropteridine in the presence of A.T.P. and magnesium ions (Mg^{++}).

The structural formulae and duration of action of some of the major sulphonamides are shown in Table 1.1.

Although there are now many antibiotics on the market, the advantages of sulphonamides i.e. their cheapness, ease of storage and administration render them important chemotherapeutic agents particularly in the treatment of meningitis. The clinically used sulphonamides may be classified into three groups on the basis of the rapidity with which they are absorbed and excreted.

TABLE 1.1

Chemical structures of some clinically useful sulphonamides

(R = $H_2NC_6H_4SO_2NH$-).

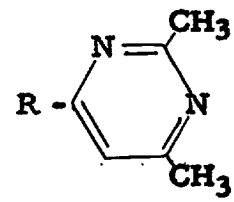
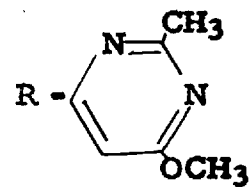
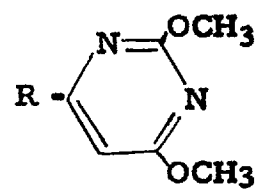
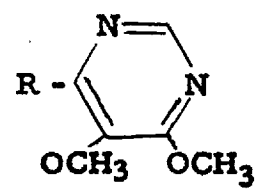
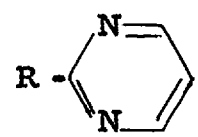
Name	Structure	Duration of Action
Sulphanilamide	RH	Short
Sulphacetamide	$RCOCH_3$	Short
Sulphasomidine (Elkosin)		Short
Sulphamethomidine		Medium-long
Sulphadimethoxine (Madribon)		Long
4,5-Dimethoxy-6-sulphanilamidopyrimidine (Fanasil)		Long
Sulphadiazine		Short

TABLE 1.1 (Continued)

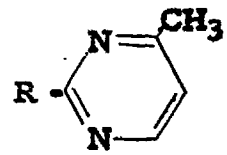
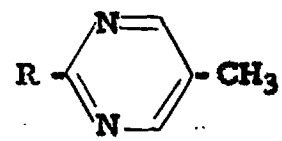
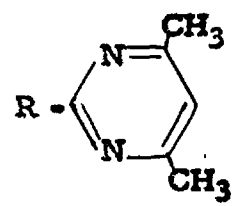
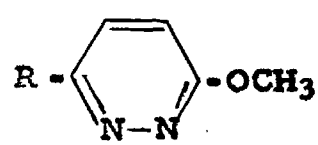
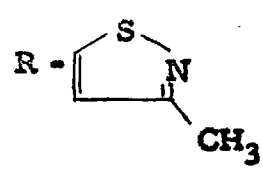
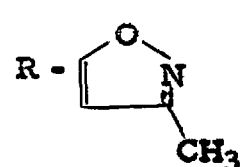
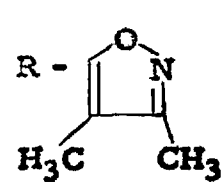
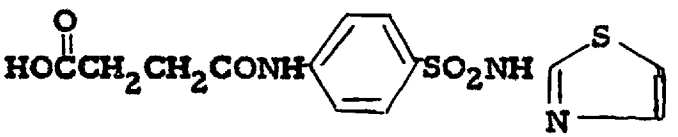
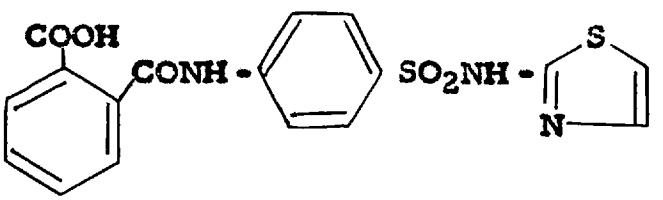
Sulphamerazine		Short
Sulphamethyldiazine		Short
Sulphadimidine		Short
Sulphamethoxypyridazine (Lederkyn)		Long
Sulphasomizole (Bidizole)		Medium-long
Sulphisemizole (Gantanol)		Medium-long
Sulphisoxazole		Short

TABLE 1.1 (Continued)

Succinylsulphathiazole	
Phthalylsulphathiazole	

Agents absorbed and excreted rapidly.

The sulphonamides such as sulphadiazine and its related compounds, sulphasomidine (elkosin) and sulphisoxazole (gantrisin) are termed short-acting sulphonamides since they rapidly attain a high blood level which is maintained for only a few hours. They are mainly used in the treatment of urinary tract diseases, but sulphadiazine and sulphasomidine are also used for meningococcal infections.

Compounds absorbed well but excreted slowly.

This class of compounds known as long-acting sulphonamides, offer the advantage of an effective plasma concentration when administered only once or twice a day. Sulphamethoxypyridazine and sulphadimethoxine are widely used in prophylactic therapy. Both sulphonamides are used for urinary tract infections caused by gram negative bacteria, and they have been used successfully in combating outbreaks of bacillary dysentery. Sulphadimethoxine has been used in the treatment of various diseases due to staphylococci, pneumococci, H-influenzae, shigella and salmonella (Weinstein, Madoff & Samet, 1960).

Sulphamethomidine, a medium-acting sulphonamide in man, has been used for acute cystitis and non-specific urethritis (Arai et al. 1961). Fanasil, which is exceptionally long-acting in man, has

been used in the treatment of malaria in Vietnam.

Poorly absorbed sulphonamides.

Some sulphonamides such as succinylsulphathiazole and phthalylsulphathiazole are poorly absorbed from the gastrointestinal tract and so tend to remain in high concentrations in the enteric fluids where they inhibit bacterial flora proliferation. They are indicated for certain intestinal infections and for the reduction of bowel flora prior to its surgical manipulation. The chief clinical use of succinylsulphathiazole is as a prophylactic agent in elective surgery of the colon. It has been used against bacillary dysentery, but is inferior in effect to fully absorbed sulphonamides.

Side effects.

The incidence of side effects ranges from 1-15% of cases treated according to the sulphonamide used and duration of treatment, (Brit. Med. J., 1964, p. 485). Those sulphonamides or their acetyl derivatives which have a very low water solubility may produce anuria by crystallizing out in the kidney. This condition is treated by giving the subject large volumes of weak alkali to enhance the solubility.

Sulphonamides are often protein bound. Occasionally, these drug-protein complexes may act as antigens, producing an antibody resulting in the development of fever and skin rash.

Various toxic effects including nausea, vomiting, headache, dizziness and some degree of mental depression have been attributed to sulphonamide therapy.

2) FACTORS AFFECTING DURATION OF ACTION.

With the introduction of long-acting sulphonamides in recent years, interest has been shown in the duration of action of these drugs and the relationship to structure. The duration of action of sulphonamides can be related to the following factors:

a) Rate of absorption and distribution.

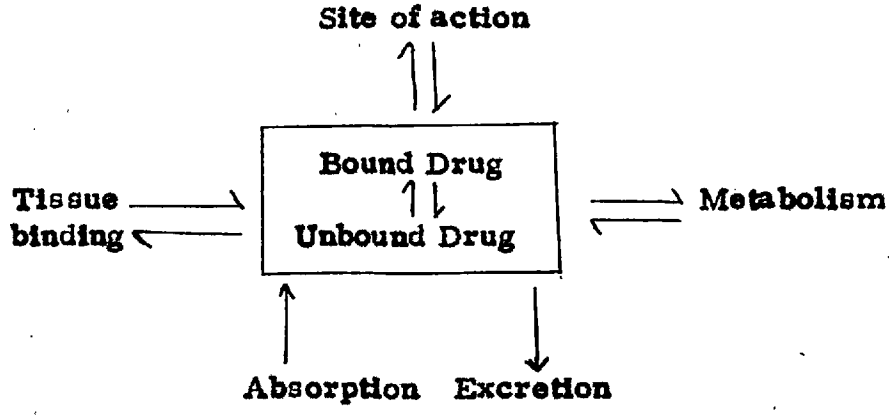
b) Nature and rate of metabolism.

c) i) Renal excretion and reabsorption.
ii) Biliary excretion and enterohepatic recirculation.

d) Tissue binding.

Fig. 1.1 depicts what happens to sulphonamides in the body, i.e. the factors that affect the plasma level of a drug and hence its duration of action.

Fig. 1.1



a) ABSORPTION AND DISTRIBUTION

The rate of absorption and tissue distribution of sulphonamides after oral or parental administration will influence their duration of action. A number of physico-chemical factors may affect the extent and rate of absorption of sulphonamides across body membranes including the nature of the membrane itself, the pH of the tissue fluids on either side of the membrane, the molecular shape, size, physical form and pKa of the compound. The passage of a compound across a cell membrane is governed mainly by physical factors. Since most membranes are lipoid in nature, fat soluble undissociated molecules tend to pass across membranes rapidly whereas lipid insoluble ionized materials only diffuse with difficulty (Brodie & Hogben, 1957). Thus, relatively weak bases and acids will be readily absorbed from the stomach and small intestine while strongly basic drugs will only be absorbed from the small intestine.

The dissociation constants of sulphonamides (1st pKa 1-2.5, 2nd pKa 5-10.5) indicate that they will be absorbed both in the stomach and small intestine. This has been confirmed in in vitro studies using perfused rat intestine (Nagami, Hanano & Yamada, 1963). Penetration across other cell membranes appears to be governed by similar factors but because of the large interfacial

area in contact with extracellular fluid even compounds with poor lipid solubility are absorbed. The ability of sulphonamides to bind with proteins must also influence the distribution of these compounds.

Appreciable amounts of sulphonamides are often found in tissues such as liver, kidney, lung and muscle (Neipp, Sackmann & Tripod, 1961) and in body fluids particularly the cerebrospinal fluid. The rate of penetration of sulphonamides into the central nervous system is largely dependent on the lipid solubility of the undissociated molecule. Sulphonamides are also located in the skin which is the cause of the Stevens-Johnson syndrome.

b) METABOLISM

The majority of foreign compounds undergo metabolic transformations in animal bodies and these may play an important role in therapeutic action or toxicity. These metabolic changes consist of a variety of oxidations, reductions, hydrolyses and syntheses, and a compound can undergo any one of these typesof reactions or any combination of them consecutively or simultaneously.

Sulphonamides undergo N-conjugations at both the N^1 and the N^4 positions, but do not appear to be deaminated in vivo. The N-glucuronides and N-sulphates are usually only minor metabolic products, whereas N^4-acetylation is the major N-conjugation reaction in most animals.

N^4-ACETYLATION.

Acetylation of the aromatic amino group was first observed with sulphanilamide (Marshall & Litchfield, 1939). The reaction occurs in most mammalian species including man, but not in the dog, fox, turtle and certain frogs (Bridges, 1963). Reports that sulphamerazine is acetylated at the N^4-position in the dog (Krebs, Sykes & Bartley, 1947) have not been confirmed by other workers. DiCarlo, Malament & Phillips (1963) found a slight difference between 'free' and 'total' drug (Bratton & Marshall, 1939, estimation method) and on this basis claimed that sulphamethomidine, sulphadimethoxine

and sulphasoxazole were acetylated in the dog. However, Kibby (1965) was unable to find any N^4-acetyl sulphadimethoxine in the urine of the dog examined chromatographically.

At least two amine acetylases have been postulated to account for the ability of the dog to acetylate glucosamine and histamine (Tabor & Mosettig, 1949) but not sulphonamides (Marshall, 1954). There are two opposing views on the arylamine acetylation process. The dog either acetylates aromatic amines which are then deacetylated before excretion, or there is something lacking in the acetylating system. Liebman & Anaclerio (1963) showed that the latter view was correct, and although the coenzyme A-acetylation system is intact, dog liver does contain an acetylation inhibitor.

DISTRIBUTION AND MECHANISM OF ACTION OF ARYLAMINE ACETYLASE.

Govier (1965) isolated reticuloendothelial cells from the livers of rabbits and showed that acetylation of sulphanilamide did occur in the reticuloendothelial system. Lung and spleen organs, known to contain a high percentage of reticuloendothelial cells, were also found to acetylate sulphanilamide and p-aminobenzoic acid. Weber & Cohen (1967) purified N-acetyl transferase from the 100,000 g. supernatent of homogenised rabbit liver, and this enzyme acetylated sulphadiazine, sulphamethazine and sulphanilamide. They also found

N-acetyl transferase activity in the intestine (jejunum), but not in the 10,000 g. supernatant fraction of heart, kidney, skeletal muscle, spleen, brain or adrenal. Bridges & Williams (1963a) found that N^4-acetylation of sulphonamides in the rabbit, coypu and chicken occurs mainly in the liver with little activity in other tissues. Bridges (1963) found acetylating enzyme activity in the mitochondrial and supernatant fractions.

The antituberculous drug isoniazid is acetylated in vivo, and it was suggested (Evans, Manley & McKusick, 1960) that the level of the acetylating system was genetically controlled in man. Frymoyer & Jacox (1963) found a bimodal pattern of acetylation of sulphadiazine in rabbits and postulated that it was under the control of a single dominant Mendelian gene. In their studies, most of the animals were rapid acetylators (dominant gene), while a few were slow acetylators (recessive gene).

The mechanism for acetylation occurs in two stages (Lipmann, 1945).

(1) $CH_3COX + CoASH \xrightarrow{A.T.P.} HX + CoASCOCH_3$

(2) $CoASCOCH_3 + HY \underset{\text{Transferase}}{\overset{\text{Acetyl}}{\rightleftharpoons}} CoASH + CH_3COY$

CH_3COX is the source of acetyl groups which may be acetate,

pyruvate or acetyl phosphate. The source of acetate combines with coenzyme A in the presence of ATP to form active acetate (acetyl CoA). The second reaction which is controlled by the enzyme acetyl transferase is the transfer of the acetyl group to HY which may be a sulphonamide.

TOXIC EFFECTS.

The $\underline{N}^4$-acetyl derivatives of sulphonamides are not only therapeutically inactive, but are usually more toxic since they tend to form insoluble deposits in the kidney leading to kidney blockage (anuria and oliguria) and renal damage. Table 1.2 gives a comparison of the solubilities of certain sulphonamides and their $\underline{N}^4$-acetyl derivatives with the occurrence of kidney blockage and their LD_{50}.

TABLE 1.2

(Data from Williams, 1959, pp. 510-511).

Sulphonamide	Solubility at 37°C mg./100 ml. water	LD_{50} in Mice g./kg.		Kidney blockage in mice.
		p.o.	i.p.	
Sulphanilamide	1500	3-8	-	-
Acetylsulphanilamide	530	2-3	-	
Sulphadiazine	13	1.8	1.6	++
Acetylsulphadiazine	20	-	0.6	
Sulphamerazine	37	3.3	1.4	++
Acetylsulphamerazine	79	-	0.7	
Sulphamethazine	75	1.9	1.9	+
Acetylsulphamethazine	115	-	1.3	
Sulphapyrazine	5	-	-	+++
Acetylsulphapyrazine	5	-	-	
Sulphathiazole	98	-	-	+++
Acetylsulphathiazole	7	-	-	

Certain additions have been made to the diet of experimental animals in an attempt to decrease the level of acetylation either by adding blocking agents or by the elimination of chemical agents which tend to potentiate acetylation. Barbituric acid (Cetrullo, 1958), vitamin D (Panagopoulos, 1959) and sodium pyrophosphate (Nashkov & Nashkova, 1960) have all been reported to reduce acetylation. The role of acetate in the diet is not clear. James (1939) found that acetate increased acetylation while Martin & Rennebaum (1943) found it decreased the acetylation of sulphanilamide.

RATES OF ACETYLATION.

In a series of three papers (Nelson, 1960, 1961; Nelson & O'Reilly, 1961) studies were conducted to determine the values of various rate constants and other parameters controlling the rate of acetylation and excretion of sulphathiazole. They showed that the disposition of these drugs depended on the result of two competitive first order reactions, one for acetylation and the other for excretion of the sulphonamide. An expression was derived for the maximum excretion rate of acetylated sulphathiazole and they suggested that this expression could be used to calculate minimum urine flow for prevention of crystalluria. However, in recent years several sulphonamides have been made commercially available which are either more soluble or poorly acetylated, and are administered at

a lower dose level and hence it is doubtful if the equation has any value in therapeutics.

Many attempts have been made to correlate the basicity of an amine with its degree of acetylation in vivo. Zehender (1943) proposed that the pKa of the amino group was related to the degree of acetylation. Smith & Williams (1948a) showed that sulphonamides with a pKa between 6 and 9 (i.e. weak acids) were the most highly acetylated while compounds with low pKa's were only poorly acetylated (Table 1.3).

TABLE 1.3

Relationship between dissociation constant and extent of acetylation in vivo of certain p-substituted anilines. (Smith & Williams, 1948a)

Compound	pKa	% Excreted as the acetyl derivative in rabbits
Sulphanilic acid	3.24	0
p-Aminobenzoic acid	4.68	23
Sulphapyrazine	6.04	87
Sulphadiazine	6.48	72
Sulphamerazine	7.06	90
Sulphathiazole	7.12	56
Sulphamethazine	7.37	85
Sulphapyridine	8.43	72
Sulphanilamide	10.43	50

It was suggested that there might be a critical level of ionisation necessary for maximal acetylation.

Weber & Cohen (1967) studying weak acids found that acetylation was relatively greater at pH values below the pKa of the drug, indicating that the uncharged form of the drug interacts more strongly with the enzyme. Perault & Pullman (1963) attempted to correlate the rate of enzymic acetylation of amines with the electronic charge of the amino nitrogen, the compounds investigated being unionized under the conditions of the experiment. However, the results were inconclusive.

The rate and degree of acetylation of sulphonamides varies from species to species. Krebs et al. (1947) suggested that this difference was quantitative rather than qualitative and he proposed that competition between acetylating and deacetylating enzymes decided the extent of acetylation in vivo. This delicate balance between acetylating and deacetylating enzymes is destroyed when tissues are minced and homogenised. Therefore, strict correlation between in vivo and in vitro results are impossible.

N^1-ACETYLATION.

In vivo acetylation of the sulphonamide nitrogen atom of sulphanilamide has been observed in mammals and plants (Bayer, Saviard & Dechauvassine, 1956; Crowdy & Jones, 1958). Bridges & Williams (1963a) showed that sulphacetamide was a minor metabolite of sulphanilamide in experimental animals. The rabbit

and guinea pig also excrete a small amount of N^1,N^4-diacetyl-sulphanilamide.

N^1-Acetylation, however, has not been observed with N^1-substituted sulphonamides. It is evident from the results of Perault & Pullman (1963) that sulphanilamides with a low electronic charge on the sulphonamide nitrogen will only be acetylated to a small extent in the N^1-position. As the N^1-substituted sulphonamides with electron withdrawing substituents will have an even lower electronic charge on this nitrogen, acetylation in this position is unlikely. Acetylation in the N^1-position is also unlikely, due to steric hinderence by the substituents.

DEACETYLATION.

Bray et al. (1950) distinguished two types of deacetylating enzyme, namely, N-acetylglycine deacetylase and acetanilide deacetylase in the liver and kidney tissues of a number of species. The aliphatic deacetylase hydrolyses the N-acetyl derivatives of certain amino acids i.e. glycine, D.L.-alanine and L-leucine, while the acetanilide deacetylase hydrolyses the acetyl derivatives of aromatic compounds including the sulphonamides.

Deacetylation activity in vivo has been observed in rats (Elson, Goulden & Warren, 1946), cats (Krebs et al., 1947), insects, dogs,

pigeons (Smith, 1964) and hen (Bridges & Williams, 1963a), whereas in man and rabbit it occurs only to a very small extent (Smith & Williams, 1948b).

Nimmo-Smith (1960) found the aromatic N-deacetylase activity of chicken kidney to be mainly in the mitochondria. Hollunger & Niklasson (1961) claimed to have solubilized and isolated a highly active acetanilide hydrolysing enzyme from rabbit liver microsomes. Franklin (1963, unpublished data) has demonstrated the presence of a deacetylase for p-acetamidobenzoic acid in the soluble fractions of kidney and liver homogenates of rat, rabbit, mice and guinea pig.

Weak N^1-deacetylating activity has been detected in many hen tissues, i.e. liver, kidney, heart and brain. Streptococcus faecalis, Lactobacillus acidophilus and Escherichia coli deacetylate N^1-acetyl sulphisoxazole, the rate increasing with pH in the range 4-9 (Uno & Kono, 1961).

N^4-SULPHATE FORMATION.

The conjugation of aromatic amines to form sulphamates has been shown to be a minor metabolic pathway in the rat, dog, rabbit and man. Sulphonamide N^4-sulphates have been reported for sulphisoxazole (Uno & Kono, 1960), sulphathiazole (Uno & Ueda, 1960), sulphadiazine (Uno, Yasuda & Sekine, 1963), sulphasomizole (Bridges

& Williams, 1963b) and sulphadimethoxine (Kibby, 1965).

Biosynthetic N^4-sulphates are usually identified by chromatography, colour reactions and a comparison with synthetic compounds. The instability of this minor metabolite of sulphonamides is the reason for the difficulty in isolating it.

Using α and β-naphthylamines as substrates, sulphamate formation was demonstrated in sheep intestinal mucosa preparations and mammalian liver (Roy, 1958, 1960 a).

The mechanism for sulphamate formation is thought to be as follows, (Roy, 1960b):

1.
$$ATP + SO_4^{--} \xrightarrow[\text{-sulphurylase}]{\text{ATP}} \text{Adenosine-5'-phosphosulphate (APS)} + \text{pyrophosphate}$$

2.
$$ATP + APS \xrightarrow[\text{phosphokinase}]{\text{APS}} \text{Adenosine-3'-phosphate-5'-phosphosulphate (PAPS)} + ADP + H^+$$

In the presence of sulphate transferase, PAPS reacts with a sulphonamide to yield sulphonamide-N^4-sulphate.

$$RNH_2 + PAPS \xrightarrow[\text{sulphokinase}]{} R\overset{H}{N}SO_3H + PAP$$

GLUCURONIDE FORMATION.

The conjugation of glucuronic acid with foreign compounds to form β-glucosiduronic acids has been recognised for a number of years as a major detoxication mechanism. The activated form of glucuronic acid can be produced in the body from carbohydrate sources in the following way. (Dutton, 1966)

1. α-glucose-1-phosphate + UTP $\longrightarrow$ UDP-glucose + pyrophosphate

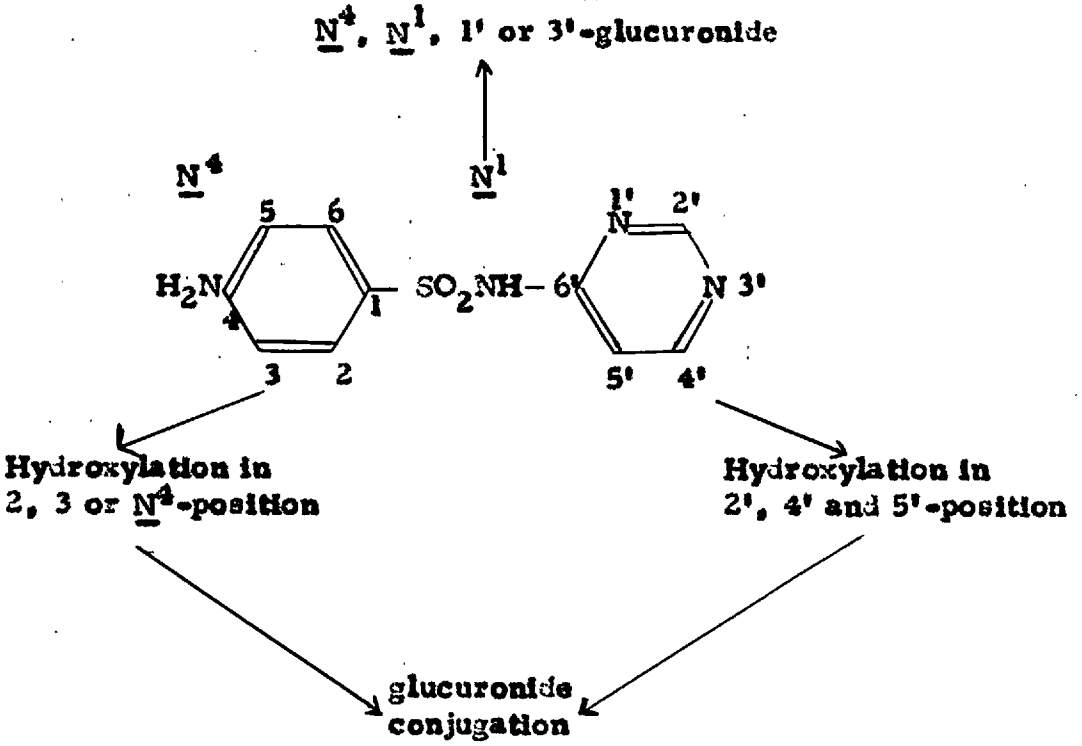
2. UDP-glucose + $2DPN^{+}$ $\xrightarrow[\text{dehydrogenase}]{\text{UDP glucose}}$ UDPGA + 2DPNH

Conjugation can then occur between UDPGA and hydroxyl, amino, carboxyl and sulphydryl groups of foreign compounds. Glucuronide formation occurs in most animals, birds, amphibia and probably reptiles, but not in insects and fish.

Five types of glucosiduronic acids are found in mammalian organisms:

1) 'Ether' glucuronides.
2) 'Ester' glucuronides.
3) 'Enol' glucuronides.
4) N-glucuronides.
5) S-glucuronides.

The following combinations are possible between glucuronic acid and an N^1-substituted 6-sulphanilamidopyrimidine.



N-GLUCURONIDES.

The work of Thierfelder (1889) showed that N-glucuronides could be formed by direct reaction of a glucuronic acid derivative with an aromatic amine.

However, the possible enzymic synthesis of N-glucuronides has been studied by several workers in the last decade with conflicting results. Axelrod, Inscoe & Tomkins (1953) showed that in vitro

aniline N-glucosiduronic acid could be formed by guinea pig liver microsomes. However, the method of estimation used was inaccurate and the evidence inconclusive (see Bridges, 1963). Isselbacher (1961) using a purified enzyme glucuronyl transferase found that it was highly effective in the in vitro synthesis of the glucuronides of o-aminophenol, p-aminophenol, anthranilic acid and bilirubin, but that it would not catalyse the formation of aniline N-glucosiduronic acid. Arias (1961) in his studies suggested that separate glucuronyl transferases were involved in the formation of N- and O-glucuronides.

Bridges (1963) showed that chemical combination between aromatic amines and sodium glucuronate occurs spontaneously in acid solution. He showed that the pH of optimum formation was approximately one pH unit above the pKa of the amine studied. Below pH 2-3 the glucuronides hydrolysed rapidly. These factors, therefore, affect the amount of N^4-glucuronides found in the urine after feeding sulphonamides. No evidence was found for the enzymic formation of the N-glucuronide of aniline, p-chloroaniline or p-nitroaniline using liver microsomes from rats, rabbits and guinea pigs, although all liver preparations were shown to be highly active in o-aminophenol glucuronide synthesis. Also β -glucuronidase (from snails) had no

effect on sulphanilamide N^4-glucuronide (Bridges & Williams, 1962).

It is probable that all sulphonamides give rise to some N^4-glucuronides, and Table 1.4 summarises some of the available data.

TABLE 1.4

Excretion of sulphonamide N^4-glucuronides.

Sulphonamide	Species	% Excreted of dose fed.	Reference
Sulphanilamide	rabbit	6-10	2
	rabbit, dog	1- 5	1
Sulphathiazole	rabbit	9-20	2
	man	0.8	3
Sulphapyridine	rabbit	35-41	4
Sulphamethylthiadiazole	man	0.3	5
Sulphasomizole	man, rat		
	rabbit, dog	0.1-1.3	7
Sulphacetamide	rat	0.1	8
Sulphadiazine	rabbit	7-13	2
Sulphadimethoxine	rabbit, rat		
	dog, guinea pig		
	monkey, man	5-17	6

REFERENCES

1. Bridges & Williams (1963a)
2. Ogiya (1960)
3. Uno & Ueda (1962)
4. Ogiya (1959)
5. Uno & Okazaki (1960)
6. Kibby (1965)
7. Bridges & Williams (1963b)
8. Bridges & Williams (1962)

Some workers in the field of sulphonamides have isolated certain glucuronide metabolites in which the glucuronic acid residue is not attached to the N^4-position, but which yields the original sulphonamide and glucuronic acid on hydrolysis by chemical or biochemical means.

In 1962, it was reported that man formed a minor metabolite of sulphathiazole (3.8% of dose fed) which contained glucuronic acid (Uno & Ueda, 1960, 1962). The structure of an N^1-glucuronide was ascribed to this metabolite on the basis of infrared and ultraviolet spectra (Uno *et al.*, 1963). This N^1-glucuronide was not found as a metabolite of sulphathiazole in the rabbit, nor was it found in *in vitro* work with perfused rabbit liver (Uno & Ueda, 1963).

DiCarlo *et al.* (1963) showed that one of the major metabolites of sulphamethomidine in man contained glucuronic acid and the sulphonamide with a ratio of 1.25:1. DiCarlo showed that this urinary glucuronide of sulphamethomidine was distinguishable from synthetic sulphamethomidine N^4-glucuronide by difference in paper chromatography and stability towards acid and paper electrophoresis. From the failure of the urinary glucuronide to form hydroxamic acid it was concluded that the metabolite was not an 'ether' glucuronide. The azo sulphamethomidine was purified and it was shown that the glucuronic acid was not attached to the benzene ring of sulphamethom-

idine because no 2-hydroxy-or 3-hydroxy-sulphanilic acid was formed after acid cleavage. DiCarlo, therefore, concluded because azosulphanilic acid was formed after diazotization and coupling of sulphamethomidine glucuronide prior to hydrolysis that the glucuronic acid was attached to the pyrimidine ring. He seemed to have overlooked the fact that it could be the $\underline{N}^1$-glucuronide that was formed.

A closely related drug to sulphamethomidine is sulphadimethoxine. It has been shown that the major metabolite of this sulphonamide in man and monkey is sulphadimethoxine-$\underline{N}^1$-glucuronide (Bridges, Kibby & Williams, 1965). This was demonstrated by isolating the biosynthetic material and comparing its properties with the synthetic material. This metabolite was also found in the urine of rat, dog and guinea pig.

In 1966, it was shown that sulphadimethoxine-$\underline{N}^1$-glucuronide was the major metabolite of sulphadimethoxine (40-70% of dose excreted in urine) in several non-human primates (Adamson, Bridges & Williams, 1966). Kibby (1965) also investigated the metabolism of several other methoxy substituted 6-sulphanilamido-pyrimidines in man, monkey, rat and rabbit, and found that except for madribon, $\underline{N}^1$-glucuronide formation was only a minor metabolic pathway. He also gave evidence for the enzymic synthesis of

madribon N^1-glucuronide in the rat and guinea pig, but found that it was not formed in the rabbit *in vitro* or *in vivo*.

OXIDATION.

In vivo oxidation followed by conjugation with glucuronic or sulphuric acid has been shown for a number of sulphonamides. With the simple sulphonamides such as *o*, *m*, and *p*-aminobenzene-sulphonamides, oxidation occurs in the benzene ring, whereas with the N^1-substituted sulphonamides oxidation occurs in the heterocyclic ring.

Sulphanilamide is metabolised in both man and rabbit to 3-hydroxysulphanilamide and it is then excreted as the sulphate conjugate (Shelswell & Williams, 1940; Williams, 1946, 1947). James (1939) found N-hydroxy-sulphanilamide in the urine of mice receiving sulphanilamide.

The extent of oxidation of a number of N^1-substituted heterocyclic sulphonamides in rabbits was estimated on the basis of increased excretion of glucuronic acid and ethereal sulphate (Smith & Williams, 1948a).

Scudi & Childress (1956) identified and isolated the heterocyclic ring oxidation product of sulphapyridine from dog urine, i.e. 5-hydroxysulphapyridine.

Other sulphonamides thought to be hydroxylated include

sulphaquinoxaline (Scudi & Silber, 1944), sulphasomizole (Bridges & Williams, 1963), sulphamethoxypyrazine (Bertalozzi, Chieli & Cicori, 1962), sulphadimethoxine (Koechlin, Kern & Engelberg, 1959) and sulphamethomidine (DiCarlo et al., 1963).

c) EXCRETION

Sulphonamides are eliminated from the body, both as the free drug and as metabolites mainly in the urine, although small amounts may be found in the faeces (Neipp, Sackmann & Tripod, 1961). Various secretions of the body may also contain sulphonamides i.e. bile, sweat, tears, saliva, milk and intestinal fluids.

i) RENAL EXCRETION AND REABSORPTION.

The urinary excretion of sulphonamides in man may vary considerably from 5% of the dose fed in the case of fanasil (Bridges, Kibby, Walker & Williams, 1969) to 75% for sulphasomidine in the first 24 hours (Prior & Saslow, 1951). The amount of sulphonamide excreted in the urine is a result of three distinct processes:

Glomerular filtration

Passive back diffusion

Tubular secretion

Glomerular filtration.

Plasma is filtered through the kidney glomerulus, and only non-protein bound drug is immediately available for filtration. Protein binding, therefore, affects the amount of 'free' drug available and consequently the rate of excretion. Weakly bound drug is in equilibrium with the free drug, and consequently will

become available for filtration when unbound drug is filtered, as a result of attempts to re-establish the equilibrium. The total blood level, extent and firmness of binding will ultimately determine the length of time for excretion of bound compounds.

Passive back diffusion.

Most drugs including sulphonamides, are weak electrolytes and hence are partially ionized in body fluids. Since the tubular epithelium is most permeable to lipid soluble unionized molecules, back diffusion will depend on the pKa of the compound, the degree to which the unionizable form is lipid soluble, the urine volume and pH (Peters, 1962). Berliner (1954) showed that a decrease in the pH of the tubule fluid decreased the excretion of weak organic acids, while a pH increase enhanced their excretion presumably by increasing the concentration of the unionized molecules.

Litchfield (1960) claimed that while renal reabsorption can prolong the action of sulphonamides, in the case of sulphadimethoxine, long action was the result of a low level of non-protein bound drug being available for renal clearance.

Tubular secretion.

Despopoulos & Callahan (1962) examined the active process of renal tubular secretion of a number of sulphonamides both in vivo and

in vitro. They suggested that the renal excretion requires a specific physiochemical interaction between the substrate and a postulated intracellular receptor molecule, and the substrate specificity of the active transport system depended on the presence of the $SO_2 - \bar{N}$ group. This group was thought to interact with the receptor only if there was a sufficient localization of charge on the nitrogen, and if the electronegativity of each oxygen was great enough for the formation of hydrogen bonds. Sulphonamides, therefore, with a pKa greater than 8.4 were not transported while those with a pKa of less than 6.4 were transported.

ii) BILIARY EXCRETION AND ENTEROHEPATIC RECIRCULATION.

This may be the major route of excretion for some drugs. Sulphonamides are known to be excreted in the bile in addition to the urine (Millburn, 1965), and a knowledge of the biliary excretion of these chemotherapeutic agents is relevant to the use of sulphonamides for the treatment of hepato-biliary infections.

Some sulphonamides, sulphacetamide, sulphaguanidine, sulphathiazole, sulphasoxazole and sulphasomizole are only excreted to a small extent (1% of dose fed) in the bile of rats whereas sulphadimethoxine (11%) and succinylsulphathiazole (42%) are excreted to a much greater extent in the same animal (Millburn, Smith & Williams, 1967). The biliary excretion of sulphaphenazole, sulphamethoxy-

pyridazine, sulphasomidine and sulphachloropyridazine has been reported to occur in rabbits following their administration by intravenous injection (Neipp, Sackmann & Tripod, 1961).

Enterohepatic recirculation of drugs excreted in the bile could be responsible for their slow and delayed excretion in the urine or faeces. However, this does not appear to be a significant factor in the long action of sulphadimethoxine (Millburn, 1965).

d) TISSUE BINDING

For a review of this aspect of the subject see Chapter 5.

3) SPECIES DIFFERENCES

Species differences in the metabolism and excretion of drugs has been known for a number of years. Species variation in response to an orally administered drug is related to differences in absorption, tissue binding, route and rate of excretion and metabolism. A number of variables may contribute to observed differences in metabolism and excretion including the dose and route of drug administration, strain of animal used, age, sex, health, diet, stress, seasonal variations, environmental temperature and other anutrient chemicals.

At a biochemical level, species differences will depend on location, amount and nature of drug metabolising enzymes, amount of endogenous inhibitors, and availability of cofactors. Also competition between different enzymes in various species for substrate and presence of enzymes which carry out the reverse reaction of product to substrate, will produce species variation in metabolic rates. It can be seen, therefore, that *in vivo* and *in vitro* work are important in the evaluation of species differences in the metabolism and excretion of drugs.

Very little work has been done with sulphonamides in this respect. Abou-El-Makarem *et al.* (1967) studied the biliary excretion of succinylsulphathiazole in a number of species. This compound was

selected because it undergoes little, if any, metabolic alteration in vivo and thus the differences in metabolism which could influence biliary excretion were obviated. There was a marked species difference in the excretion of this compound, which was given intravenously. The rat, dog and hen were good biliary excretors (20-29% excreted of dose fed), while the guinea pig, rabbit and monkey were limited in this respect (1% approximately excreted). The cat and sheep were intermediate between the two groups.

Bridges (1963) studied the metabolism and urinary excretion of sulphanilamide in the fox, dog, cat, guinea pig, hen, turkey, pigeon, rat, mouse, coypu, rabbit, monkey and man. Sulphanilamide is rapidly excreted in all these species (70-90% of the dose fed is excreted in 24 hours). The main metabolite is the N^4-acetyl derivative which was found in all species except the dog and fox, but only low levels of this metabolite were found in the cat (8.4%) and guinea pig (6.6%). Small amounts of minor metabolites such as sulphanilamide-N^4-glucuronide and N^1-acetyl sulphanilamide were also found in the urine of these animals. The N^1,N^4-diacetyl-sulphanilamide was found in all species except the dog and fox. It appears, therefore, that different types of $-NH_2$ groups may require different acetylases for their acetylation, and one or other of these may be deficient in some species. Other workers have confirmed

that the dog is unable to acetylate the aromatic amino group of sulphonamides.

The metabolism and excretion of sulphasomizole has also been studied (Bridges & Williams, 1963a) and the results are given in Table 1.5.

TABLE 1.5

Metabolism and Excretion of Sulphasomizole.

	Chinchilla rabbits	Wistar rats	Corgi dogs	Man
Number of animals	3 ♀	3 ♀	2 ♂	5 ♂
Oral dose of drug mg./kg.	150	150	500	30
% dose excreted in 24 hrs.	79.5	71.5	35.9	59.7
Metabolites found in urine.				
Unchanged drug	26.9	68.1	90.2	61.3
N^4-Acetyl derivative	68.3	29.0	0.0	36.8
N^4-Glucuronide	1.1	1.8	1.4	0.7
N^4-Sulphate	0.0	1.4	3.9	0.1
'Oxidation product'	2.2	0.0	4.2	0.0

The major metabolic product of sulphasomizole is the N^4-acetyl derivative in rats, rabbits and man. The extent of acetylation varies with the species, two-thirds of the excreted dose is acetylated in the rabbit, one-third in the rat and man and none at all in the dog. Both

the N^4-sulphate and N^4-glucuronide are minor metabolites of sulphasomizole, the latter probably arising as an artefact in the urine.

An interesting piece of work has been done on the metabolism and excretion of 2,4-dimethoxy-6-sulphanilamidopyrimidine (sulphadimethoxine) in a number of species (Adamson, Bridges & Williams, 1966 - unpublished data) Table 1.6.

There is considerable species variation in the nature of the urinary metabolites. In the rabbit and guinea pig the major metabolite is N^4-acetylsulphadimethoxine. In the dog there is no acetylation, and the major part of the drug is excreted unchanged. In the rat the unchanged drug and N^4-acetyl derivative are excreted in roughly equal amounts. The primates, however, differ from the other species in that the major metabolite is the N^1-glucuronide. The other species, except the rabbit, only produce small amounts of the glucuronide. When it was found that the monkey and man metabolised sulphadimethoxine in a similar way, it might have been predicted that the isomers of the sulphonamide would behave like the parent drug in the monkey and the results could be translated to man. Table 1.7 shows that this is not true and that although they are metabolised similarly, none of the other derivatives form an N^1-glucuronide in major amounts in any of the species examined (Bridges, Kibby, Walker & Williams, 1969).

TABLE 1.6

Metabolism and excretion of sulphadimethoxine in various species.

Order	Species	% dose excreted 24 hrs.	% composition of 24 hr. urine N^1-glucuronide	N^4-acetyl
Rodentia	Rat	9	7	46
	Guinea pig	20	5	66
Lagomorpha	Rabbit	43	0	94
Carnivora	Dog	23	19	0
Primates	Man	25	70	21
Old World	Rhesus monkey	42	70	21
	Green monkey	12	30	61
	Baboon	42	72	16
New World	Squirrel monkey	9	51	37
	Capuchin	8	48	15
	Giant bushbaby	26	48	2
	Slow loris	19	62	35
Doubtful Primates	Tree shrew	26	52	18

TABLE 1.7

N^1-Glucuronide formation in various species given methoxy-6-sulphanilamidopyrimidines.

Portion of methoxy groups	% of compound excreted in 24 hrs. as N^1-glucuronide			
	Man	Monkey	Rat	Rabbit
2	-	5	2	0
4	18	2	3	0
5	-	2	3	0
2,4	62	70	7	0
2,5	-	1	0	0
4,5	0	2	0	0

It appears, therefore, that 2,4-substitution of the pyrimidine ring has something to do with N^1-glucuronide formation in man and other primates. The problem was further investigated by looking at the metabolism of two other 2,4-disubstituted 6-sulphanilamidopyrimidines, namely, sulphamethomidine (2-methyl-4-methoxy) and sulphasomidine (2,4-dimethyl).

The aim of investigations into species differences is to find an animal or animals which respond to drugs in a similar way to man. So far not enough information has been accumulated to give an answer to this problem. The nearest we might hope to get to the answer is to find a species which will respond to a particular group of drugs i.e. the sulphonamides, or at least to a particular chemical grouping in a similar way to man.

CHAPTER 2

SPECIES DIFFERENCES IN THE METABOLISM AND EXCRETION OF SULPHAMETHOMIDINE AND SULPHASOMIDINE.

1) INTRODUCTION.

2) MATERIALS AND METHODS.

a) Syntheses
b) Chromatography and Colour Reactions
c) Spectra
d) Quantitative Estimation
e) Animals
f) Isolation of Metabolites
g) Hydrolysis of Sulphamethomidine and its Metabolites.

3) RESULTS.

4) DISCUSSION.

Metabolism

Structure of the N^1-glucuronide

Biliary Excretion.

.....

1. INTRODUCTION

The long acting sulphonamide drug, sulphadimethoxine (2,4-dimethoxy-6-sulphanilamidopyrimidine) shows a remarkable species difference in metabolism (Bridges, Kibby, Walker & Williams, 1968) in that it is excreted in man and other primates, but not in several lower species, mainly as an N^1-glucuronide (Adamson, Bridges & Williams, 1966). It was shown that this formation of an N^1-glucuronide did not occur in man and the rhesus monkey if the positions of the methoxyl groups were altered, for 2-, 4- or 5-methoxy and 2,5- or 4,5-dimethoxy-6-sulphanilamidopyrimidines did not form appreciable amounts of N^1-glucuronide (Bridges, Kibby, Walker & Williams, 1969). It appeared, therefore, that formation of N^1-glucuronide was associated with the 2,4-disubstitution of the pyrimidine ring in this series of drugs.

This chapter describes the metabolism and excretion of two other drugs of this series containing 2,4-disubstituted rings, in man, monkey, rat and rabbit. These are 2,4-dimethyl-6-sulphanilamidopyrimidine (sulphasomidine; Elkosin) a short acting antibacterial drug and 2-methyl-4-methoxy-6-sulphanilamidopyrimidine (sulphamethomidine), a medium-long acting drug in man (Cooper, Madoff & Weinstein, 1962). Sulphasomidine is very water soluble, and is well absorbed and rapidly excreted in man (80-90% of the dose fed appearing in the 48 hour urine). It is excreted mainly as the unchanged drug, the extent of acetylation

being low (5-15% of the amount excreted) (Prior & Saslow, 1951). Walter (1960) reported that 10-35% of the dose may be excreted as a glucuronide.

Sulphamethomidine is well absorbed in the rabbit and dog, but poorly so in the rat. It is excreted mainly as the acetyl derivative in the rabbit and rat but poorly acetylated in the dog (DiCarlo, Malamont, Haynes & Phillips, 1962). In man, about 26% of an oral dose is excreted in the urine in 24 hours, mainly as a glucuronide which is claimed to be a conjugate of a hydroxy-sulphamethomidine in which the hydroxyl group occurs in the pyrimidine ring (DiCarlo, Malamont & Phillips, 1963). It has been reported briefly (Bridges, Walker & Williams, 1967) that this glucuronide is an $\underline{N}^1$-glucuronide similar to that formed in man from sulphadimethoxine (Bridges, Kibby & Williams, 1965) and in this chapter evidence is given for this structure.

2. MATERIALS AND METHODS.

2-Methyl-4-methoxy-6-sulphanilamidopyrimidine (sulphamethomidine), m.p. 177-178°, was a gift from Warner-Lambert, Morris Plains, New Jersey. 2,4-Dimethyl-6-sulphanilamidopyrimidine (sulphasomidine; Elkosin) m.p. 240-241°, 2,4-dimethoxy-6-sulphanilamidopyrimidine (sulphadimethoxine; Madribon), m.p. 200-201°, and N^4-acetylsulphadimethoxine m.p. 210-211° were given by Dr. R. Long, Roche Products Ltd., Welwyn Garden City, Herts. 4-Amino-3-hydroxybenzene-sulphonic acid, m.p. 266° (decomp.) and 4-amino-2-hydroxybenzene-sulphonic acid, m.p. 275° (decomp.) were given by May & Baker Ltd., Dagenham, Essex. Sulphanilic acid, 288° (decomp.), sulphanilamide, m.p. 164-166° and barbituric acid, m.p. 245°, were purchased (Hopkin & Williams Ltd.). Sulphadimethoxine-N^1-glucuronide (NH_4 salt), m.p. 150-160° (decomp.), and sulphadimethoxine-N^4-glucuronide (Na salt), m.p. 149° (decomp.), were synthesised according to Bridges et al. (1965).

a) SYNTHESES.

Sulphamethomidine-N^1-glucuronide. Sodium sulphamethomidine (3 g.) and methyl 2,3,4-tri-O-acetyl-1-bromoglucuronate (3.3 g.) m.p. 106-109°, (Bollenback, Long, Benjamin & Linlquist, 1955) were dissolved in a water (6 ml.) and acetone (25 ml.) mixture and kept at 37° for 4 hr. The resulting dark-red solution was then kept overnight at 0°. It was

then treated with propan-2-ol (12 ml.) followed by water (100 ml.) and the tri-O-acetyl methyl ester of sulphamethomidine-N^1-glucuronide separated. This was filtered and sucked dry to a buff powder (3.2 gm.), m.p. 115-122°. The ester in acetone (5 ml.) was then passed through a column (2.5 cm. x 20 cm.) of activated alumina (type H; 100-200 mesh) (P. Spence Ltd., Widnes, Lancs.) and eluted with acetone. The first 50 ml. was collected and evaporated to dryness, and the product recrystallized from propan-2-ol to give methyl 2,3,4-tri-O-acetyl-1-deoxy-1-[$N^{1'}$-(4''-methoxy-2''-methylpyrimidin-6''-yl)sulphanilamido-glucosid]uronate, as white needles, m.p. 108-110° and $[\alpha]_D^{20}$ + 67° (c = 1 in methanol). (Found: C, 49.5; H, 5.1; N, 8.8; S, 5.0%. $C_{25}H_{30}O_{12}N_4S$ requires C, 49.2; H, 4.9; N, 9.2; S, 5.2%). The above ester (1 g.) in methanol (25 ml.) was treated with 0.5N-barium methoxide in methanol (2.5 ml.) and the mixture kept for 3 days at 0°. 2N-H_2SO_4 (0.5 ml.) and water (12.5 ml.) were then added, and the solution passed through a column (2.5 cm. x 10 cm.) of Amberlite CG-120 (H^+ form; 100-200 mesh) (British Drug Houses Ltd., Poole, Dorset). The column was washed with water (10 ml.) and then eluted with 2N-ammonia solution (75 ml.) and finally water (50 ml.). The combined eluates were evaporated at 45° in vac. to a white solid. This was dissolved in methanol (5 ml.) and the solution banded on a thin layer plate of fluorescent silica gel, which was irrigated with propan-1-

ol/ammonia solution (sp. gr. 0.83) (7:3, by vol.) for 3 hr. The band corresponding to sulphamethomidine-N^1-glucuronide was eluted with 0.2N-ammonia solution and the eluate evaporated to dryness at 45° in vac. This process was repeated and the ammonium 1-deoxy-1-[$N^{1'}$-(4''-methoxy-2''-methylpyrimidin-6''-yl)sulphanilamido-glucosid]uronate was recrystallized from methanol to give white needles, m.p. 140-150° (decomp.) $[\alpha]_D^{20}$ + 4.0° (c = 5 in water). (Found: C, 42.5; H, 5.1; N, 13.8; S, 6.6%. $C_{18}H_{25}N_5O_9S.H_2O$ requires C, 42.8; H, 5.3; N, 13.9; S, 6.3%).

Sulphamethomidine N^4-glucuronide. Sulphamethomidine (1.1 g.) in dimethyl formamide (10 ml.) was added to a solution of sodium glucuronate (0.6 g.) in ethylene glycol (20 ml.). The mixture was heated for 10 min. at 70° and then kept in the dark for 24 hr. at room temp. On addition of excess acetone a gel formed, so sufficient water to just dissolve the gel was added. The solution was then kept for several days at room temp. and white crystals, m.p. 156-157°, of the sodium salt of sulphamethomidine N^4-glucuronide separated. The compound which readily reduced Benedict's and Fehling's solution on warming, was very labile and was not obtained entirely free of sulphamethomidine. It could, however, be used for chromatographic purposes (see Table 2.1) and was easily distinguishable from sulphamethomidine-N^1-glucuronide on paper. It gave a purple colour on paper after 5 mins. when sprayed with p-dimethylaminocinnamaldehyde

made up in acetic acid (2N).

N^4-Acetyl sulphamethomidine. Sulphamethomidine (0.5 g.) was dissolved in a 1:1 mixture of glacial acetic acid and acetic anhydride (10 ml.). The mixture was boiled for 30 min. and then allowed to cool. The solution was diluted with 50% ethanol (25 ml.) and warmed to dissolve any precipitate. On cooling, white crystals formed and the product was recrystallized from 25% acetic acid (v/v) (0.36 g.) m.p. 218-220°. This derivative had to be hydrolysed with 2N-HCl at 100° for 40 min. before it could be conjugated with N^1-α-naphthylethylenediamine in the Bratton & Marshall reaction. It gave a purple colour on paper with p-dimethylaminocinnamaldehyde on standing overnight (Table 2.1). The infrared spectrum was taken of this compound.

Sulphamethomidine-N^4-sulphate. Chlorosulphonic acid (1.15 ml.) was added dropwise with stirring, to anhydrous pyridine (25 ml.) cooled on ice. After allowing the mixture to stand for 30 min., sulphamethomidine (2.9 g.) was added, and the mixture shaken vigorously until all the solid material was dissolved. The solution was then kept in the dark at room temp. for 24 hours. Potassium hydroxide (0.2N, 100 ml.) was then added, and the solution extracted with ether (4 x 500 ml.) to remove the pyridine. The aqueous solution on evaporation to dryness at 50° in vac. gave a yellow residue.

After dissolving this material in a minimal amount of water (20 ml.) acetone was added (100 ml.) to precipitate inorganic salts and the solution filtered. This filtrate was evaporated to a small volume (8 ml.) which was then banded on to Whatman's 17 mm. chromatography paper and run against reference spots in a solvent of butan-1-ol: ammonia solution (sp. gr. 0.83):water (10:1:1, by vol.). The strip corresponding to an R_F value of 0.00 to 0.10 was cut out and eluted with ammonium hydroxide (0.2N). The eluate was evaporated to dryness and the residue dissolved in a minimum amount of absolute ethanol from which the N^4-sulphate was precipitated. The N^4-sulphate was labile and so was not obtained free of uncombined sulphamethomidine.

An acidified solution of the salt gave no test for sulphate with barium nitrate until boiled. On diazotisation and then coupling with alcoholic N^1-α-naphthylethylenediamine, it gave a red colour and on paper it was labile enough to give a colour with p-dimethylaminocinnamaldehyde after 10 min.

N^4-Acetyl sulphasomidine. Sulphasomidine (0.5 g.) was dissolved in a 1:1 mixture of glacial acetic acid and acetic anhydride (10 ml.). The mixture was boiled for 20 min. and then allowed to cool. The precipitated N^4-acetyl sulphasomidine was dissolved in dimethyl sulphoxide (10 ml.) and water (10 ml.) was added. On standing overnight at 0°, white crystals formed, m.p. 295°.

Sulphasomidine-N^4-glucuronide and sulphasomidine-N^4-sulphate were prepared as described for sulphamethomidine. They were difficult to purify, but could be used to determine their R_F values and colour reactions (see Table 2.1).

b) CHROMATOGRAPHY AND COLOUR REACTIONS.

The R_F values of sulphasomidine, sulphamethomidine and other relevant compounds were measured in three solvents and the results are given in Table 2.1. The solvent systems used were:

Solvent A : Butan-1-ol:ammonia soln. (sp. gr. 0.88):water (10:1:1, by vol.)

Solvent B : Propan-1-ol:ammonia solution (sp. gr. 0.88) (7:3, by vol.)

Solvent C : Butan-1-ol:acetic acid:water (4:1:2, by vol.)

Descending chromatography on Whatman No. 1 paper for 12 hours.

p-Dimethylaminocinnamaldehyde test (DMAC). DMAC was dissolved in 2N hydrochloric acid (0.2% solution) and diluted with ethanol (4 volumes). Weak N-conjugates such as the N^4-glucuronides and N^4-sulphates gave a purple spot after a few minutes. N^4-acetyl conjugates showed up as purple spots on keeping the chromatogram for 12 hours. The sulphonamides themselves and the N^1-conjugates gave a colour reaction immediately.

Naphthoresorcinol test (NR). The paper was sprayed with 1% (w/v) naphthoresorcinol in acetone spray to which 10% phosphoric acid (4:1 v/v) was added just before use. On heating at 140° for 10 min. the N^4-glucuronides showed up as a bright blue spot. Both the synthetic and biosynthetic N^1-glucuronide of sulphamethomidine isolated from monkey urine gave very weak blue-brown spots.

c) SPECTRA.

Ultraviolet light. Chromatograms were examined under the ultra-violet light (254 mμ) from a Hanovia 'Chromatolite' lamp (Engelhard Industries Ltd., Slough, Bucks.). Some compounds such as sulphanilic acid or sulphanilamide showed a weak violet fluorescence while others such as sulphasomidine and sulphamethomidine quenched the background fluorescence of the paper and appeared as dark spots.

Ultraviolet spectra. These were determined with the Unicam Spectrophotometer S.P. 500.

Infrared spectra. These were determined with the Perkin Elmer Infracord Spectrophotometer (KBr disc).

d) QUANTITATIVE ESTIMATION.

Determination of free and total aromatic amines in urine and bile. The method of Bratton & Marshall (1939) was used. Urine or bile (0.01-10 mg. aromatic amine/100 ml.) was diluted with water to contain 10-30 μg. of sulphonamide/ml. Free amine, which in this case includes free drug, the N^1-glucuronide of sulphamethomidine

and the N^4-glucuronide and N^4-sulphate conjugates (the N^4-glucuronide and N^4-sulphate groups are removed during diazotisation) were determined directly by the Bratton & Marshall method. The recovery of drug or its N^1-glucuronide added to urine or bile was 99-100%. For total aromatic amine the diluted urine or bile (1 ml.) was mixed with 2N-HCl (1 ml.) and heated on a boiling water bath for 40 min., cooled, and the total amine determined by the Bratton & Marshall method. The recovery of N^4-acetyl sulphamethomidine and N^4-acetyl sulphasomidine added to urine (10 mg./100 ml.) was 99-100%.

Determination of metabolites on chromatograms. Urine or bile (0.1-0.5 ml.) were banded across Whatman 3 mm. paper (5" bands) dried and chromatographed in propan-1-ol:ammonia solution (solvent B) against reference spots of possible metabolites by the descending technique for 12 hours. Strips corresponding to the reference spots (detected by ultraviolet light and DMAC spray) were cut from the chromatogram and eluted with dilute ammonia solution (0.2N). The eluate was neutralized with hydrochloric acid (2N), made up to a known volume and free and total amines were determined as described above. Recoveries of synthetic compounds estimated in this way were from 95-100%. The remainder of the chromatogram was eluted and estimated similarly. No free or total diazotisable material was found in this portion of the chromatogram with sulphasomidine or

sulphamethomidine.

e) ANIMALS.

The animals used were female Wistar albino rats (Porton strain), female New Zealand white rabbits and female Macacca rhesus monkeys.

Human subjects (healthy male volunteers) took the powdered drugs orally in rice paper sachets. The monkeys (4 kg.) were given the powdered drug concealed in honey. Rabbits (4 kg.) were given the sulphonamides as an aqueous suspension by stomach tube. Rats (250-300 g.) were given the drugs orally as a suspension (1 ml.) in aqueous sodium carboxy methyl cellulose (0.5%). Animals were fed with pellets and given water ad libitum. Urine was collected for 48 hours in two 24 hour batches. Sulphonamides were administered at a dose level of 100 mg./kg.

Preparation of rats with biliary fistulae. Female albino rats (250 ± 10 g.) were anaesthetized by the intraperitoneal injection of hexobarbitone sodium (100 mg./kg.: 25 mg./ml. water). The abdominal cavity was then opened up and the bile duct exposed. A polythene cannula (0.40 mm. and 0.80 mm. internal and external diameters respectively) was inserted into the common bile duct and tied in position, the tip being about 3 mm. from the junction of the right and left bile ducts. The cannula was sewn in position on the abdominal wall and the cavity closed. The rats were kept for 24 hours

in Bollman restraining cages (Bollman, 1948), with free access to a solution consisting of 5% w/v glucose and 1% w/v NaCl. Bile was collected at room temp. in a measuring cylinder and urine was collected in polythene trays fitted underneath the restraining cages.

f) ISOLATION OF METABOLITES.

N^4-Acetylsulphamethomidine. The 24-hr. urine (185 ml.) of a rabbit (5 kg.) which had received sulphamethomidine (0.5 g.) was brought to pH 5.5 with N-HCl and then shaken with ethyl acetate (400 ml.). The extract was evaporated at 45° to a paste which was then dissolved in N-Na_2CO_3 (10 ml.). The solution was treated with 2N-HCl (10 ml.) and the solid which formed was collected and dissolved in a little aqueous ethanol. The solution was banded on a thin-layer plate (fluorescent silica gel) and developed for 3 hr. in solvent B (Table 2.1). The area containing the N^4-acetyl derivative was located and the metabolite extracted with 0.2N-NH_4OH. The extract was evaporated at 45° and then N^4-acetylsulphamethomidine (45 mg.) had m.p. and mixed m.p. 218-220° after recrystallization from 25% acetic acid. It was characterized by R_F values and i.r. spectrum.

Sulphamethomidine N^1-glucuronide. The 24-hr. urine (300 ml.) of a monkey given the drug (0.5 g.) was concentrated to 50 ml. in a rotary evaporator. Ethanol (50 ml.) containing 2N-NH_4OH (5% v/v)

was added and the solution, filtered from solid material, was freeze-dried. The residue in a little aqueous ethanol was banded on Whatman 3MM paper and chromatographed in solvent B (Table 2.1) for 12 hr. The area containing the N^1-glucuronide was cut out and eluted with 0.2N-NH_4OH. The chromatography and elution was repeated. The eluate was evaporated to dryness at 45° and the solid recrystallized from aqueous ethanol. The sulphamethomidine N^1-glucuronide was obtained as white needles (8 mg.) m.p. 140-150° (decomp.). It was not quite pure but its chromatographic properties, colour reactions and u.v. and i.r. spectra were in agreement with those of the synthetic compound. It did not reduce Fehling's or Benedict's solutions.

g) HYDROLYSIS OF SULPHAMETHOMIDINE AND ITS METABOLITES.

Acid Hydrolysis. The drug or its N^4-acetyl derivative (0.5 g.) dissolved in ethanol (5 ml.) was mixed with 2N-HCl (5 ml.) and heated at 100° for 1 hr. The residual solution (2 ml.) was placed as a band (10cm.) on Whatman 3MM paper and chromatographed with solvents A, B or C (Table 2.1). Three main products were detected, namely sulphamethomidine, sulphanilic acid and an unknown suspected to contain the pyrimidine moiety. The areas corresponding to sulphanilic acid and the unknown were eluted with 2N-NH_4OH. The eluates were evaporated to dryness at 45° in vac. and i.r. spectra of the residues examined. The sulphanilic acid residue, m.p. 288° (decomp.) was identified but not the unknown.

Aqueous solutions of biosynthetic or synthetic sulphamethomidine N^1-glucuronide (1 mg./ml.) were heated with an equal vol. of 2N-HCl at 100° for 1 hr. The hydrolysates were then chromatographed as above and the products identified by R_F values and colour reactions.

β-glucuronidase. An aqueous solution (0.8 ml.) of the NH_4 salt of synthetic sulphamethomidine N^1-glucuronide (1 mg./ml.) free of sulphamethomidine (by chromatography) was added to each of 3 tubes. To one tube was added 1000 Fishman units of β-glucuronidase (Ketodase - William R. Warner & Co. Ltd.) in acetate buffer pH 5 (0.2 ml.). To the second the same amount of boiled enzyme and to the third 0.2 ml. water. The tubes were plugged and incubated for 24-hr. at 37°. The contents of the tubes were then chromatographed in solvents A, B and C (Table 2.1). The experiment was repeated with the biosynthetic glucuronide. The first tube in both cases gave spots corresponding to glucuronic acid, sulphamethomidine and a small amount of unchanged sulphamethomidine N^1-glucuronide (Table 2.4). The control tubes gave only one spot corresponding to unchanged glucuronide.

Table 2.1. R_F values and colour reactions of sulphasomidine and sulphamethomidine and related compounds.

Whatman No. 1 paper was used. The solvents (descending) were, A, butan-1-ol-ammonia solution (sp. gr. 0.88)-water (10:1:1); B, propan-1-ol-ammonia solution (sp. gr. 0.88) (7:3); C, butan-1-ol-water-acetic acid (4:2:1, all by vol.), run for 12 hr. DMAC = dimethylaminocinnamaldehyde and NR = naphthoresorcinol spray; - means no colour; q means quenching of background fluorescence of paper.

Compound	R_F values in A	B	C	Colour reactions DMAC	NR	Fluorescence in 254 mμ.
Sulphasomidine (SS)	0.45	0.66	0.9[illegible]	purple	-	q
N^4-Acetyl-SS	0.60	0.79	0.9[illegible]	- (purple after 24 hr.)	-	q
SS-N^4-Sulphate (K salt)	0.05	0.30	0.53	purple after 10 min.	-	q
SS-N^4-Glucuronide (Na salt)	0.00	0.15	decomp.	purple	blue	q
Sulphamethomidine (SM)	0.50	0.65	0.9[illegible]	purple	-	q

Continued/...

Table 2.1 (continued).

N^4-Acetyl-SM	0.62	0.76	0.94	- (purple after 24 hr.)	-	q
SM-N^4-Sulphate (K salt)	0.05	0.32	0.54	purple after 10 min.	-	q
SM-N^4-Glucuronide (Na salt)	0.00	0.12	decomp.	purple	blue	q
SM-N^1-Glucuronide (NH_4 salt)	0.10	0.39	0.63	purple	blue-brown	q
Sulphanilic acid	0.15	0.63	0.41	red	-	violet
Sulphanilamide	0.63	0.82	0.76	red	-	violet
Barbituric acid	0.09		0.54	-	-	q
4-Amino-3-hydroxy-benzenesulphonic acid	0.06	0.36	0.35	red	-	violet
4-Amino-2-hydroxy-benzenesulphonic acid	0.11	0.58	0.41	red	-	violet
Glucuronic acid (Na salt)	0.00	0.12	0.23	-	blue	q

Table 2.2. The excretion of total amine after the administration of sulphasomidine and sulphamethomidine to various species.

The drugs were administered orally (dose 100 mg./kg.; except in man where the dose was 30 mg./kg.). The urine was analysed daily for total aromatic amine by the Bratton & Marshall method (see text). The results are averages with ranges in parentheses. The number of animals used and their sex, M = male, F = female, are given in parentheses in the first column.

Species	Day after dosing	% of dose excreted after	
		Sulphasomidine	Sulphamethomidine
Rat (3F)	1	77(70-87)	41(36-49)
	2	80(75-90)	49(43-59)
Rabbit (3F)	1	76(66-85)	69(60-78)
	2	80(70-89)	74(65-83)
Monkey (6F)	1	63(58-68)	54(43-69)
	2	68(62-73)	58(50-72)
Man (2M)	1	74, 70	29, 22
	2	86, 80	54, 41

Table 2.3. **Urinary metabolites of sulphasomidine and sulphamethomidine in various species.**

The metabolites of sulphasomidine (SS) and sulphamethomidine (SM) were separated chromatographically and determined as described in the text. The urine collected during the first 24-hr. after dosing was analysed and the amount of each metabolite is expressed as a percentage of the amount excreted in 24 hr. The figures are the averages for 3 animals.

Metabolite	% of 24 hr. excretion in Rat		Rabbit		Monkey		Man*	
Drug fed	SS	SM	SS	SM	SS	SM	SS	SM
Unchanged drug	72	19	82	16	87	5	96	13
N^4-Acetyl	19	79	15	75	11	59	4	16
N^1-Glucuronide	0	0	0	0	0	32	0	68
N^4-Glucuronide	6	1	0	9	0	0	0	0
N^4-Sulphate	3	1	3	0	2	4	0	3

* Two subjects.

Table 2.4. Acid and enzymic hydrolysis of sulphamethomidine and its metabolites.

The compounds were hydrolysed as described in the text and the products identified by chromatography and colour reactions.

Compound	Hydrolysing agent	R_F value of products in solvent A†	Nature of product
Sulphamethomidine or N^1-Acetylsulphamethomidine	N-HCl	0.15	Sulphanilic acid
		0.50	Sulphamethomidine
		0.86	unknown ‡
Sulphamethomidine N^1-glucuronide, synthetic or biosynthetic	N-HCl	0.15	Sulphanilic acid
		0.00	Glucuronic acid
		0.86	unknown ‡
	β-Glucuronidase*	0.50	Sulphamethomidine
		0.00	Glucuronic acid
		0.10	Sulphamethomidine N^1-glucuronide (small amounts)

* The control tubes gave one spot R_F 0.10 in A, 0.39 in B and 0.63 in C, i.e. the unchanged glucuronide.

† See Table 2.1. Solvents B and C were also used to confirm the identifications.

‡ The unknown spot is probably that of the pyrimidine moiety which showed up on paper as a dark spot in u.v. light.

Table 2.5 **Biliary excretion of sulphasomidine (SS), sulphamethomidine (SM) and their N^4-acetyl derivatives in the rat.**

Compounds (100 mg./kg.) in dimethyl sulphoxide (0.5 ml.) were injected i.p. into biliary cannulated rats. The bile was collected for 24 hours. The biliary metabolites were separated chromatographically and estimated as described in the text. Average figures are quoted, the number of animals used being given in parenthesis.

Compound administered	Mol. wt.	% of the administered dose appearing in the bile in 24 hrs. as Unchanged Drug	N^1-glucuronide	N^4-glucuronide	N^4-sulphate	N^4-acetyl
SS (3)	278	2.1	0	0	0	4.4
N^4-acetyl SS (3)	320	0	0	0	0	4.6
SM (3)	294	1.3	1.6	0.3	0	3.3
N^4-acetyl SM (3)	336	0	0	0	0	4.0

4. DISCUSSION

Metabolism. The excretion of 2,4-dimethyl-6-sulphanilamido-pyrimidine (sulphasomidine) and 2-methyl-4-methoxy-6-sulphanilamido-pyrimidine (sulphamethomidine) is shown in Table 2.2. In the four species studied, sulphasomidine is more rapidly excreted in the urine than sulphamethomidine since about 70% of the former is excreted in 24-hr. and about 80% in 2 days, whereas with the latter drug the 24-hr. excretion is 20-30% in man, 40-50% in the rat, 50-60% in the monkey, but nearly 70% in the rabbit. The nature of the urinary metabolites of the two drugs is shown in Table 2.3. Sulphasomidine is largely excreted unchanged in the four species, for more than 70% of the 24-hr. excretion is unchanged drug and less than 20% is $\underline{N}^4$-acetylated, the other metabolites i.e. $\underline{N}^4$-sulphate and $\underline{N}^4$-glucuronide, being of little or no importance. With sulphamethomidine, however, the proportion of unchanged drug excreted is less than 20% of the 24-hr. excretion and the major metabolite in the rat, rabbit and monkey is $\underline{N}^4$-acetylsulpha-methomidine (60-80% of the 24-hr. excretion). However, in man the acetyl derivative is relatively minor (16%) and the major metabolite is now the $\underline{N}^1$-glucuronide (68% of the 24-hr. excretion). This glucuronide is also an important metabolite in the monkey (32%), but it is quanti-tatively less than the $\underline{N}^4$-acetyl compound (59%). In the rabbit and rat,

no N^1-glucuronide was detected in the urine. Small amounts of the N^4-sulphate of sulphamethomidine were found in rat, monkey and human urine.

It appears that in the four species studied, there is no marked difference in the metabolism and excretion of sulphasomidine (Fig. 2.1; $R_2 = R_4 = CH_3$) and although this drug is substituted with CH_3 groups in the 2 and 4 positions of the pyrimidine ring there is no formation of N^1-glucuronide in man and the monkey as in the case of sulphadimethoxine ($R_2 = R_4 = OCH_3$) which has OCH_3 groups in these positions (Bridges et al., 1968). However, with sulphamethomidine ($R_2 = CH_3$, $R_4 = OCH_3$) which has CH_3 in position 2 and OCH_3 in 4, a marked species difference again appears, for N^1-glucuronide formation occurs in man and the monkey but not in the rat and rabbit. With 2-methoxy-6-sulphanilamidopyrimidine ($R_2 = OCH_3$, $R_4 = H$)

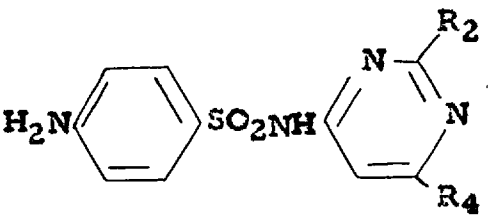
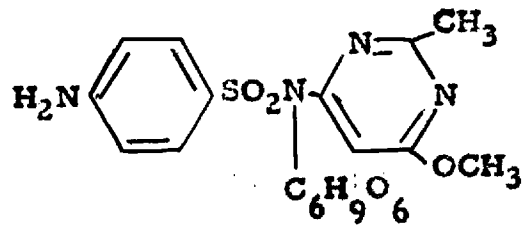


Fig. 2.1

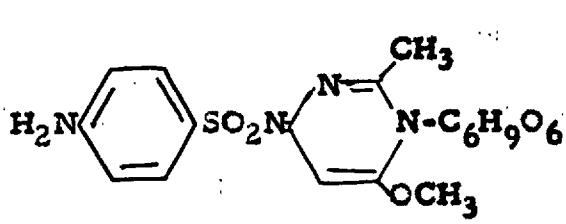
and sulphamonomethoxine ($R_2 = H$, $R_4 = OCH_3$) in which one OCH_3 group occurs in the 2 or 4 position, respectively, formation of N^1-glucuronide does not occur (Bridges et al., 1969).

Structure of the N^1-glucuronide. The identification of this metabolite as sulphamethomidine N^1-glucuronide is in conflict with DiCarlo et al. (1963) who proposed that the glucuronic acid was attached to the hydroxylated pyrimidine ring. The urinary glucuronide was slowly hydrolysed by β-glucuronidase to sulphamethomidine and glucuronic acid (Table 2.4) and by N-HCl to sulphanilic acid, glucuronic acid and an unidentified pyrimidine derivative. The 2- and 3- hydroxy-sulphanilic acids (Table 2.1) were not found as acid hydrolysis products and so it was concluded that the glucuronide was not an o-glucuronide of a hydroxy-sulphamethomidine, but an N-glucuronide. The N^4-glucuronide can be eliminated because it readily reduced Benedict's and Fehling's solution whereas the urinary metabolite did not. Also the two gave very different R_F values in the three solvents and colour reactions (Table 2.1). The urinary glucuronide was similar in all respects to a sulphamethomidine N^1-glucuronide synthesised from sodium sulphamethomidine and methyl 2,3,4-tri-O-acetyl-1-bromo-glucuronate. From the infrared spectra it could be seen that both the synthetic and biosynthetic glucuronides of sulphamethomidine had an SO_2 symmetrical stretching frequency at 1160 $cm.^{-1}$ which, according to Uno, Machida, Hanai, Ueda & Sasaki (1963) indicated that the conjugate existed in the amido form (Fig. 2.2a) and not the imido form (Fig. 2.2 b and c).

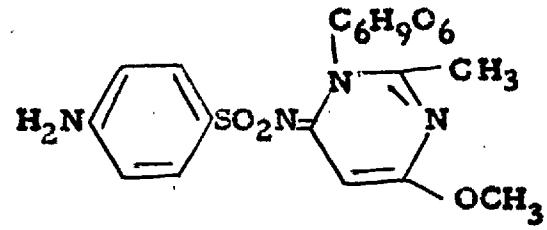
Fig. 2.2



(a)



(b)



(c)

Biliary excretion. The biliary excretion of sulphasomidine and sulphamethomidine and their acetyl derivatives was examined in rats (Table 2.5). The compounds were injected intraperitoneally into biliary cannulated rats at a dose level of 100 mg./kg. In the case of sulphasomidine, 6.5% of the dose appeared in the bile in 24 hours, whereas with N^4-acetyl sulphasomidine, 4.6% of the dose appeared in the bile unchanged. With sulphamethomidine, 6.5% also appeared in the bile in 24 hours of which 1.6% was as the N^1-glucuronide. Thus, in the rat, although no N^1-glucuronide appears in the urine, a small amount is formed and excreted in the bile. N^4-Acetyl sulphamethomidine appeared in the bile to an extent of 4% of the dose as the unchanged compound. The biliary excretion of these compounds in the rat is of a low order and this would have been expected on the

hypothesis put forward by Millburn, Smith & Williams (1967) concerning polarity and molecular weight and the extent of biliary excretion. However, the tri-O-acetyl-methyl ester of sulphamethomidine N^1-glucuronide when injected (i.p.) into rats, was excreted to an extent of 66% of the dose given in the bile in 24 hours. This compared favourably with sulphadimethoxine N^1-glucuronide (78% of the dose excreted unchanged in the bile in 24 hours) (Bridges *et al.*, 1968).

It can be seen, therefore, that the N^4-acetyl derivatives of these sulphonamides have a low affinity for biliary excretion, whereas the N^1-glucuronides of sulphamethomidine and sulphadimethoxine are efficiently excreted in the bile of rats. The mechanism by which these glucuronides are excreted is probably an active one, since the hepatic transfer system responsible for the excretion of sulphadimethoxine N^1-glucuronide in the bile has been shown to be saturated by large doses of this compound (Millburn, 1965). When the N^4-sulphate of sulphadimethoxine was administered to rats very little was excreted in the bile whereas when the N^4-glucuronide was given it was excreted in the bile to an extent of 42% (Bridges *et al.*, 1968). These results suggest that biliary excretion is a highly selective process for these compounds which depends not only on water solubility, molecular weight and polarity, but also on other unknown factors such as active transport.

CHAPTER 3

IN VITRO SYNTHESIS OF SULPHADIMETHOXINE N^1-GLUCURONIDE AND THE N^4-ACETYL DERIVATIVES OF SOME SUBSTITUTED 6-SULPHANILAMIDOPYRIMIDINES.

1) INTRODUCTION.

2) MATERIALS AND METHODS.

a) Estimation of [^{35}S]sulphadimethoxine
b) In vitro acetylation rates
c) In vitro synthesis of sulphadimethoxine-N^1-glucuronide.

3) RESULTS.

Fig. 3.1a Formation of N^4-acetyl sulphadimethoxine by rabbit liver homogenate with time.

Fig. 3.1b Velocity - pH curve for the formation of N^4-acetyl sulphadimethoxine by rabbit liver homogenate.

Table 3.1 Metabolism and excretion of some substituted 6-sulphanilamidopyrimidines in the rabbit and monkey.

Table 3.2 In vitro acetylation of some substituted 6-sulphanilamidopyrimidines by liver homogenates.

Table 3.3 Synthesis of sulphadimethoxine N^1-glucuronide by liver preparations from various species.

Table 3.4 Electronic charge and free valence of the N^1- and N^4- positions of the substituted 6-sulphanilamidopyrimidines.

4) DISCUSSION.

Acetylation in vitro.

Enzymic synthesis of the N^1-glucuronide.

.....

1) INTRODUCTION

It was shown in the previous chapter that sulphasomidine was excreted mainly as the unchanged drug while sulphamethomidine was chiefly acetylated in all of the species examined. The *in vitro* acetylation of these two drugs was investigated in the rabbit and monkey to see whether this factor would explain the species difference in metabolism.

The fates of six methoxy substituted 6-sulphanilamidopyrimidines in man, monkey, rat and rabbit have already been investigated (Bridges, Kibby, Walker & Williams, 1969)[1]. The urinary excretion of the substituted 6-sulphanilamidopyrimidines is shown in Table 3.1. The rabbit rapidly excretes all the sulphonamides studied when administered orally, between 43 and 82% of the dose being excreted in the first 24 hrs. The rate of excretion of these sulphonamides was lower in the monkey than in the rabbit and also showed a greater variation. The 4-methoxy (12%), 5-methoxy (17%), and 4,5-dimethoxy (25%) derivatives are slowly excreted, whereas the 2-methoxy (57%), the 2-methyl-4-methoxy (54%) and the 2,4-dimethyl (63%) derivatives are rapidly excreted by the monkey. These compounds vary in their rates of excretion not only with species, but also with the position of the substituents in the pyrimidine ring in an apparently haphazard manner.

The rates of excretion could be related to the nature of the metabolites, the amounts of which in a 24 hr. excretion are shown in Table 3.1. The important metabolites quantitatively are the free drugs, the $\underline{N}^4$-acetyl derivatives and the $\underline{N}^1$-glucuronides. In the rabbit, all the drugs, except the 2,5-dimethoxy and the 2,4-dimethyl derivatives are extensively acetylated. The amount of acetylated 2,5-dimethoxy and 2,4-dimethyl is low (7% and 15% of the 24 hr. excretion respectively), most of the drug being excreted unchanged. The rabbit forms no $\underline{N}^1$-glucuronide with any of the drugs.

In the monkey, the 4-methoxy (89%), 5-methoxy (86%), 4,5-dimethoxy (85%) and 2-methyl-4-methoxy (59%) compounds are highly acetylated, whilst the 2-methoxy (17%), 2,4- (21%) and 2,5- (3%) dimethoxy and 2,4-dimethyl (11%) are poorly acetylated *in vivo*. The mono- and di-substituted methoxy compounds were therefore included in the *in vitro* acetylation study in order to find a relationship between structure and metabolic fate.

An interesting species difference observed is that sulphamethomidine (see Chapter 2) and sulphadimethoxine (Adamson, Bridges & Williams, 1966) are metabolised to the $\underline{N}^1$-glucuronide in primates (Table 3.1), whereas this metabolite is only found in small amounts in other species. The *in vitro* rate of formation of sulphadimethoxine $\underline{N}^1$-glucuronide in the monkey, rat and rabbit was therefore studied in order to evaluate the role in determining species differences in metabolism.

2) MATERIALS AND METHODS.

[^{35}S]Sulphadimethoxine m.p. 200-201°, (sp. activity 35 uc./mg.), 4,5-dimethoxy-6-sulphanilamidopyrimidine (sulphorthodimethoxine, fanasil) m.p. 194-197°, 2,5-dimethoxy-6-sulphanilamidopyrimidine m.p. 190-193°, 2-methoxy-6-sulphanilamidopyrimidine m.p. 186-187°, and 5-methoxy-6-sulphanilamidopyrimidine m.p. 200-201°, were the gifts of Dr. R. Long, Roche Products Ltd., Welwyn Garden City, Herts. 4-Methoxy-6-sulphanilamidopyrimidine m.p. 201-203° was given by the Imperial Chemical Industries, Pharmaceutical Division, Alderley Park, Cheshire. Sulphadimethoxine-N^1-glucuronide (ammonium salt) m.p. 150-160° (decomp.) was synthesised by M.R. Kibby (cf. Bridges, Kibby & Williams, 1965).

a) ESTIMATION OF [^{35}S]MADRIBON.

i) Scintillation technique. [^{35}S]Sulphadimethoxine was examined for purity by chromatography in solvents A, B & C. [^{35}S] Activity was measured in a Tri-Carb Scintillation Spectrometer (Model 3214) manufactured by Packard Instrument Co., Wembley, Middlesex. All counting was done at 0°C.

Liquid scintillators. These consist of highly fluorescent compounds dissolved in a suitable solvent. The following scintillator systems were used:

The 'Dioxan' system - 60 g. naphthalene, 4 g. PPO (2,5-diphenyloxazole), 200 mg. POPOP [2,2-p-phenylenebis (5-

phenyloxazole)], 100 ml. 'Analar' methanol and 20 ml. of ethanediol made up to one litre with dioxan. This system was used for the counting of aqueous samples.

The 'Gel' system -a 5% suspension of Cab-o-Sil (a thixotropic gelling agent supplied by Packard Instrument Co.) in the 'Dioxan' scintillator. This was used for counting heterogenous samples such as faecal homogenates and tissue homogenates.

Preparation of urine, faeces and tissue samples. Urine (0.5 ml.) and bile (0.5 ml.) were usually counted in 20 ml. of 'Dioxan' scintillator. Faeces were homogenized with a 1:1 mixture of dioxan and methanol, and aliquots (0.5-1 ml. of the homogenates) were suspended in 20 ml. of 'Gel' scintillator. Animal tissues were either counted in solution or as homogenates. Tissues were dissolved in 25% (w/v) sodium hydroxide and aliquots of the solutions were counted in 'Gel' scintillator. Homogenates of the tissues in dioxan and methanol (1:1) were also suspended in 'Gel' scintillator. Whole rats (wt. approx. 250 g.) were dissolved in 25% (w/v) sodium hydroxide (1.5-2 litres) and heated to 50°C for 24 hours. Aliquots of the solutions were counted in 'Gel' scintillator. Recoveries of 97-98% were achieved when rat carcases containing known amounts of radioactivity (5 uc.) were counted by the procedure described.

Counting procedure. The counting efficiencies of individual samples were determined by the 'Channel Ratio' method. The absolute counting efficiency of any sample can be read from a standard graph by this method, when the ratio of counts given by the sample in the two channels of the counter is known.

Each sample was counted several times and the d.p.m. were calculated for each count. It was found that even for quenched samples (about 40% counting efficiency) the variation in the absolute count for any one sample was less than 1%. The agreement found when different volumes of the same sample (0.5-1 ml. urine for example) were counted, was usually within 1%. Some of the samples, especially faecal, tissue homogenates, and sodium hydroxide samples, gave very high counting rates at first, shown by high red/green ratio. This was probably due to chemiluminescence. A dark adjustment period of 24-48 hours in the freezer compartment of the counter was sufficient to enable the true counting rate of the sample to be determined.

ii) Estimation of metabolites by strip scanner. Urine or bile (0.1-0.2 ml.) containing 0.01 uc collected from an animal that had been fed with [^{35}S]sulphadimethoxine, or an in vitro incubation mixture (0.1-0.2 ml.) was banded (1" band) on a strip (1.5" wide) of Whatman No. 1 paper. These strips were chromatographed in solvents A, B

and C by the descending technique for 12 hours. Strips with reference spots were chromatographed at the same time. The metabolites of [^{35}S]sulphadimethoxine were identified by ultraviolet light, R_F values and colour reactions. The amounts of each metabolite were estimated by running the strip through a radiochromatogram scanner (Packard Model 7200). These strips were run through at a rate of 50 cm./hr., time constant 30 secs., at 1.2 Kv. (for best signal/noise ratio with gas used 98.7% Helium and 1.3% Butane). Counting was performed at two sensitivities, a low one (300) to determine accurately the major metabolites, and a high one (100) to estimate the minor metabolites on the recording chart. For accurate estimations of minor metabolites the appropriate part of the strip was cut out and counted in the Tri-Carb Scintillation Spectrometer using 'Dioxan' scintillator.

b) IN VITRO ACETYLATION RATES.

Animals were killed by cervical dislocation in the case of rabbits and by a lethal i.v. injection of pentobarbital (150 mg./kg.) in the case of monkeys. Livers were rapidly removed and placed in crushed ice. Portions of the liver (10 g.) were homogenized in 0.25M sucrose solution (30 ml.) and spun in an M.S.E. super-speed '40' centrifuge (Measuring & Scientific Equipment Ltd., London, S.W.1.) at 2,000 g. for 10 minutes. Acetylation rates were determined by incubating the sulphonamide (0.3 uM in 1 ml. 0.1M citrate - 0.2M

phosphate buffer, pH 7.4) and the 2,000 g. supernatant (1.0 ml.) in stoppered centrifuge tubes at 37° for 1 hour in a shaking water bath. Trichloroacetic acid (1 ml. of 20% w/v) and water (7 ml.) were then added to each tube, which were then centrifuged at 2,000 g. for 5 minutes. Samples of the supernatant (4 ml.) were withdrawn from each tube and free and total amine determined by the Bratton & Marshall method (1939). The effect of time and pH on the acetylation rate of sulphadimethoxine was also investigated.

c) IN VITRO SYNTHESIS OF SULPHADIMETHOXINE-N^1-GLUCURONIDE.

i) Tissue preparations. Monkeys were killed by a lethal i.v. injection of pentobarbital, rabbits and rats by cervical dislocation. The livers were rapidly removed and placed in crushed ice. Portions of the liver (10 g.) were homogenized with ice-cold 0.25M sucrose (30 ml.) in a Potter tube with a Teflon pestle. The 2,000 g. and 10,000 g. supernatants were obtained by centrifuging for 10 minutes in a M.S.E. 'super-speed 40' centrifuge.

Liver slices (0.5-1.0 mm. thick) were cut by hand with a stainless steel blade and immediately placed in the incubate.

ii) Incubations and estimations. Incubation mixtures consisted of liver fraction (1 ml.), [^{35}S]sulphadimethoxine (1.3 µM in 0.1 ml. water containing a little 2N-NaOH to dissolve the drug), 0.5M tris buffer pH 7.4 (0.2 ml.), UDPGA (7 µM in 0.4 ml.) and water (0.3 ml.) in unstoppered centrifuge tubes. Control tubes contained either boiled

liver homogenate or sodium glucuronate (7 μM in 1 ml. of 0.9% NaCl) in place of the normal tissue preparation. Additional tubes containing o-aminophenol in place of [^{35}S]sulphadimethoxine were also run. All tubes were incubated in a shaking water bath (H. Mickle Ltd., Gomshall, Surrey) at 37°C for 1 hour.

Tubes containing o-aminophenol were then analysed for o-aminophenol glucuronide using the method of Levy & Storey (1949). Tubes containing sulphadimethoxine were plunged into a boiling water bath for 1 minute, cooled in running water and centrifuged at 2,000 g. for 5 minutes. Aliquots (0.1 ml.) of the supernatant were analysed using the radiochromatogram scanner as described above. The peak corresponding to sulphadimethoxine-N^1-glucuronide was cut from the paper, eluted with dilute ammonium hydroxide (0.2N), and the eluate was chromatographed in solvents A, B and C. The incubation solution (1 ml.) was also extracted with ethyl acetate saturated with water (2 x 2.5 ml.) to remove free sulphadimethoxine, and portions of the supernatant (0.1 ml.) were withdrawn, banded on Whatman No. 1 paper, chromatographed in solvents A and B, and estimated in a radiochromatogram scanner. No N^1-glucuronide was found with rabbit liver, but in the case of rat liver, the N^1-glucuronide detected on chromatograms was cut out and counted on the paper in dioxan in a scintillation spectrometer.

A typical incubation with liver slices was as follows, each determination being performed in triplicate.

Into each of three 25 ml. conical flasks was pipetted 2 mls. of Krebs bicarbonate ringer solution (pH 7.4). To the first flask was added monkey liver slices (approximately 100 mg. wet weight) and [^{35}S]sulphadimethoxine (1.3 µM in 0.1 ml.), and to the second [^{35}S]sulphadimethoxine (1.3 µM in 0.1 ml.) and sodium glucuronate (7 µM in 0.1 ml. of 0.9% NaCl). Similar incubation solutions were prepared to determine o-aminophenol conjugation. Each flask was gased with oxygen containing carbon dioxide (5%), immediately stoppered and placed in a shaking water bath at 37° for 1 hour. Liver slices were then immediately withdrawn from all flasks, drained on filter papers and weighed. The supernatants were poured into centrifuge tubes, placed in boiling water for 1 min., cooled in running water and then estimated as above.

Fig. 3.1a

FORMATION OF N^4-ACETYL SULPHADIMETHOXINE BY RABBIT LIVER HOMOGENATE WITH TIME.

pH 7.4

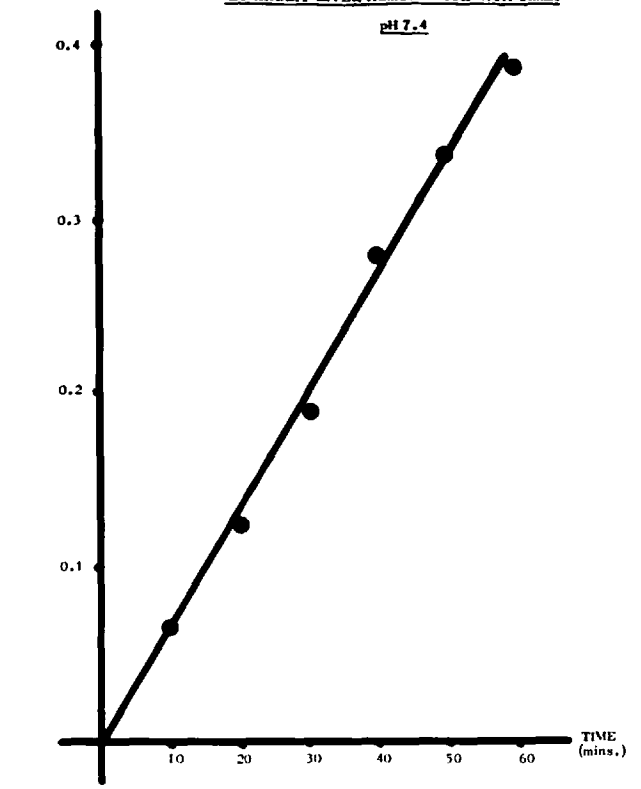


Fig. 3.1b

VELOCITY - pH CURVE FOR THE FORMATION OF N^4-ACETYL SULPHADIMETHOXINE BY RABBIT LIVER HOMOGENATE.

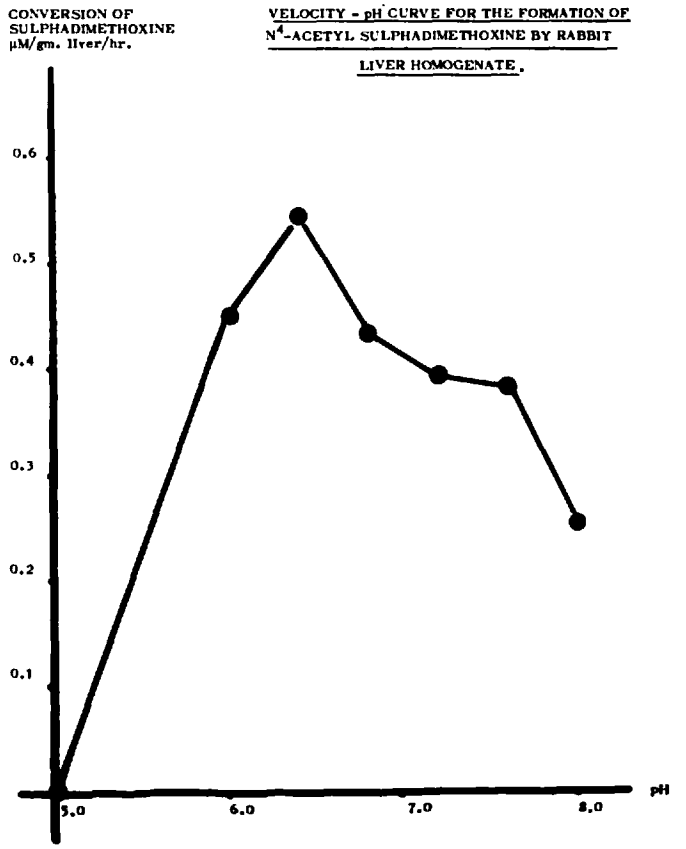


Table 3.1. **Metabolism and excretion of some substituted 6-sulphanilamidopyrimidines in the rabbit and monkey (Bridges, Kibby, Walker & Williams, 1969.).**

Dose level 100 mg./kg. p.o.

	Position of substituents							
Rabbit	2-methoxy	4-methoxy	5-methoxy	2,4-dimethoxy	2,5-dimethoxy	4,5-dimethoxy	2-methyl 4-methoxy	2,4-dimethyl
Amount excreted in 24 hrs.	67	75	82	43	66	63	69	76
% composition of 24 hr. excretion								
Free drug	33	2	6	1	92	9	16	32
N^1-glucuronide	0	0	0	0	0	0	0	0
N^4-acetyl	67	98	92	92	7	89	75	15
Monkey								
Amount excreted in 24 hrs.	57	12	17	42	42	65	54	63
% composition of 24 hr. excretion								
Free drug	76	7	8	6	94	8	5	37
N^1-glucuronide	3	4	2	70	1	2	32	0
N^4-acetyl	17	89	86	21	3	85	59	11

Table 3.2. **In vitro acetylation of some substituted 6-sulphanilamidopyrimidines by liver homogenates**

Incubations consisted of the sulphonamide (0.3 umoles in 1 ml. of 0.1M citrate, 0.2M phosphate buffer pH 7.4) and 2,000 g. liver supernatant (equivalent to 330 mg. in 1 ml. of 0.25M sucrose). Controls contained boiled liver preparations. Results given are the average of three animals with ranges in parentheses.

Substituents	Rate of acetylation (umoles of sulphonamide converted/g. liver/hr.)	
	Rabbit	Monkey
2-Methoxy	0.26 (0.21-0.30)	0.235 (0.20-0.27)
4-Methoxy	0.32 (0.30-0.34)	0.31 (0.29-0.35)
5-Methoxy	0.28 (0.23-0.31)	0.29 (0.26-0.34)
2,5-Dimethoxy	0.07 (0.04-0.11)	0.13 (0.09-0.17)
4,5-Dimethoxy	0.085 (0.05-0.13)	0.155 (0.125-0.19)
2,4-Dimethoxy	0.235 (0.20-0.27)	0.165 (0.14-0.205)
2-Methyl-4-methoxy	0.165 (0.13-0.235)	0.195 (0.17-0.24)
2,4-Dimethyl	0.190 (0.165-0.225)	0.175 (0.13-0.205)

Table 3.3. Synthesis of sulphadimethoxine N^1-glucuronide by liver preparations from various species

The incubates consisted of either [^{35}S]sulphadimethoxine or o-aminophenol (1.3 uM in 0.1 ml. buffer), 0.5M-tris buffer, pH 7.4 (0.2 ml.), liver (equivalent to 330 mg. in 1 ml. 0.25M sucrose, UDPGA (7 µM in 0.4 ml. water) and water (0.3 ml.). Controls contained boiled liver preparation or sodium glucuronate (7 µM in 1 ml. of 0.9% NaCl).

Animal	Preparation	Glucuronide formed (µM/g. liver/hr.) from Sulphadimethoxine	o-aminophenol
Monkey (2 females)	slices	0.1, 0.09	0.63, 0.67
	2,000 g. supernatant	1.46, 1.46	1.82, 1.68
	10,000 g. supernatant	1.38, 1.42	1.78, 1.64
	boiled 10,000 g. supernatant	0, 0	0, 0
	sodium glucuronate	0, 0	0, 0
Rat (4 females)	10,000 g. supernatant*	0.08, 0.03	0.46, 0.40
		0.04, 0.05	0.40, 0.80
Rabbit (3 females)	10,000 g. supernatant*	0, 0, 0	0.95, 0.87, 0.91

* No activity was found with the boiled preparation.

Table 3.4. Electronic charge and free valence of the N^1- and N^4-positions of the substituted 6-sulphanilamidopyrimidines

Molecular orbital calculations were carried out on an I.C.T. 1905 computer (International Computers Ltd.) using the parameters and programme of Krüger-Thiemer & Hansen (1966).

6-Sulphanilamido-pyrimidine	Charge on N^4 (amino)	Charge on N^1 (imido)	Free valence N^4 (amino)	Free valence N^1 (imido)
2-Methoxy	1.84	1.635	1.010	0.489
4-Methoxy	1.84	1.637	1.010	0.491
5-Methoxy	1.84	1.635	1.010	0.493
2,4-Dimethoxy	1.84	1.637	1.010	0.491
2,5-Dimethoxy	1.84	1.635	1.010	0.493
4,5-Dimethoxy	1.84	1.638	1.010	0.497
2-Methyl-4-methoxy	1.84	1.637	1.010	0.491
2,4-Dimethyl	1.84	1.636	1.010	0.490

4) DISCUSSION

The structural requirements for acetylation among the substituted 6-sulphanilamidopyrimidines appears to be partly dependent on the position of the substituents in the pyrimidine ring. Differences in the in vivo acetylation between the various isomers could be due to differences in their distribution in the body (which is largely dependent on their physical properties - discussed in Chapter 4) and in their acetylation rates.

Acetylation in vitro. The rate of acetylation of these eight drugs in vitro by liver homogenates is shown in Table 3.2. All are acetylated by both rabbit and monkey liver preparations. The in vitro acetylation rate of sulphadimethoxine by rabbit liver homogenate (2,000 g. supernatant) was studied at various pH's. The optimum pH was found to be 6.4 (Fig. 3.1b). The variation in N^4-acetylation with time at pH 7.4 was also studied, and the rate was found to be constant over the first sixty minutes (Fig. 3.1a). The monomethoxy compounds are well acetylated (0.26-0.32 uM/g. liver/hr.) by both species whereas the disubstituted compounds are less well acetylated. The 2,5- and 4,5-dimethoxy compounds are the least acetylated of all the drugs studied. It can be seen that the good in vivo acetylation of 2-methyl-4-methoxy-6-sulphanilamidopyrimidine in the monkey

and rabbit and the poor *in vivo* acetylation of the 2,4-dimethyl derivative in these two species (Table 3.1) is not related to their rates of *in vitro* acetylation (Table 3.2). In these two species, the 2,4-dimethyl is acetylated *in vitro* at a rate which is not very different from that of the 2-methyl-4-methoxy and the 2,4-dimethoxy compounds. It would appear that *in vivo* the 2,4-dimethyl sulphonamide may be cleared from the kidneys at a rate that does not allow extensive acetylation. According to Perault & Pullman (1963), the rate of enzymic acetylation of aromatic amines *in vitro* parallels the electronic charge on the NH_2 group. This does not appear to be true in the series of compounds studied here, for the electronic charge on the aromatic amino nitrogen is the same for all of them (Table 3.4), yet there is up to a four-fold variation in the rate of *in vitro* acetylation (Table 3.2). It appears probable that some unknown steric factors are involved.

Enzymic synthesis of the N^1*-glucuronide.* The results of experiments on the *in vitro* synthesis of sulphadimethoxine N^1-glucuronide are shown in Table 3.3, which shows that glucuronyl transfer to the sulphonamide nitrogen of sulphadimethoxine occurs in the 2,000 g. and 10,000 g. supernatants of monkey liver homogenates, fortified with UDPGA. In monkey liver slices the synthesis is small, possibly because the drug does not penetrate readily under the conditions of

the experiment. It can be seen, therefore, that the low acetylation rate of sulphadimethoxine *in vivo* (Table 3.1) in the monkey could be due to the competing metabolic reaction of N^1-glucuronide formation.

Synthesis of the N^1-glucuronide also occurs to a slight extent in the 10,000 g. supernatant of rat liver homogenates but no synthesis at all was detected in similar preparations from rabbit liver. The liver homogenates from all three species were able to conjugate *o*-aminophenol. It would appear, therefore, that the enzyme forming the stable N^1-glucuronide is different from that forming *o*-aminophenylglucuronide. Another possibility is that the glucuronyl transferase of monkey liver can catalyse glucuronyl transfer to the sulphonamide nitrogen of sulphadimethoxine and to the hydroxyl group of *o*-aminophenol, whereas that of rabbit liver can only transfer to *o*-aminophenol in these experiments. These *in vitro* findings support those found in the whole animals, especially the rhesus monkey and the rabbit. It should be noted that the stable sulphadimethoxine N^1-glucuronide is different from the unstable *N*-glucuronides of aromatic amines which can be formed spontaneously from glucuronic acid and the free amine. The N^1-glucuronide cannot be formed spontaneously in this way. The question of the occurrence of more than one UDP-glucuronyltransferase has been discussed by Dutton (1966) and our findings suggest that in primates at least two of these may occur.

CHAPTER 4

THE IN VIVO DISTRIBUTION AND PHYSICAL PROPERTIES OF SOME SUBSTITUTED 6-SULPHANILAMIDOPYRIMIDINES.

1) INTRODUCTION.

2) MATERIALS AND METHODS.

a) Physical Data Determination.

i) Solubilities
ii) pKa values
iii) Partition coefficients.

b) Distribution of [^{35}S]Sulphadimethoxine in the Mouse and Rat.

i) Blood levels
ii) Body tissues
iii) Autoradiography.

c) Route and Media of Administration as Factors Affecting Metabolism and Excretion in the Rat.

3) RESULTS.

4) DISCUSSION.

Physical data
Distribution
Excretion and Metabolism.

.....

1) INTRODUCTION

The duration of action of sulphonamides is related to both their metabolism, absorption and distribution throughout the body. The pKa, water solubility and lipid-water partition coefficient of a drug affect the passage of the drug across cell membranes and therefore influence its absorption, distribution and excretion. These properties were measured for a series of substituted 6-sulphanilamido-pyrimidines and their N^4-acetyl derivatives and the results used to explain differences between *in vivo* and *in vitro* acetylation rates.

The effect of the route and medium of administration of [^{35}S]-sulphadimethoxine on the metabolism and excretion of this drug was investigated in the rat. The distribution of this sulphonamide was studied both by isolation and estimation of rat tissues after oral and parenteral administration of the drug, and also by autoradiography in the mouse.

2) MATERIALS AND METHODS

[^{35}S] 4,5-dimethoxy-6-sulphanilamidopyrimidine m.p. 194-197° (sp. activity 35 uc./mg.) was a gift from Dr. R. Long, Roche Products Ltd., Welwyn Garden City, Herts. The N^4-acetyl derivatives of the following substituted 6-sulphanilamidopyrimidines were synthesised from acetic anhydride and the corresponding free drug:

4-methoxy-6-sulphanilamidopyrimidine	m.p. 219-220°
5-methoxy-6-sulphanilamidopyrimidine	m.p. 215-217°
2,5-dimethoxy-6-sulphanilamidopyrimidine	m.p. 158-160°
2,4-dimethoxy-6-sulphanilamidopyrimidine	m.p. 210-211°
4,5-dimethoxy-6-sulphanilamidopyrimidine	m.p. 214-215°

a) PHYSICAL DATA DETERMINATION.

i) Solubilities. The sulphonamide or its N^4-acetyl derivative was added to 0.1M citrate, 0.2M phosphate buffer (20 ml.) of pH 4.2, 5.2, 6.2, 7.2 or 7.4 in a conical flask which was then shaken for 2 hours on a Griffin shaking machine (Griffin & George, Wembley, Middlesex). After equilibration, the undissolved sulphonamide was filtered off and the amount of sulphonamide in solution determined on samples of the filtrate using the method of Bratton & Marshall (1939).

ii) pKa Values. The pH of the filtrate from the previous experiment was checked using a Pye Master pH meter. The pKa's were determined from the solubility results at various pH values (measured

at room temperature 20°C) using the following equation (Krebs & Speakman, 1945).

$$\text{Log}_{10} \ (S/S_o - 1) = pH - pKa \qquad (1)$$

where S = solubility at any pH value,
So = solubility of free acid,
pKa = apparent dissociation constant.

"So" can be obtained by extrapolating the plot of S against $1/[H^+]$ to $[H^+] = \infty$

$$\text{i.e. } S = So + [1/_{H^+}] \qquad (2)$$

OR

If S_1 = solubility at pH = X_1

and S_2 = solubility at pH = X_2

and $X_2 - X_1$ = 1 pH unit

$$\text{Then } 10S_1 - S_2 = 9So \qquad (3)$$

Hence "So" can be obtained from these equations. Then from equation (1) a graph of $\text{Log}_{10} (S/S_o - 1)$ against pH should be a straight line intercepting the pH axis at the pKa value of the sulphonamide.

iii) Partition coefficients of the sulphonamides and their N^4-acetyl derivatives. These were obtained by shaking a saturated solution of the sulphonamide in citrate-phosphate buffer pH 7.4 (10 mls.) with an equal volume of chloroform for 1 hour and redetermining the concentration of the sulphonamide in the aqueous layer.

b) DISTRIBUTION OF [^{35}S]SULPHADIMETHOXINE IN THE MOUSE AND RAT.

i) Blood levels. Thirty female mice (I.C.I. strain, 30 g.) were injected i.p. with [^{35}S]sulphadimethoxine in dimethylsulphoxide (DMSO) (0.05 ml.) at a dose level of 100 mg./kg. (0.15 μc.). After time intervals of 15 mins., 30 mins., 1 hr., 2 hrs., 4 hrs., 6 hrs., 8 hrs., 10 hrs., 12 hrs. and 24 hrs., three mice had their throats cut. The blood collected (3 ml.) mixed with heparin (0.05 ml.) was centrifuged at 2000 g. for 5 mins., and the plasma (0.2 ml.) counted in dioxan gel using a scintillation counter.

Twenty-two female rats (Wistar albino, 300 g.) were injected i.p. with [^{35}S]sulphadimethoxine in DMSO (0.5 ml.) at a dose level of 100 mg./kg. (1.5 μc.). After time intervals of 30 mins., 1 hrs., 2 hrs., 4 hrs., 8 hrs., 12 hrs., 18 hrs., 25 hrs., 30 hrs., 72 hrs. and 168 hrs., pairs of rats were stunned and their throats cut. The blood collected (10 mls.) mixed with heparin (0.1 ml.) was centrifuged and counted as above.

ii) Body tissues. Female rats (300 g.) were given [^{35}S]sulphadimethoxine (100 mg./kg., 5 μc.) in DMSO (0.5 ml.) by i.p. injection. Urine and faeces were collected every 24 hrs. for three days. After three days the rats were killed, the tissues dissected out and estimated, together with the carcass, for [^{35}S] activity by the method already described (see Chapter 3).

iii) Autoradiography. Male mice (20 gm.) were injected i.p. with the sodium salt of [^{35}S]sulphadimethoxine or [^{35}S]sulphorthodimethoxine (dose level 100 mg./kg., 8 µc.) in water (0.1 ml.). After 2 hrs. the animal was anaesthetised, suspended in a gel of carboxymethylcellulose, and immersed in liquid nitrogen. Whole sections were cut longitudinally through the mouse with a microtome. The section of the whole animal (20 microns thick) was placed in contact with a photographic film for 14 days. The developed film showed the pattern of distribution of the radioactive sulphonamide (Fig. 4.4 and 4.5).

c) RATE AND MEDIA OF ADMINISTRATION AS FACTORS AFFECTING METABOLISM AND EXCRETION IN THE RAT.

i) Sulphamethomidine. Female rats (250 g.) were given sulphamethomidine at a dose level of 100 mg./kg. as a suspension in aqueous sodium carboxymethylcellulose (0.5% solution) either orally or intraperitoneally. Female rats were also given the sodium salt of sulphamethomidine (100 mg./kg.) in water (1.25 ml.) by injection via the femoral vein over a period of two minutes. Urine was collected in two 24 hr. batches and examined for free and total drug by the Bratton & Marshall method (1939). Urines were also examined chromatographically for individual metabolites.

ii) [^{35}S]Sulphadimethoxine. Separate groups of female rats (250 g.) were given [^{35}S]sulphadimethoxine (dose level 100 mg./kg., 5 µc.) as a suspension in warm propane 1.2.diol (0.5 ml.) either orally or

intraperitoneally. Other groups of rats were given the same drug using DMSO as the medium for administration. Urine was collected in two 24 hr. batches and examined for [^{35}S] activity and individual metabolites using the radiochromatogram scanner.

Fig. 4.1. VARIATION IN THE CONCENTRATION OF [^{35}S] SULPHADIMETHOXINE IN RAT PLASMA AFTER SINGLE I.P. INJECTION.

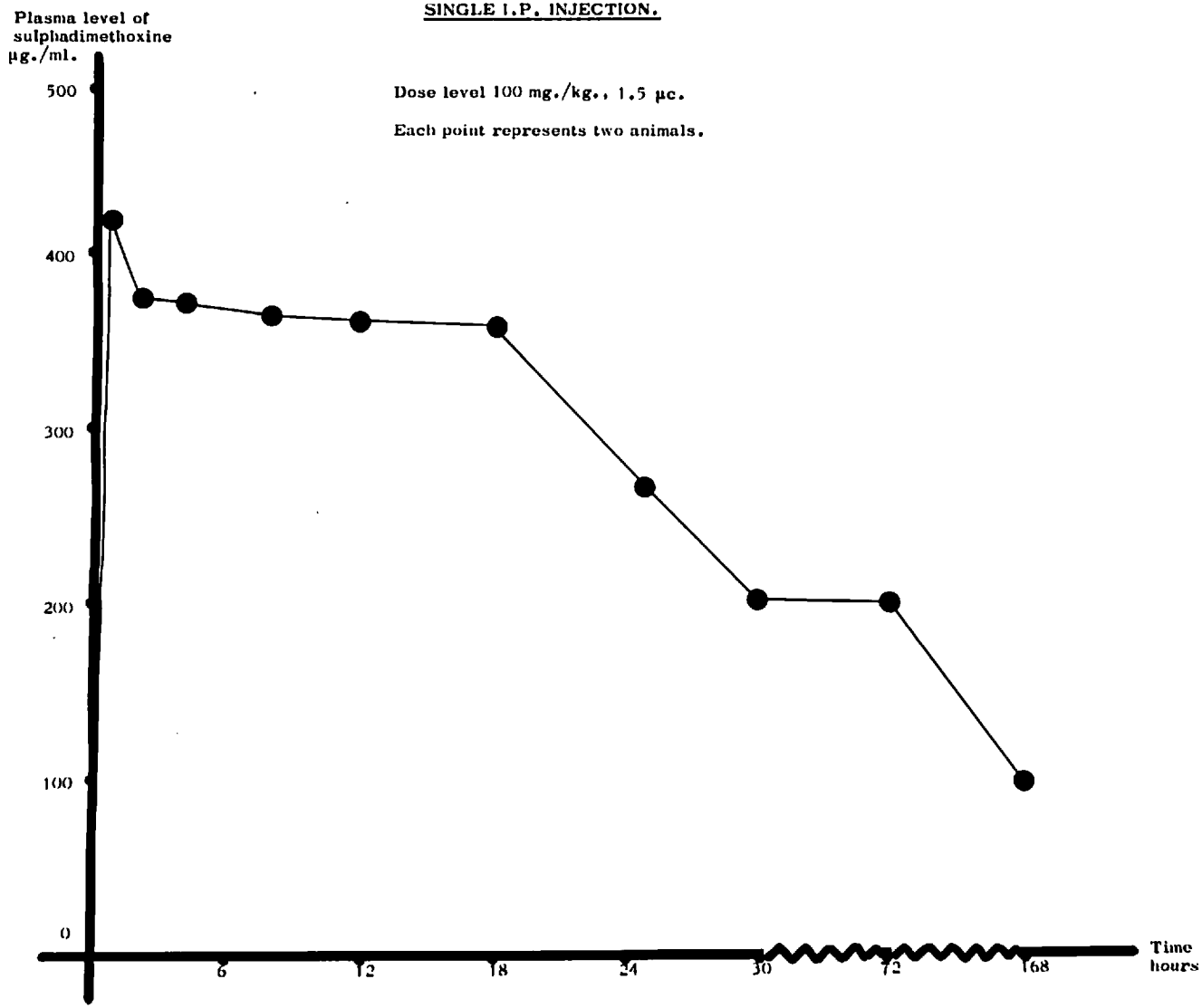


Fig. 4.2. VARIATION IN THE CONCENTRATION OF [^{35}S] SULPHADIMETHOXINE IN MOUSE PLASMA AFTER SINGLE I.P. INJECTION.

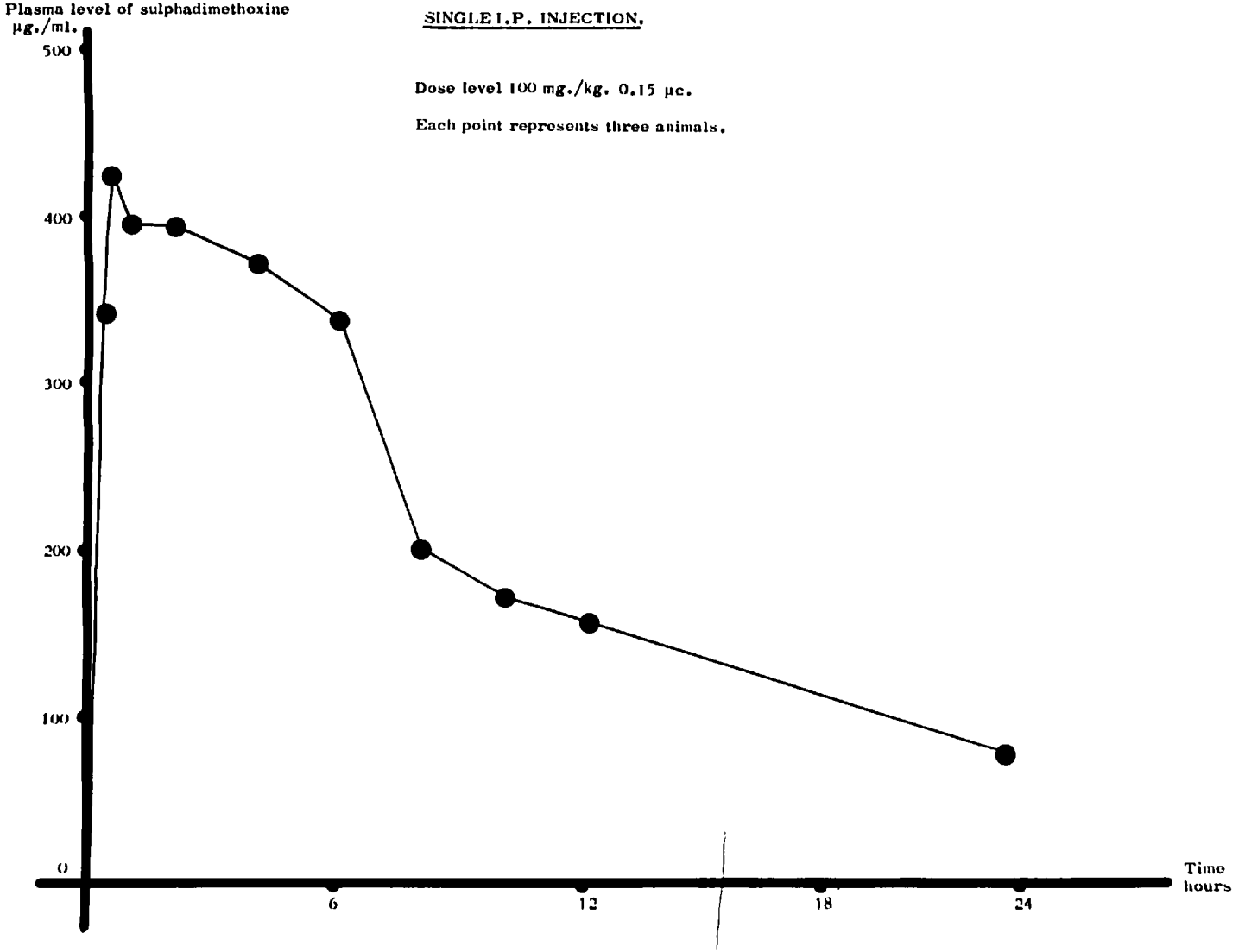


Fig. 4.3 DISTRIBUTION OF ^{35}S SULPHADIMETHOXINE IN RAT TISSUES. AFTER A SINGLE INJECTION (i.p.).

Dose 100 mg./kg. (5 μc).

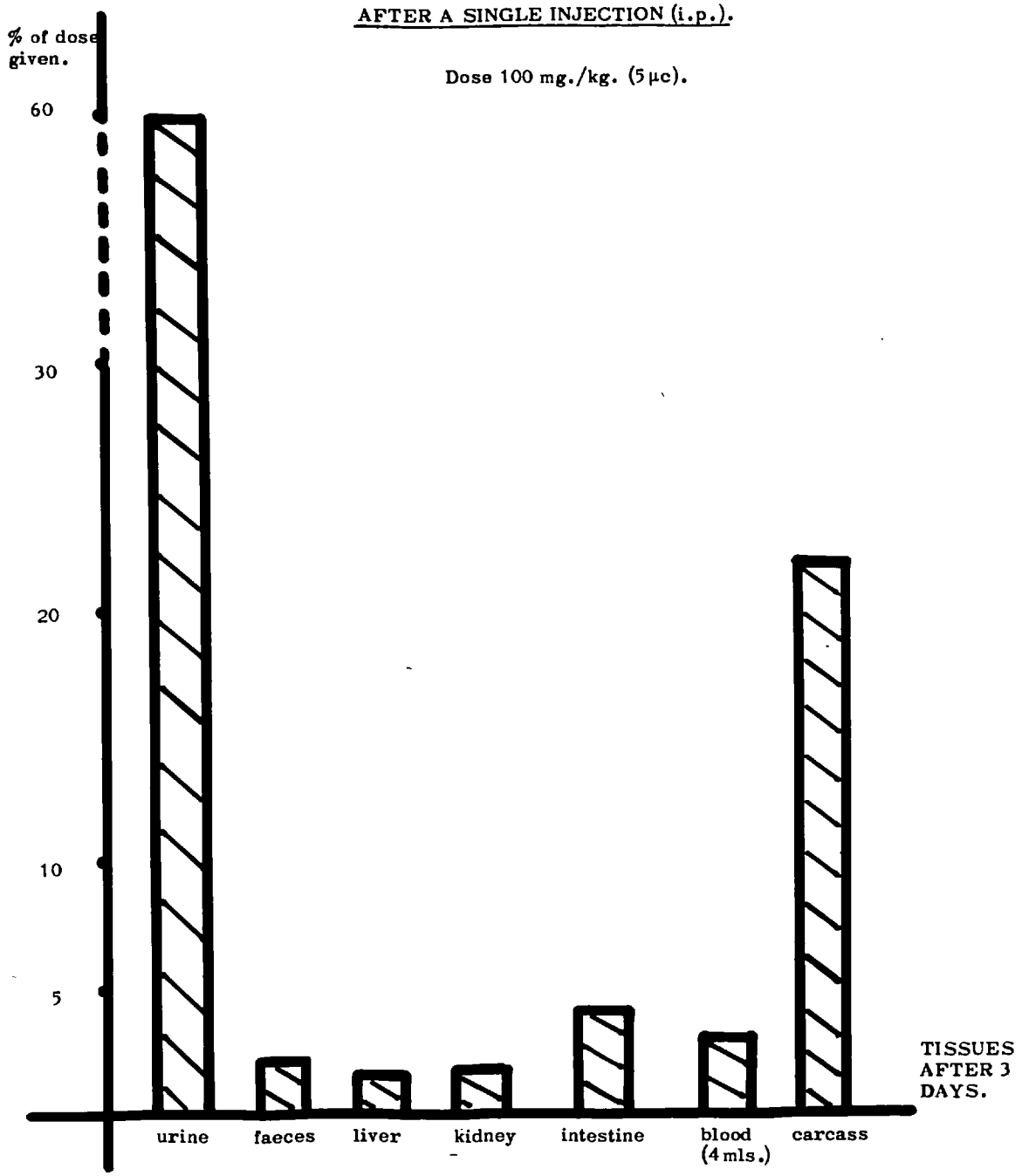


Fig. 4.4

AUTORADIOGRAM OF A LONGITUDINAL SECTION THROUGH A MOUSE 2 HRS. AFTER AN INJECTION OF ^{35}S SULPHADIMETHOXINE (i.p.).

Dose 100 mg./kg. (8μc.).

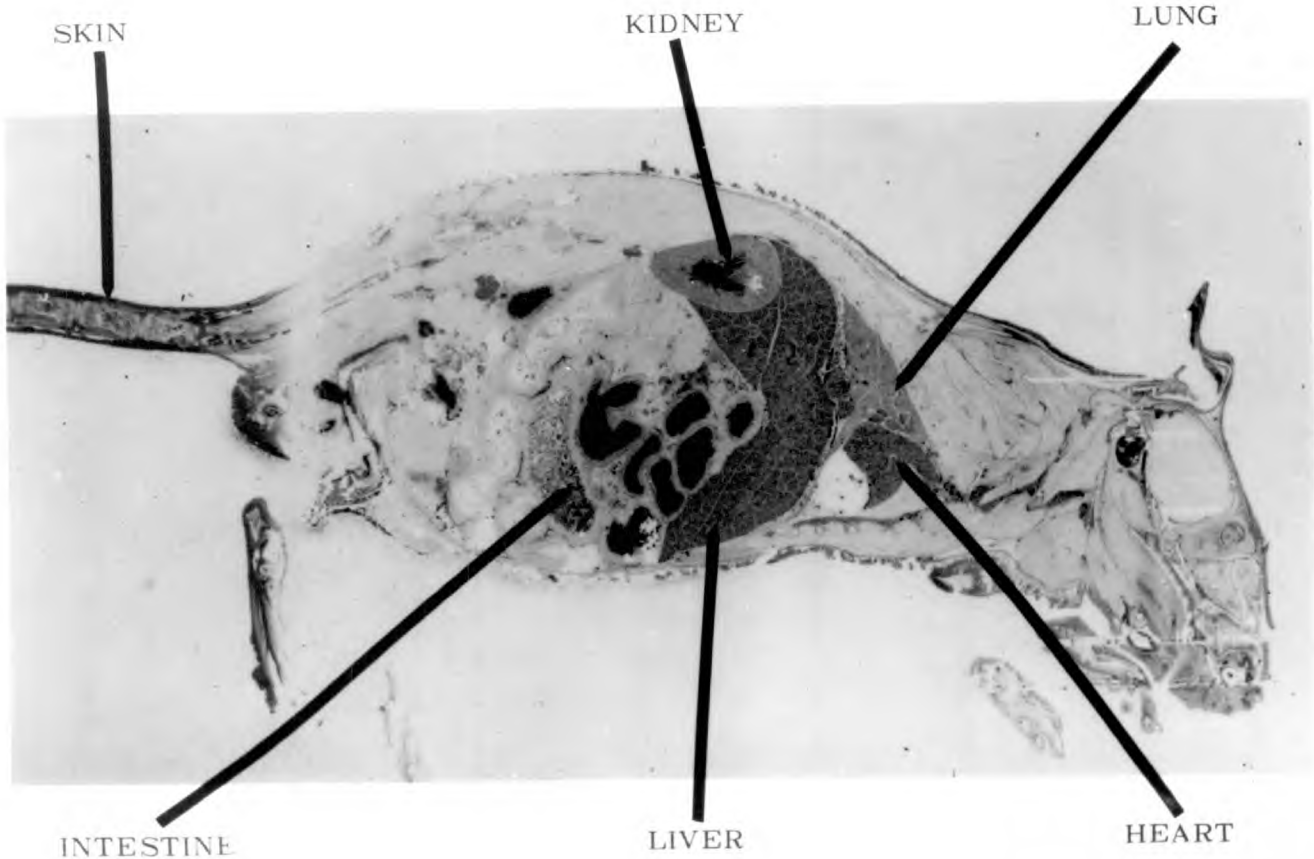


Fig. 4.5

AUTORADIOGRAM OF A LONGITUDINAL SECTION THROUGH A MOUSE 2HRS. AFTER AN INJECTION OF ^{35}S SULPHORTHODIMETHOXINE (i.p.).

Dose 100 mg./kg. (8μc).

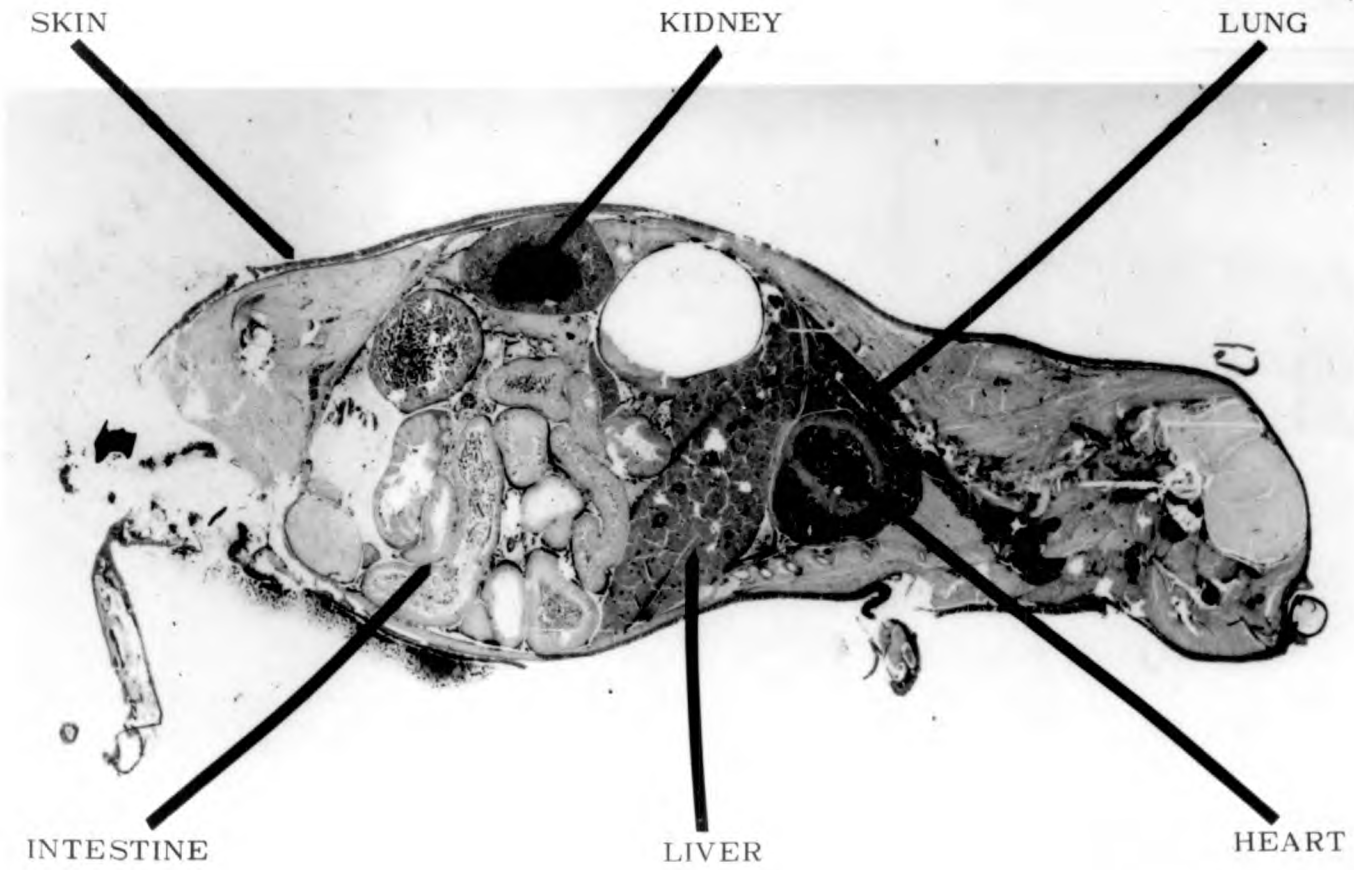


TABLE 4.1

Comparison of the fate of sulphamethomidine when administered orally, intraperitoneally or intravenously

Three female Wistar albino rats (250 g.) were used in each experiment (dose level 100 mg./kg.).

		Route of administration		
		p.o.	i.p.	i.v.
% of dose fed excreted in urine	24 hrs.	41±7	49±10	57±3
	48 hrs.	49±7	59±15	66±3
% composition of 24 hr. urine	N^2-glucuronide	1±1	1±1	1±1
	N^4-sulphate	1±1	1±1	1±1
	N^1-glucuronide	0	0	0
	Unchanged drug	19±2	14±3	12±2
	N^4-acetyl	79±2	84±3	86±2

TABLE 4.2

Effect of medium and route of administration on the metabolism and excretion of [^{35}S]sulphadimethoxine in the rat.

Dose level 100 mg./kg.

Medium		Propane 1.2.diol		DMSO	
Route of administration		p.o.	i.p.	p.o.	i.p.
Number of animals used		5 ♀	5 ♀	6 ♀	6 ♀
% of dose fed excreted in urine	24 hrs.	15±1	18±4	26±5	31±2
	48 hrs.	28±3	34±5	52±8	45±1
% composition of 24hr. urine*	N^4-glucuronide	5±1	5±1	2±1	5±1
	N^4-sulphate	6±2	4±1	8±1	3±1
	N^1-glucuronide	7±3	8±2	9±1	6±2
	Unchanged drug	20±4	19±3	22±1	11±4
	N^4-acetyl	62±2	64±3	59±3	75±6

*Average for three animals.

TABLE 4.3

Solubilities and pKa values of the substituted 6-sulphanilamidopyrimidines and their N^4-acetyl derivatives.

Solubilities in citrate-phosphate buffer, pH 7.4 (see text).

6-Sulphanilamidopyrimidine	pKa	Solubility in buffer mg./l.	Solubility in buffer at pKa mg./l.	Distribution ratio $CHCl_3$/buffer
2-Methoxy	6.40	1479	331	0.08
N^4-Acetyl-2-methoxy	6.28	-	-	-
4-Methoxy	6.36	267	45	0.23
N^4-Acetyl-4-methoxy	-	900	-	0.09
5-Methoxy	6.24	1048	124	0.52
N^4-Acetyl-5-methoxy	-	768	-	0.19
2,4-Dimethoxy	6.26	343	51	5.25
N^4-Acetyl-2,4-dimethoxy	6.16	190	-	0.38
2,5-Dimethoxy	6.18	4507	394	0.21

Continued/...

TABLE 4.3 (continued)

N^4-Acetyl-2,5-dimethoxy	-	3429	-	0.03
4,5-Dimethoxy	6.00	2428	156	2.03
N^4-Acetyl-4,5-dimethoxy	-	2050	-	0.24
2-Methyl-4-methoxy	7.04	843	496	1.00
N^4-Acetyl-2-methyl-4-methoxy	-	260	-	0.18
2,4-Dimethyl	7.62	1615	2016	0.15
N^4-Acetyl-2,4-dimethyl	-	44	-	0.10

4) DISCUSSION

Physical data. The solubilities, partition coefficients between chloroform and citrate-phosphate buffer (pH 7.4) and the pKa's of some substituted 6-sulphanilamidopyrimidines and their N^4-acetyl derivatives are given in Table 4.3. As the methyl groups of sulphasomidine are replaced by methoxyl groups with a positive mesomeric effect, so the pKa of the sulphonamide decreases (sulphasomidine pKa 7.62, sulphamethomidine pKa 7.04 and sulphadimethoxine pKa 6.26).

The order of the methoxyl substituted 6-sulphanilamidopyrimidines in terms of decreasing pKa values is 2-, 4-, 2,4-, 5-, 2,5- and 4,5-. This indicates that when the methoxyl substituent is in the '2' position, it exerts the least mesomeric effect on the amido nitrogen because it is shielded by the two nitrogens in the ring. However, the greatest mesomeric effect is observed when the methoxyl substituent is in the '5' position, the position being the most truely aromatic in the pyrimidine ring.

The order of this series of isomers in terms of decreasing solubility in citric acid-phosphate buffer at pH 7.4 is 2,5-dimethoxy-, 4,5-dimethoxy-, 2,4-dimethyl-, 2-methoxy-, 5-methoxy-, 2-methyl-4-methoxy-, 2,4-dimethoxy-, 4-methoxy-6-sulphanilamidopyrimidines. In every case except the 4-methoxy- compound the N^4-acetyl derivative is far less water soluble than the parent drug. It can be seen that as

the methyl groups of sulphasomidine are replaced by methoxyl groups so the water solubility of the sulphonamide decreases. Correspondingly, the partition coefficient between chloroform and buffer at pH 7.4 which gives an indication of the lipid/water partition coefficient, increases from sulphasomidine to sulphadimethoxine due to the addition of lipophillic methoxy groups. In each case, the N^4-acetyl derivatives have much lower partition coefficients.

It was seen that with rabbit liver homogenates, the 2-, 4- and 5-methoxy and 2,4-dimethoxy derivatives were acetylated 3-4 times faster than the 2,5- and 4,5-dimethoxy compounds, whereas with the monkey liver the monomethoxy were acetylated at about twice the rate of the dimethoxy compounds (Table 3.2). The *in vitro* results were in good agreement with the *in vivo* results (Table 3.1), except for the fact that *in vivo* the 4,5-compound was highly acetylated whereas the 2,5-compound was hardly acetylated at all. However, *in vivo*, other factors are involved such as distribution, tissue binding and excretion by the kidney, which in turn are related to the physical properties of the drug. Table 4.3 shows that the 2,5-dimethoxy compound has a much greater water solubility at pH 7.4 than the others, and at the same time its distribution ratio indicates that it is poorly lipid soluble. This would suggest that it would be more readily excreted by the kidneys in the unchanged state than the other drugs.

Both the 2,4- and 4,5- dimethoxy compounds are relatively lipid soluble (their distribution ratios are 25 and 10 times respectively, that of the 2,5-dimethoxy compound) and would tend to be reabsorbed in the kidney tubule whereas their less lipid soluble acetyl derivatives would be more readily excreted. It is probable, therefore, that the very poor acetylation of the 2,5-dimethoxy compound *in vivo* can be partly explained in terms of its relatively high water and low lipid solubility, and also its slow rate of acetylation.

Sulphasomidine and sulphamethomidine were both acetylated to the same extent *in vitro* (Table 3.2) but *in vivo* sulphasomidine was poorly acetylated both in the monkey and rabbit (Table 3.1). Again, this can be explained in terms of physical constants. Sulphasomidine is more water soluble and far less lipid soluble than sulphamethomidine (Table 4.3) and so will be rapidly excreted unchanged by the kidney and not reabsorbed.

Distribution. A high plasma level (425 µg./ml.) was rapidly obtained after an intraperitoneal injection (Fig. 4.1 and 4.2) which indicates that [^{35}S]sulphadimethoxine is quickly absorbed from the peritoneal cavity of the rat and mouse. The persistance of this drug in these two species differs markedly. In the mouse, after 12 hrs., the concentration of the sulphonamide in the plasma had dropped to 150 µg./ml. and after 24 hrs. it was 75 µg./ml., a total of 60%

of the dose given having been excreted. In the rat, initially there was a small drop within 2 hrs., but after that the plasma level was maintained fairly constant for 16 hrs., after which it fell again. However, even after 7 days, the plasma level was 100 µg./ml. This species difference may be explained in terms of the protein binding of this drug which in turn affects metabolism and excretion. Sulphadimethoxine binds more extensively to plasma proteins in the rat than in the mouse at the same concentration (see Chapter 5).

The distribution of [^{35}S]sulphadimethoxine in the rat after 3 days is shown in Fig. 4.3 (average of 3 animals). A total of 60% of the dose given was excreted via the kidneys, but only 2% was found in the faeces. When the organs of the rat were dissected out after 3 days, small amounts of radioactivity were found in the liver, kidney, intestine and blood, the remainder of the [^{35}S] activity was found in the carcass (total radioactivity recovered was 94 $\pm$ 3%). It seems that the drug remains bound not only to plasma proteins but also to tissue proteins as small amounts of radioactivity were found in these organs after 3 weeks.

Fig. 4.4 shows the distribution of [^{35}S] sulphadimethoxine in the mouse 2 hrs. after an i.p. injection of the sulphonamide. The labelled material is found in such tissues as the liver, kidney, heart, lung and skin. A similar autoradiogram was seen when [^{35}S]sulphortho-

dimethoxine was injected into a mouse (Fig. 4.5).

Excretion and metabolism. When sulphamethomidine was given intraperitoneally to rats, more of the dose was excreted than when the drug was fed orally (Table 4.1). The highest rate of excretion was achieved when the drug was given intravenously, although the metabolism was unaffected by the route of administration. Most of the drug was excreted as the N^4-acetyl derivative (79-86%) while the remainder, apart from the minor metabolites (N^4-glucuronide and N^4-sulphate), was excreted unchanged. No N^1-glucuronide was detected in the urine of rats receiving sulphamethomidine.

A similar pattern was observed with [^{35}S]sulphadimethoxine in the rat (Table 4.2). However, the medium used does affect the amount excreted. When [^{35}S]sulphadimethoxine in DMSO was given either orally (26%) or intraperitoneally (31%) to the rats, a larger percentage of the dose fed was excreted in the first 24 hrs. than when the drug was given in propane 1.2.diol (15% excreted when given p.o. and 18% when given i.p.). The amounts of minor metabolites excreted (N^1-glucuronide, N^4-sulphate and N^1-glucuronide) were approximately the same whatever the medium used or route of administration. There was a little more variation in the amounts of N^4-acetyl derivative and unchanged drug excreted, but these differences did not appear to be statistically important.

CHAPTER 5

THE BINDING OF SULPHASOMIDINE, SULPHAMETHOMIDINE AND SULPHADIMETHOXINE TO PLASMA PROTEINS.

1) INTRODUCTION.

- a) Absorption and Distribution
- b) Nature of binding
- c) Effect of Binding on Kidney Excretion
- d) Drug Interaction
- e) Metabolism
- f) Species Differences.

2) METHODS.

- a) Ultrafiltration
- b) Equilibrium Dialysis
- c) Calculations.

3) RESULTS.

4) DISCUSSION.

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1) INTRODUCTION

A fundamental premise in pharmacology is that the biological activity of a drug is related to the unbound concentration of the substance in the plasma, that is, to the level in plasma water. This fluid is the common matrix through which therapeutic agents, after oral and parental administration, are transported to sites of action, excretion and metabolism.

Since the initial experiments of Davis (1942) it has been known that sulphonamides may be bound to plasma proteins. Later experiments performed by Anton (1960) and Newbould & Kilpatric (1960) have demonstrated (in vitro) the ability of albumin to bind sulphonamides. They have also shown that the protein bound drug was without bacteriostatic effect. Clausen (1966) showed by micro-immunoelectrophoresis that plasma sulphonamides are not only bound to albumin, but also to other proteins. Changes in amounts and the composition of plasma proteins will effect the binding of sulphonamides which in turn may alter the elimination and distribution of the drug.

The ability of sulphonamides to combine with plasma proteins differs greatly from one sulphonamide to another. In vitro incubation of 10 mg. of sulphonamide with 100 ml. plasma led to the following percentages of protein binding with each sulphonamide: sulphanilamide 20%; sulphapyridine 40%; sulphadiazine 55%; sulphamerazine 85%;

sulphamethazine 85% (Truhaut, 1965).

a) ABSORPTION AND DISTRIBUTION.

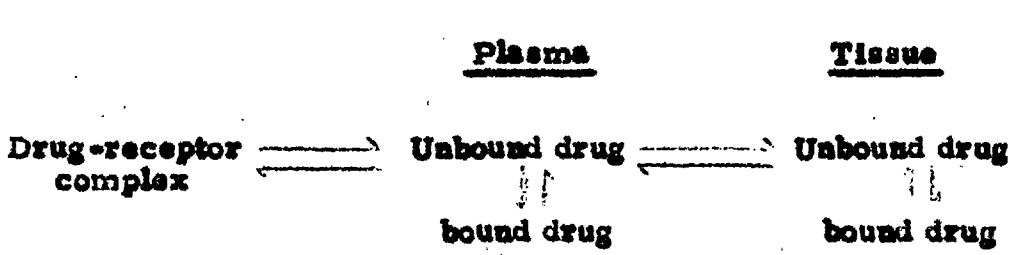
With highly lipid-soluble crystalline drugs the limiting factor in the rate of absorption into the blood stream from the intestine may be the rate of solution of the crystals. Attachment to plasma proteins withdraws large amounts of the drug from the plasma water, thus preserving a high concentration gradient in favour of dissolution and absorption from the intestine of the drug. The material bound to plasma proteins can be considered as a reservoir of the drug. As unbound drug is metabolised and excreted so the dynamic equilibrium results in a dissociation of bound drug. The duration of action of the drug is therefore prolonged.

The binding of drugs to plasma proteins means that they do not have to be given so frequently, which in turn stops the concentration of the drug in the plasma oscillating between therapeutic and toxic levels. Newbould & Kilpatric (1960) subdivided sulphonamides into those with prolonged and those with short-lived activity. In the former group, sulphaphenylpyrazole and sulphamethoxypyridazine are the ones which bind themselves more extensively to proteins.

When a lipoid membrane separates two fluid compartments i.e. plasma and cerebrospinal fluid, the plasma protein and its attached drug cannot diffuse across the membrane. When a drug is lipid

soluble the concentration of drug in the cerebrospinal fluid will normally be identical to that of the unbound drug in the plasma. With some drugs a comparison of the concentration in the cerebrospinal fluid and the plasma is a simple way of measuring protein binding. Reider (1963) showed that sulphonamide concentration in the plasma ultrafiltrate is far nearer to the level in most of the extravascular body media than the whole plasma concentration. Since the more a sulphonamide is bound to plasma proteins, the lower will be its plasma ultrafiltrate level, the significance of protein binding in the distribution of a drug in the body is apparent. Reider also concluded that tissue proteins bind sulphonamides in a similar manner to plasma proteins. Drugs that are bound to proteins are distributed as shown in Fig. 5.1.

Fig. 5.1



b) NATURE OF BINDING.

Binding consists of the interaction of ionized, polar or non-polar groups of a drug with corresponding groups of the protein. The energy of the binding depends on the number and nature of these interactions involved in each binding site (Thorp, 1964). Brodie (1965a)

points out that ionic bonds are only a small part of the story. The non-polar part of the molecule is also important, thus, in a series of barbiturates, all having the same acidic strength (pKa 7.6) barbital itself is hardly bound, but the binding increases as the side chain is lengthened, reaching 55% with pentobarbital. The forces describing this increase in binding are called London or Van der Waals forces. Merely coining a name for such a binding does not mean that we understand the nature of the binding forces. We do know, however, that binding to plasma proteins increases with lipid solubility for some series of drugs. Some substances do not need an ionic bond if they are very lipid soluble i.e. xenon and methylcholanthrene are highly bound.

Klotz, (1950, 1957) has indicated the complementary relationship between the amino acid composition of a protein and its ability to bind molecules of a particular structure and charge. Thus, the relatively high affinity of albumin for anions in spite of the negative charge at pH 7.4 is explicable in terms of the hydrogen bonds. These are formed preferentially between the hydroxyl or carboxyl groups of amino acids, serving to leave unbonded cationic groups available for binding with anions.

At present, it is doubtful that the binding of any drug to plasma protein is fully understood (Brodie, 1965a). The number of primary

binding sites (those with the highest association constant) of albumin for organic anions is generally no more than five and often only one. The number of secondary sites is about twenty. Small changes in the structure of an anion can alter its binding to albumin. Nakagaki et al. (1963) studied the mechanism of binding of sulphamonomethoxine to bovine serum albumin in comparison with that of sulphadimethoxine to see the effect of the methoxyl group on the binding of these sulphonamides. They concluded that in the binding of albumin with sulphonamides, the electrostatic force is the most important factor.

A consequence of a single binding site for a drug, is the limitation in carrying capacity of plasma for a drug to one molar equivalent of its albumin content. This is of the order 7×10^{-4}M which for a compound of molecular weight of 280 (characteristic of most sulphonamides) is equivalent to a concentration in the plasma of 200 γ/ml. Beyond this concentration, the fraction of unbound drug begins to increase rapidly and will be available for diffusion to sites for metabolism and excretion.

Newbould & Kilpatric (1960) found a striking increase in the binding of sulphonamides as pH changed from acidity to alkalinity (pH 5→9). They found that this increase was greater with less extensively bound compounds (i.e. sulphadimidine). Protein binding is dependent on pH, both as it affects the ionization of the protein and the drug. Reider (1963) made three measurements in connection with protein binding. The first was the E.P.B. values of a series of sulphonamides

which gave the percentage of the protein bound sulphonamide to total plasma sulphonamide. The second was Langmuir's α constant (also referred to as 'K' in this chapter), which is inversely proportional to the binding strength of the plasma protein - sulphonamide bond, and thirdly Langmuir's β constant which is a measure of the binding capacity of the proteins for the sulphonamides. These three measurements should be made in any comparison of the plasma protein binding of sulphonamides.

c) EFFECT OF BINDING ON KIDNEY EXCRETION.

The binding of sulphonamides might be expected to affect their duration of action because protein bound drug is not removed from the kidney by glomerular filtration. However, if dissociation of the drug-protein complex is very rapid, the rate of removal of drug will not be limited because of binding. The rate constant for dissociation of the drug-protein complex shows that dissociation occurs with a half life of 20 milli-seconds ($R_1 = 35$ sec.$^{-1}$). The rate of association or dissociation does not limit the transport of the drug out of the plasma (Thorp, 1964). Phenol red is 80% bound to proteins and yet the dye is completely removed in a single passage through the kidney (Brodie, 1965a). Protein bound dye is fully available for tubular secretion since the unbound dye is secreted so rapidly and the rate constant R has a high absolute value.

However, the rapid establishment of an equilibrium between bound and unbound drug has not been shown for all groups of compounds, and may in fact not be the case with some sulphonamides. Also there may be two types of binding for some drugs, strongly bound and weakly bound to plasma proteins. The weakly bound drug may be in equilibrium with the unbound drug, but the strongly bound drug may not be reversibly bound at all and will only be metabolised and excreted on the breakdown of plasma protein. It is only if the degree of binding is very extensive that complete removal is hindered, and of course if the binding is 100% (that is irreversible) there is no way in which the kidney can excrete the drug.

Reider (1963) did not find any correlation between the rate of elimination of sulphonamides from the plasma and any of the three criteria for plasma protein binding, either in the rabbit or in humans. There was no correlation found for the five sulphonamides tested in the rabbit between any of the protein binding values and the rate of renal elimination in the urine. In the healthy kidney, excretion in the urine may be expected to be dependent on protein binding only in the rare cases in which a sulphonamide is extracted from the plasma chiefly by glomerular filtration, and not reabsorbed or secreted by the tubules to any notable extent.

d) DRUG INTERACTION

Anton (1961) found that the distribution of certain sulphonamides

in the rat could be modified by interfering with their binding to plasma proteins by another drug. Highly bound acidic drugs compete for a limited number of binding sites on plasma albumin. The administration of a second drug modified the distribution of the first, thereby altering its activity, toxicity and duration of action. Phenylbutazole, sulphinpyrazole ethyl biscoumacetate and ionphenoxic acid were found to be the most active in displacing a sulphonamide from plasma albumin.

The displacing agent altered the plasma and tissue concentration only of those sulphonamides that showed more than 45% plasma protein binding. Since these sulphonamides are not readily metabolised or excreted, the displaced unbound molecules diffuse from plasma into tissues. The result being that plasma levels decline, but the levels of drug in skeletal muscle C.S.F. and brain show a pronounced rise, and the antibacterial activity of the sulphonamides is profoundly enhanced.

A drug may be made more toxic by displacement from protein. Such displacement is especially dangerous when most of the drug in the body is attached to plasma proteins and the unbound drug comprises only a minor fraction of the total. Displacement of only a few percent can double or treble the level at the drug receptor. So the administration of a long acting sulphonamide such as sulphaphenazole has thus been shown to induce hypoglycaemic attacks in cases of Tolbutamide-treated diabetics (Brodie, 1965b).

In premature babies, the albumin concentration is low and readily saturated with bilirubin, and a number of other organic acids compete for the same binding sites (Odell, 1959). At one time, premature babies were routinely treated with sulphisoxazole and penicillin to protect them against infection. In a certain study, premature babies were given either tetracycline or the combination of penicillin and the sulphonamide. A large percentage of the babies treated with the sulphonamide mixture died (Silverman et al., 1956). Death was associated with Kernicterus and it is now required that neonates should not be given sulphonamides with other displacing agents.

c) METABOLISM.

Anton & Boyle (1964) studied the alteration of the acetylation of sulphonamides by protein binding. They found that bovine albumin interfered with the acetylation of sulphamethoxypyridazine. This effect was due to the binding of the sulphonamide to the protein, since a similar effect was not observed with bovine globulin to which this sulphonamide was not bound. Neither protein affected the acetylation of sulphanilamide whose binding to albumin was much less than that of sulphamethoxypyridazine (12% cf. 60% respectively). Newbould & Kilpatric (1963) also found that acetylation rates depended on the diffusible concentration of sulphonamides. Using perfused rabbit liver, they found that the highly bound sulphonamides were

only slightly acetylated. Anton & Boyle stated in their paper that there appeared to be no consistant correlation among a number of sulphonamides between their binding to proteins, lipid solubility, pKa and acetylation.

f) SPECIES DIFFERENCES.

Using the method of equilibrium dialysis, Genazzani (1963) estimated the binding of sulphathiazole, sulphamethoxypyridazine, sulphadimethoxine, sulphamethoxypyrazine and sulphisoxazole to the serum of the horse, sheep, pig, ox, turkey and man in vitro. Human serum had the most extensive binding capability followed by the serum of ox, sheep, pig, horse and turkey. He believed the higher binding capability of human serum to be related to the higher percentage of albumin. But no evidence was given of precise correlation between albumin percentage and binding capability for the serum of the other animals. He assumed that the binding capability must be related not only to the quantity of serum albumin, but also to the different reactivity of the protein macro-molecules of different sera.

However, Genazzani (1963) only measured protein binding at concentrations 3×10^{-5}M (10 γ/ml.) and 1×10^{-4}M (33 γ/ml.). These concentrations are far below those obtained in vivo. So in order for a true comparison to be made, binding at higher concentrations

should be evaluated.

Reider (1963) studied the protein binding of sulphadimethoxine, sulphisoxazole, sulphamethoxazole, sulphamethoxypyridazine and sulphadiazine in rabbits. He found that Langmuir's α and β constants for plasma protein binding were lower in the rabbit for all five sulphonamides than in man. Rabbit plasma proteins thus have a higher affinity for the sulphonamides tested, but can bind only smaller quantities than those in human plasma.

In view of the fact that protein binding affects the absorption, distribution, metabolism and excretion of sulphonamides and hence is intimately related to their duration of action, it was decided to investigate this aspect in relation to sulphasomidine, sulphamethomidine and sulphadimethoxine. The plasma protein binding of these three sulphonamides was measured at different concentrations in human plasma and the constants, K, β and n were calculated from the results obtained. A comparison of the binding of sulphadimethoxine to plasma protein in human, rabbit and rat was made in order to evaluate the species differences between these three animals. The protein binding of the $\underline{N}^4$-acetyl derivatives and the $\underline{N}^1$-glucuronides of sulphamethomidine and sulphadimethoxine were also measured in human plasma to find out something about the site of binding on the sulphonamide molecule.

2) METHODS

a) ULTRAFILTRATION.

Blood taken from the species under investigation was mixed with heparin (Weddel Pharmaceuticals, London) (0.1 ml. of heparin to 5 ml. of blood) and centrifuged at 2,000 g. for 10 minutes. A known weight of sulphonamide was then dissolved in the plasma and a known volume of 0.1M citrate - 0.2M phosphate buffer pH 7.4 (1/5 of the volume of plasma) was added. The solution was centrifuged and the supernatant (5 mls) was then pipetted into dialysis bags made from cellophane tubing (15 cm. in length and 3 cm. flat width), which had been previously soaked in distilled water for 24 hrs. and dried. The ends of the tubing were knotted and the bag was placed with a flat surface against a sintered disc (porocity 1) at the bottom of a polythene tube (10 cm. length, 2.7 cm. diameter). The tube was spun at 3,000 g. for 2 hrs. in a thermostatically controlled centrifuge (20°). The contents of the bag were analysed for total drug (bound and unbound) by the addition of trichloroacetic acid (1 ml. of 20%) to 1 ml. plasma, centrifugation of the precipitated proteins (2,000 g. for 5 mins.) and analysis of the supernatant by the Bratton and Marshall (1939) method. The ultrafiltrate which had collected in the bottom of the polythene tube (0.3-0.5 ml.) was analysed and this represents the amount of

unbound drug (in an equal volume of solution) present in the bag.

Animals. In order to obtain reliable results for each sulphonamide, the amount of the sulphonamide bound to plasma protein was estimated at four concentrations in each species under investigation. This experiment was then repeated three times, the estimation of the percentage bound at each concentration was performed in quadruplicate. Blood obtained from three 'A' rhesus positive human males, six New Zealand white rabbits and seventy-two Wistar albino rats was used for each set of experiments.

b) EQUILIBRIUM DIALYSES.

The same method as already described was adopted to introduce 5 mls. of a solution containing plasma, buffer pH 7.4 and the sulphonamide under investigation into a dialysis bag made from cellophane tubing. This bag was placed in a solution of 0.1M citrate - 0.2M phosphate buffer pH 7.4 (10 mls.) in a conical flask (20 mls.). The unit was agitated for 18 hrs. in a temperature controlled shaking water bath (20°). This allowed equilibration of the diffusible constituents (unbound drug) between plasma and buffer. At the end of the experiment, sulphonamide concentrations were measured in the inner and outer fluids. The concentration outside the bag is that of unbound drug and the concentration of drug inside the bag is that of

bound and unbound sulphonamide.

Controls were performed in both these experiments using buffer and drug only inside the bag. In these cases, the concentration of sulphonamide inside the bag was the same as that outside. This latter method was used in order to compare the results obtained by equilibrium dialysis with those obtained in ultrafiltration. This was done with the binding of sulphadimethoxine to human plasma protein and the two methods agreed within the limits of experimental error (±1%).

c) CALCULATIONS.

Interaction of a drug with the unoccupied binding sites of a protein may be considered as a reversible reaction obeying the law of mass action.

$$P_F + X \underset{R_1}{\overset{R_2}{\rightleftharpoons}} P_F X$$

$$K = \frac{[P_F]\,[X]}{[P_F X]} \qquad (1)$$

where $[P_F]$ = concentration of free binding sites of protein.

$[X]$ = concentration of unbound sulphonamide.

$[P_F X]$ = concentration of combined binding sites

K (=q) = dissociation constant.

If n = number of binding sites on each protein molecule, and

P = molar concentration of total protein (albumin)

then from (1) $$V = \frac{n[X]}{K + [X]} \qquad (2)$$

where V = moles sulphonamide bound per mole total protein. This expression is identical in form with the Langmuir isotherm.

By rearrangement of (2)

$$\frac{1}{V} = \frac{K}{n} \cdot \frac{1}{[X]} + \frac{1}{n} \qquad (3)$$

From a straight line plot of $1/V$ against $1/[X]$, K can be calculated from the slope of the line and n from the intercept on the $1/V$ axis (Fig. 5.1). K and n are affected by the pH, temperature, ionic strength and dielectric constant of the solution.

The β constant is a measure of the binding capacity of proteins for sulphonamides and can be measured by plotting V against X (Fig. 5.2). As the concentration of sulphonamide in plasma increases so the total amount bound increases. Eventually, a state is reached where a further increase in sulphonamide concentration merely increases the amount of unbound sulphonamide present, the total number of moles of sulphonamide bound per mole of protein remains constant.

It has been assumed for these experiments, that the sulphonamides are only bound to any significant extent (quantitatively) to plasma albumin.

Values for plasma albumin content are as follows (Martin, 1961):

Human	0.63 mmoles/litre
Rabbit	0.69 mmoles/litre
Rat	0.62 m moles/litre

The molecular weight of plasma albumin has been taken as 69,000. As these values are only calculated and not estimated exactly, the results for $K_{1/2}$ and n obtained can only be used in a comparative manner.

Graphs such as Figs. 5.1 and 5.2, were plotted for sulphadimethoxine in human, rabbit and rat plasma, and also for sulphamethomidine and sulphasomidine in human plasma. The results obtained are given in Tables 5.1 and 5.2.

The plasma protein binding of the major metabolites of these three sulphonamides were also measured at 0.33 μmoles/ml. However, N^4-acetyl sulphadimethoxine was poorly soluble in human plasma and so the concentration used for this substrate was 0.1 μmoles/ml.

Fig. 5.1. BINDING OF SULPHADIMETHOXINE BY HUMAN PLASMA PROTEIN.

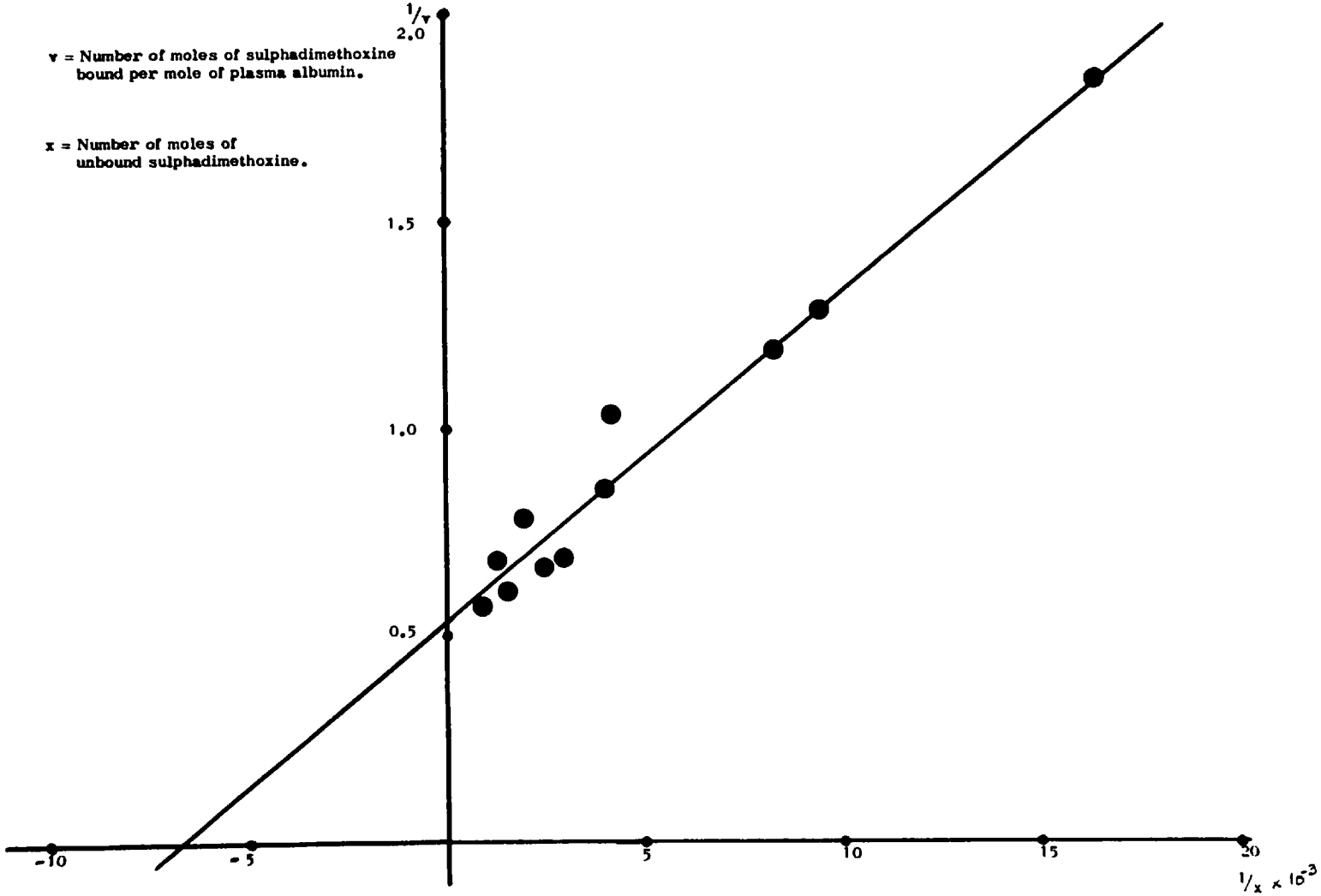


Fig. 5.2. BINDING OF SULPHADIMETHOXINE BY HUMAN PLASMA PROTEIN.

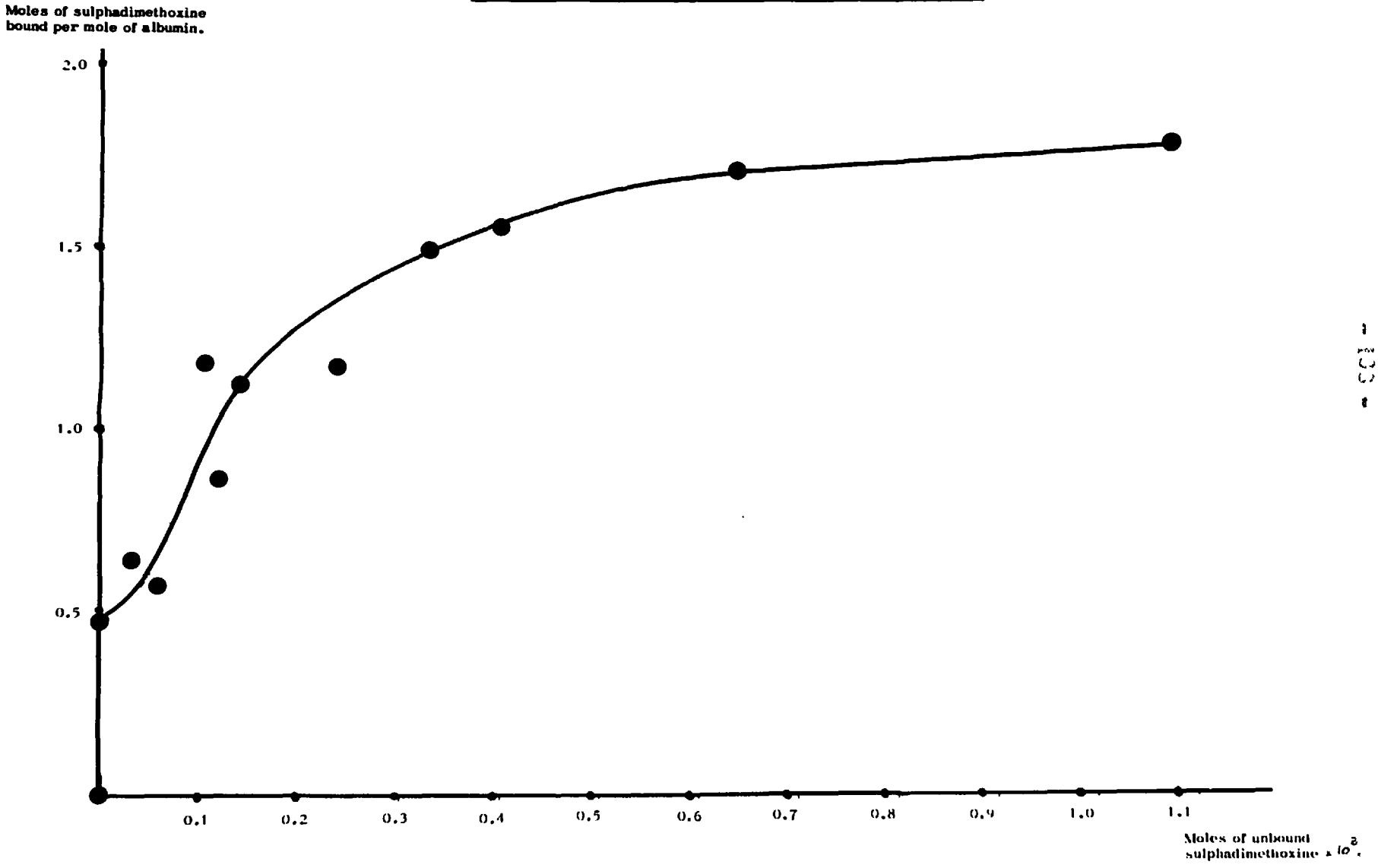


TABLE 5.1

A comparison of the binding of sulphadimethoxine, sulphamethomidine and sulphasomidine to human plasma protein

		Sulphadimethoxine	Sulphamethomidine	Sulphasomidine
% Bound at concentration	0.33 μmoles/ml.	92%	90%	67%
	0.67 μmoles/ml.	85%	85%	58%
	1.00 μmoles/ml.	80%	79%	44%
	$K \times 10^{-3}$	0.147	0.163	0.314
	β	1.77	1.73	1.03
	n	1.9	2.1	1.1

TABLE 5.2

A comparison of the binding of sulphadimethoxine by human, rabbit and rat plasma proteins

		Human	Rabbit	Rat
% Bound at concentration	0.33 μmoles/ml.	92%	98%	100%
	0.67 μmoles/ml.	85%	83%	94%
	1.00 μmoles/ml.	80%	64%	81%
	$K \times 10^{-3}$	0.147	0.083	0.019
	β	1.77	1.20	1.64
	n	1.9	1.2	1.6

TABLE 5.3

Protein binding of some sulphonamides and their metabolites in human plasma

Substrate concentration 0.33 μmoles/ml.

Substrate	% Bound
Sulphasomidine (SS)	67
Sulphamethomidine (SM)	90
Sulphadimethoxine (SD)	92
N^4-Acetyl SS	78
N^4-Acetyl SM	88
N^4-Acetyl SD*	90
SM-N^1-Glucuronide	33
SD-N^1-Glucuronide	28

* concentration 0.1 μmoles/ml.

4) DISCUSSION

A comparison of the binding of sulphadimethoxine (SD), sulphamethomidine (SM) and sulphasomidine (SS) to human plasma protein is given in Table 5.1. At all concentrations, sulphadimethoxine was the most highly protein bound, although very similar results were obtained for sulphamethomidine. Sulphasomidine is the least protein bound at 0.33 umoles/ml. nearly four times as much unbound drug being available for metabolism and excretion in vivo compared with the other two sulphonamides (67% compared with 92% for sulphadimethoxine and 90% for sulphamethomidine). In vivo these two long acting sulphonamides in man are excreted to an extent of 25% of the dose fed in the first 24 hrs., whereas 72% of the dose of sulphasomidine fed is excreted in the same time (see chapter 2). These facts can now be explained partly on the basis of protein binding.

Plasma albumin has only one site for binding sulphasomidine, but two for sulphadimethoxine and sulphamethomidine, hence the total binding capacity of albumin for sulphasomidine in man is markedly less than that for sulphamethomidine and sulphadimethoxine (β constant for SS = 1.03; SM = 1.73; SD = 1.77). Thus, as the concentration of sulphasomidine is increased from 0.33 umoles/ml. so the percentage bound rapidly falls (56% at 0.67 umoles/ml., 44% at 1.00 umoles/ml.).

The replacing of the methoxyl group in the '2' position in sulphadi-

methoxine by a methyl group does not appear, therefore, to affect the percentage bound, the number of binding sites, the constant or the value of K (SD = 0.147; SM = 0.163). However, when the methoxyl group in '4' position of sulphadimethoxine is also replaced by a methyl group then the constants alter (K value for sulphasomidine = 0.314). This means that the strength of the bond in the drug-protein complex is much less than for the other two sulphonamides.

Table 5.2 gives a comparison of the binding of sulphadimethoxine to human, rabbit and rat plasma proteins. At a concentration of 0.33 μmoles/ml. the rat binds 100% of the drug, the rabbit 98% and the human 92%. The species difference between these three species can be seen in the value of the β constants (1.77 for human, 1.20 for rabbit and 1.64 for rat). This means the binding capacity of the rabbit for sulphadimethoxine is less than for the rat and human, and this fact is related to the number of binding sites of albumin in the different species. The same pattern is seen in the results obtained for sulphamethomidine and sulphasomidine.

The K constants which are inversely proportional to the binding strength of the plasma protein - sulphonamide bond, decrease from 0.147 (human), 0.083 (rabbit) to 0.019 (rat). Sulphadimethoxine is, therefore, very tightly bound to rat plasma albumin. Thus, because at low concentrations (0.33 μmoles/ml.) sulphadimethoxine is also 100%

bound, rats do not rapidly excrete this sulphonamide after an intraperitoneal injection. Even after three weeks a significant amount remains in the blood, none being available for excretion.

The protein binding of these three sulphonamides and their major metabolites to human plasma protein is given in Table 5.3.

When sulphadimethoxine and sulphamethomidine are acetylated in the N^4-position, then the percentage bound (concentration 0.33 umoles/ml.) is unaffected. However, when sulphasomidine (67% bound) is acetylated the percentage bound to plasma protein increases (78%). It is known that acetylation causes a small decrease in the pKa value of the sulphonamide nitrogen (Table 4.3). If the plasma albumin attaches to the sulphonamide in the N^1-position, then a small alteration of the pKa by acetylation may affect the protein binding of the substrate.

The attachment of a glucuronic acid molecule to the N^1-position of sulphadimethoxine and sulphamethomidine results in these metabolites being poorly protein bound (28% and 33% respectively, at low concentrations). This may be due to the molecule being sterically hindered from attaching itself to the binding site of plasma albumin. The low protein binding together with the high water solubility of the N^1-glucuronides allow them to be rapidly excreted in vivo.

CHAPTER 6

THE EFFECT OF PHENOBARBITONE PRETREATMENT ON THE METABOLISM AND EXCRETION OF SULPHAMETHOMIDINE AND [^{35}S]SULPHADIMETHOXINE.

1) INTRODUCTION.

2) MATERIALS AND METHODS.

- i) Pretreatment with phenobarbitone
- ii) Blood levels
- iii) Uptake by kidney slices
- iv) Competition between sulphonamides for excretion
- v) Plasma protein binding
- vi) Biliary excretion.

3) RESULTS.

4) DISCUSSION.

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1) INTRODUCTION

The metabolism of various lipid soluble drugs is accelerated in animals treated previously with a number of structurally related or unrelated compounds. Some activators such as phenobarbitone, as well as being oxidised themselves, stimulate the enzyme systems responsible for the hydroxylation and dealkylation of foreign compounds. Remmer (1962) found that the enzymic activity was clearly reduced 2-4 hrs. after administration of phenobarbitone. On continued administration, the increase in enzymic activity reached a maximum on the second and third day, declining to normal values after 5-7 days. Remmer (1962) concluded from his experiments that activation could occur when the stimulating drug was given in one large or in several smaller doses over a period of time. This increase in drug metabolising enzymes is reported to be the result of a quantitative increase in smooth endoplasmic reticulum (Remmer & Merker, 1963). Gelboin & Sokoloff (1961) showed that previous treatment of rats with phenobarbitone stimulated the incorporation of amino acids into microsomal proteins of cell-free liver preparations.

Remmer (1964) reported that pretreatment with phenobarbitone affected the metabolism of sulphadimethoxine in the rat and dog. Kibby (1965) found that when sulphadimethoxine was administered to rats, pretreatment increased the renal excretion of all metabolites.

Also pretreatment appeared to stimulate glucuronide synthesis and excretion in the bile.

The acceleration of the metabolism and excretion of sulphonamides might easily be induced in therapy because of the wide use of many different drugs which may activate enzymes in liver microsomes. Because of this interest in the interaction of drugs, we decided to investigate the effect of phenobarbitone on the metabolism and excretion of [^{35}S]sulphadimethoxine. In order to explain the effect of this interaction between phenobarbitone and sulphadimethoxine, blood levels, protein binding and uptake by kidney slices were investigated.

2) MATERIALS AND METHODS

1) PRETREATMENT WITH PHENOBARBITONE.

Phenobarbitone sodium (30 mg./kg.) was administered in aqueous solution intraperitoneally to female rats (300 g.) or female mice (30 g.) once daily for three days prior to the administration of [^{35}S]sulphadimethoxine. Control groups of female rats or mice were pretreated with water (1.0 ml. and 0.1 ml. respectively) intraperitoneally daily for three days.

A comparison of the metabolism and excretion of sulphamethomidine and [^{35}S]sulphadimethoxine in normal and pretreated rats was carried out. [^{35}S]sulphadimethoxine (100 mg./kg., 5 µc.) was injected (i.p.) in dimethylsulphoxide (0.5 ml.) 2 hours after the final injection of phenobarbitone sodium. Urine was collected in two twenty-four hour batches, and the total amount of radioactivity excreted was estimated using the scintillation counter. Urinary metabolites were estimated using the radiochromatogram scanner as previously described (see chapter 3). Sulphamethomidine (100 mg./kg.) was given i.p. in DMSO (0.5 ml.) to pretreated rats 2 hrs. after the final injection of phenobarbitone sodium. Urine was collected in two twenty-four hour batches and analysed for free and total drug by the Bratton & Marshall (1939) method. Urinary metabolites were estimated chromatographically

by the method described in chapter 2.

The effect of using propane 1.2.diol, instead of DMSO as the medium, on the metabolism and excretion of [^{35}S]sulphadimethoxine administered orally to normal and pretreated rats was investigated. The species difference in the excretion of [^{35}S]sulphadimethoxine given i.p. in DMSO to normal and pretreated rats and mice was also investigated. On this occasion, a single injection intraperitoneally of phenobarbitone sodium (30 mg./kg.) was also given to one group of rats and one group of mice 2 hrs. prior to the administration of [^{35}S]sulphadimethoxine (100 mg./kg.).

ii) BLOOD LEVELS.

Female rats (300 g.) pretreated for 3 days by a single intraperitoneal injection (1 ml.) of phenobarbitone sodium (30 mg./kg.) were injected (i.p.) with [^{35}S]sulphadimethoxine (100 mg./kg., 1.5 μc.) together with a control group of rats pretreated with water (1 ml.) i.p. daily for 3 days. After time intervals of 30 mins., 1 hr., 2 hrs., 4 hrs., 8 hrs., 12 hrs., 13 hrs., 25 hrs. and 30 hrs. two normal rats and two pretreated rats were stunned and their throats cut. The blood collected (10 ml.) was mixed with heparin (0.1 ml.), centrifuged at 2000 g. for 5 mins. and the plasma (0.2 ml.) counted in dioxan gel (Fig. 6.1).

iii) UPTAKE BY KIDNEY SLICES.

Female rats (250 g.) were killed by a blow on the back of the neck, and the kidneys immediately removed and placed in Krebs-Ringer phosphate solution (0.154M$_{\equiv}$, pH 7.4) in chilled beakers. Slices of the kidney were cut (100 mg. wet weight) and placed in 2 ml. of Krebs-Ringer solution containing [^{35}S]sulphadimethoxine (400 µg., 0.6 µc.). The mixtures were shaken (100 oscillations/minute) in 20 ml. conical flasks at 37° in an atmosphere of 95% O_2 + 5% CO_2 for 1 hr. At the end of this time period, the slices were removed from the incubation medium, blotted on moist filter paper and weighed.

The slices were digested with 5 ml. of an alcoholic sodium hydroxide solution (12 ml. 33% $^{w}/_{v}$ aqueous NaOH diluted to 50 ml. with 95% $^{v}/_{v}$ ethanol) at 38-40° for 1 hr. 1.4M$_{\equiv}$-Acetic acid (5 ml.) was then added and the neutralized solution estimated for [^{35}S] activity. Aliquots (1 ml.) of neutralized kidney solution and the supernatant were counted in dioxan gel, and the slice/medium (^{s}/m) ratio calculated. The experiment was performed in triplicate. Three normal rats, three pretreated rats and three rats given a single i.p. injection of phenobarbitone sodium (30 mg./kg.) 2 hrs. prior to the animals being killed, were used in this experiment. Controls had [^{35}S]sulphadimethoxine (400 µg., 0.6 µc.) added at the end of the incubation period.

iv) COMPETITION BETWEEN SULPHONAMIDES FOR EXCRETION.

Female rats (250 g.) were given sulphamethomidine (100 mg./kg.) intraperitoneally as a suspension in propane 1.2.diol (0.6 ml.). Two hours later, [^{35}S]sulphadimethoxine (100 mg./kg., 5 μc.) was given (i.p.) in DMSO (0.4 ml.). Urine was collected after 24 hrs. and 48 hrs. Total sulphonamide present was estimated by the Bratton & Marshall method (1939). Urine was also examined chromatographically for individual metabolites. The amount of [^{35}S]sulphadimethoxine present in the first 24 hr. urine sample and its individual metabolites were estimated as already described.

v) PLASMA PROTEIN BINDING.

The effect of phenobarbitone sodium on the binding of [^{35}S]sulphadimethoxine to rat plasma proteins was determined in vivo and in vitro. Two groups of female rats (250 g.) were taken and one group (6 rats) were pretreated with phenobarbitone sodium daily for 3 days as previously described. Then both groups of rats were injected (i.p.) with [^{35}S]sulphadimethoxine (100 mg./kg., 1.5 μc.) in DMSO (0.5 ml.) 2 hrs. after the final injection of phenobarbitone. One hour after the injection of [^{35}S]sulphadimethoxine, the rats were stunned, their throats cut and blood was taken from each group of rats as previously described. The binding of [^{35}S]sulphadimethoxine to plasma proteins was estimated by an ultrafiltration method (see chapter 5).

Blood taken from female rats (250 g.) was centrifuged at 2000 g. for 10 mins. and [^{35}S]sulphadimethoxine added to the plasma to give concentrations of 0.33, 0.67 and 1.00 μmoles/ml. Phenobarbitone sodium was added to samples of plasma from each group to give concentrations of 100 μg./ml. and 300 μg./ml. The plasma protein binding for these solutions was measured by ultrafiltration and the results compared with those already obtained for normal rat plasma.

vi) BILIARY EXCRETION.

Female rats (250 g.) were pretreated with phenobarbitone as already described. A control group of rats were pretreated with water (1 ml.) i.p. daily for 3 days. Normal and pretreated rats were cannulated 2 hrs. after final pretreatment injection by the method already described (see Chapter 2). Both groups were given an intraperitoneal injection of [^{35}S]sulphadimethoxine (100 mg./kg., 2 μc.) in DMSO (0.5 ml.) and the bile collected for 24 hrs. The experiment was repeated using propane 1.2.diol as the medium of administration. The bile was analysed for total drug and individual metabolites by the method already described.

Female rats (250 g.) were pretreated with phenobarbitone sodium (50 mg./kg.) daily for 3 days and then left for 24 hrs. A control group of female rats was treated similarly and left for 24 hrs. after the final i.p. injection of water (1 ml.). The two groups of rats (3 rats in each

group) were biliary cannulated. [^{35}S]Sulphadimethoxine (100 mg./kg., 2 μc.) in DMSO (0.5 ml.) was injected i.p. and bile collected at intervals of 15 mins., 30 mins., 45 mins., 60 mins., 75 mins., 90 mins., 2 hrs., 2½ hrs., 3 hrs., 3½hrs., 4 hrs., 5 hrs., 6 hrs., 10 hrs. and 24 hrs. Each sample was analysed for [^{35}S] activity and a graph plotted (Fig. 6.2).

TABLE 6.1

Metabolism and excretion of sulphamethomidine and [^{35}S]sulphadimethoxine in normal and pretreated rats.

Dose level 100 mg./kg. injected i.p. in DMSO. (Average for 3 animals ♀.)

		Sulphamethomidine		Sulphadimethoxine		
		N	P	N	JP	P
% of dose fed excreted in	24 hrs.	52±6	44±12	31±2	43±4	44±7
	48 hrs.	60±6	57±10	45±1	57±1	55±5
% of 24 hr. urine excreted as	N^4-glucuronide	Trace	0	5±1	1±1	6±1
	N^4-sulphate	1±1	1±1	3±1	2±1	15±7
	N^1-glucuronide	0	0	6±2	3±1	8±1
	Unchanged drug	14±2	18±3	11±4	29±7	21±2
	N^4-acetyl	85±3	81±4	75±6	65±4	50±10

N = Normal

P = Pretreated for 3 days with phenobarbitone sodium (30 mg./kg.).

JP = Pretreated with phenobarbitone sodium (30 mg./kg.) just 2 hrs. prior to injection of [^{35}S]sulphadimethoxine i.p.

TABLE 6.2

Metabolism and excretion of [^{35}S]sulphadimethoxine administered orally to normal and pretreated rats in propane 1.2.diol or DMSO.

Dose level 100 mg./kg. (5 μc.).

		DMSO		Propane 1.2.diol	
		N	P	N	P
Number of animals used		6 ♀	3 ♀	5 ♀	3 ♀
% of dose fed excreted in	24 hrs.	26±5	43±3	15±1	30±5
	48 hrs.	52±3	60±3	28±3	42±3
	5 days	67±1	68±1	-	-
% of 24 hr. urine excreted as	N^2-glucuronide	2±1	6±1	5±1	4±1
	N^4-sulphate	8±1	15±1	6±2	7±4
	N^1-glucuronide	9±1	8±2	7±3	9±2
	Unchanged drug	22±1	38±1	20±4	35±5
	N^4-acetyl	59±3	33±1	62±2	45±4

TABLE 6.3

Biliary excretion of [^{35}S]sulphadimethoxine in normal and pretreated rats.

Dose level 100 mg./kg. injected i.p. Average of 3 Q.

		DMSO		Propane 1.2.diol	
		N	P	N	P
% of dose fed excreted in	24 hrs.	9±3	11±5	12±1	11±2
% of 24 hr. bile excreted as	N^4-glucuronide	23±12	26±1	8±1	18±2
	N^4-sulphate	0	0	0	0
	N^1-glucuronide	45±13	31±4	68±3	53±1
	Unchanged drug	25±13	24±2	21±3	25±1
	N^4-acetyl	7±7	19±7	3±1	6±1

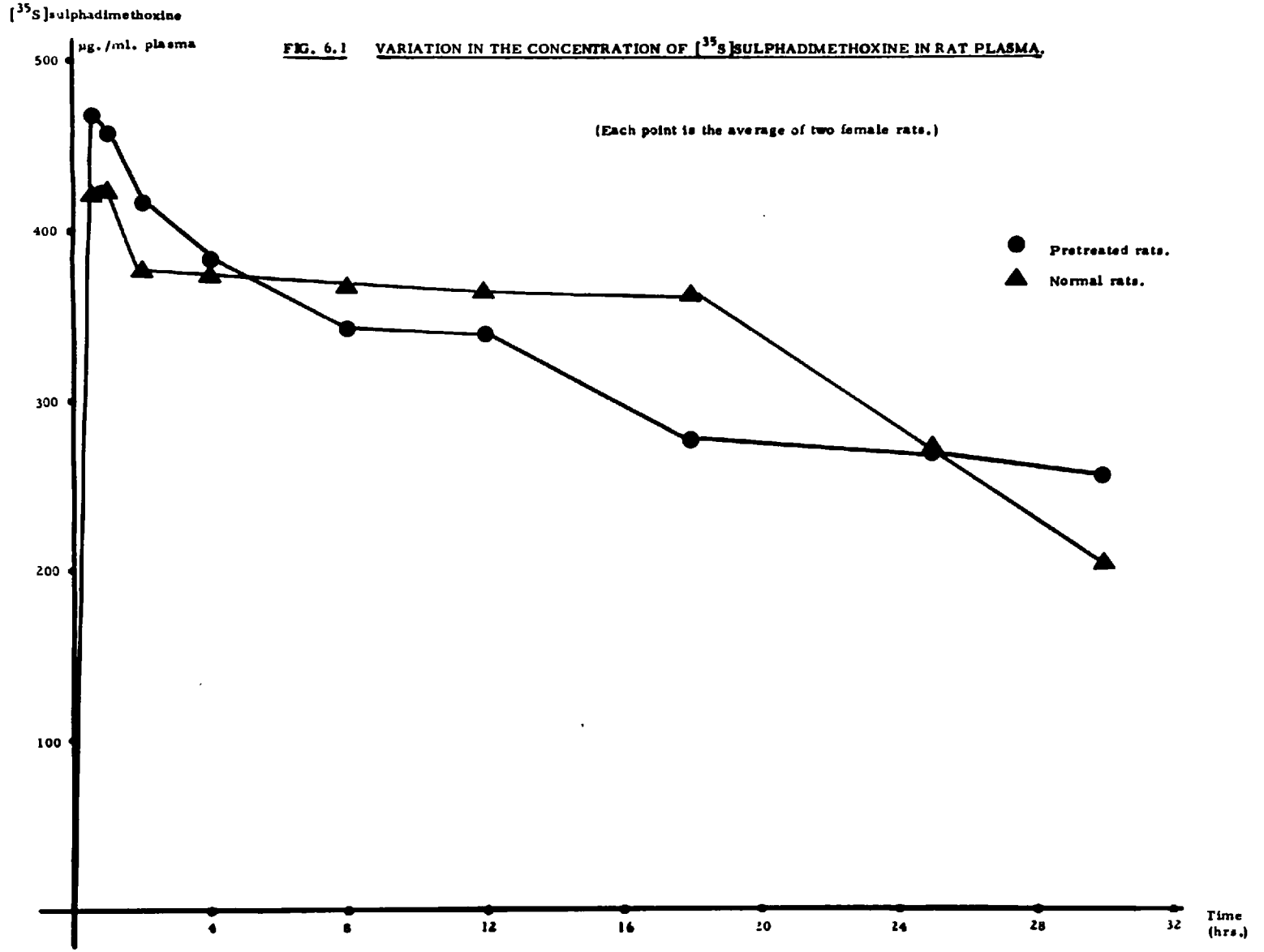
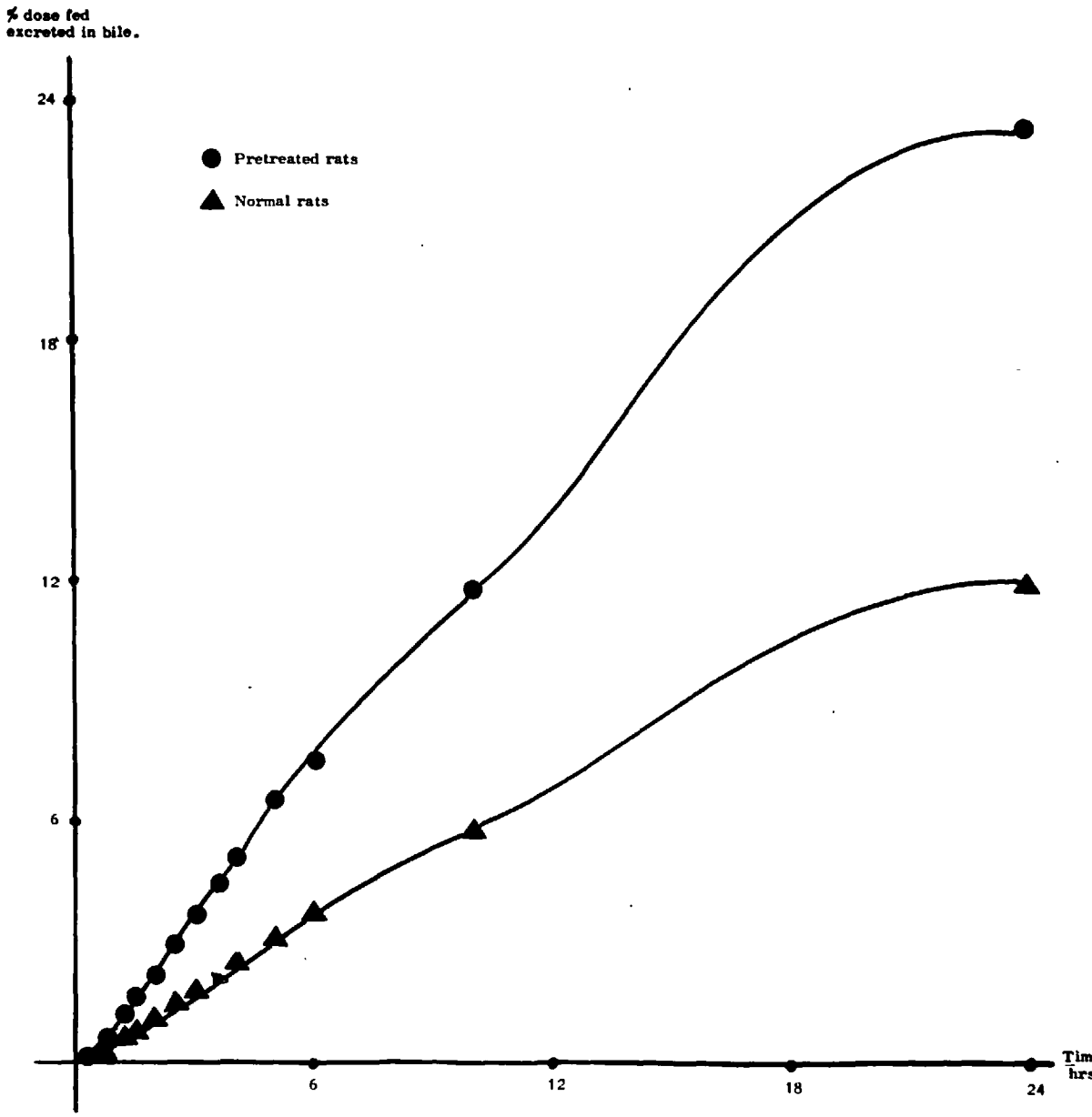


FIG. 6.1 VARIATION IN THE CONCENTRATION OF [^{35}S]SULPHADIMETHOXINE IN RAT PLASMA.

(Each point is the average of two female rats.)

FIG. 6.2 BILIARY EXCRETION OF [S^{35}] SULPHADIMETHOXINE IN NORMAL AND PRETREATED FEMALE RATS.

(Each point is the average of three animals.)



4) DISCUSSION

A comparison of the metabolism and excretion of sulphamethomidine and [^{35}S]sulphadimethoxine in normal and pretreated female rats can be seen in Table 6.1. Injection of phenobarbitone sodium (30 mg./kg. daily for 3 days) has no statistically significant effect on the excretion or metabolism of sulphamethomidine. However, pretreatment of rats with phenobarbitone does increase the excretion of [^{35}S]sulphadimethoxine from 31% to 44% of the dose given intraperitoneally in DMSO. The excretion of this sulphonamide was also increased to 43% after a single i.p. injection of phenobarbitone 2 hrs. prior to the administration of [^{35}S]sulphadimethoxine. The effect of phenobarbitone on the metabolism of this compound is seen in the decrease in the percentage of $\underline{N}^4$-acetyl sulphadimethoxine excreted as a proportion of the 24 hr. urine. There was an increase in the amount of free drug excreted whichever way the pretreatment was carried out (from 11% to 29% for rats just pretreated and 21% for those pretreated for 3 days). The amounts of $\underline{N}^4$-glucuronide and $\underline{N}^1$-glucuronide excreted as a percentage of the 24 hr. urine were unaltered whereas there was an increase in the $\underline{N}^4$-sulphate excreted (3% to 15%). Even after 48 hrs. the pretreated animals had excreted 10% more sulphadimethoxine than the normal rats (45% compared with 55%).

A similar pattern is seen when [^{35}S]sulphadimethoxine was given orally to normal and pretreated rats (Table 6.2). The total amount excreted in 24 hrs. increased from 26% to 43% of the dose fed. Again the amount of $\underline{N}^4$-acetyl derivative decreased from 59% to 33% of the 24 hr. urine, although the actual amount excreted in mg. is about the same in each case. There was an increase in the amount of unchanged drug excreted from 22% to 38% of the 24 hr. urine (1.9 - 4.9 mg.). This accounts for the increase in total amount excreted. The quantity of $\underline{N}^4$-sulphate increased from 8% to 15% of the 24 hr. excretion.

When [^{35}S]sulphadimethoxine in propane 1.2. diol was fed orally to rats then again there was increased excretion (15% to 30%). The differences in metabolism follow the same pattern as when DMSO was used as the medium. The effect of DMSO and phenobarbitone on excretion are effects which reinforce one another. DMSO increased the excretion of this drug from 15% to 26% and pretreatment with phenobarbitone increased this to 43% of the dose fed.

The effect of phenobarbitone on the metabolism and excretion of sulphadimethoxine in the rat was not found in the mouse. The difference in percentage excretion in normal and pretreated mice was not significant. (Normal: 55 $\pm$ 4%, just pretreated: 48 $\pm$ 5% and pretreated for

3 days: 61 ± 1%, an average of 5 mice in each group.) Some explanation is therefore needed for this phenomenon in the rat, particularly as a single injection of phenobarbitone 2 hrs. prior to the administration of sulphadimethoxine is sufficient to alter the excretion pattern. In addition, the metabolism and excretion of the closely related drug sulphamethomidine, is unaffected by phenobarbitone in the rat. Phenobarbitone, as well as affecting the microsomal enzymes, might affect the permeability of cell membranes and this may be the explanation of the increased excretion.

Fig. 6.1 shows the effect of pretreatment on the plasma level of [^{35}S]sulphadimethoxine in the rat. A higher plasma level (475 µg./ml.) was more rapidly attained in pretreated rats than in normal ones. This higher plasma level was maintained for about 4 hrs. after which the pattern in each group of animals was similar. Presumably the initial high plasma level enables the compound to be more rapidly excreted in the first few hours.

The possibility that phenobarbitone was affecting the rate of excretion of sulphadimethoxine by the kidney was investigated by examining the uptake of this drug by kidney slices of normal, pretreated and just pretreated rats. A slice/medium ratio ($^{s}/_{m}$) of greater than 1 (i.e. 5-10) indicates that sulphadimethoxine may be actively secreted by the renal tubules. However, the results for the experiment were

as follows:

	N.	J.P.	P.
$^{s}/_{m}$ ratio	1.17 ± 0.14	1.28 ± 0.12	1.37 ± 0.15

This showed that pretreatment had no significant effect on the uptake of sulphadimethoxine by kidney slices in vitro.

The effect of a single injection of sulphamethomidine instead of phenobarbitone two hours prior to the administration i.p. of [^{35}S] sulphadimethoxine was also studied. It was found that, like phenobarbitone, sulphamethomidine increased the excretion of [^{35}S]sulphadimethoxine (from 30% to 40% of the dose fed). However, the metabolism of sulphadimethoxine was unaltered, and the excretion and metabolism of sulphamethomidine remained normal. The possibility that sulphamethomidine increased the excretion of sulphadimethoxine by competition for protein binding sites was examined and this competition was shown to occur in an in vitro system. The effect of phenobarbitone on the protein binding of [^{35}S]sulphadimethoxine to rat plasma albumin was investigated and found to be negligible.

The effect of pretreatment for three days on the biliary excretion of [^{35}S]sulphadimethoxine can be seen in Table 6.3. Regardless of the medium of administration (propane 1.2.diol or DMSO) there was no increase in the biliary excretion of this compound given 2 hrs.

after the final injection of phenobarbitone sodium. The percentage of unchanged drug excreted remained the same, while there were minor changes in the percentage of $\underline{N}^1$-glucuronide, $\underline{N}^4$-glucuronide and $\underline{N}^4$-acetyl derivatives excreted. The $\underline{N}^4$-sulphate was not excreted in the bile. However, when the pretreated rats were left for 24 hrs. after receiving phenobarbitone sodium daily for three days, then the biliary excretion increased from 12% to 23% of the dose fed (Fig. 6.2). One possibility is that when [^{35}S]sulphadimethoxine is given 2 hrs. after the last injection of phenobarbitone sodium then there is competition for excretion. But when the pretreated animals are left for 24 hrs. after the last injection of phenobarbitone, then the stimulation effect is not counteracted by a competition effect and the result is an increase in the percentage excreted. It is obvious, therefore, that there is still much to learn about the role of phenobarbitone and its effect on the metabolism and excretion of foreign compounds. For although pretreatment of rats with phenobarbitone is known to accelerate the metabolism and excretion of drugs, the mechanism by which a single dose of phenobarbitone increases the excretion of sulphadimethoxine is as yet unknown.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS.

1) SPECIES DIFFERENCES.

2) FORMATION OF N^1-GLUCURONIDES.

3) DURATION OF ACTION.

4) PHENOBARBITONE INDUCTION.

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1) SPECIES DIFFERENCES.

A comparison of the metabolism and excretion of sulphasomidine and sulphamethomidine in man, monkey, rabbit and rat has shown that there is little species variation in response to the short-acting sulphasomidine. This drug is excreted mainly as the unchanged compound in all species examined, the N^4-acetyl derivative being a minor metabolite (4-19% of dose excreted in the first 24 hours). After the ingestion of sulphamethomidine, the N^4-acetyl derivative was the main metabolite in the monkey, rabbit and rat (59-79% of first 24 hour excretion), whereas sulphamethomidine-N^1-glucuronide was a major metabolite in man and monkey (68% and 32% of first 24 hour excretion respectively). This metabolite was not seen in the urine of the rabbit or the rat. Sulphadimethoxine-N^1-glucuronide was not found in the urine of rabbits given sulphadimethoxine, and only small amounts were seen in the urine of the rat (Bridges et al., 1968). However, N^1-glucuronide formation was a major metabolic pathway in primates (Adamson, Bridges & Williams, 1966). It appears, therefore, that the rhesus monkey most closely resembles man in the metabolism and excretion of these sulphonamides, and also in the proportion and nature of metabolites of other substituted 6-sulphanilamidopyrimidines except the 4,5-dimethoxy compound (Bridges et al., 1969).

This similarity between these two species is in conflict with Brodie (1964) who stated, without giving any examples to support his argument, that the monkey generally does not treat foreign compounds in a similar way to man.

It would seem from these studies of species differences that the monkey is a very suitable animal in which to study the metabolism and excretion of new sulphonamides prior to clinical trial as they frequently metabolise these drugs in a similar way to man. Other metabolic reactions that have been reported as being peculiar to man i.e. the aromatization of (-) quinic acid to benzoic acid, the conjugation of aryl acetic acids with glutamine, have also been found to occur in the rhesus monkey (Adamson, Bridges & Williams, 1966; Bridges, King & Williams, 1964 - unpublished data). However, these reactions do not occur to any great extent in the lower animals.

The difference found in the metabolism and excretion of substituted 6-sulphanilamidopyrimidines in various animals indicates the difficulty in predicting species differences in the future from our present limited knowledge of drug metabolism. Not enough information is available to answer the question: which animal or animals may respond to a group of drugs or a chemical grouping in a similar way to man? However, as a result of these investigations it does seem as if the rhesus monkey is the animal of choice for some sulphonamides and maybe many other foreign compounds.

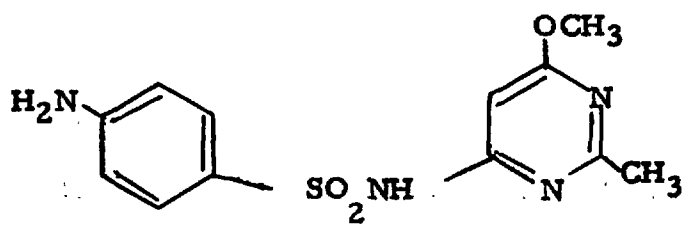
2) FORMATION OF N^1-GLUCURONIDES.

The *in vitro* synthesis of sulphadimethoxine-N^1-glucuronide was investigated in the rat, rabbit and monkey using [^{35}S]sulphadimethoxine as the substrate. These results confirmed those obtained *in vivo* i.e. no N^1-glucuronide could be detected in the rabbit, whereas considerable amounts were found in the monkey. This glucuronide formation is associated with only 2,4 disubstituted 6-sulphanilamidopyrimidines in which a methoxyl group is present in the '4' position of the pyrimidine ring. When the '2' position is unsubstituted (sulphamonomethoxine) then only 8% of the dose excreted in the first 24 hours in man is the N^1-glucuronide. N^1-Glucuronides of sulphathiazole (Uno & Ueda, 1962) and sulphisomazole (Ueda & Kuribayashi, 1964) have been reported as minor metabolites in man (less than 5% of dose excreted in 24 hours). However, the N^1-glucuronides of sulphamethomidine and sulphadimethoxine are major metabolites in primates (32-70%). It would be interesting to know whether other 2,4 disubstituted derivatives (2-methoxy-4-methyl-, 2,4-diethoxy-6-sulphanilamidopyrimidines) would form an N^1-glucuronide *in vivo*. Further investigations using these substrates including 2,4-dimethyl-6-sulphanilamidopyrimidine should be performed *in vitro* to ascertain whether the failure of this latter compound to form an N^1-glucuronide *in vivo* is due to its rapid clearance from the

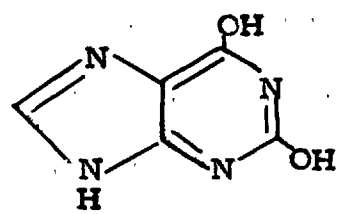
liver or its unsuitability as a substrate. Such studies on the formation of N^1 -glucuronides using various related substrates would be a useful tool for elucidating the nature of the site of enzyme action.

It is possible that this major route of detoxication in the monkey and man is due to the resemblance of sulphamethomidine (Fig. 7.1a) and sulphadimethoxine to naturally occuring compounds involved in nutrient metabolism. These two sulphonamides contain a pyrimidine ring which is found in many biologically important compounds i.e. the purines are structurally similar particularly xanthine (Fig. 7.1b).

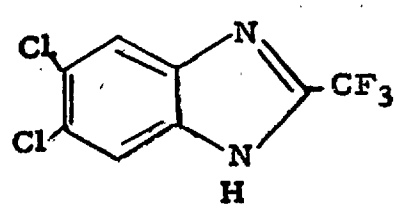
Fig. 7.1



(a)



(b)



(c)

As well as (a) and (b) being structurally similar, the acidic hydrogen atom of the sulphonamide group is analogous to the hydrogen of the imidazole ring of xanthine and other purines. However, there is no evidence at present that purines form glucuronides in the monkey. It would be interesting in this context to investigate the fate of various anutrient purines i.e. caffeine, and theophylline, to see if these also formed N^1-glucuronides. It has recently been found that 5,6-dichloro-2-trifluoromethyl-benzimidazole (Fig. 7.1c) is metabolised to an N^1-glucuronide in the rat and rabbit (6% and 9% of the dose fed respectively in the first 24 hours) (Flockhart, Smith & Williams, 1963 - unpublished data).

Further studies could be performed in lower species to see whether sulphonamide N^1-glucuronyltransferase activity is absent in these species or just suppressed in the same way that o-amino-phenylglucuronyltransferase activity is inhibited in the gunn rat.

3) DURATION OF ACTION.

Various parameters have been studied in order to explain the long action of some sulphonamides, but it seems that there is no single answer to the problem. However, in the series of isomers studied, the long-acting sulphonamides 2,4-dimethoxy-, 4,5-dimethoxy- and 4-methoxy-2-methyl-6-sulphanilamidopyrimidines all contain a lipophilic methoxyl group in the '4' position of the heterocyclic ring.

When this methoxyl group is shifted in the pyrimidine ring or replaced by a methyl group, then the sulphonamide becomes shorter acting. It would seem that there is a difficulty in designing drugs from existing knowledge, because a series of sulphonamides with many similarities in structure are metabolised and excreted very differently.

The physical properties of sulphadimethoxine, sulphorthodimethoxine and sulphamethomidine show that they are all comparatively highly soluble in organic solvents indicated by the chloroform-buffer partition coefficients (i.e. 2,4-dimethoxy:5, 4,5-dimethoxy:2, and 4-methoxy-2-methyl:1). This means that long-acting sulphonamides should be able to penetrate lipoprotein membranes of cells and be absorbed at lipophilic sites in the body more easily than short-acting compounds. They will therefore diffuse from the renal tubules and become reabsorbed into the blood stream and then metabolised before being finally excreted.

It has been suggested that long-acting sulphonamides underwent a different metabolic fate from short-acting sulphonamides which enabled them to be excreted at a slower rate. The sulphonamides described above were all metabolised to some extent to the N^4-acetyl derivatives, but there was no apparent correlation between long action and metabolism. The *in vitro* synthesis of these metabolites was studied for a series of substituted 6-sulphanilamidopyrimidines in

the rabbit and monkey. The differences between the *in vivo* and *in vitro* results could be largely explained by the physical properties (solubility, pKa and partition coefficient). However, some, but not all, long-acting sulphonamides form water-soluble glucuronides in man and monkey, and these being extensively formed *in vitro* (sulphadimethoxine-N^1-glucuronide formation in monkey) are rapidly excreted. Sulphorthodimethoxine, a very long-acting sulphonamide in man (5% of dose fed excreted in 24 hours) is not metabolised in this way.

A comparison of the binding of sulphamethomidine, sulphadimethoxine and sulphasomidine to human plasma proteins showed some important differences. The long-acting drugs were more highly and tightly bound at all concentrations studied. Plasma protein binding seems to be an important field that has received very little systematic attention. The problem of long action could be further investigated by altering the structure of the sulphonamide, measuring the binding at different concentrations and correlating the results with those obtained *in vivo*. The duration of action of the long-acting sulphonamides seems to be determined by their physical properties and protein binding which in turn affects their metabolism and excretion.

4) PHENOBARBITONE INDUCTION.

Pretreatment with phenobarbitone sodium daily for 3 days or a

single intraperitoneal injection just 2 hours prior to the ingestion of sulphadimethoxine decreased the duration of action of this sulphonamide in the rat. This phenomenon was not observed with sulphamethomidine. Phenobarbitone may have an effect on cell permeability which would affect the metabolism and excretion of a drug, and this aspect needs to be investigated.

Further work should be done on the effect of phenobarbitone on the excretion of sulphadimethoxine in vitro using perfused rat kidneys. The interaction of foreign compounds will continue to be of increasing interest as more anutrient compounds enter man's diet and combinations of drugs are administered during therapy.

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Corrigendum

2-Methyl-4-methoxy-6-sulphanilamidopyrimidine (sulphamethomidine) should be correctly named as 4-methoxy-2-methyl-6-sulphanilamidopyrimidine throughout this thesis.