

THE METABOLISM OF SOME HETEROCYCLIC SULPHONAMIDE DRUGS IN
MAN AND OTHER ANIMALS

By

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ABSTRACT

1. A short history of the antibacterial sulphanilamide derivatives relates their development from Prontosil to the present day. Recent advances in the understanding of the absorption, distribution and excretion of sulphonamides are discussed. Previous work on their metabolism by acetylation, oxidation and conjugation with sulphate and glucuronate is reviewed, introducing the work described herein.

2. The glucuronide of sulphadimethoxine formed in man has been isolated from urine by the use of ion-exchange resins, purified by chromatography, and finally characterised as the S-benzylthiouronium salt. On hydrolysis with β -glucuronidase the glucuronide yields sulphadimethoxine and glucuronic acid; with acid or alkali it yields glucuronic acid and the hydrolysis products of sulphadimethoxine-sulphanilamide, sulphanilic acid and barbituric acid.

3. Comparison of the isolated glucuronide with synthetic sulphadimethoxine N⁴-glucuronide shows the two compounds to be dissimilar. The biosynthetic glucuronide is identical with a glucuronide formed from sodium sulphadimethoxine and methyl acetobromoglucuronate.

4. The identity of the latter compound is shown to be sulphadimethoxine N¹-glucuronide by infrared spectroscopy. Ultraviolet spectroscopy is shown to have no application in the resolution of the structure.

5. Sulphadimethoxine exhibits extensive species differences in metabolism. Whereas in man and the rhesus monkey the N¹-glucuronide forms the major urinary metabolite, this is only a minor component in

the urine from the dog, rat and guinea-pig. The dog excretes principally the unchanged compound. The rat and guinea-pig excrete the N^4 -acetyl derivative; the rabbit excretes almost exclusively this conjugate. Sulphadimethoxine N^1 -glucuronide is the major metabolite in rat bile.

6. Pretreatment of rats with phenobarbitone extensively alters the excretion of sulphadimethoxine and its derivatives. Following administration of sulphadimethoxine the renal excretion of all metabolites increases but in the bile only the excretion of the N^1 -glucuronide increases. Following administration of sulphadimethoxine N^1 -glucuronide excretion in the urine and bile decreases. In the light of in vitro results the suggestion is made that phenobarbitone activates systems for sulphadimethoxine N^1 -glucuronide transport.

7. The metabolism of structural analogues of sulphadimethoxine (2-methoxy-, 4-methoxy-, 5-methoxy-, 2,5-dimethoxy-, and 4,5-dimethoxy-6-sulphanilamidopyrimidine) in the monkey, rat and rabbit (some also in man) shows that none of the five compounds is extensively conjugated with glucuronic acid in the N^1 -position. In general the sulphonamides are highly acetylated by the rabbit and less so by the rat, monkey and man. Those sulphonamides containing a 2-methoxy substituent are less acetylated than those lacking one. 2,5-Dimethoxy-6-sulphanilamidopyrimidine is scarcely acetylated in any species but 4-methoxy- and 4,5-dimethoxy-6-sulphanilamidopyrimidine are highly acetylated. The latter compound is only slowly excreted by man; this effect is not observed in the other species.

8. Colorimetric, radiometric and chromatographic methods indicate that liver slices, but not liver suspensions, conjugate sulphadimethoxine, although at a slow rate. This is taken to indicate a requirement for organised cellular elements.

9. Kinetic studies on the action of β -glucuronidase on sulphadimethoxine $\underline{\text{N}}^1$ -glucuronide show that the substrate has only slight affinity for the enzyme.

10. The foregoing results are discussed and hypotheses proposed for the specificity of $\underline{\text{N}}^1$ -glucuronide formation, structural effects in in vivo acetylation and the long action of some sulphonamides.

To Elspeth

PREFACE

Sulphonamides are the drug of choice in the treatment of meningococcal meningitis and their use is to be preferred in infections of the respiratory and urinary tracts. Antibiotics, although potent, are better reserved for other acute infections to avoid both premature induction of bacterial resistance and exposure of the patient to side-effects often associated with these compounds.

Many new sulphonamides have been introduced in the last 10 years. Although their toxicological and clinical evaluation has been adequate, their ultimate metabolic fate has often remained obscure. The literature abounds with references to unspecified water-soluble conjugates. The work described in this thesis is an attempt to elucidate the metabolic routes taken by sulphadimethoxine and some of its close structural analogues.

I wish to thank Professor R. T. Williams for giving me the opportunity to work at St. Mary's and for his interest and advice deriving from past experience with sulphonamides. I also wish to thank the staff, Dr. D. V. Parke, Dr. J. H. Smith, Dr. R. L. Smith, Dr. P.J. Creaven, Mr. P. Milburn and particularly Dr. J. W. Bridges, also the research fellows and my fellow students for providing a stimulating atmosphere in which to work. I also thank Mr. Audas and his technical staff for their valuable assistance.

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INDEX

Abstract	2
Preface	6
Chapter 1. Introduction	10
Chapter 2. The structure of the glucuronide of sulphadimethoxine formed in man.	38
Chapter 3. The metabolism of sulphadimethoxine: species differences.	62
Chapter 4. The metabolism of sulphadimethoxine: effect of phenobarbitone pretreatment.	74
Chapter 5. The metabolism of the methoxy-6- sulphanilamidopyrimidines.	88
Chapter 6. The <u>in vitro</u> synthesis of sulphonamide <u>N</u> ¹ -glucuronides.	105
Chapter 7. The action of β -glucuronidase on sulphadimethoxine <u>N</u> ¹ -glucuronide.	115
Chapter 8. Discussion.	125
Appendix	143
Bibliography	145

'The time has come', the Walrus said,
'To talk of many things:
Of shoes - and ships - and sealing-wax -
Of cabbages - and kings -
And why the sea is boiling hot -
And whether pigs have wings.'

(Lewis Carroll)

CHAPTER 1

INTRODUCTION

HISTORY OF SULPHONAMIDES

Ehrlich's work on acridines and other antibacterial dyestuffs (Ehrlich and Hata, 1910; Ehrlich and Benda, 1913) indicated the potentialities of chemotherapy and ultimately led to the testing of the dye Prontosil. This azosulphanilamide had outstanding antibacterial properties in experimental animals (Domagk, 1935) but lacked any activity ^{in vitro}. Trefouel, Trefouel, Mittl and Bovet (1935) suggested that the azo link was reduced in vivo liberating sulphanilamide, which they had found to be active both in vivo and in vitro. In confirmation Fuller (1937) found sulphanilamide in the urine of patients treated with Prontosil.

Many derivatives of sulphanilamide have since been synthesised in the course of improving the original drug. It was soon found that substitution of the free amino group led to reduction or abolition of the antibacterial activity of sulphanilamide. All important derivatives of sulphanilamide have involved substitution of the amido group.

The marked activity of N¹-heterocyclic sulphanilamides against both gram-positive and gram-negative organisms combined with the ease of administration and low toxicity compared with the parent compound established them as chemotherapeutic agents. There quickly followed sulphapyridine (M and B 693) (Ewins and Phillips, 1938; Whitby, 1938), sulphathiazole and its derivatives (Fosbinder and Walter, 1939; Bergheim, Coy and Lott, 1940; Hartmann, 1940; Meier and Alleman 1941). These were superseded for most purposes by sulphadiazine and its derivatives

(Roblin, Williams, Winnek and English, 1940; Roblin, Winnek and English 1942). The favourable properties of sulphanilamide-pyrimidines, including high activity and low toxicity gave impetus to the synthesis of many related compounds, including sulphamerazine and sulphomethazine (Roblin, Winnek and English, 1942; Schmidt, Sesler and Hughes, 1944; Schmidt, Hughes, Badger and Schmidt, 1944).

The theory of Woods (1940) and Fildes (1940) on the mode of action, combined with the studies of Bell and Roblin (1942, 1943) on the most efficient heterocyclic substituents of sulphanilamide focussed attention on the sulphadiazines in general as the most useful sulphonamides. Northey (1948) stated with foresight that "further developments in the field of sulphonamide therapy would be confined to the synthesis of compounds which were better tolerated and had improved absorption, distribution and excretion characteristics".

Compounds with extended duration of action were found in the series of 60 described by Van Dyke, Tupikova, Chow and Walker (1945). The most promising compound, 4,6-dimethoxy-2-sulphanilamidopyrimidine, was also discovered independently by Gage, Martin, Rose, Spinks and Tuey (1947). However, when submitted to clinical trial it was found to be poorly absorbed from the intestine and to exhibit toxic effects due to the low solubility of the drug and its principal metabolites (Bevan and Luxton, 1947).

The following ten years showed a marked decline in interest in sulphonamides² due to the success of penicillin and other antibiotics. Revival began when it became clear that antibiotics had certain disadvantages, manifest as both primary toxicity and secondary effects. Induced bacterial resistance also proved to be a major problem. At this time research was directed into the investigation of sulphonamide combinations. The theoretical advantages were first pointed out by Lehr (1945); two, three or more components would each dissolve in urine regardless of the presence of the others, reducing toxicity due to the insolubility of the sulphonamide and its metabolite. However, in practice this was counterbalanced by the multiplication of other toxic reactions.

The continued use of sulphonamides has been due largely to the development of long-acting compounds, with their associated simple dosage regime. The first of these, and subsequently the most widely used, were sulphamethoxyypyridazine (Clark et al. 1958; Nichols, Jones and Finland, 1956) and sulphadimethoxine (Bretschneider and Kloetzer, 1955; Semenitz, 1954; Schnitzer, De Lorenzo, Grunberg and Russomanno, 1958). They have since been followed by many others.

²Throughout this work 'sulphonamide' indicates the antibacterial sulphanilamide derivatives. It does not include those compounds whose principal pharmacological effect is diuresis or hypoglycaemia.

The most recent development has been the discovery of an extra-long-acting sulphonamide, sulphorthodimethoxine. This has a half-life in man of 100-200 hr. (Madsen, 1963, 1964).

Although the newest sulphonamides include some that are highly active, safe and easy to administer, medical opinion (Anonymous, 1964) holds that their principal uses to-day are for meningococcal meningitis, for which they are the drug of choice, and some urinary, intestinal and respiratory tract infections.

ABSORPTION, DISTRIBUTION AND EXCRETION OF SULPHONAMIDES

Absorption

Absorption of sulphonamides after an oral dose has been followed in man and experimental animals by measurement of blood levels, almost since the inception of sulphonamide therapy. Since most sulphonamides are well absorbed when given by the oral route, many investigations of this kind have been performed. After reviewing the results reached with several modern sulphonamides, Bagdon (1964) concludes that comparisons of blood concentrations of various sulphonamides require a detailed consideration of the dose employed, the species and the specimens assayed. Extrapolating these data to other species may be of limited value.

Simple diffusion has been demonstrated to be the mode of absorption of sulphonamides from the perfused rat intestine, the rate for each compound being related to the lipid solubility (Nogami, Hanano and Yamada, 1963; Koizumi, Arita and Kakemi, 1964a, 1964b).

Distribution and tissue binding

Sulphonamides vary in their distribution within the body quite markedly, see Table 1.1. Volumes of distribution are less than the theoretical 65%, indicating that they are localised in some part of the body water. This^{is} usually the plasma with sulphonamides due to protein binding.

TABLE 1.1

Volume of distribution of four sulphonamides
after a single intravenous dose of 25 mg./kg. Data from
Litchfield (1960).

Sulphonamide	Volume of distribution (%)
Sulphadiazine	46
Sulphamethoxypyridazine	34
Sulphisoxazole	31
Sulphadimethoxine	20

Many sulphonamides are bound more or less reversibly to plasma protein, erythrocytes and other tissues. This has a profound effect on the behaviour of this class of compounds.

Plasma protein binding reduces the amount of sulphonamide diffusible into the tissues (Davis, 1943). Thus less sulphonamide is able to reach the site of infection.

Tissue binding, although concentrating sulphonamide in certain organs, may reduce the concentration available to fight a bacterial infection. Protein bound sulphonamide has been shown not to be antibacterially active

(Anton, 1960), suspected since Davis (1942, 1943) found that protein interfered with the determination of in vitro activities. However, Madsen, Oythus and Boe (1963) found that the antibacterial potency of serum of patients corresponded closely to the concentration of free sulphonamide regardless of the degree of protein binding.

Plasma protein binding may reduce the amount of sulphonamide reaching the metabolising enzyme sites (Newbould and Kilpatrick, 1960). It certainly reduces the amount of sulphonamide filterable at the renal glomeruli.

Binding to protein or other tissue may also prolong the duration of action of a compound by acting as a reservoir (e.g. Neipp, Sackmann and Tripod, 1961).

Finally, administration of sulphonamides to premature infants is contra-indicated due to the risk of kernicterus, a form of jaundice causing brain damage. The sulphonamide competes successfully for binding sites on plasma protein, liberating free bilirubin (Odell, 1959); it is not possible for the bilirubin to be rendered less toxic by conjugation due to the lack of glucuronyl transferase in the prematurely born. Davis (1943) found that sulphonamides were bound to plasma albumin but not globulin.

Other examples of competition for plasma protein binding sites have been given by Anton (1961). The action of sulphonamides in vivo and in vitro was potentiated by compounds such as sulphipyrazone; not all

compounds with a high affinity for protein displaced sulphonamides, demonstrating that binding is to specific sites. An example of the latter is suramin, which binds so tightly to proteins that it causes denaturation (Town, Wills, Wilson and Wormald, 1950).

Investigators of the physical chemistry of protein binding have in general assumed a reversible interaction of the enzyme-substrate type. However, Kawerau (1964) has shown that part of the plasma bound fraction of sulphadimethoxine is held very tightly, being resistant to exhaustive dialysis. He believes that in vivo the sulphonamide may not be released until degradation of the protein molecule occurs.

Excretion

Sulphonamides are known to be excreted in bile in addition to urine, some in high concentration; sulphadimethoxine (Fust, Boehni, Schnitzer, Rieder and Struller, 1960; Preziosi, Loscalzo, Marmo and Miele, 1959) and sulphaphenazole (Neipp, Padowetz, Sackmann and Tripod, 1958a, 1958b; Preziosi et al., 1959). Other sulphonamides were excreted to an extent of less than 1% of the dose. Neipp et al. (1961) found that after an intravenous dose of 100 mg./kg. 6.3% of sulphachloropyridazine, 3.3% of sulphaphenazole, 2.4% of sulphamethoxypyridazine and 1.3% of sulphisomidine were excreted in the bile in 24 hr. These differences are relevant to the use of sulphonamides in biliary tract infections and to their possible enterohepatic recirculation, which would have the effect of prolonging their duration of action. However, Preziosi, Loscalzo,

Marmo and Miele (1963) found that enterohepatic circulation was not a significant factor in the long action of sulphadimethoxine.

Secretion of organic ions into the urine can be attributed to one of two systems, the organic base mechanism (Peters, 1960) and the organic acid (hippurate) mechanism (Sperber, 1959). Many sulphonamides have been shown to be secreted by the tubules (Despopoulos and Callahan, 1962); the mechanism was shown to be related to the renal hippurate transport system. Sulphonamides with a pK_a greater than 8.4 were not transported, those with a pK_a less than 6.4 were.

In addition to glomerular filtration and tubular secretion, sulphonamides have been shown to reach the renal tubules by passive diffusion, the rate being related to the lipid solubility of the sulphonamide (Koizumi, Arita and Kakemi, 1964c).

METABOLISM OF SULPHONAMIDES

N^t-Acetylation

In common with many aromatic amines, sulphonamides are acetylated at the amino group in vivo. This was first detected soon after the inception of therapy with sulphanilamide and its successors (Marshall, Cutting and Emerson, 1937; Marshall and Litchfield, 1939). The reaction occurs in most mammalian species including man, but excluding the dog and fox. Sulphamerazine was reported to be acetylated at the N^t-position in the dog (Krebs, Sykes and Bartley, 1947). Marshall (1954) was unable to detect any N^t-acetyl derivatives on feeding dogs with either sulphanilamide or sulphamerazine. Bridges and Williams (1963a) were unable to find any N^t-acetyl sulphanilamide in the urine of the dog or fox by separation of the metabolites chromatographically. Dog liver extract contains a non-dialysable inhibitor of N^t-acetylation of sulphanilamide (Liebman and Anaclerio, 1961, 1962).

Sulphonamides produce a number of toxic effects (see Goodman and Gilman, 1955), of which the principal and characteristic one is renal damage due to deposition of crystals of the sulphonamide and its N^t-acetyl derivative. Interest in the metabolism of sulphonamides by acetylation stems from the decreased solubility of the metabolite in many cases potentiating the danger of renal toxicity (Table 1.2). As sulphonamide drugs and their N^t-acetyl derivatives are much more soluble at higher pH, reduction of side-effects involves ensuring the excretion of a large volume of alkaline urine. A high urinary pH assists the excretion of N^t-acetyl

compounds by enhancing their passive diffusion from the blood plasma to the renal tubules (Braun, Adlung and Malorney, 1964).

TABLE 1.2

Comparison of the solubilities of certain sulphonamides and their N^t-acetyl derivatives with the occurrence of kidney blockage and their LD50. Data from Williams (1959, pp. 510, 511).

Sulphonamide	Solubility at 37° (mg./100 ml. water)	LD50 for mice (g./kg.)		Kidney blockage
		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	0.9	+
Acetylsulphamethazine	115	-	1.3	
Sulphapyrazine	5	-	-	+++
Acetylsulphapyrazine	5	-	-	
Sulphathiazole	98	-	-	+++
Acetylsulphathiazole	7	-	-	

In a series of papers (Nelson, 1960, 1961; Nelson and O'Reilly, 1961) it was shown that the acetylation and excretion of sulphathiazole and other sulphonamides could be described as two competing first order reactions, one for the acetylation and the other for the excretion of the sulphonamide. It was suggested that the expression obtained could be used to calculate safe dose levels for sulphonamide drugs. However, recently introduced compounds are more soluble and administered at lower dose levels, rendering the expression of more theoretical interest. White (1958) had found no significant variation in the proportion of acetylated sulphadiazine excreted by the rabbit in the dose range 0.05 - 1.2 g.

The decreased solubility and increased toxicity of many N⁴-acetyl sulphonamides compared with the parent drug led some workers to attempt to decrease the level of acetylation in vivo by additions to the diet. The effect of acetate is not clear, some finding acetylation increased (e.g. James, 1940) and others decreased (e.g. Martin and Rennebaum, 1943). Sodium pyrophosphate (Nashkov^{and Nashkova}, 1960), barbituric acid (Cetrullo, 1958) and vitamin D (Panagopoulos, 1959) have all been reported to reduce acetylation.

Recently Anton and Boyle (1964) have shown in vitro the reduction of acetylation of sulphonamides in the presence of serum proteins and its reversal by sulphinpyrazone and suramin.

Acetylation is the major mode of metabolism of the antituberculous drug isoniazid (Hughes, Schmidt and Biehl, 1954). Evans, Manley, and McKusick (1960) suggested there was a genetically controlled variation in the level of the acetylating system in man. Rapid inactivators were thought to possess the dominant gene. Frymoyer (1962) and Frymoyer and Jacox (1963a) have put forward the hypothesis that the acetylation of both isoniazid and sulphadiazine in rabbits is under the control of a single dominant Mendelian characteristic in which fast acetylation was dominant to slow. In man (Evans, 1963) the acetylation of isoniazid and sulphadimidine is under the control of the same alleles. In a limited study of six subjects Peters, Gordon and Brown (1965) found that the acetylation of sulphamethazine closely parallels that of isoniazid but that the acetylation of sulphanilamide did not. The suggestion that there is a fundamental difference in the mechanism should be treated with extreme reservation since the results may reflect differences in the rate of absorption of the oral dose.

A sulphadiazine acetylase was found in high or intermediate concentrations in liver preparations from rapidly acetylating rabbits, but not in those from rabbits which slowly acetylated sulphadiazine in vitro (Frymoyer and Jacox, 1963b).

The large discrepancies in the rate constants of acetylation of sulphamethylthiadiazole between subjects from the United States and Japan were probably due to ethnic differences in the distribution of fast and slow acetylators (Naito and Nelson, 1963). However, the

possibility of this distribution of subjects by chance was not excluded.

In common with other drug metabolising enzymes, acetylation of aromatic amines including sulphonamides takes place largely in the liver (e.g. Klein and Harris, 1938). Van Winkle and Cutting (1940) demonstrated that the cat spleen also contains arylamine acetylating activity. Acetylation has also been demonstrated in the blood - the activity of rat reticulocytes was 40 - 60 times greater than reticulum-free cells (Matte^his and Pfefferko^rn, 1960). Bridges and Williams (1963a) found that N⁴- acetylation of sulphonamides in the rabbit, coypu and chicken occurs mainly in the liver with little, if any, in other tissues.

Little work has been performed on the acetylating activity of cell fractions. Bridges (1963) found acetylating enzymes in the mitochondrial and supernatant fractions. In this respect arylamine acetylase differs from most other drug metabolising enzyme systems, which are localised in the microsomes.

At least two amine acetylases have been postulated to account for the ability of the dog to acetylate glucosamine and histamine (Tabor and Mosettig, 1949) but not sulphonamides (Marshall, 1954). Enzymic acetylation of amines runs parallel to the electronic charge of the amino nitrogen, provided that the compound is not protonated under the experimental conditions (Perault and Pullman, 1963). Some sulphonamide examples are given in Table 1.3.

TABLE 1.3

Arylamines as acetyl acceptors. Data from Perault
and Pullman (1963)

Amine	Relative rate of acetylation		Electronic charge of N atom	Free valency of N atom
	(1)	(2)		
Aniline	0.79		1.851	1.023
Sulphanilamide	0.17	1.00	1.841	1.010
Sulphathiazole		0.30	1.841	1.011
Sulphadiazine		0.20	1.836	1.012

Other attempts had been made to correlate the basicity of an amine with its degree of acetylation in vivo (e.g. Zehender, 1943). Smith and Williams (1948a) found that those sulphonamides with a pK_a between 6 and 9 were the most highly acetylated (Table 1.4).

TABLE 1.4

Relationship between the dissociation constant and extent
of acetylation in vivo of certain p-substituted anilines

(Smith and Williams, 1948a)

Compound	pK_a	Excreted as acetyl derivative (%)
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

Enzymes performing the hydrolytic removal of acetyl groups from
amines are widely distributed in animal species. Deacetylation in the
dog has been observed with acetanilide (Brodie and Axelrod, 1948) and
the N⁴-acetyl derivatives of sulphanilamide, sulphapyridine, sulphathiazole

and sulphadiazine (Shaffer and Bieter, 1950). Man and the rabbit are far less efficient deacetylators of acetanilide (Brodie and Axelrod, 1948; Smith and Williams, 1948b). Deacetylases appear to be active in birds, e.g. the pigeon (Efimochkina, 1951), chick (Shaffer and Bieter, 1950) and hen (Bridges and Williams, 1963a). Bray, James, Thorpe and Wasdell (1950) have distinguished acetyl glycine deacetylase and acetanilide deacetylase. The former appears specific for acetylated aminoacids, the latter deacetylates aromatic compounds. The enzymes were found in both the liver and kidney, the relative amounts depending on the species. Dog liver was more efficient deacetylating aromatic acetamido compounds whereas rabbit liver was more active with aliphatic compounds. Bridges and Williams (1963a) found acetylsulphanilamide deacetylating activity in the kidney, strong in the hen and weak in the rabbit, but not in the liver.

Nimmo-Smith (1960) investigated the aromatic N-deacetylase of chick kidney mitochondria. It has a wide specificity. Bridges (1963) showed deacetylation of various substrates to be independent of the pK_a of the amine. The solubilisation and isolation of a highly active acetanilide hydrolysing enzyme from rabbit liver microsomes has been claimed (Hollunger and Niklasson, 1964).

The extent of excretion of acetylated amines has been suggested to be the resultant of acetylating and deacetylating activity (Krebs et al., 1947).

N¹-Acetylation

Acetylation of the sulphonamide nitrogen of sulphanilamide was reported by Boyer, Saviard and Dechauvassine (1956). It has not been observed with N¹-substituted sulphonamides. The correlation of electronic charge on the nitrogen atom with acetylation (Perault and Pullman, 1963) indicates that sulphanilamide (charge 1.736) should be scarcely acetylated at the N¹-position and N¹-heterocyclic substituted sulphonamides even less so.

Study of the metabolism of sulphanilamide in experimental animals (Bridges and Williams, 1963a) confirmed that N¹-acetylsulphanilamide (sulphacetamide) was a minor metabolite. The dog excreted about 9% of the dose in this form, the rabbit 1% and also 4% of N¹,N⁴-diacetylsulphanilamide; the guinea-pig excreted 9 and 7% respectively. In vitro, N¹-acetylation occurred in the liver of the rabbit and coypu but not the hen.

Weak N¹-deacetylating activity was detected in many hen tissues, e.g. liver, kidney, heart, brain. The intestinal bacteria Escherichia coli, Lactobacillus acidophilus and Streptococcus faecalis deacetylated N¹-acetylsulphisoxazole, the rate increasing with pH in the range 4-9 (Uno and Kono, 1961).

N⁴-Sulphonation

Aromatic sulphamates, analogous to sulphate esters, have been detected as metabolites of a number of aromatic amines in man, rat, rabbit and dog. β -Naphthylamine and aniline derivatives (Boyland,

Manson and Orr, 1957) are the best examples of this class of conjugate. Sulphonamide N^+ -sulphonates have been reported for sulphisoxazole (Uno and Kono, 1960), sulphathiazole (Uno and Ueda, 1960), sulphamethylthiadiazole (Uno and Okazaki, 1960), sulphadiazine (Uno, Yasuda and Sekine, 1963) and sulphasomizole (Bridges and Williams, 1963b). Since they are only minor metabolites and unstable, none of the sulphonamide N^+ -sulphonates has yet been isolated; identification has been based on chromatography and comparison with synthetic compounds.

Sulphamate formation has been demonstrated in mammalian liver and sheep intestinal mucosa preparations using α - and β -naphthylamines as substrates (Roy, 1958, 1960; Kent and Pasternak, 1958). A particular enzyme, arylamine sulphokinase, has been suggested to catalyse the reaction, although steroids appear to inhibit the reaction by 'partial competition' (Roy, 1961) or purely competitively (Roy, 1964).

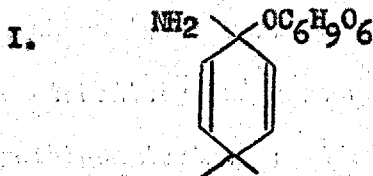
Formation of N^+ -glucuronides

The possible occurrence of carbohydrate N -conjugates was first recorded by Schoorl (1900) who observed that urea and glucose occurred in equimolecular proportions in the blood (0.006M). A modification of the theory stated that l-ureido-glucuronic acid was the transport form (Neuberg and Neimann, 1905).

An N -sulphonate or N -glucuronide was postulated as the water-soluble conjugate found in the urine after administering azobenzene to rats and which yielded benzidine on acidification (Elson and Warren, 1944). McChesney (1947) isolated a labile glucuronic acid containing metabolite

of 3',5'-dibromosulphanililide which has subsequently been suggested to be an N⁴-glucuronide (Williams, 1959, p.506).

Smith and Williams (1949a) proposed that the increased glucuronic acid output on feeding aniline was due to the conjugate I.



A similar conjugate was also suggested for phenetidine. Labile glucuronides were suggested for being responsible for the increased output of glucuronic acid on feeding benzonitrile and *o*-toluonitrile (Bray, Hybs and Thorpe, 1951). Pollack and Eades (1954) found chromatographic evidence for the excretion in normal urine of aspartic and glutamic acids as N-glucuronides.

The first N-glucuronide isolated was that formed from 4,4'-diaminodiphenylsulphone (Bushby and Wolwod, 1955, 1956). Apart from its characterisation by chromatography and ionophoresis, some evidence was found for the occurrence of the metabolite in blood.

The N-glucuronide derived from β -naphthylamine was reported by Boyland, and Manson (1955) and isolated from the urine of rabbits (Boyland, Manson and Orr, 1957). Infrared evidence indicated that the structure of the conjugate was that of an aryl glucuronylamine and not a Schiff's base. The N-glucopyranoside structure (probably β) of aniline N-glucuronide was confirmed by synthesis of methyl aniline-N-

(2,3,4-tri-O-acetyl-D-glucopyranosyl)uronate by two routes (Ishidate, Takitani and Kishi, 1959).

Table 1.5 collects the available data on the excretion of N⁺-glucuronides of sulphonamides. The values obtained vary greatly between different workers; this is entirely consistent with the non-enzymic nature of their formation (see below). Probably all sulphonamides give rise to some N⁺-glucuronide; in many cases it has not been searched for.

It is known that substitution of the free amino group of sulphonamides greatly reduces their bacteriostatic activity. This has been confirmed with sulphonamide N-glucuronides (Ogiya and Suzuki, 1961).

The enzymic synthesis of N-glucuronides has been a subject of debate. Axelrod and his associates claimed to have detected the formation of aniline glucuronide by guinea-pig microsomes (Axelrod, Insooe and Tomkins, 1957, 1958; Arias, 1961). The evidence for this was indirect and the method of estimation inadequate (for a critical discussion see Bridges, 1963). The use of more suitable substrates has failed to confirm these results, e.g. p-chloroaniline (Bridges and Williams, 1962) and sulphapyridine (Ogiya, Hashimoto, Kataoka and Monden, 1963).

It has long been known that N-glucuronides can be formed by direct reaction of an aromatic amine and a glucuronic acid derivative (Thierfelder, 1889). Takitani (1959) showed that an aqueous solution of equimolar amounts of amine and glucuronic acid approached an equilibrium position. Solutions of the N-glucuronides mutarotated, reaching the same equilibrium mixture. In general, the N-glucuronides were stable above pH 7.

TABLE 1.5

Excretion of sulphonamide N^4 -glucuronides

Sulphonamide	Species	% of the dose	References
Sulphanilamide	Rabbit	5-10	2
	Rabbit, dog, etc.	1-5	1
Sulphathiazole	Rabbit	9-20	2
	Man	0.8	3
Sulphapyridine	Rabbit	35-41	4
Sulphamethylthiadiazole	Man	0.3	5
Sulphisoxazole	Man	?	6
Sulphasemizole	Man, rat,		
	rabbit, dog	0.1-1.3	7
Sulphacetamide	Rat (blood)	0.1	8
Sulphadiazine	Rabbit	7-13	2

References:

- | | |
|----------------------------------|---------------------------------|
| 1. Bridges and Williams (1963a). | 5. Uno and Okazaki (1960). |
| 2. Ogiya (1960). | 6. Uno and Kono (1960). |
| 3. Uno and Ueda (1962) | 7. Bridges and Williams (1963b) |
| 4. Ogiya (1959). | 8. Bridges and Williams (1962) |

The spontaneous formation of a number of N-glucuronides, including sulphanilamide, appeared to have an optimum pH approximately one pH unit above the pK_a of the amine (Bridges and Williams, 1962). No appreciable synthesis occurred with glucuronolactone. Below pH 7 synthesis readily took place, but below pH 2-3 the glucuronides also hydrolysed rapidly.

Summarising, the evidence for the non-enzymic formation of N-glucuronides is sufficient to account for the appearance of these conjugates as metabolites. The evidence for their enzymic synthesis is extremely tenuous.

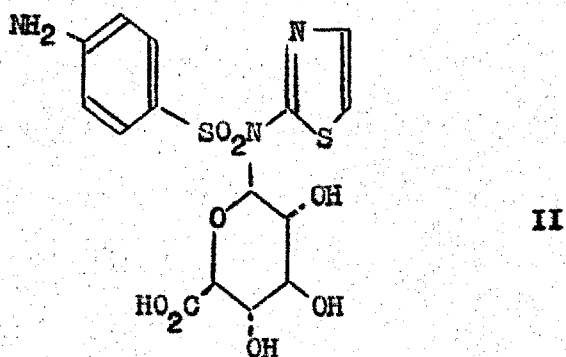
Since N-glucuronides rapidly reach equilibrium under hydrolytic conditions, the action of enzymes such as β -glucuronidase is difficult to detect. No evidence for the enzymic hydrolysis has been found with aniline glucuronide (Axelrod et al., 1958) and sulphanilamide N⁴-glucuronide (Bridges and Williams, 1962).

Formation of other N-glucuronides

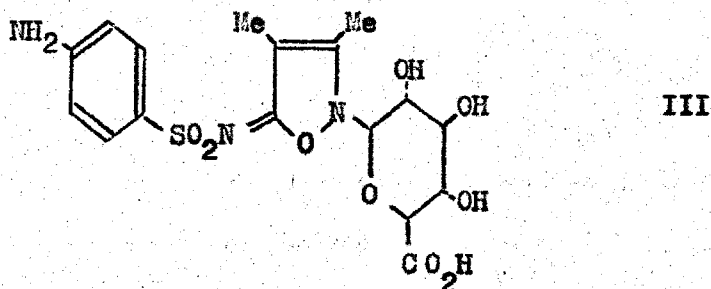
Recently Uno and his co-workers have isolated glucuronide metabolites of certain sulphonamides in which the glucuronic acid residue is not attached to the N¹-position, but which on hydrolysis yield only the original sulphonamide and glucuronic acid. These workers have concluded that they are glucuronides of the sulphonamide group or its tautomers.

Man was reported to form a minor metabolite of sulphathiazole containing glucuronic acid (Uno and Ueda, 1960), amounting to 3.8% of the dose (Uno and Ueda, 1962). On the basis of its infra-red spectrum (Uno, Machida, Hanai and Ueda, 1963) and by comparison of its ultra-violet

spectrum with the spectra of analogous compounds it was ascribed the structure of an N^1 -glucuronide (II). Sulphathiazole N^1 -glucuronide was not found to be a metabolite of sulphathiazole in the rabbit, nor was it found in rabbit liver perfusates (Uno and Ueda, 1963b).



Sulphisoxazole was reported to form a glucuronide in man (Uno and Kono, 1960) which proved to be dissimilar to synthetic sulphisoxazole N^1 -glucuronide (Uno and Kono, 1962). On this basis the compound was thought to be sulphisoxazole 2'-glucuronide (III).



The only evidence for this structure is from the ultra-violet spectrum, and should be treated with reservation especially since the same group of workers report that the close analogue of sulphisoxazole, sulphisomezole,

forms an N^1 -glucuronide. (Ueda and Kuribayashi, 1964).

Hydroxylation

There are many references in the literature to the excretion of oxidation products of sulphonamides, but in very few cases have they been isolated and characterised. Evidence for their formation often depends on the measurement of increases in the excretion of glucuronic acid and ethereal sulphate. This is unreliable since there are daily variations of these quantities in animals maintained on a regular diet, and even if 'equilibration' is ensured, stress caused by administration of the compound may upset this equilibrium. Also the compound may induce the excretion of other glucuronides or sulphates. With the use of sulphonamide drugs which are more easily extractable into organic solvents, the method of determining the non-extractable part of an excreted dose has come into being. There are many water-soluble metabolites which will be classed as oxidation products by those unfamiliar with the complexities of sulphonamide metabolism (see Table 1.4).

3-Hydroxysulphanilamide is found as a metabolite of sulphanilamide in both the rabbit and man as the sulphate conjugate (Shelswell and Williams, 1940; Williams, 1946, 1947).

Sulphapyridine is extensively oxidised and conjugated with glucuronic acid in the dog (20% of the excreted dose). Both the aglycone (5'-hydroxysulphapyridine) and the conjugate have been isolated (Weber, Lalich and Major, 1943; Scudi, 1944; Scudi and Childress, 1956).

Administration of sulphadiazine to the rabbit was reported to increase the excretion of glucuronic acid (Bray, Lake and Thorpe, 1951). However, Uno, Yasuda and Sekine (1963) failed to detect any glucuronides chromatographically in human urine.

Other sulphonamides thought to be hydroxylated include sulpha-quinoxaline (Soudi and Silber, 1944), sulphapyrazine (Bray et al., 1951), sulphasomizole (Bridges and Williams, 1963), sulphamethomidine (DiCarlo, Malament and Phillips, 1963), sulphadimethoxine (Koechlin, Kern and Engelberg, 1959) and sulphamethoxypyrazine (Bertalozzi, Chieli and Ciceri, 1962).

Smith and Williams (1947) studied the oxidation and acetylation of sulphonamides in the rabbit. With some sulphonamides, e.g. sulphamerazine and sulphathiazole, oxidation occurred in the free drug but not the acetylated form, whereas with sulphapyridine both forms were oxidised. Although the product was not isolated, it was concluded that oxidation occurred in the pyrimidine ring of sulphamethazine.

Examples of the data obtained with the newer sulphonamides are shown in Table 1.6.

The most interesting of the new compounds appeared to be sulphadimethoxine, a widely used efficient and safe long-acting sulphonamide. Koechlin et al. (1959) reported that it was excreted by man to the extent of 80% as a glucuronide. The glucuronide was not isolated in a pure state but it was shown not to be a conjugate of sulphadimethoxine hydroxylated in the benzene ring.

The large proportion of glucuronide made it likely that a unique type of metabolite was formed. Having found the nature of this metabolite, we investigated the structural specificity of sulphonamides for this pathway by observing the metabolic fate of related methoxy-6-sulphanilamidopyrimidines. This work is reported in the following chapters.

TABLE 1.6

Urinary metabolites of some newer sulphonamides in man.

Data expressed as proportion of the excreted dose (Walter, 1963).

Sulphonamide	Acetylated* (%)	*Free* (%)	*Oxidation product* (%)
Sulphadiazine	20-30	60-70	10
Sulphadimethyloxazole	45-70	30-40	20
Sulphaphenazole	15-30	10	70
Sulphamethoxydiazine	20-30	40-50	30
Sulphamethoxypyridazine	35-60	15-30	14-20
Sulphadimethoxine	8-12	10-25	80-85
Sulphamethyldiazine	30-40	30-40	25

CHAPTER 2

THE STRUCTURE OF THE GLUCURONIDE OF SULPHADIMETHOXINE FORMED IN MAN

Sulphadimethoxine (2,4-dimethoxy-6-sulphanilamidopyrimidine; Madribon) was synthesised by Bretschneider & Kloetzer (1955) and its antibacterial properties reported by Semenitz (1954) and Schnitzer, Delorenzo & Grunberg (1959). It has the advantage that its major metabolite in man is a relatively water-soluble glucuronide, whereas many other sulphonamides tend to form relatively insoluble acetyl derivatives which could produce renal damage. Previous attempts to isolate and characterize this glucuronide have been made (e.g. Koechlin, Kern & Engelberg, 1959) but only a crude product was obtained which contained sulphonamide and glucuronic acid in equimolecular amounts. This product was not hydrolysed by β -glucuronidase but with acid it gave sulphanilic acid.

EXPERIMENTAL

Materials and synthesis of reference compounds

Sulphadimethoxine m.p. 200-201⁰, was donated by Roche Products Ltd., Welwyn Garden City, Herts., 4-methoxy-6-sulphanilamidopyrimidine m.p. 201-203⁰ was donated by Imperial Chemical Industries (Pharmaceutical Division), Alderley Park, Cheshire, and N⁴-acetylsulphanilyl chloride m.p. 149⁰ by May and Baker Ltd., Dagenham, Essex., 4,6-dihydroxypyrimidine m.p. 230⁰, was purchased from Koch-Light Ltd., Colnbrook, Bucks.

Synthesis of sulphadimethoxine N¹-glucuronide. The method of Okumura (1962) was modified. Sodium sulphadimethoxine (6.0 g.) and acetobromoglucuronic acid methyl ester (7.7 g.; m.p. 106-109⁰; Bollenback, Long, Benjamin and Lindquist, 1955) were dissolved in a mixture of water (12 ml.)

and acetone (50 ml.) and the solution kept at 37° for 4 hr. The dark red solution was kept at 0° overnight and then treated with isopropanol (25 ml.) followed by water (200 ml.) whereby the triacetyl methyl ester of sulphadimethoxine-N¹-glucuronide was precipitated and then filtered and dried at the pump to give a buff coloured powder (6.5 g.), m.p. 110-120°. The crude ester in acetone (5 ml.) was passed through a column (2.5 x 25 cm.) of activated alumina (type H 100-200 mesh, P. Spence Ltd., Widnes, Lancs.) and eluted with acetone (300 ml.). The first fraction (100 ml.) was collected and evaporated to dryness. The product was recrystallized from isopropanol to give white platelets of the pure methyl [1-N¹-(2",4"-dimethoxypyrimidin-6"-yl)sulphanil-amido-2,3,4-tri-O-acetyl-1-deoxyglucosid]uronate, m.p. 154-155°, $\left[\alpha\right]_D^{20} + 87^\circ$ (c = 1 in methanol) (Found: C, 47.4; H, 5.2; N, 8.6; C₂₅H₃₀N₄O₁₃S requires C, 47.9; H, 4.8; N, 8.9%).

The above ester (1.84 g.) in methanol (50 ml.) was treated with 0.5N-barium methoxide in methanol (5 ml.). After keeping for 3 days at 0, 2N-H₂SO₄ (1.25 ml.) and water (25 ml.) were added. The BaSO₄ which had separated was removed and the solution passed through a column (2.5 x 10 cm.) of the H⁺ form of Amberlite CG-120 (100-200 mesh; British Drug Houses Lt., Poole, Dorset). The column was washed with water (10 ml.) and eluted with 2N-ammonia solution (150 ml.) followed by water (100 ml.). The combined eluates were evaporated to dryness in vacuo at 45° to give a solid (1.1 g.) which was then dissolved in methanol (10 ml.). Addition of isopropanol to the solution

precipitated the ammonium salt of the glucuronide which was recrystallized from methanol. Ammonium $\left[1-N^{1'}-(2'',4''\text{-dimethoxypyrimidin-6''-yl})\text{sulphanilamido-1-deoxyglucosid}\right]$ uronate (0.7 g.) was obtained as clusters of small prisms, m.p. 150-160° (decomp.) and $\left[\alpha\right]_D^{23} + 4.5$ (c = 5 in water) (Found: C, 40.4; H, 5.2; N, 12.9; S, 5.8; loss at 2 mm. at room temp., 4.4. $C_{18}H_{25}N_2O_5S \cdot 1.5 H_2O$ requires C, 40.7; H, 5.3; N, 13.2; S, 6.0; H_2O , 5.1%). This compound did not reduce Benedict's or Fehling's solution.

The salt (90 mg.) was dissolved with warming in a saturated aqueous solution of S-benzylthiuronium chloride (5 ml.). The solution was filtered (charcoal) and allowed to crystallize. S-Benzylthiuronium $\left[1-N^{1'}-(2'',4''\text{-dimethoxypyrimidin-6''-yl})\text{sulphanilamido-1-deoxyglucosid}\right]$ uronate (42 mg.) had m.p. 178-179° and $\left[\alpha\right]_D^{19} -13$ (c = 1 in water) (Found: C, 46.5; H, 4.8; N, 12.3; S, 10.3; loss at 110°, 2.8%. $C_{26}H_{32}N_2O_5S \cdot H_2O$ requires C, 46.6; H, 5.1; N, 12.5; S, 9.6; H_2O , 2.7%). This salt was also non-reducing.

Attempts to prepare sulphadimethoxine N¹-glucuronide by fusing sodium sulphadimethoxine with glucuronic acid or by heating concentrated aqueous solutions of glucuronic acid and sulphadimethoxine with sulphuric acid according to the method of Tsukamoto, Yoshimura and Tatsumi (1963b) for the preparation of meprobamate N-glucuronide, were unsuccessful.

Methylation of sulphadimethoxine. The drug (2.5 g.) was mixed with ether (2 ml.) and an excess of diazomethane in ether was distilled into the stirred mixture (cf. Shepherd, Bratton and Blanchard (1942)

on the methylation of sulphapyridine). After keeping at room temperature overnight, the mixture was warmed to remove excess diazomethane and filtered. The solid residue was now extracted with ether containing 3% ethanol (2 x 100 ml.) and the extracts evaporated to dryness in vacuo. The white crystalline residue was dissolved in methanol and on keeping N¹-methylsulphadimethoxine (0.95 g.) crystallized (m.p. 150-152°). The residue from the extraction was now shaken with 10% NaOH to remove unchanged drug and the residue from this was heated with 6N-acetic acid (50 ml.) for 5 min., filtered hot and the residue retained. The filtrate on cooling yielded a mixture of methylated sulphadimethoxines from which N¹-methylsulphadimethoxine was removed by extraction with hot methanol (0.25 g. m.p. 148-151°). The two crops of N¹-methylsulphadimethoxine were combined and recrystallized from methanol. 2,4-Dimethoxy-6-(N^{1'}-methylsulphanilamido)pyrimidine formed white needles, m.p. 151-152° (Found: C, 48.5; H, 5.0; N, 17.3; S, 10.3. $C_{13}H_{16}N_4O_4S$ requires C, 48.1; H, 4.9; N, 17.3; S, 9.9%).

The residue from the acetic acid extraction and that from the second crop of N¹-methylsulphadimethoxine above were combined and washed with hot methanol to remove any N¹-methyl isomer still present. This residue (0.5 g.) was now recrystallized from 6N-acetic acid and there was obtained 0.46 g. of small white plates, m.p. 169-173° (Found: C, 47.8; H, 4.8; N, 17.1; S, 9.9. $C_{13}H_{16}N_4O_4S$ requires C, 48.1; H, 4.9; N, 17.3; S, 9.9%), which appears, from infrared spectra (see below) to be a mixture of the two ring N-methylsulpha-

dimethoxines i.e., 2,4-dimethoxy-1- and -3-methyl-6-sulphanilamidopyrimidine.

Attempts to separate these were not successful.

N¹-Acetyl-N¹-methylsulphadimethoxine. (1) N¹-Methylsulphadimethoxine (0.5 g.) was dissolved with warming in an equal mixture of acetic anhydride and pyridine. The solution was kept at room temperature for 4 hr. and then diluted with 50% aqueous ethanol (50 ml.) with warming to dissolve any separated material. On keeping 2,4-dimethoxy-6-(N¹-acetyl-N¹-methylsulphanilamido)pyrimidine separated and was recrystallized from methanol. It formed small white plates (0.28 g.) m.p. 142-144° (Found: C, 49.1; H, 4.9; N, 15.2; S, 8.8. $C_{15}H_{18}N_4O_5S$ requires C, 49.2; H, 4.9; N, 15.3; S, 8.7%).

(2) 2,4-Dichloro-6-methylaminopyrimidine, m.p. 184-186°, was synthesized from barbituric acid via 2,4,6-trichloropyrimidine according to Winkelman (1927) and Baddily and Topham (1944). It (0.5 g.) was dissolved in hot methanol (10 ml.) and added to sodium methoxide in absolute methanol (0.24 g. Na in 5 ml. MeOH). The mixture was heated in a sealed tube for 5 hr. at 120°. The methanol was evaporated and the residue recrystallized from water to give 2,4-dimethoxy-6-methylaminopyrimidine (0.22 g.) m.p. 134-136° (Found: C, 49.5; H, 6.1; N, 25.1. $C_7H_{11}N_3O_2$ requires C, 49.7; H, 6.5; N, 24.9%). Bredereck, Kupsch and Wieland (1959) prepared it by another route and gave m.p. 135-136°. 2,4-Dimethoxy-6-methylaminopyrimidine (0.1 g.) and N-acetylsulphanilyl chloride (0.2 g.) were dissolved in pyridine (0.5 ml.) and the solution kept at room temperature for 24 hr. Water (5 ml.) was added and the

solution warmed to dissolve the precipitate which had formed. On standing N⁴-acetyl-N¹-methylsulphadimethoxine separated and was recrystallized from methanol, m.p. 142-144° (yield, 47 mg.) (Found: C, 49.1; H, 5.0; N, 15.2; S, 8.9%). This sample was identical in all respects (mixed m.p. and infrared spectrum) with the sample obtained by acetylation of N¹-methylsulphadimethoxine.

Methylation of 4-methoxy-6-sulphanilamidopyrimidine. This compound was methylated as described above for sulphadimethoxine. 4-Methoxy-6-(N¹-methylsulphanilamido)pyrimidine gave white needles from methanol, m.p. 152° (Found: C, 48.9; H, 4.9; N, 19.0; S, 11.0. $C_{12}H_{14}N_4O_2S$ requires C, 49.0; H, 4.8; N, 19.0; S, 10.9%). Ring-N-methyl-4-methoxy-6-sulphanilamidopyrimidine was recrystallized from 6N-acetic acid to give white platelets, m.p. 179-180°, of 4-methoxy-1- or -3-methyl-6-sulphanilamidopyrimidine (Found: C, 49.0; H, 5.0; N, 18.9; S, 11.0. $C_{12}H_{14}N_4O_2S$ requires C, 49.0; H, 4.8; N, 19.0; S, 10.9%). The material from its infrared spectrum and m.p. appeared to contain only one isomer but the position of the methyl group was not determined.

4-Methoxy-6-(N⁴-acetyl-N¹-methylsulphanilamido)pyrimidine.

(1) N¹-Methyl-4-methoxy-6-sulphanilamidopyrimidine (1.0 g.) was dissolved in an equal mixture of acetic anhydride and pyridine and the solution kept at room temperature overnight. Treatment with ice-water gave crystals which were recrystallized from 40% aqueous methanol to give white needles (0.68 g.) of 4-methoxy-6-(N⁴-acetyl-N¹-methylsulphanilamido)pyrimidine

m.p. 178-179° (Found: C, 49.7; H, 4.8; N, 16.5; S, 9.6. $C_{14}H_{16}N_4O_4S$ requires C, 50.0; H, 4.8; S, 9.5%).

(2) 4-Methoxy-6-methylaminopyrimidine, m.p. 82-83°, was synthesised from 4,6-dihydroxypyrimidine via 4,6-dichloropyrimidine (m.p. 64-65°) and 6-methylamino-4-chloropyrimidine (m.p. 137-138°) according to Hull (1951) and Brown (1955). It (1.5 g.) was dissolved in pyridine (6 ml.) together with N-acetylsulphanilyl chloride (3 g.) and kept overnight at room temperature. On pouring into ice-water crystals formed which on recrystallization from 40% aqueous methanol gave white needles m.p. 178-179° (yield 1.2 g.) (Found: C, 50.0; H, 5.1; N, 16.5; S, 9.7%), identical in all respects with the sample obtained by acetylation of N¹-methyl-4-methoxy-6-sulphanilamidopyrimidine. N⁴-Acetyl-ring-N-methyl-4-methoxy-6-sulphanilamidopyrimidine was obtained by treatment of ring-N-methyl-4-methoxy-6-sulphanilamidopyrimidine (0.2 g.) with acetic anhydride-pyridine mixture. The compound was recrystallized from 6N-acetic acid to give 4-methoxy-1- or -3-methyl -6-(N⁴-acetylsulphanilamido)-pyrimidine m.p. 235-236° (Found: C, 49.8; H, 4.9; N, 16.5; S, 9.7. $C_{14}H_{16}N_4O_4S$ requires C, 50.0; H, 4.8; N, 16.7; S, 9.5%). This compound was shown to be different from the N⁴-acetyl-N¹-methyl isomer by infrared, mixed m.p. and chromatography.

Sulphadimethoxine-N⁴-glucuronide. Sulphadimethoxine (6.3 g.) dissolved in dimethylformamide (20 ml.) was mixed with a solution of sodium glucuronate (2.4 g.) in ethylene glycol (30 ml.). Glacial acetic acid

(0.5 ml.) was added to bring the pH to 3-4 and the solution was kept at 70° for 10 min. On keeping overnight at 0°, crystals (3.9 g.) separated which were filtered and washed with dimethylformamide, then acetone and finally ether. Attempts to purify the compound were made by dissolving it in 0.02 N-ammonia (10 ml.) and adding acetone (25 ml.). On keeping at 0° overnight, crystals separated and these were filtered and washed with acetone (yield, 2.1 g.; m.p. 149° decomp.). The compound which was expected to be sodium 1-p-(2',4'-dimethoxypyrimidin-6'-yl-sulphamoyl)-anilino-1-deoxyglucosiduronate (sulphadimethoxine-N⁴-glucuronide Na salt) was very labile and was never obtained free of uncombined sulphadimethoxine. However, it was used as such for chromatographic purposes (see Table 1) and on paper it could easily be distinguished from sulphadimethoxine N⁴-glucuronide. It reduced Benedict's and Fehling's solutions readily on warming and the ratio of sulphadimethoxine to glucuronic acid in the compound was 1.03.

METHODS

Chromatography

The R_F values of sulphadimethoxine and other relevant compounds are shown in Table 2.1. The following tests were used:

p-Dimethylaminocinnamaldehyde test (DMAC). DMAC was dissolved in 2N-HCl (0.2 g./100 ml.) and diluted with ethanol (4 vol.). On spraying, aromatic amines showed up immediately as red-purple spots. Weak N-conjugates such as the N⁴-glucuronide did not react immediately but a

purple spot developed within 1-5 min. N^t-Acetyl conjugates, barbituric acid and N-methylbarbituric acid showed up as purple spots on keeping the chromatogram overnight.

Naphthoresorcinol test (NR). The paper was sprayed with 1% (w/v) naphthoresorcinol in acetone to which 10% phosphoric acid (4:1 v/v) was added just before use. On heating at 140°, the N^t-glucuronide showed up as a bright blue spot. Both the synthetic N¹-glucuronide and the biosynthetic glucuronide isolated from human urine gave very weak blue-brown spots.

Ferric chloride test. The phenols gave red, brown or green colours, when sprayed with 0.1% (w/v) aqueous ferric chloride solution.

Diazotised sulphanilic acid (DSA). (Elliott, 1959). Phenols gave red-pink spots with this reagent.

Gibbs reagent. (see Elliott, 1959). Certain phenols gave red-blue spots with this reagent.

Ultra violet 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 and sulphanilamide showed a weak purple fluorescence while others such as sulphadimethoxine quenched the background fluorescence of the paper and appeared as dark spots.

Infrared and ultra violet spectra. These were determined with the Perkin Elmer infracord spectrophotometer and the Unicam spectrophotometer SP 500, respectively.

QUANTITATIVE ESTIMATIONS

Free and total aromatic amines. Free amine was determined using the method of Bratton and Marshall (1939) directly. Total aromatic amine was estimated after hydrolysis of the sample with an equal volume of 2N-HCl in a water bath for 20 min. (Recoveries were between 99.5-100%).

Glucuronides. These were determined by the method of Paul (1951).

ISOLATION OF SULPHADIMETHOXINE GLUCURONIDE FROM HUMAN URINE

Two male volunteers each took 2 g. of sulphadimethoxine orally and their urine (3 l.) was collected for 24 hr. This was evaporated in 500 ml. batches under reduced pressure at 50° to a syrup (75 ml.). An equal volume of ethanol containing 5% of 2N-ammonia solution was added and the precipitated inorganic material was filtered off and discarded. A strongly basic anion exchange resin column 2 x 35 cm. ('De-Acidite' FF 100-200 mesh, 2-3% cross-linked - British Drug Houses Ltd.) was loaded with the filtrate, washed with water (500 ml.) and the washings discarded. It was then eluted with a solution of sulphur dioxide (prepared by bubbling the gas through water for 4 hr.) and the first 350 ml. fraction retained after the first appearance of the sulphite in the elute. The fraction was degassed under reduced pressure, and passed through a strongly acidic cation exchange column 2.5 x 25 cm. ('Amberlite' IR-120, -16 + 25 mesh H⁺ form, British Drug Houses Ltd.). After washing with water (250 ml.) it was eluted with 2N-ammonia solution and the first 300 ml. fraction retained after the first appearance of ammonia in the elute. The fraction was evaporated to dryness under reduced pressure at 45° to give 120 mg. of crude ammonium sulphadimethoxine glucuronide. This material was about

40% pure as shown by analysis, the impurities being mainly amino acids. The salt (60 mg.) in methanol (2 ml.) was banded on a thin-layer plate (30 x 30 cm.) of cellulose (MN - cellulose powder 300G Camblab Ltd., Cambridge) and developed in butan-1-ol-acetic acid-water (4:1:2 by vol.) against a reference spot of the original urine. The band corresponding to the glucuronide (identified by examining the plate under ultra violet light and also by taking a print from the wet plate onto Whatman No.4 paper and spraying with naphtharesorcinol and p-dimethylaminocinnamaldehyde) was scraped from the plate, suspended in water (10 ml.) and centrifuged at 2000 g. for 10 min. The supernatant was evaporated to dryness under reduced pressure to give the NH_4 salt of sulphadimethoxine glucuronide (20 mg.) which was 90% pure. This material (5 mg.) was recrystallized from methanol and 3 gm. of the salt (m.p. $150-160^\circ$) was obtained which was 99% pure. Further attempts to purify the salt resulted in decomposition. The 90% pure material was therefore dissolved in water (2 ml.) and treated with solid S-benzylthiouronium chloride (50 mg.). On standing yellow prisms of the S-benzylthiouronium salt of the glucuronide appeared (13 mg.; m.p. 164° decomp.). On recrystallization from water (charcoal) the m.p. was raised to $179-180^\circ$ (decomp.) (Found: C, 46.5; H, 4.8; N, 12.3; $\text{C}_{26}\text{H}_{32}\text{N}_6\text{O}_{10}\text{S}_2 \cdot \text{H}_2\text{O}$ requires C, 46.6; H, 5.1; N, 12.5%). The glucuronide did not reduce Benedict's solution on warming.

The ammonium salt of the glucuronide (0.46 mg. 99% pure) was hydrolysed with 2N-hydrochloric acid (1 ml.) at 100° for 20 min., and glucuronic acid and aromatic amine in the hydrolysate determined. It was found to contain 35% glucuronic acid and 56% sulphadimethoxine (estimated as sulphanilic

acid), which gives a molar ratio of glucuronic acid to sulphadimethoxine of 1.0.

Hydrolysis of sulphadimethoxine glucuronide

1. With β -glucuronidase. The NH_4 salt of sulphadimethoxine glucuronide isolated from human urine was dissolved in water (0.33 mg./ml.). The sample used was 90% pure and was free from sulphadimethoxine as indicated by paper chromatography. Three test-tubes were set up each containing 0.3 ml. of the glucuronide solution and 0.1 ml. of 0.1 M -sodium acetate buffer, the pH of the mixture being 4.2. To one tube there were added 15,000 Fishman units of mollusc β -glucuronidase solution (0.1 ml.) (Koch-Light Ltd.), to the second the same amount of boiled enzyme, and to the third 0.1 ml. water. The tubes were plugged and incubated for 24 hr. at 38° . The contents of each tube were then chromatographed in solvents A, B and C. The experiment was repeated with the ammonium salt of synthetic sulphadimethoxine N^1 -glucuronide. The first tube in both cases gave spots corresponding to glucuronic acid, sulphadimethoxine, and unchanged sulphadimethoxine N^1 -glucuronide. The control tubes gave, in each case, only one spot corresponding to the unchanged glucuronide.

A sample (50 mg.) of the 40% pure ammonium salt of sulphadimethoxine glucuronide isolated from human urine was dissolved in 0.2 M -sodium acetate buffer pH 4.3 (10 ml.). This sample of glucuronide was free of sulphadimethoxine. The solution was treated with 30,000 Fishman units of β -glucuronidase and then incubated at 38° for 96 hr. The mixture was extracted with ethyl acetate and the extract was chromatographed as a band on Whatman No.1 paper using the top layer of the solvent mixture, butan-1-

ol-ammonia solution (s.g. 0.88)-water (4:1:5 by vol.). The band R_f 0.4 was eluted with ethyl acetate. On evaporation, the eluate yielded pale yellow crystals (1 mg.) which were identified as sulphadimethoxine (m.p. 200° and mixed m.p. 201°) by infrared spectrum, chromatography in solvents A, B and C, and its electrophoresis properties at pH 1 ($0.1N-HCl$) and 9 ($0.1M-NaHCO_3-NaOH$).

2. With acid and alkali. Aqueous solutions (0.02% w/v) of the biosynthetic or synthetic sulphadimethoxine N^1 -glucuronide were hydrolysed by heating at 100° for 1 hr. with an equal volume of $2N-HCl$ or $2.5N-NaOH$. The hydrolysates were chromatographed in solvents A and C (Table 21). Aqueous suspensions (0.5% w/v) of sulphadimethoxine, N^4 -acetylsulphadimethoxines were similarly hydrolysed and chromatographed. The results are shown in Table 22. The products of the hydrolyses were identified by R_f values and colour reactions.

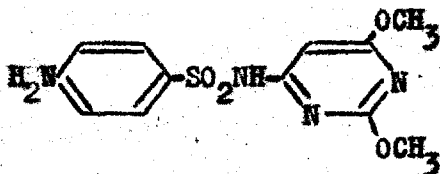
Hydrolysis of sulphadimethoxine. The drug (0.25 g.) in $N-HCl$ (10 ml.) was heated on a boiling water bath for 1 hr. A 2 ml. portion of hydrolysate was put on an alumina column (2 x 20 cm.; Whatman standard grade powder) and eluted with solvent A. The eluate was collected in 50 ml. portions and the fifth eluate was evaporated to yield 5 mg. of sulphanilamide which on recrystallization from water had m.p. $163-164^{\circ}$ and mixed m.p. $162-164^{\circ}$. Its identity was checked by its infrared spectrum. The rest of the hydrolysate (8 ml.) on keeping overnight yielded 30 mg. of colourless crystals which were identified as barbituric acid (m.p. $247-249^{\circ}$, mixed m.p. $246-248^{\circ}$).

Stability of sulphadimethoxine N¹-glucuronide at various pH values.

The NH₄ salt of the synthetic glucuronide (5 mg.) was dissolved in 1 ml. of N-HCl and various buffers of pH 3, 5, 7 and 9. The solutions were shaken at 37° for various intervals of time up to 24 hr. Samples at intervals of 1, 3, 5 and 24 hr. were chromatographed on Whatman No. 1 paper in solvent A and the spots corresponding to the N¹-glucuronide and sulphanilic acid were eluted with water and analysed by the Bratton and Marshall method. The glucuronide was found to be stable at pH 3, 5, 7 and 9 but at pH 0 it slowly hydrolysed and in 24 hr. 92% had disappeared. About 73% was converted to sulphanilic acid.

DISCUSSION

The major urinary metabolite of sulphadimethoxine in man is a glucuronide which has been isolated and characterized. A perusal of the formula of sulphadimethoxine (I) shows that several structures could be postulated for this glucuronide. If it were an O-glucuronide, it



I

could be derived from a sulphadimethoxine hydroxylated in the benzene ring or position 5 of the pyrimidine ring, or demethylated at one of the methoxyl groups in the pyrimidine ring. These types of structures can be eliminated because hydrolysis of the glucuronide with β -glucuronidase yields sulphadimethoxine and with acid sulphanilic acid and some sulphanil-

TABLE 2.1

Chromatography and colour reactions of sulphadimethoxine and related compounds

Descending chromatography on Whatman No.4 paper was used. The solvent systems employed were: A, butan-1-ol-ammonia solution (s.g. 0.880)-water (10:1:1 by vol.); B, 80% aqueous ethanol; C, butan-1-ol-acetic acid-water, (4:1:2 by vol.) Chromatograms were run for 6 hr. in solvent A and C and 4 hr. in solvent B.

Compound	R _F values in solvent			Fluorescence in u.v. light (254 mμ)	Colour reactions with		
	A	B	C		DSA ^a	DMAC ^a	NR ^a
Sulphadimethoxine	0.40	0.95	0.95	q*	none	purple	none
N ⁴ -Acetylsulphadimethoxine	0.65	0.95	0.95	q	none	none ^c	none
Sulphadimethoxine N ⁴ -glucuronide Na salt	0.00	0.25	- ^b	q	none	purple slowly	blue
Sulphadimethoxine N ¹ -glucuronide NH ₄ salt (synthetic) ⁴	0.15	0.55	0.80	q	none	purple	blue-brown
Sulphadimethoxine glucuronide NH ₄ salt (from urine) ⁴	0.15	0.55	0.80	q	none	purple	blue-brown
Glucuronic acid	0.00	0.37	0.23	q	none	none	blue
Glucuronolactone	0.03	0.65	0.39	q	none	none	blue
N ¹ -Methylsulphadimethoxine	0.83	0.82	0.85	q	none	purple	none
Ring-N-methylsulphadimethoxine	streaks in all solvents			q	none	purple	none
Sulphanilamide	0.62	0.72	0.77	violet	none	red	
Sulphanilic acid	0.11	0.57	0.32	violet	none	red	none
Barbituric acid	0.03	0.35	0.48	q	none	none ^c	none
N-Methylbarbituric acid	0.09	0.50	0.65	q	none	none ^c	none
4-Amino-3-hydroxybenzenesulphonic acid	0.19		0.06	violet	deep orange	red	none
4-Amino-2-hydroxybenzene sulphonic acid	0.27		0.08	violet	deep orange-red	red	none

*quenches background fluorescence of paper.

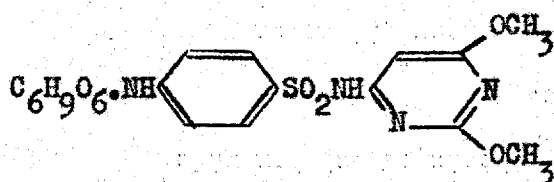
^aDSA = diazotized sulphanilic acid; DMAC = p-dimethylaminocinnamaldehyde; NR = naphtharesorcinol (see text)

^bhydrolyses to free amine in this solvent.

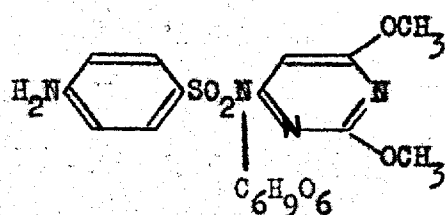
^cpurple spot on leaving paper overnight.

amide. No hydroxysulphanilic acid or hydroxysulphanilamide was detected (see Table 2.2). Sulphadimethoxine may not be readily demethylated in vivo for we found that when it was incubated in vitro at 38° with the rabbit-liver O-demethylating system of Takemori and Mannering (1958) no formaldehyde was produced during 1 hr. The drug was not hydroxylated or demethylated by the ascorbic acid-iron system of Brodie, Axelrod, Shore and Udenfriend (1954).

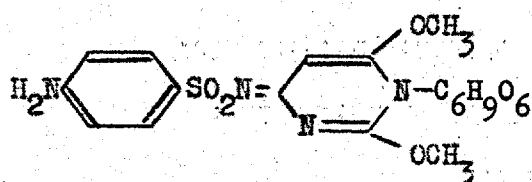
The glucuronide must therefore be an N-glucuronide and four of these are possible (II-V).



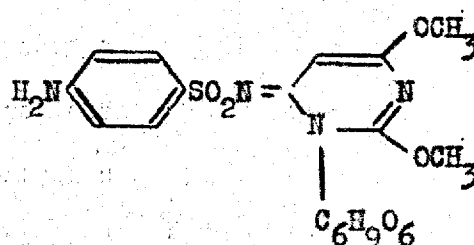
II



III



IV



V

The N⁴-glucuronide, II, can be readily eliminated. Aromatic amine N-glucuronides are labile, reduce alkaline copper reagents and are not

TABLE 2.2

Hydrolysis products of sulphadimethoxine and its derivatives

The compounds were hydrolysed with acid or alkali as described in the text. The hydrolysis products were identified by R_F values and colour reactions (see Table 2.1).

Compound hydrolysed	Products of hydrolysis by	
	$2N-HCl$	$2.5N-NaOH$
Sulphadimethoxine, N^4 -Acetylsulphadimethoxine	{barbituric acid sulphanilic acid (sulphanilamide*)	barbituric acid sulphanilic acid sulphanilamide
N^1 -Methylsulphadimethoxine	barbituric acid sulphanilic acid	sulphanilic acid (trace) [†] sulphanilamide (trace)
Ring- N -Methylsulphadimethoxine	N -methylbarbituric acid sulphanilic acid	sulphanilic acid (trace) [†] sulphanilamide (trace) [†]
Synthetic sulphadimethoxine N^1 -glucuronide NH_4 salt	{barbituric acid sulphanilic acid sulphanilamide* glucuronic acid (glucuronolactone	barbituric acid sulphanilamide glucuronic acid
Sulphadimethoxine glucuronide NH_4 salt from human urine	{barbituric acid sulphanilic acid glucuronic acid (glucuronolactone	barbituric acid sulphanilamide glucuronic acid

*A minor product.

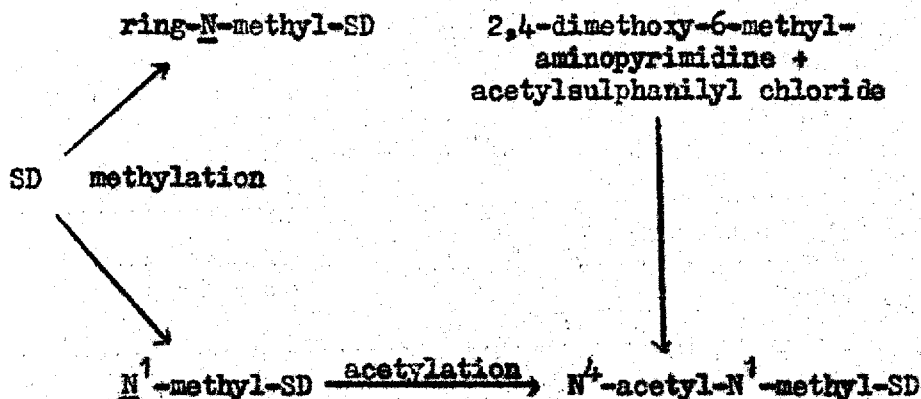
† The methylsulphadimethoxines appear to be relatively stable under these conditions of hydrolysis.

hydrolysed by β -glucuronidase (Bridges and Williams, 1962). A sample of sulphadimethoxine N^4 -glucuronide was synthesized and it was found to be labile, to reduce Benedict's and Fehling's solution, and to give a colour slowly with p -dimethylaminocinnamaldehyde (see Table 2.1). These properties did not agree with the urinary glucuronide which is stable, non-reducing and gives a colour immediately with p -dimethylaminocinnamaldehyde. R_f values were also different (Table 2.1). The urinary glucuronide was similar in all respects with a sulphadimethoxine glucuronide synthesized from sodium sulphadimethoxine and 1-bromo-2,3,4-tri- O -acetylglucuronic acid methyl ester (see Tables 2.1 and 2.4). The S -benzylthiouronium salt of the urinary glucuronide was identical with that of the synthetic glucuronide.

A glucuronide synthesized as above could have one of three structures (III, IV, V) as a result of the tautomerism of the sulphanilamidopyrimidines. A basis for distinguishing the amido structure III from the imido structures, IV and V, has been suggested by Uno, Machida, Hanai, Ueda and Sasaki (1963) who showed that the amido and imido forms of pyridine and thiazole sulphonamides could be distinguished by their SO_2 symmetrical stretching frequencies. In the imido forms this frequency occurs in the range 1120-1145 cm^{-1} whereas in the corresponding amido forms it occurs in the range 1145-1170 cm^{-1} . Methyl derivatives of sulphadimethoxine and 4-methoxy-6-sulphanilamidopyrimidine were synthesized containing both these structures. The structures of the methyl derivatives containing the amido link were proved by synthesis i.e., 4-methoxy and 2,4-dimethoxy-6-(N^4 -methylsulphanilamido) pyrimidines and their N^4 -acetyl derivatives. The structures of the

corresponding ring-N-methyl derivatives were then deduced by inference.

In the case of sulphadimethoxine (SD), the routes were as follows:-



The SO_2 symmetrical stretching frequencies of these compounds are shown in Table 2.3, and on this evidence it is clear that the synthetic and urinary sulphadimethoxine glucuronides have the amido structure, II.

The ring-N-methylsulphadimethoxine obtained by methylation of sulphadimethoxine appeared to be a mixture of two compounds which we failed to separate. This conclusion was based on the infrared spectrum which showed extensive doubling of the absorption bands. Methylation of 4-methoxy-6-sulphanilamidopyrimidine appeared to give only one ring N-methyl derivative in which the doubling of infrared absorption bands was absent. Acid hydrolysis of the ring N-methyl sulphadimethoxine yielded N-methylbarbituric acid, whereas that of the N¹-methyl derivative gave barbituric acid. Acid hydrolysis appears to demethylate all the methoxypyrimidine derivatives (see Table 2.2). The products of hydrolysis of the methylated 4-methoxy-6-sulphanilamidopyrimidines could not be identified.

TABLE 2.3

SO₂ symmetrical stretching frequencies of sulphadimethoxine and related compounds

Infrared spectra in KBr pellets determined in a Perkin-Elmer "Infracord" spectrometer.

Compound	SO ₂ -stretching frequency cm. ⁻¹
Sulphanilamide	1150
2,4-Dimethoxy-6-sulphanilamidopyrimidine (sulphadimethoxine)	1150
2,4-Dimethoxy-6-(N ⁴ -acetylsulphanilamido)pyrimidine	1150
Sulphadimethoxine glucuronide NH ₄ salt from urine	1160
Sulphadimethoxine N ¹ -glucuronide NH ₄ salt, synthetic	1160
2,4-Dimethoxy-6-(N ¹ -methylsulphanilamido)pyrimidine	1150
2,4-Dimethoxy-6-(N ⁴ -acetyl-N ¹ -methylsulphanilamido)pyrimidine	1155
4-Methoxy-6-sulphanilamidopyrimidine	1155
4-Methoxy-6-(N ¹ -methylsulphanilamido)pyrimidine	1150
4-Methoxy-6-(N ⁴ -acetyl-N ¹ -methylsulphanilamido)pyrimidine	1155
2,4-Dimethoxy-1- and -3-methyl-6-sulphanilamidopyrimidine*	1125
4-Methoxy-1- or -3-methyl-6-sulphanilamidopyrimidine †	1130
4-Methoxy-1- or -3-methyl-6-(N ⁴ -acetylsulphanilamido)-pyrimidine †	1130

*This is a mixture of the two possible ring methyl forms.

† These are single compounds, but the position of the N-methyl group in the pyrimidine ring is either 1 or 3.

It has been claimed that ultraviolet absorption spectra can be used to distinguish the N^1 -methyl and ring- N -methyl derivatives of sulphapyridine and sulphathiazole (Shepherd, Bratton and Blanchard, 1942; Sheinker, Postovskii, Voronina and Kushkin, 1957; Sheinker and Kuznetsova, 1957). The N^1 -methyl (amido) forms in ethanol shows one absorption peak, whereas the ring- N -methyl (imido) forms show two peaks, the major of which occurs at longer wavelengths than the single peak of the amido forms. The absorption peaks (above 220 $m\mu$) of a number of sulphanilamidopyrimidines were determined (see Appendix 1). It is clear that, in this series of compounds, ultraviolet spectra cannot be used to distinguish amido and imido forms. Most of the compounds in this table show one peak and there is no separation of the absorptions due to the sulphanilamide and pyrimidine portions of the molecules. The N^1 -methyl and ring- N -methyl derivatives of 2,4-dimethoxy- and 4-methoxy-6-sulphanilamidopyrimidines have similar spectra showing an inflection in the 255-265 $m\mu$ region and a peak in the 275-285 $m\mu$ region. These amido and imido forms are thus not readily distinguishable by ultraviolet spectra. The sulphadimethoxine glucuronide isolated from human urine showed a spectrum identical with that of synthetic sulphadimethoxine N^1 -glucuronide (Table 2.4).

Sulphadimethoxine N^1 -glucuronide could be an α - or β -glycoside. Specific optical rotations (triacetyl methyl ester, + 87° in methanol; NH_4 salt + 4.5° in water; S -benzylthiouronium salt - 13° in water) gave no indication of the configuration. The glucuronide, however, is slowly

hydrolysed by β -glucuronidase and the hydrolysis is inhibited by saccharo-1,4-lactone (see Chapter 7) a known inhibitor of β -glucuronidase (Levy, 1952). This suggests that the compound has the β -configuration and can be tentatively described as [1-deoxy-1-N^{1'}-(2'',4''-dimethoxy-pyrimidin-6''-yl)sulphanilamido- β -D-glucosid]uronic acid. The comparatively high positive rotation for a β -glucuronide can possibly be explained by steric hindrance. The sulphonamide nitrogen atom has three bulky substituents and may take up a preferential orientation, causing a contribution to optical rotation as in the case of the sulphonamide benzenesulphonyl-(8-nitro-1-naphthyl)glycine which has been resolved (Mills and Elliott, 1928).

The glucuronide showed only about one-hundredth of the activity of the parent drug against Staphylococcus aureus Oxford (tested by Dr. McFazean of May and Baker Ltd., Dagenham, Essex).

There have been reported in the literature several types of N-glucuronides which are formed in vivo. These include the labile N-glucuronides of aromatic amines, the stable N-glucuronides of carbamates of which two are known, namely those of meprobamate (2-methyl-2-propyl-1,3-propanediol dicarbamate) Tsukamoto, Yoshimura and Tatsumi, 1963b) and of sulphisoxazole (Uno and Kono, 1962) and the N¹-glucuronides of sulphonamide drugs of which two have been previously known, namely, of sulphathiazole (Uno and Ueda, 1963) and sulphisomezole (Ueda and Kuribayashi, 1964).

TABLE 2.4

Ultraviolet spectra of sulphadimethoxine, its glucuronide metabolite and its N¹-glucuronide. Solvent: ethanol.

Wavelengths to nearest μ and $\epsilon_{\max}$ to nearest 100.

Compound	$\lambda_{\max}$	$\epsilon_{\max} \times 10^{-3}$
Sulphadimethoxine	261, 273	19.0, 23.0
Sulphadimethoxine glucuronide NH_4 salt (from urine)	269	21.4
Sulphadimethoxine <u>N</u> ¹ -glucuronide NH_4 salt (synthetic)	269	21.8

CHAPTER 3

THE METABOLISM OF SULPHADIMETHOXINE: SPECIES DIFFERENCES

Chronic toxicity tests in the rat and dog (Randall, Bagdon and Engelberg, 1959) showed sulphadimethoxine to be non-toxic up to doses of 200 mg./kg. Acute toxicities have been summarised see Table 3.1.

TABLE 3.1

LD50 values of sulphadimethoxine in various species.

Data cited by Fust, Boehni, Schnitzer, Rieder and Struller (1960).

Species	Route	LD50 (mg./kg.)
Mouse	p.o.	16,000+
	s.c.	791
	i.p.	866
	i.v.	844
Rat	p.o.	20,000+
Rabbit	p.o.	1,000+
	i.v.	1,000
Dog	p.o.	3,200+

Detailed investigations of the metabolism of sulphadimethoxine have not been previously reported. In the rat (Randall et al. 1959), rabbit (unpublished data cited by Bagdon, 1964) and cow (Stowe and Sisodia, 1963) the major metabolite in urine has been shown to be the N^4 -acetyl derivative, whereas in man the major metabolite is a glucuronide (Koechlin, Kern and Engelberg, 1959) which has been shown to be an N^1 -glucuronide (Chapter 2).

EXPERIMENTAL

Materials and synthesis of reference compounds

N⁴-Acetylsulphadimethoxine m.p. 210-211° was donated by Roche Products Ltd., Welwyn Garden City, Herts.

Sulphadimethoxine-N⁴-sulphamate. Chlorosulphonic acid (2.0 g.) was added dropwise with stirring to anhydrous pyridine (25 ml.) which was cooled in ice. After allowing to stand for 30 min. sulphadimethoxine (6.24 g.) was added, the mixture shaken vigorously until all solid material had dissolved, and the solution was then kept in the dark at room temperature for 24 hr. 0.4N-KOH (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 in vacuo at 45° gave a white residue (6.8 g.). After dissolving this material in a minimal amount of water (12 ml.) acetone was added (100 ml.) to precipitate inorganic salts and the solution was filtered. The filtrate on evaporation to dryness in vacuo yielded a white amorphous residue (2.8 g.) which on recrystallization from absolute ethanol gave white platelets of the pure dipotassium 1-p-(2'4'-dimethoxypyrimidin-6'-yl-sulphamoyl)benzene sulphamate (sulphadimethoxine-N⁴-sulphate dipotassium salt) Found: C, 29.9; H, 3.0; N, 11.5; K, 16.1; loss in vacuo at 100° 4.1; 3.5; $C_{12}H_{12}K_2N_4O_7S_2 \cdot H_2O$ requires C, 29.7; H, 2.9; N, 11.6; K, 16.1; H₂O 3.7%. The compound which was very soluble in water gave no immediate reaction on paper with dimethylaminocinnamaldehyde in 0.1N-HCl but on standing for 30 min. a purple spot developed. Addition of Ba(NO₃)₂ to an acidified solution of the compound only gave a ppt. of BaSO₄ on boiling. The material did

however diazotise and couple with N^1 -naphthylethylenediamine directly.

METHODS

Free and total aromatic amines in urine and bile. These were determined by the method of Bratton and Marshall (1939). Urine or bile containing 0.01-10.0 mg./100 ml. was diluted with water so as to contain between 10-30 μ g./ml. Free aromatic amine (which includes N^4 - and N^1 -glucuronides and N^4 -sulphate) in this diluted urine or bile was determined directly by the Bratton and Marshall method. Recovery of sulphadimethoxine and sulphadimethoxine- N -glucuronide from urine and bile was 99-100 per cent. To determine total aromatic amine, diluted urine or bile (1 ml.) was mixed with 2N-hydrochloric acid (1 ml.) and heated on a boiling water bath for 20 min. and the total aromatic amine determined by the Bratton and Marshall method. The recovery of N^4 -acetylsulphadimethoxine added to urine (10 mg./100 ml.) was 99 per cent.

Determination in Faeces. Faeces from rats, rabbits and guinea pigs dosed with sulphadimethoxine were also analysed. Faeces were homogenized with 10%-NaOH (1 ml./g.) and water and centrifuged. Free and total aromatic amines were determined on the supernatant. The recovery of sulphadimethoxine added to faeces (0.1-10 mg./g. of air-dried faeces) was 66-92 per cent. Recovery of sulphadimethoxine- N^1 -glucuronide (2 mg./g. air-dried faeces) was 85-92 per cent.

Determination of metabolites on strip chromatograms. Urine or bile (0.1-2 ml.) was banded across Whatman No.1 paper (15 cm. wide). Papers were chromatographed in butan-1-ol-ammonia solution (sg. 0.880) + water

(10:1:1) for 17 hrs. against reference spots of the possible metabolites and of the original urins. Strips corresponding to the reference spots (detected by ultraviolet light and dimethylaminocinnamaldehyde spray (Chapter 2) were cut from the chromatogram and eluted with dilute ammonia solution (0.02N). The eluate was made up to a known volume and free and total amines were determined as described above.

Chromatography spray reagents. The p-dimethylaminocinnamaldehyde and naphoresorcinol spray reagents have been described (Chapter 2). The barium chloride - sodium rhodizonate spray (Smith, 1961) was applied after treating the chromatogram with 2N-HCl and heating at 70° for 5 m. The sulphate released by hydrolysis appeared as a white spot on a pink ground.

Animals and human subjects. Human subjects were healthy male volunteers aged 18-26. Sulphadimethoxine was administered by mouth in tablet form. Monkeys (Macacca rhesus) were given powdered sulphadimethoxine concealed in fruit or mixed with honey. Dogs were of mixed strains; sulpha-dimethoxine tablets were administered with food. Rats (Wistar albino, 180-200 g.): sulphadimethoxine was suspended in aqueous glycerol for administration by feeding cannula or dissolved in warm propane-1,2-diol for intraperitoneal injection. Guinea-pigs (English) were administered an aqueous suspension of sulphadimethoxine by feeding cannula. Rabbits (Chinchilla) were given sulphadimethoxine as an aqueous suspension by stomach tube.

Detection and isolation of metabolites

Isolation of N^4 -acetylsulphadimethoxine

A Chinchilla rabbit (3.9 kg.) was given orally 0.5 g. of sulphadimethoxine. The 24 hr. urine (175 ml.) was collected, its pH adjusted to pH 5.5, and extracted with ethyl acetate (350 ml.). The ethyl acetate was evaporated to dryness in vacuo at 45°. The gum-like residue was dissolved in 1N-sodium carbonate (5 ml.) and reprecipitated by the addition of 2N-hydrochloric acid (6 ml.). The precipitate was filtered off and recrystallized from glacial acetic acid to give clusters of small white crystals m.p. 207-208° mixed m.p. with synthetic N^4 -acetylsulphadimethoxine 208-210°. Yield 8 mg. The infrared spectrum of the isolated metabolite proved to be identical with synthetic N^4 -acetylsulphadimethoxine.

Isolation of sulphadimethoxine

A mongrel dog (10 kg.) was given orally 1 g. of sulphadimethoxine. The 24 hr. urine was collected (400 ml.) acidified to pH 5 and extracted with ethyl acetate (200 ml.). The ethyl acetate was extracted with half its volume of 1N-sodium hydroxide. On neutralizing the aqueous phase with glacial acetic acid, a dense white precipitate appeared (290 mg.). This was filtered off and recrystallized twice from 50 per cent ethanol to give 200 mg. material m.p. 197°, mixed m.p. with synthetic sulphadimethoxine 198°.

Isolation of sulphadimethoxine- N^1 -glucuronide

The detection, isolation and characterization of sulphadimethoxine- N^1 -glucuronide from the urine of volunteers receiving 2 g. of sulphadimethoxine is described in Chapter 2.

Formation of sulphadimethoxine N^4 -glucuronide in urine

Sulphadimethoxine (2-20 mg./100 ml.) was added to freshly voided human urine. The urine was then examined quantitatively as above to determine the N^4 -conjugate formed.

RESULTS

Sulphadimethoxine N^4 -glucuronide was identified as a metabolite by its similar chromatographic properties to the synthetic material (Table 3.2). On spraying with p-dimethylaminocinnamaldehyde reagent it characteristically appeared in about 5-10 m. in solvents A and B due to slow hydrolysis releasing the free amino group. It did not appear in the acidic solvent C due to hydrolysis. The constitution of the original spot was therefore confirmed by elution and rechromatography in solvent C, when it gave rise to sulphadimethoxine and glucuronic acid.

The glucuronide occurred in all urines and bile investigated, as a minor metabolite accounting for less than 10% of the excreted dose except in the case of the dog, where it accounted for 17% of the excreted dose (Table 3.3).

Addition of sulphadimethoxine to normal human urine also yielded the N^4 -glucuronide. The highest proportion (19%) was found at a sulphadimethoxine concentration of 2 mg./100 ml., the lowest (3%) at 20 mg./100 ml.

Sulphadimethoxine N^4 -sulphonate was identified chromatographically by comparison with the synthetic compound. On spraying with the p-dimethylaminocinnamaldehyde reagent a spot only appears after one hour. It has

TABLE 3.2

Chromatography and colour reactions of sulphadimethoxine and its metabolites

Descending chromatography on Whatman No.4 paper was used. The solvent systems employed were: A, butan-1-ol-ammonia solution (s.g. 0.880)-water (10:1:1 by vol.); B, 80% aqueous ethanol; C, butan-1-ol-acetic acid-water (4:1:2 by vol.); D, propan-1-ol-ammonia solution (s.g. 0.880) (7:3 by vol.). Chromatographs were run for six hours in solvents A, C and D and 4 hours in solvent B.

Compound	R _F values in solvent				Fluorescence in light (254 mμ)	Colour reaction		
	A	B	C	D		DMAC ^a	NR ^a	BaRh ^a
Sulphadimethoxine-N ⁺ -glucuronide sodium salt	0.00	0.25	^b	0.11	q [*]	purple (5-10 m)	blue	none
Sulphadimethoxine-N ⁺ -sulphate dipotassium salt	0.05	0.79	0.66	0.42	q	purple (30 m.)	none	white
Sulphadimethoxine-N ⁺ -glucuronide ammonium salt	0.15	0.80	0.55	0.54	q	purple	blue-brown	none
Sulphadimethoxine	0.40	0.95	0.95	0.72	q	purple	none	none
N ⁺ -Acetylsulphadimethoxine	0.65	0.95	0.95	0.84	q	none ^c	none	none
Sulphanilamide	0.62	0.72	0.77	0.75	violet	red	none	none
Sulphanilic acid	0.11	0.57	0.32	0.48	violet	red	none	none

*quenches background fluorescence of paper

^aDMAC = p-dimethylaminocinnamaldehyde; NR = naphtharesorcinol (see text); BaRh = barium chloride-sodium rhodisonate

^bhydrolyses to free amine in this solvent.

^cdeveloped a purple spot after keeping overnight

a very different R_F value from the N^4 -acetyl conjugate, which reacts similarly. The N^4 -sulphonate diazotises and couples without preliminary hydrolysis.

Significant amounts of the N^4 -sulphonate were found only in rat urine (2%). It occurred in traces below the limits of estimation in the urine from some other species.

The isolation and identification of sulphadimethoxine N^1 -glucuronide in human urine has been discussed in Chapter 2. It was detected in the urines and bile of other species by its chromatographic properties (Tables 3.2 and 3.3).

Unchanged sulphadimethoxine was excreted by all species examined. It was isolated from dog urine in which it was the principal metabolite (64%) (Table 3.3). In other species it was identified by its R_F value in several solvents and its immediate reaction with p -dimethylamino-cinnamaldehyde reagent.

N^4 -Acetylsulphadimethoxine was isolated from the urine of a rabbit receiving sulphadimethoxine. On chromatograms of the urine of other species it was identified by R_F values and slow reaction with p -dimethylaminocinnamaldehyde. It appeared in the urines of all species examined except the dog.

In rat bile sulphadimethoxine N^1 -glucuronide was the principal component (68%) (Table 3.4).

Recoveries of sulphonamide varied from 76% of the dose in three days in the rabbit in the urine, none being in the faeces, to 43% in

the guinea-pig, and 17% in two days in the rat (Table 3.3). Only traces of sulphonamide were found in the faeces at this dose level (100 mg./kg.). Recoveries in the rat did not differ significantly between oral and intraperitoneal administration (Table 3.5). Sulphonamide was detected in rat faeces at very high dose levels (400-800 mg./kg. p.o.). No trace of sulphonamide was found during qualitative examination of the tissues of a rat six days after administration of sulphadimethoxine.

TABLE 3.4

Fate of sulphadimethoxine in rats with biliary fistula

The sulphonamide was administered intraperitoneally at 100 mg./kg.

Except where indicated otherwise the data is expressed as a proportion of the dose

No. of animals	3
'Free' amine in 24 hr. bile	12 $\pm$ 1
Total amine in 24 hr. bile	12 $\pm$ 1
(N ⁴ -glucuronide	7 $\pm$ 1
% of the excreted (N ⁴ -sulphonate	0
dose in 24 hr. (N ¹ -glucuronide	68 $\pm$ 3
bile as (sulphonamide	21 $\pm$ 3
(N ⁴ -acetyl	3 $\pm$ 1
'Free' amine in 48 hr. bile	3.5 $\pm$ 0.3
Total amine in 48 hr. bile	3.5 $\pm$ 0.3
Total recovery	15 $\pm$ 1

TABLE 3.5

Fate of sulphadimethoxine in the rat.

The sulphonamide was administered either orally or intraperitoneally at 100 mg./kg. Except where indicated otherwise, data is expressed as a proportion of the dose.

Route	p.o.	i.p.
No. of animals	3	3
'Free' amine in 24 hr. urine	3.9 ± 0.5	5.3 ± 0.9
Total amine in 24 hr. urine	8.6 ± 1.3	12.7 ± 2.3
% of the excreted dose in 24 hr. urine as	(N ⁺ -glucuronide	5 ± 1
	(N ⁺ -sulphate	1 ± 1
	(N ⁺ -glucuronide	8 ± 1
	sulphonamide	38 ± 8
	(N ⁺ -acetyl	48 ± 8
'Free' amine in 48 hr. urine	7.4 ± 1.9	6.6 ± 1.4
Total amine in 48 hr. urine	20 ± 2	18 ± 5
'Free' amine in days 3-6 urine	10 ± 2	9.9 ± 1.5
Total amine in days 3-6 urine	17 ± 1	18 ± 5
Total recovery in 6 days	45 ± 3	50 ± 9

TABLE 3.3

The fate of sulphadimethoxine in various species after oral administration

(Figures represent the percentage of the dose fed recovered unless otherwise stated)

Species	Rabbit	Guinea Pig	Rat	Dog	Monkey		Man
Sex	♀	♂	♀	♀	♀		♂
Dose mg./kg	100	100	100	200	100	33	33
No. of animals or subjects	3	2	3	3	2	4	3
'Free' amine in 24 hr. urine	2.6 ± 2.5	68	3.9 ± 0.5	23. ± 5	32	24 ± 5	19 ± 1
Total amine in 24 hr. urine	43 ± 5	20	8.5 ± 1.3	23 ± 5	42	25 ± 5	25 ± 3
% of (N ⁺ -glucuronide	7 ± 1	13	5 ± 1	17	3	11 ± 3	8 ± 2
excreted (N ⁺ -sulphate	0	0	2 ± 1	0	trace	trace	0
dose in (N ⁺ -glucuronide	0	5	7 ± 3	19	70	62 ± 12	62 ± 2
24 hr. (Free sulphadimethoxine	1 ± 0	16	40 ± 3	64	6	21 ± 12	8 ± 4
urine as (N ⁺ -acetylsulpha-							
dimethoxine	92 ± 2	66	46 ± 1	0	21	6 ± 6	21 ± 4
'Free' amine in 48 hr. urine		6.2	4 ± 1.9	-	19	9.4	-
Total amine in 48 hr. urine	Free 1.4	15.5	20 ± 2	-	21	9.4	-
'Free' amine in 72 hr. urine	Total 33.5	4.3	-	-	2.8	-	-
Total amine in 72 hr. urine		7.6	-	-	2.8	-	-

CHAPTER 4

**THE METABOLISM OF SULPHADIMETHOXINE:
THE EFFECT OF PHENOBARBITONE PRETREATMENT**

Remmer (1964_g) reported that pretreatment with phenobarbitone affected the metabolism of sulphadimethoxine in the rat and the dog. Although he made an attempt at identifying the glucuronide of sulphadimethoxine, Remmer was unaware of its true nature. He probably assumed, following Koechlin, Kern and Engelberg (1959), that it was a conjugate of an oxidation product of sulphadimethoxine. Phenobarbitone, in common with many other compounds, has been known to stimulate the enzyme systems responsible for the hydroxylation and dealkylation of foreign compounds (see Remmer and Merker, 1965).

Once the true nature of sulphadimethoxine glucuronide became known to us it indicated a potential new reaction subject to activation by phenobarbitone.

EXPERIMENTAL

Pretreatment with phenobarbitone. Phenobarbitone sodium (15 mg./kg.) was administered in aqueous solution intraperitoneally to rats twice daily for three days prior to administration of sulphadimethoxine. This was one of the regimes used by Remmer (1964).

Sulphadimethoxine N¹-glucuronide (NH₄ salt) was administered as an aqueous solution.

Incubation of intestinal contents with sulphadimethoxine N¹-glucuronide.

The caecal and small intestinal contents of a freshly-killed rat were each suspended in 0.1M-phosphate buffer pH 7.4 (Booth and Williams, 1963).

Sulphadimethoxine N^1 -glucuronide (NH_4 salt) was added to aliquots of the suspensions to give a final concentration 1 mg./ml. and the whole incubated at 37° without shaking. At intervals samples were withdrawn and analysed for hydrolysis products as described for urine (Chapter 3).

RESULTS

Fate of sulphadimethoxine in normal and pretreated rats

The relevant data are shown in Table 4.1.

Recoveries. The recovery data for each period (day 1, day 2, days 3-6) were the same whether sulphadimethoxine was given by oral or intraperitoneal routes to normal rats. Similarly the route did not affect recoveries for each period after pretreatment of the rats with phenobarbitone.

Compared with control rats, the pretreated rats excreted a larger proportion of the dose in the first day (30 against 10%). However, total recoveries were statistically indistinguishable whether the rats were pretreated or not.

Metabolites. No significant changes occurred in the relative amounts of day 1 metabolites on alteration of the route of administration or pretreatment of the rats with phenobarbitone. The principal metabolite was N^4 -acetylsulphadimethoxine (45-49% of the excreted dose), followed by unchanged sulphonamide (36-40%), with small amounts of the N^1 -glucuronide (7-9%), N^4 -sulphonate (1-4%) and N^4 -glucuronide (5%).

Fate of sulphadimethoxine N^1 -glucuronide in normal and pretreated rats

The relevant data are shown in Table 4.2.

Recoveries. The urinary recovery of sulphonamide derivatives from normal rats was much lower when sulphadimethoxine N^1 -glucuronide was given orally

than when given intraperitoneally. Day 1 recoveries were 16 and 43% respectively with total recoveries of 51 and 70%.

Faecal recovery was somewhat higher after oral administration, but the difference is not significant.

Pretreatment with phenobarbitone depressed urinary recovery after administration of the glucuronide by either route, compared with the control animals. After oral administration, day 1 recovery decreased from 16 to 8.2%; intraperitoneal, from 43 to 17%. Total recoveries were also lower.

Faecal recovery remained the same (24%) in pretreated animals after oral administration, but decreased (18 to 5%) in pretreated rats compared with controls after intraperitoneal injection.

Metabolites. After oral administration, the principal urinary metabolites were sulphadimethoxine (41-51%) and its N^4 -acetyl derivative (35-36% of the excreted dose). Differences in the proportions of metabolites between pretreated and control animals are probably not significant.

After intraperitoneal administration, the principal urinary metabolite was unchanged sulphadimethoxine N^1 -glucuronide (74-75%). Again, pretreatment had no significant effect on the proportion of metabolites.

No sulphadimethoxine N^4 -sulphonate was observed.

Effect of pretreatment on bile-fistula rats

The relevant data are shown in Table 4.3.

Sulphadimethoxine administered. Total recovery of drug in the bile was greatly enhanced after phenobarbitone pretreatment (12 to 44%).

This was largely due to the increase in the amount of sulphadimethoxine

N¹-glucuronide excreted (68 to 89% of the excreted dose). Free sulphadimethoxine excretion remained unchanged (2.5 ± 0.6 and $2.2 \pm 0.8\%$ of the dose in normal and pretreated animals respectively).

Sulphadimethoxine N¹-glucuronide administered. Recovery in the bile after phenobarbitone pretreatment was halved (78 to 38%). This was due entirely to depression in the excretion of unchanged glucuronide; this being almost the sole component (100 and 99% of the excreted dose in normal and pretreated animals respectively).

Intestinal β -glucuronidase

Caecal contents hydrolysed 2% of the added sulphadimethoxine N¹-glucuronide after 5 hr. and 5% after 32 hr. The contents of the small intestine hydrolysed at a similar rate.

DISCUSSION

Enterohepatic recirculation

On administering sulphadimethoxine N¹-glucuronide orally, 24% of the dose appeared in the faeces, possibly unchanged. Part of the fraction absorbed was excreted in the urine, the ratio of metabolites being very similar to that observed after administration of sulphadimethoxine and dissimilar to the metabolites found after intraperitoneal administration of the glucuronide. It follows that the glucuronide was almost completely hydrolysed to sulphadimethoxine on absorption.

Milburn (1965) introduced both bile from sulphadimethoxine-treated rats and sulphadimethoxine N¹-glucuronide into the duodena of bile-fistula rats, finding little absorption (7.6 and 11.6% respectively) or hydrolysis.

We found that caecal and small intestinal contents, known to contain an active β -glucuronidase (Marsh, Alexander and Levvy, 1953), did not break down sulphadimethoxine N^1 -glucuronide at a rate fast enough to account for the observed hydrolysis in vivo.

When given by intraperitoneal injection the principal urinary metabolite, with good recovery, was unchanged glucuronide. Other glucuronides given intraperitoneally are not hydrolysed (e.g. Garton and Williams, 1949). Endogenous β -glucuronidase does not appear to be a significant factor.

Sulphadimethoxine N^1 -glucuronide when given orally may be hydrolysed within the intestinal mucosa since this tissue is known to contain β -glucuronidase (Fishman, 1961). The data of Milburn (1965) appears to indicate that this mode of hydrolysis is a very minor one.

Although at pH 3 and 37° sulphadimethoxine N^1 -glucuronide was stable, it was hydrolysed rapidly by normal acid (Chapter 2). This is consistent with stomach acid being the principal hydrolytic agent in vivo. In the intact rat glucuronide excreted in the bile may be subjected to stomach acid by regurgitation or zones of incomplete neutralisation in the duodenum.

A fraction (25%) of the sulphonamide derivatives appearing in the urine after intraperitoneal injection of sulphadimethoxine N^1 -glucuronide represents products of its hydrolysis. It follows that at least 25% of the excreted dose (13% of the dose administered) has been present in

the gastrointestinal tract and after hydrolysis has been reabsorbed. The fraction 13% approximates to the proportion of the administered dose which has undergone one cycle of the enterohepatic circuit in 24 hr. Since excretion in the bile following treatment with sulphadimethoxine N^1 -glucuronide represents 78% of the dose, one-sixth of the glucuronide excreted in the bile can be expected to be reabsorbed. When sulphadimethoxine glucuronide is administered, recirculation can be expected to be significant.

Since much smaller quantities of the glucuronide are excreted in the bile of a rat receiving sulphadimethoxine, it is unlikely that more than 2.5% of the dose undergoes enterohepatic recirculation. This value was calculated by assuming complete reabsorption of sulphadimethoxine excreted in the bile and is in good agreement with the conclusion of Milburn (1965). It is unlikely that this mechanism is responsible for the long action of sulphadimethoxine. Preziosi, Loscalzo, Marmo and Miele (1963) also discounted enterohepatic recirculation as a significant factor in the long action of sulphadimethoxine. They found that tying off the bile duct did not affect the plasma half-life of the drug nor its urinary excretion.

Enterohepatic recirculation has long been thought to be a contributory factor in the long action of sulphadimethoxine and other long-acting sulphonamides (see discussion by Williams, Milburn and Smith, 1965), despite data showing excretion in the bile to be a comparatively minor pathway (Fust, Boehni, Schnitzer, Rieder and Struller, 1960; Preziosi, Loscalzo, Marmo and Miele, 1959).

Phenobarbitone pretreatment

There are three observed phenomena caused by pretreatment which require interpretation:

1. When sulphadimethoxine is administered, pretreatment increases renal excretion of all metabolites.
2. When sulphadimethoxine is administered, pretreatment appears to stimulate glucuronide synthesis and excretion in the bile.
3. When sulphadimethoxine N^1 -glucuronide is administered, pretreatment depresses excretion in the urine and bile.

Pretreatment with phenobarbitone and other stimulators of drug metabolism is held to be principally due to the induction of the enzyme systems responsible for the metabolism of foreign compounds. Induction is accompanied by changes in the endoplasmic reticulum with which these systems are associated (see Remmer and Merker, 1965).

It is but a short step to suggest that other cell membranes are also susceptible to the influence of phenobarbitone, in particular the cell walls. Many cells contain active transport systems located within the boundary membranes. Those of the liver and renal tubules are of particular interest to biochemical pharmacologists. It is proposed that the systems concerned with the active transport of sulphadimethoxine N^1 -glucuronide are stimulated by phenobarbitone.

Milburn (1965) found that the biliary excretion of sulphadimethoxine N^1 -glucuronide exhibits saturation at high concentrations, confirming that an active transport system for this compound does exist in the liver.

Its presence is invoked to explain the requirement of intact cells for the synthesis of sulphadimethoxine N^1 -glucuronide in vitro (Chapter 7). Stimulation of the system by phenobarbitone would account for the increased biliary excretion of sulphadimethoxine N^1 -glucuronide after administration of sulphadimethoxine.

An active transport system for organic anions exists in the kidney (Sperber, 1959). Many sulphonamides have been shown to be transported by a related mechanism (Despopoulos and Callahan, 1962). Presumably glucuronides are also actively transported by this system. Stimulation of active transport by phenobarbitone pretreatment would increase the renal excretion of all sulphadimethoxine metabolites.

If an active transport system exists in other tissues, also susceptible to stimulation by phenobarbitone, it is possible that the volume of distribution available to sulphadimethoxine N^1 -glucuronide is greatly increased. Since this compound is not very lipid-soluble, it will normally be confined to the extracellular water. Increasing the distribution of the glucuronide will reduce its concentration, reducing the amount available to the liver and kidney cells. The reduced excretion following administration of sulphadimethoxine N^1 -glucuronide may be interpreted in this way. An additional factor reducing renal excretion may be the decreased enterohepatic recirculation caused by a lower bile concentration.

Since recoveries do not change significantly in rats receiving sulphadimethoxine after pretreatment with phenobarbitone, it is reasonable

to assume that pretreatment does not affect that part of the dose unaccounted for, thought to be irreversibly tissue bound. This assumption probably holds for rats receiving sulphadimethoxine N^1 -glucuronide orally, since the glucuronide is hydrolysed on absorption. The recovery data bear this out. Intraperitoneal injection of the glucuronide presents a different case; recovery of sulphonamide derivatives is halved. The reason for this is possibly the increased volume of distribution suggested above.

Pretreatment reduces the excretion of metabolites after oral administration of sulphadimethoxine N^1 -glucuronide, although the latter is thought to be hydrolysed to sulphadimethoxine, the excretion of which is enhanced in these circumstances. This is perhaps an indication that the intestinal mucosa plays a more significant part in the hydrolysis of the glucuronide than has been previously supposed. If the glucuronide were taken up into the mucosal cells prior to hydrolysis, alteration of active transport at this site might reduce the rate of absorption and ultimately the rate of excretion.

The foregoing discussion makes it clear that pretreatment with phenobarbitone has a more profound and widespread effect than has been thought. Previous workers have concentrated on the induction of drug-metabolising enzymes, overlooking other effects which are just as significant in the influence of one drug on another.

More experimentation is required to substantiate the hypothesis propounded above. However, phenobarbitone and allied compounds may well be useful tools in increasing the understanding of active transport.

TABLE 4.1

Fate of sulphadimethoxine in normal and pretreated rats

Treatment Route of sulphonamide administration	None p.o.	Phenobarbitone p.o.	None i.p.	Phenobarbitone i.p.
No. of animals	3	3	5	3
Free amine in 24 hr. urine	3.9 ± 0.5	15 ± 3	5.3 ± 0.9	16 ± 5
Total amine in 24 hr. urine	8.5 ± 1.3	29 ± 7	13 ± 2	31 ± 10
% of drug excreted in 24 hr. as	(N ⁴ -glucuronide	5 ± 1	1 ± 1	5 ± 1
	(N ⁴ -sulphate	2 ± 1	1 ± 1	1 ± 1
	(N ¹ -glucuronide	7 ± 3	8 ± 1	9 ± 1
	Sulphadimethoxine	40 ± 3	38 ± 8	36 ± 4
	(N ⁴ -acetylsulphadimethoxine	46 ± 1	48 ± 8	49 ± 5
'Free' amine in 48 hr. urine	7.4 ± 1.9	26 ± 2	6.6 ± 1.4	8.2 ± 2.9
'Total' amine in 48 hr. urine	20 ± 2		18 ± 5	15 ± 7
Free amine in day 3-6 urine	10 ± 2		9.9 ± 1.5	1.5 ± 0.9
Total amine in day 3-6 urine	17 ± 1		18 ± 3	3.9 ± 1.9
Total recovery in 6 days from urine	45 ± 3	55 ± 9	50 ± 9	51 ± 19

TABLE 4.2

Fate of sulphadimethoxine-N¹-glucuronide in normal and pretreated rats

Treatment Route of sulphonamide administration	None p.o.	Phenobarbitone p.o.	None i.p.	Phenobarbitone i.p.
No. of animals	3	3	3	3
'Free' amine in 24 hr. urine	10 ± 2	4.9 ± 0.5	16 ± 5	42 ± 4
'Total' amine in 24 hr. urine	16 ± 3	8.2 ± 1.0	17 ± 5	43 ± 4
(N ¹ -glucuronide	10	7	5	4 ± 1
% of (N ¹ -sulphate	0	0	0	0
drug excreted (N ¹ -glucuronide	13	7	74 ± 3	75 ± 2
in 24 hr. as Sulphadimethoxine	41	51	15 ± 2	12 ± 1
(N ¹ -acetylsulphadimethoxine	36	35	6 ± 1	9 ± 1
'Free' amine in 48 hr. urine	7.3 ± 0.8	4.1 ± 1.5	7.2 ± 2.7	6.0 ± 0.3
'Total' amine in 48 hr. urine	11 ± 2	8.7 ± 3.5	13 ± 3	8.6 ± 0.2
Pooled faeces (Free amine	15 ± 4	21 ± 9	4.6 ± 0.4	10 ± 1
of 1st 48 hr. (Total amine	24 ± 5	24 ± 8	5.0 ± 0.5	18 ± 4
Total recovery in 2 days	51 ± 2	41 ± 4	35 ± 4	70 ± 6

TABLE 4.3

Fate of sulphadimethoxine and sulphadimethoxine-N¹-glucuronide in
normal and pretreated biliary cannulated rats

Compound		Sulphadimethoxine		Sulphadimethoxine-N ¹ -glucuronide	
No. of animals		3	3	3	3
Treatment		None	Phenobarbitone	None	Phenobarbitone
Free amine in 24 hr. bile		12 ± 1	44 ± 7	78 ± 6	38 ± 3
Total amine in 24 hr. bile		12 ± 1	44 ± 7	78 ± 6	38 ± 3
% of drug excreted in 24 hr bile as	(N ⁴ -glucuronide	8 ± 1	4 ± 1	0	0
	(N ⁴ -sulphate	0	0	0	0
	(N ¹ -glucuronide	68 ± 3	89 ± 2	100 ± 0	99 ± 1
	Sulphadimethoxine	21 ± 3	5 ± 1	0	1
	(N ⁴ -acetylsulphadimethoxine	3 ± 1	2 ± 1	0	0

CHAPTER 5

THE METABOLISM OF THE METHOXY-6-SULPHANILAMIDOPYRIMIDINES

2-Methoxy-6-sulphanilamidopyrimidine* was first synthesised by Winnek and Roblin (1947) who reported it to be therapeutically active. A second synthesis was achieved by Bretschneider, Kloetzer, Spitteller and Dehler (1961). It has not attained clinical use. There appears to be no published toxicity data.

4-Methoxy-6-sulphanilamidopyrimidine (sulphamonomethoxine) was synthesised by Shepherd, Taft and Krazinski (1961). It was up to twice as potent as sulphadiazine on a blood level basis in standardised infections in the mouse (Redin and McCoy, 1962). Clarkson and Martin (1961) attribute its high activity in mice compared with sulphadimethoxine and sulphamethoxy-pyridazine to a high intrinsic activity and persistence in the mouse combined with lower plasma protein binding.

Acute toxicities in the mouse were: intraperitoneal LD50 2800 mg./kg.; oral LD50 greater than 8000 mg./kg. (Redin and McCoy, 1962).

The sulphonamide has been reported in clinical use in Japan; in man it appeared to be very little acetylated (Oshima, Kasahara and Shibata, 1962). Its introduction in this country has been delayed due to untoward toxic reactions in the monkey (Spinks, 1963).

5-Methoxy-6-sulphanilamidopyrimidine. The synthesis has been reported (Budesinsky, Bydzovsky, Kopecky, Svab and Vavrina, 1961). According to

*Strictly, some sulphonamides in this series should be named as the corresponding 4-sulphanilamido derivatives. For clearness in comparison the sulphonamido group will be given the 6-position. The 4- and 6-positions on the pyrimidine ring are equivalent.

Budesinsky and Bydzovsky (1961) the sulphanilamido group q- to the 5-methoxy group on the pyrimidine ring strongly inhibited antibacterial activity.

2,5-Dimethoxy-6-sulphanilamidopyrimidine. The synthesis has been reported by Budesinsky et al. (1961). It also would not be expected to be highly active on the above considerations.

4,5-Dimethoxy-6-sulphanilamidopyrimidine (sulphorthodimethoxine).

A patent for the synthesis of this compound has been issued to Hoffman-La Roche and Co. (1964). The substance can be regarded as an extra-long-acting sulphonamide, with a half-life in man of 100-200 hr. (Madsen, 1964). It has recently been claimed to be effective in the treatment of malaria (Laing, 1964, 1965) and Leprosy (Languillon and Clary, 1964).

EXPERIMENTAL

Materials and the synthesis of reference compounds

2-Methoxy-6-sulphanilamidopyrimidine m.p. 186-187°, 5-methoxy-6-sulphanilamidopyrimidine m.p. 200-201°, 2,5-dimethoxy-6-sulphanilamidopyrimidine m.p. 190-193°, 4,5-dimethoxy-6-sulphanilamidopyrimidine m.p. 194-197° were donated by Roche Products Ltd., Welwyn Garden City, Herts., 4-methoxy-6-sulphanilamidopyrimidine m.p. 201-203° was donated by Imperial Chemical Industries (Pharmaceutical Division), Alderley Park, Cheshire.

N⁴-Acetyl derivatives. The sulphonamide (0.5 g.) was dissolved in 10% (w/v) Na₂CO₃ solution (5 ml.) and acetic anhydride (0.5 ml.) added. When the gentle reaction had subsided, the mixture was kept at room temperature (1 hr.), and the crystals of the N⁴-acetyl derivative filtered

TABLE 5.1

Melting points and analyses of the N^4 -acetyl derivatives of the 6-sulphanilamidopyrimidines

6-Sulphanil- amidopyrimidine	m.p. (°)	Solvent	Formula	Analyses				Required (%)			
				C	H	N	S	C	H	N	S
2-Methoxy-	225-7	2	-	-	-	-	-	-	-	-	-
4-Methoxy-	219-20	2	$C_{13}H_{14}N_4O_4S \cdot \frac{1}{2}H_2O$	47.1	4.6	16.7	9.7	47.1	4.6	16.9	9.7
5-Methoxy-	215-7	3	$C_{13}H_{14}N_4O_4S \cdot \frac{1}{2}H_2O$	47.1	4.6	16.7	9.5	47.1	4.6	16.9	9.7
2,5-Dimethoxy-	158-60	1	-	-	-	-	-	-	-	-	-
4,5-Dimethoxy-	214-5	4	$C_{14}H_{16}N_4O_5S$	47.7	4.7	16.0	9.0	47.7	4.6	15.9	9.1

Recrystallisation solvents:

1. Acetic acid.
2. Acetic acid (50% v/v).
3. Acetic acid (25% v/v).
4. Ethanol.

off. Yields were 90-100%. In each case this was a new route to the known compounds. Properties and analyses are shown in Table 5.1.

N⁴-Glucuronides. Small-scale syntheses (0.5-1.0 g. sulphonamide) were attempted using the method described for sulphadimethoxine (Chapter 2). Only impure crystals of the N⁴-glucuronides were obtained, with the exception of sulphorthodimethoxine which crystallised as disodium 1-deoxy-1-[p-(4',5'-dimethoxypyrimidin-6'-ylsulphamoyl)anilino]glucosiduronate from acetone-0.2N-NaOH (20:1 v/v), decomp. 138°. (Found: C, 38.7; H, 4.0; N, 9.1; Na 7.5. $C_{18}H_{20}N_4Na_2O_{10}S_2 \cdot 2H_2O$ requires C, 38.2; H, 4.3; N, 9.9; Na, 8.1%). The impure preparations, as with sulphadimethoxine, could be used for chromatographic purposes.

N⁴-Sulphonates. These conjugates were synthesised from 4-methoxy-, 5-methoxy- and 4,5-dimethoxy-6-sulphanilamidopyrimidines by the method used for sulphadimethoxine (Chapter 3). The products were recrystallised from absolute ethanol. Dipotassium 1-p-(4'-methoxypyrimidin-6'-ylsulphamoyl)benzene sulphamate. (Found: C, 29.6; H, 2.7; K, 16.1; N, 11.7.

$C_{11}H_{10}K_2N_4O_6S_2 \cdot \frac{1}{2}H_2O$ requires C, 29.6; H, 2.5; K, 17.6; N, 12.6%).

Dipotassium 1-p-(5'-methoxypyrimidin-6'-ylsulphamoyl)benzene sulphamate.

(Found: C, 29.6; H, 2.7; K, 16.3; N, 11.7. $C_{11}H_{10}K_2N_4O_6S_2 \cdot \frac{1}{2}H_2O$ requires C, 29.6; H, 2.5; K, 17.6; N, 12.6%). Dipotassium

1-p-(4',5'-dimethoxypyrimidin-6'-ylsulphamoyl)benzene sulphamate

(Found C, 29.1; H, 3.4; K, 14.9; N, 10.6. $C_{12}H_{12}K_2N_4O_7S_2 \cdot 2H_2O$ requires C, 28.6; H, 3.2; K, 15.6; N, 11.1%).

N¹-Glucuronides. Due to the small quantity of some sulphonamides available, attempts were made to synthesise only sulphamonomethoxine N¹-glucuronide and sulphorthodimethoxine N¹-glucuronide.

The sodium salt of sulphamonomethoxine (3.16 g.) and acetobromoglucuronic acid methyl ester (4.13 g.) were dissolved in aqueous acetone (75% v/v) (50 ml.) and kept 6 days. The solution was passed through a column (2.5 x 25 cm.) of activated alumina (type H; 100-200 mesh) (P. Spence Ltd., Widnes, Lanes.) and eluted with acetone. The eluate (200 ml.) was evaporated to dryness and crystallised from propan-2-ol to give white needles of the pure methyl 2,3,4-tri-O-acetyl-1-deoxy-1-[N¹-(4''-methoxypyrimidin-6''-yl)sulphanilamidoglucosid]uronate, m.p. 183-184° and $\left[\alpha\right]_D^{17.5} + 68^\circ$ (c 1.05 in acetone). (Found: C, 47.0; H, 4.9; N, 9.1; S, 5.4. $C_{24}H_{28}N_4O_{12}S \cdot H_2O$ requires C, 46.9; H, 4.9; N, 9.1; S, 5.2%).

The above ester was treated with barium methoxide in a similar way to the ester of sulphadimethoxine N¹-glucuronide. The crude yellow ammonium salt of sulphamonomethoxine N¹-glucuronide (100 mg.) could not be induced to crystallise. However, it could be used as such for chromatographic purposes.

The potassium salt of sulphorthodimethoxine when treated as above gave no recognisable product. The reaction mixture darkened with keeping and could not be purified on an alumina column. On treating the brown oil (obtained by concentration) with barium methoxide, only sulphorthodimethoxine was isolated. Chromatography indicated the presence of sulphorthodimethoxine N¹-glucuronide in the reaction mixture.

METHODS

Experimental methods and animals were as described previously (Chapter 3). Four monkeys were used in rotation for all the experiments; a period of at least 14 days elapsed between successive doses in the same animal. Not all sulphonamides could be administered to man because the toxicities of some were not known.

Isolation of major metabolites. The 24 hr. urine from animals receiving 100 mg./kg. sulphonamide orally was adjusted to pH 5 with acetic acid and extracted twice with an equal volume of ethyl acetate. The two phases were separated by centrifugation where necessary, and the aqueous layer discarded. The ethyl acetate extracts were combined and the metabolite taken into saturated NaHCO_3 solution ($\frac{1}{2}$ vol.). On acidification, the metabolite crystallised. Metabolites were recrystallised and characterised by m.p., mixed m.p., infrared spectrum and chromatographic properties.

TABLE 5.2

Isolated metabolites of the methoxy-6-sulphanilamidopyrimidines.

6-Sulphanilamido- pyrimidine	Metabolite	Species	m.p.
2-Methoxy-	N^4 -Acetyl	Rabbit	224-5
4-Methoxy-	N^4 -Acetyl	Rabbit	217-9
5-Methoxy-	Unchanged	Monkey	200
2,5-Dimethoxy-	Unchanged	Rabbit	189-92
4,5-Dimethoxy-	N^4 -Acetyl	Rabbit	212-3

RESULTS

Identification of metabolites. Apart from those major metabolites isolated, identification has been largely dependent on chromatography, comparison with the synthesized compounds and analogy with the metabolites of sulphadimethoxine. Table 5.3 shows the close similarity of R_F values of the series of compounds. The bases of identification have been discussed in Chapter 3.

Metabolism of 2-methoxy-6-sulphanilamidopyrimidine. Recoveries in the first 24 hr. are high in the rabbit (67%) and the monkey (57%). The value obtained with the rat (19%) is lower but significantly higher than the corresponding value for sulphadimethoxine (8.5%). The major metabolite in the rabbit is the N^4 -acetyl derivative, whereas in the rat and monkey the unchanged sulphonamide predominates (Table 5.4).

Metabolism of 4-methoxy-6-sulphanilamidopyrimidine. The good recovery of this sulphonamide in the rabbit (83% in two days) could possibly be accounted for by the almost exclusive excretion of the N^4 -acetyl derivative in the urine (98% in the excreted dose). However, the rat shows good recoveries (65% in two days) but excretes only 40% of the recovered dose in the urine as the acetylated compound (Table 5.5). There is a trace of N^1 -glucuronide excreted. As with sulphadimethoxine, this metabolite is found in much larger quantities in rat bile (Table 5.9, 22% of the excreted dose).

Like the rabbit, the monkey highly acetylates this sulphonamide (89% of the excreted dose), but whereas the rabbit showed no untoward

TABLE 5.3

Colour reactions of the metabolites of the methoxy-6-sulphanilamidopyrimidines and their R_F values in the solvent system D [propan-2-ol-ammonia solution (s.g. 0.880) (7:3 by vol.)]

All compounds quenched the background fluorescence of the paper under ultraviolet light (254 mμ).

Metabolite	Position of methoxy substituents						Colour reaction ^a		
	2	4	5	2,4	2,5	4,5	DMAC	NR	Barh
N^4 -Glucuronide	0.10	0.07	0.08	0.11	0.07	0.09	Purple	Blue	None
N^4 -Sulphonate	^b	0.36	0.30 ^c	0.42	0.30 ^c	0.20	Purple (5-10 m.)	None	White
N^1 -Glucuronide	0.35 ^c	0.48	^b	0.54	^b	0.32	Purple (30 m.)	Blue- brown	None
Sulphonamide	0.64	0.63	0.68	0.72	0.60	0.56	Purple	None	None
N^4 -Acetyl	0.76	0.75	0.77	0.84	0.72	0.71	None*	None	None

* Developed a purple spot after keeping overnight.

^a DMAC = p-dimethylaminocinnamaldehyde NR = naphtharesorcinol.

Barh = barium chloride - sodium rhodizonate (see Chapters 2 and 3).

^b Metabolite not observed.

^c Not compared with synthetic compound.

TABLE 5.4

Fate of 2-methoxy-6-sulphanilamidopyrimidine in various species after oral administration (100 mg./kg.).

Recoveries represented as proportion of the dose (%).

Species		Rabbit	Rat	Monkey
Sex		Female	Female	Female
No. of animals		3	3	2
'Free' amine in 24 hr. urine		25 $\pm$ 5	3.8 $\pm$ 1.4	51
Total amine in 24 hr. urine		67 $\pm$ 8	19 $\pm$ 7	57
% of the	(N ⁴ -glucuronide	0	2 $\pm$ 1	2
excreted	(N ⁴ -sulphonate	0	0	0
dose in 24 hr.	(N ¹ -glucuronide	0	2 $\pm$ 2	5
urine as	(sulphonamide	33 $\pm$ 7	76 $\pm$ 9	84
	(N ⁴ -acetyl	67 $\pm$ 7	20 $\pm$ 6	9
'Free' amine in 48 hr. urine		0.6 $\pm$ 0.1	8.2 $\pm$ 1.3	1.3
Total amine in 48 hr. urine		3.7 $\pm$ 0.1	10.6 $\pm$ 2.4	1.6

TABLE 5.5

Fate of 4-methoxy-6-sulphanilamidopyrimidine in various species after oral administration.

Recoveries represented as proportion of the dose (%).

Species	Rabbit	Rat	Monkey	Man
Sex	Female	Female	Female	Male
No. of animals or subjects	3	3	2	1
Dose level (mg./kg.)	100	100	100	8
'Free' amine in 24 hr. urine	3.0 $\pm$ 0.0	19 $\pm$ 7	1.5 ^a	17
Total amine in 24 hr. urine	76 $\pm$ 5	40 $\pm$ 12	12 ^a	29
% of the excreted dose in 24 hr. urine as	(N ⁺ -glucuronide	0	1	3
	(N ⁺ -sulphonate	0	1	0
	(N ⁺ -glucuronide	0	2	18
	(sulphonamide	2 $\pm$ 1	7	18
	(N ⁺ -acetyl	98 $\pm$ 1	89	61
'Free' amine in 48 hr. urine	0.7 $\pm$ 0.1	10*	0.3 ^a	-
Total amine in 48 hr. urine	7.4 $\pm$ 3.3	25*	2.3 ^a	-

* One animal died

^a Animals showed oliguria

TABLE 5.6

Fate of 5-methoxy-6-sulphanilamidopyrimidine in various species after oral administration (100 mg./kg.).

Recoveries represented as proportion of the dose (%).

Species	Rabbit	Rat	Monkey
Sex	Female	Female	Female
No. of animals	3	3	2
'Free' amine in 24 hr. urine	5.8 $\pm$ 1.1	9.1 $\pm$ 1.3	3.0
Total amine in 24 hr. urine	82 $\pm$ 3	20 $\pm$ 3	17
% of the excreted dose in 24 hr. urine as	(N ⁺ -glucuronide	1 $\pm$ 0	3 $\pm$ 0
	(N ⁺ -sulphonate	1 $\pm$ 0	0
	(N ⁺ -glucuronide	0	3 $\pm$ 0
	(sulphonamide	6 $\pm$ 1	31 $\pm$ 9
	(N ⁺ -acetyl	92 $\pm$ 1	63 $\pm$ 8
'Free' amine in 48 hr. urine	0.7 $\pm$ 0.1	-	2.1
Total amine in 48 hr. urine	2.3 $\pm$ 0.1	-	12

TABLE 5.7

Fate of 2,5-dimethoxy-6-sulphanilamidopyrimidine in various species after oral administration (100 mg./kg.).

Recoveries represented as proportion of the dose (%).

Species	Rabbit	Rat	Monkey
Sex	Female	Female	Female
No. of animals	3	3	2
'Free' amine in 24 hr. urine	66 ± 12	58 ± 12	42
Total amine in 24 hr. urine	66 ± 12	58 ± 12	42
% of the excreted dose in 24 hr. urine as	(N ⁴ -glucuronide	1 ± 0	1
	(N ⁴ -sulphonate	0	1
	(N ¹ -glucuronide	0	1
	sulphonamide	92 ± 0	94
	(N ⁴ -acetyl	7 ± 0	3
'Free' amine in 48 hr. urine	4.6 ± 0.5	-	1.7
Total amine in 48 hr. urine	5.9 ± 0.5	-	1.7

TABLE 5.8

Fate of 4,5-dimethoxy-6-sulphanilamidopyrimidine

in various species after oral administration.

Recoveries represented as proportion of the dose (%).

Species	Rabbit	Rat	Monkey	Man
Sex	Female	Female	Female	Male
No. of animals or subjects	3	3	3	2
Dose level (mg./kg.)	100	100	100	17
'Free' amine in 24 hr. urine	15 ± 3	8.0 ± 1.0	5.0 ± 0.9	7.6
Total amine in 24 hr. urine	63 ± 4	19 ± 1	25 ± 4	22
% of the excreted dose in 24 hr. urine as	(N ⁴ -glucuronide	2 ± 1	4 ± 1	4
	(N ⁴ -sulphonate	0	5 ± 1	1
	(N ¹ -glucuronide	0	0	2
	sulphonamide	9 ± 2	26 ± 3	8
	(N ⁴ -acetyl	89 ± 2	66 ± 4	85
				69
'Free' amine in 48 hr. urine	2.9 ± 1.1	8.1 ± 0.7	-	4.8
Total amine in 48 hr. urine	12 ± 3	27 ± 2	-	13

TABLE 5.9

**Excretion of sulphamonomethoxine and sulphorthodimethoxine
in rat bile after intraperitoneal administration (100 mg./kg.).**

Sulphonamide	Sulphamonomethoxine	Sulphorthodimethoxine
No. of animals	3	3
'Free' amine in 24 hr. bile	3.8 ± 1.3	5.4 ± 0.6
Total amine in 24 hr. bile	3.9 ± 0.8	6.4 ± 0.2
% of the $(N^4\text{-glucuronide})$	2	4 ± 3
excreted $(N^4\text{-sulphonate})$	0	1 ± 1
dose in $(N^1\text{-glucuronide})$	22	0
24 hr. (sulphonamide)	73	89 ± 6
bile as $(N^4\text{-acetyl})$	3	6 ± 6

symptoms, possibly due to the higher pH of its urine and consequent greater solubility of the metabolite, all monkeys given this compound showed extensive oliguria, excreting about 50 ml. urine per day compared with a more normal 150-200 ml. per day. This effect was undoubtedly due to partial kidney blockage by crystals of the metabolite. Renal damage probably accounts for the high toxicity of 4-methoxy-6-sulphanilamido-pyrimidine in the monkey (Spinks, 1963).

The compound has been tested clinically in Japan (Oshima et al., 1962), being reported to be only moderately acetylated. The one human subject tested (Table 5.5) at a very low dose level (8 mg./kg.) acetylated the compound to the extent of 61%. This can be considered to be high for man.

N¹-glucuronide was a minor metabolite in the monkey (2% of the excreted dose) and a more significant one in the one human subject examined (18%). Since the synthetic N¹-glucuronide was available, this identification was definite. The amount of glucuronide formed is unlikely to ameliorate possible toxic effects in man due to the high degree of acetylation.

Metabolism of 5-methoxy-6-sulphanilamidopyrimidine. Table 5.6.

This sulphonamide is very highly acetylated in all three species examined. Kidney function did not appear to be affected in any of the animals. Again recovery was good in the rabbit (82% in one day, 92% of the excreted dose acetylated). Recovery was average for this group of compounds in the rat and monkey (20 and 17% in one day respectively).

Traces of a metabolite suspected of being an N¹-glucuronide were observed in rat and monkey urine (2-3% of the excreted dose). Since

the synthetic compound was not available, it was not identified with certainty.

Metabolism of 2,5-dimethoxy-6-sulphanilamidopyrimidine. If this compound were active bacteriostatically, it would appear to be an ideal short-acting sulphonamide. It was rapidly excreted by the rabbit, rat and monkey (Table 5.7). Moreover, the only significant product in the urine was the unchanged sulphonamide (92, 99 and 94% of the excreted dose in rabbit, rat and monkey, respectively).

Metabolism of 4,5-dimethoxy-6-sulphanilamidopyrimidine. The metabolism of sulphorthodimethoxine is of great interest since the compound will probably be widely used prophylactically due to its long half-life in man.

Recovery of the sulphonamide was markedly low only in man (5.5% first day, 6.0% second day, see Table 5.8). In other species recovery in 24 hr. urine was average for this class of compounds in the rabbit, rat and monkey.

The difference in the excretion rate between man and other species is paralleled by the degree of acetylation, being high in the rabbit, rat and monkey (89, 66 and 85% respectively) but low in man (29%). This compound of the series formed much greater amounts of N^4 -sulphonate than the others in the rat, monkey and man (5, 16 and 9% of the excreted dose respectively). This sulphate conjugate should be isolatable; no sulphonamide N^4 -sulphonate has yet been isolated.

Unchanged sulphorthodimethoxine predominates in rat bile (Table 5.9), contrasting with the metabolic picture in rat urine.

CHAPTER 6

THE IN VITRO SYNTHESIS OF SULPHONAMIDE N¹-GLUCURONIDES

The biological occurrence of sulphonamide N^1 -glucuronides has only recently been recognised. Those detected by Japanese workers, derivatives of sulphathiazole and sulphisomezole, have only occurred as trace metabolites in human urine (Uno, Machida, Hanai and Ueda, 1963; Ueda and Kuribayashi, 1964). Sulphadimethoxine N^1 -glucuronide, however, occurs to the extent of 70% of the excreted dose in human and monkey urine. It is also found in the urine of the dog, rat and guinea-pig (Chapter 3). Sulphadimethoxine appeared to be an ideal substrate with which to investigate the synthesis in vivo of N^1 -glucuronides.

EXPERIMENTAL

Methods

Chromatography. In addition to the solvents listed in Table 2.1, propan-1-ol-ammonia solution (s.g. 0.880) (7:3 by volume) was also used. Spray reagents were as in Chapter 2.

Tissue preparations. Female rat and guinea-pig tissues were removed immediately after death from a blow on the neck and placed in crushed ice. Livers were homogenised in a Potter tube with a glass or Teflon plunger with three times their volume of ice-cold 1.15% KCl solution. The 10,000 g supernatant was obtained by centrifuging for 10 m. in an M.S.E. "Super-speed 40" centrifuge (Measuring and Scientific Equipment, Ltd., London, S.W.1). Liver slices (0.5-1.0 mm. thick) were cut by hand with a stainless steel blade and immediately placed in the incubate. Kidney and adrenal tissue was treated in a similar way to liver. Small intestine was washed with ice-cold KCl to remove its contents, chopped finely and

homogenised thoroughly. Tissue homogenates and 10,000 g supernatants were used as soon as possible.

Incubations and estimations. o-Aminophenol conjugation was determined by the method of Dutton and Storey (1962) for both tissue homogenates and slices. The substrate solution was prepared from o-aminophenol hydrochloride; the latter was found to be easier to purify than the free base.

Incubates to determine sulphadimethoxine conjugation were prepared similarly except that an equimolar amount of sulphadimethoxine replaced the o-aminophenol substrate. The addition of ascorbic acid was unnecessary. A typical incubation with a tissue suspension was as follows:

Into each of three 15 ml. centrifuge tubes was pipetted the tissue preparation (1.0 ml.), 0.14 mM-sulphadimethoxine in sufficient NaOH to achieve solution (0.2 ml.), 0.5 mM-UDPGA (0.2 ml.), water 1.4 ml.) and 0.5 M-tris buffer pH 7.4 (0.2 ml.). Control tubes contained either boiled tissue preparation or 0.028 mM-sodium glucuronate (1.0 ml.). After incubation at 37° for 1 hr. in a shaking water-bath (H. Mickle Ltd., Gomshall, Surrey), the tubes were plunged into a boiling water-bath for 60 sec., cooled in running water and centrifuged at 2000 g for 10 m. Acetic acid (0.1 ml.) was added to part of the supernatant (2.0 ml.) and the solution then extracted with ethyl acetate saturated with water (2 x 2.5 ml.) to remove free sulphadimethoxine (the tubes being centrifuged to break any emulsion formed). 2 N-HCl (1.0 ml.) was added to the aqueous phase and the solution centrifuged at 2000 g for 5 m. Part of the supernatant (2.5 ml.) was then treated by the Bratton and Marshall method.

A calibration curve was prepared using mixtures of sulphadimethoxine and sulphadimethoxine N^1 -glucuronide. Mean recovery in the range of 10-100% conjugation was $98 \pm 3\%$.

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 ml. of Krebs bicarbonate Ringer solution pH 7.4. To the first flask rat liver slices (approximately 100 mg. wet weight) and 0.1 ml. sulphadimethoxine (0.5 μ moles in 0.9% NaCl) was added, to the second rat liver slices and 0.1 ml. of 0.9% NaCl and to the third sulphadimethoxine (0.5 μ moles) plus 0.1 ml. of sodium glucuronate solution (0.5 μ mole in 0.9% NaCl). Each flask was gassed with O_2 containing 5% CO_2 , immediately stoppered and placed in a shaking water bath at 37° (H. Kickle Ltd., Gosshall, Surrey) for 80 min. At this time 0.5 μ moles of sulphadimethoxine was added to the second flask, liver slices were then immediately withdrawn from all flasks, drained on filter papers and weighed. The supernatants were poured into centrifuge tubes, placed in a boiling water bath for 1 min., cooled in running water, and then estimated as above. Formation of sulphadimethoxine- N^1 -glucuronide by the rat liver slices was demonstrated qualitatively by paper chromatography in butan-1-ol-ammonia solution-water, butan-1-ol-acetic acid-water and propan-1-ol-ammonia solution.

The synthesis of glucuronide was also studied by following the incorporation of $[^{14}C]$ -glucose into sulphadimethoxine- N^1 -glucuronide. The procedure used was as follows: rat liver slices were incubated

with 2 ml. of Krebs bicarbonate Ringer solution pH 7.4 containing 5×10^5 d.p.m. of $[^{14}\text{C}]$ -glucose (0.06 μ moles) and sulphadimethoxine as described above. After incubating for 1 hr. at 37° the liver slices were immediately withdrawn from the flasks, drained and homogenized in dioxan (10 ml.). Controls were prepared as above except that sulphadimethoxine was added after incubation. Samples of this homogenate (1 ml. and 2 ml.) were pipetted into dioxan, PPO, POPOP scintillator containing 5% Carbosil gel and their radioactive content determined in a Tri-Carb scintillation counter model no. 3214 (Packard Instrument Co. Ltd., Wembley, Middlesex). Disintegrations per minute were then calculated using the channel ratio method, (Davies, 1965). The glycogen content of the liver was determined by the method of Hassid and Abraham (1957). To the supernatant from each incubate 100 mg. of sulphadimethoxine- N^1 -glucuronide (NH_4 salt) was added which dissolved on warming. The solutions were placed in a boiling water bath for 1 min. to deproteinize and centrifuged at 2500 g. for 5 min. The supernatant was decanted off into other tubes and β -benzylthiouronium chloride (0.3 g.) was added to each of these with warming. On standing crystals of the β -benzylthiouronium salt of sulphadimethoxine- N^1 -glucuronide were formed (100 mg.) which on recrystallising twice from water yielded the pure β -benzylthiouronium salt (30-50 mg.). This material was counted in dioxan PPO, POPOP scintillator containing 5% Carbosil gel and its true radioactive content determined by the channels ratio method.

RESULTS

In vitro conjugation of sulphadimethoxine. Tissue homogenates from the small intestine, kidneys, adrenals and liver appeared unable to catalyse the conjugation of sulphadimethoxine. The trace of colour obtained in the estimation method could be ascribed to non-enzymic formation of the N^4 -glucuronide, since controls containing glucuronate in place of the tissue suspension gave similar results. The tissue suspensions were all active in the conjugation of o-aminophenol (Table 6.1).

Liver slices from both the rat and guinea-pig were active in sulphadimethoxine N^1 -glucuronide formation compared with controls. The rate of conjugation with guinea-pig tissue was approximately 10% of that for o-aminophenol. Evidence to support the colorimetric results was difficult to obtain chromatographically because there was little conjugate synthesised and the chromatograms of tissue extracts showed extensive streaking of the spots, in spite of deproteinisation and desalting. Nevertheless there appeared to be a spot corresponding to sulphadimethoxine N^1 -glucuronide in solvents C and D.

Confirmatory evidence was sought by means of isotope dilution. A small amount of labelled glucose was added to the incubates; it was assumed that this equilibrated with the glucose pool within the cells of the liver slice, some label proportionately being incorporated into the UDPGA and thence into sulphadimethoxine N^1 -glucuronide. The labelled glucuronide was diluted with carrier, a derivative crystallised, purified and counted. Dilution from test incubations showed higher activity

than those from control incubations (1840 compared with 1320 disintegrations/m./g. liver, difference significant at the 1% level). Uptake of label from the medium by the slices showed no significant difference (test 19 200 d.p.m., control 17 200 d.p.m.). The rate of conjugation was calculated on the basis the above assumptions (Table 6.1).

Phenobarbitone pretreatment of the animal prior to preparation of the liver slices had a slight effect on the in vitro synthesis of sulphadimethoxine N^1 -glucuronide (Table 6.1). The difference was not statistically significant.

Other sulphonamide substrates. 2-Methoxy- and 4-methoxy-6-sulphanilamido-pyrimidine were tested in the sulphadimethoxine conjugating system. The 4-methoxy derivative was conjugated at 10-40% the rate of sulphadimethoxine and the 2-methoxy derivative at 0-10%.

DISCUSSION

The evidence in favour of an enzymic synthesis of N^1 -glucuronides derives from several sources.

1. Colorimetric analyses indicate an increase in the water-soluble and diazotisable material on incubation with sulphadimethoxine with liver slices, but not liver homogenates. Sodium glucuronate in place of the slices did not produce these results, showing non-enzymic formation of the N^1 -glucuronide was not responsible.

2. Chromatography shows the presence of a spot corresponding to sulphadimethoxine N^1 -glucuronide.

TABLE 6.1

Ability of rat and guinea-pig liver preparations
to conjugate sulphadimethoxine and o-aminophenol. Data given
as μ moles glucuronide formed/g. liver/hr.

Species	Preparation	Substrate	
		Sulphadimethoxine	o-Aminophenol
Rat	Slices	0.28*	-
	Slices	0.29 ± 0.03	-
	Homogenate	0	-
	10 000 g supnt.	0	0.66
Guinea-pig	Slices	0.07 ± 0.01	8.2 ± 0.5
	Slices ^a	0.11 ± 0.04	29 ± 3
	Homogenate	0	4.0

* Approximate value derived from isotope dilution.

^a Slices prepared from the livers of animals pretreated with phenobarbitone.

3. Isotope dilution confirms the colorimetric results, both qualitatively and quantitatively.

4. Incubation of other sulphonamide substrates gave much lower rates of conjugation, in agreement with the in vivo results (Chapter 5). If the colorimetric measurement were an artifact, then other sulphonamides would be expected to give a similar result.

Since liver suspensions do not conjugate sulphadimethoxine, but liver slices appear to do so, it seems that an organised cell system is essential for the synthesis of sulphadimethoxine N^1 -glucuronide. The system appears to be more complex than that required for the conjugation of o-aminophenol. Phenobarbitone pretreatment greatly increased the rate of synthesis of o-aminophenol glucuronide (briefly reported by Inscoc and Axelrod, 1960), but did not significantly affect sulphadimethoxine glucuronide synthesis. Phenobarbitone pretreatment is thought to induce the synthesis of drug-metabolising enzymes (e.g. Remmer and Merker, 1965). In spite of the greater concentration of enzyme available, the rate of conjugation of sulphadimethoxine did not increase significantly. Some other process must be rate-limiting. Access of sulphadimethoxine to the conjugating site cannot be the rate-limiting step, because liver homogenates would then be expected to be more active and not less, as was observed.

If it is assumed that the free energy change is sufficiently great in passing from sulphadimethoxine to sulphadimethoxine N^1 -glucuronide, then the reaction:

sulphadimethoxine + UDPGA = sulphadimethoxine N^1 -glucuronide + UDP
will be in equilibrium. An active transport system will be required to

remove the product and displace the equilibrium in favour of the synthesis of the glucuronide. In the absence of some system for the removal of the glucuronide, only undetectable quantities of the glucuronide will be formed. This is possibly the situation in tissue suspensions.

In the intact liver, specific systems are thought to transport excreted products into the bile. Such an active transport system has been indicated for sulphadimethoxine N¹-glucuronide.

CHAPTER 7

THE ACTION OF β -GLUCURONIDASE ON SULPHADIMETHOXINE N¹-GLUCURONIDE

Koechlin, Kern and Engelberg (1959) found that β -glucuronidase did not hydrolyse the glucuronide of sulphadimethoxine found in human urine. We found that the enzyme did catalyse the hydrolysis of sulphadimethoxine N^1 -glucuronide, although at a slow rate (Chapter 2). Both the synthetic and biosynthetic material yielded sulphadimethoxine and glucuronic acid.

This chapter describes in vitro experiments to investigate the reaction more fully, since it is the first example of an N -glucuronide being hydrolysed by the enzyme.

EXPERIMENTAL

Materials

Sulphanilamide N^4 -glucuronide (sodium salt) had been previously synthesised (Bridges, 1963), m.p. 195-205° (decomp.). β -Glucuronidase was derived from beef liver (Warner-Chilcott 'Ketodase'). Phenolphthalein glucuronide (Koch-Light Ltd.) and potassium hydrogen saccharate (British Drug Houses Ltd.) were purchased. A solution of saccharo-1,4-lactone was obtained by boiling a solution of potassium hydrogen saccharate (Levy, 1952).

Methods

Estimation of the action of β -glucuronidase on sulphadimethoxine

N^1 -glucuronide. A typical procedure was as follows. Ketodase (0.5 ml.), 0.1M-sodium acetate buffer pH 5.0 (1.0 ml.), 0.5mM-ammonium sulphadimethoxine N^1 -glucosiduronate (1.0 ml.) and water (0.5 ml.) were incubated at 37° for 120 m. in a 15 ml. round-bottomed centrifuge tube without shaking. At the end of the incubation period the residual sulphadimethoxine N^1 -glucuronide was estimated.

The reaction was stopped by adding acetic acid (0.1 ml.). The mixture was extracted twice with ethyl acetate (2.5 ml.) with centrifuging if necessary to break any emulsion formed. Trichloroacetic acid (30% w/v) (1.0 ml.) was added to the aqueous layer and the mixture centrifuged for 5 m. at 2 000 g. Two samples of the upper layer were taken (1.0 ml.) and each diazotised and coupled by the Bratton and Marshall procedure with 0.1% sodium nitrite (1.0 ml.), 0.5% ammonium sulphamate (1.0 ml.) and 0.1% N-1-naphthylethylenediamine dihydrochloride (1.0 ml.). The solution was allowed to stand 15 m. and read at 540 μ in the Unicam SP 600 spectrophotometer.

A calibration curve for the hydrolysis of sulphadimethoxine N¹-glucuronide was prepared by adding known amounts of sulphadimethoxine and the glucuronide to 'incubates' and estimating in the same way. Recoveries were 90-95%.

Separate calibration curves were prepared for estimations in the presence of sulphanilamide and sulphanilamide N⁴-glucuronide.

Estimation of the hydrolysis of phenolphthalein glucuronide by β -glucuronidase

This reaction was estimated by the method of Levvy and Marsh (1959). A calibration curve was constructed from phenolphthalein in glycine-sodium hydroxide buffer pH 10.7.

RESULTS AND DISCUSSION

Action of β -glucuronidase on sulphadimethoxine N¹-glucuronide.

A progress curve for the hydrolysis of sulphadimethoxine N¹-glucuronide shows that the rate is approximately constant over a period of 120 m. Thus this period can be used for the determination of initial reaction

velocities. This is consistent with the assumption that initial reaction rates are constant while less than 20% of the substrate has been consumed (Dixon and Webb, 1964).

A plot of enzyme concentration against velocity of the reaction yields a straight line (Figure 7.1). The possibility of an endogenous inhibitor being present (see below) can be discounted on the basis of experiments conducted in the presence of the non-ionic surface-active agent Triton X-100. This agent frees the enzyme from inhibition derived from the tissue used in the preparation of the enzyme (Walker and Levy, 1953). No effect was observed (Figure 7.1).

The effect of substrate concentration on the velocity of the reaction is shown in Figure 7.2. The curve is approximately linear but cuts the horizontal axis at about 0.01 mM , rather than passing through the origin. This abnormality is reflected in the Lineweaver-Burke plot (Figure 7.3). The deviation from normal cannot be explained by the presence of endogenous inhibitors often associated with β -glucuronidase.

Similar unexplained abnormalities have been found in the action of β -glucuronidase on other substrates (Marsh, 1954). The non-linearity of the Lineweaver-Burke plot prevents a direct determination of K_m . However, an approximate extrapolation indicates that the curve cuts the horizontal axis very near the origin. Thus the value of K_m is very high. As the equilibrium conditions for the Michaelis-Menten equation presumably hold for this very slow reaction, $K_m = K_s$ the dissociation constant for the enzyme substrate complex. Hence $1/K_m$ is a measure of the affinity

of the substrate for the enzyme, i.e. the affinity of sulphadimethoxine N^1 -glucuronide for β -glucuronidase is very low. The slow rate of hydrolysis of the glucuronide is thus due to the low affinity of the substrate for the enzyme and not to the slow rate of breakdown of the enzyme-substrate complex to products.

An alternative determination of K_m from the substrate concentration which produces half the maximum velocity is not possible since the maximum velocity of the reaction has not been determined.

Action of inhibitors. Saccharo-1,4-lactone. Complete inhibition of the enzyme at a substrate concentration of 0.1mM was obtained by boiled potassium hydrogen saccharate (0.0004mM), a substrate-inhibitor ratio of 250.

The inhibition of the hydrolysis of sulphadimethoxine N^1 -glucuronide by saccharo-1,4-lactone is confirmation of the enzyme performing the hydrolysis being β -glucuronidase. α -glucuronidase has been shown not to be inhibited by saccharolactone (Levy and Marsh, 1959). This is additional evidence for the configuration of sulphadimethoxine N^1 -glucuronide being the β -form.

Sulphanilamide N^4 -glucuronide. The effect of sulphanilamide N^4 -glucuronide on the hydrolysis was tested in an attempt to detect any interaction between the latter and β -glucuronidase, which had not been previously demonstrated. No effect was shown up to a substrate-inhibitor ratio of 0.5 (concentration of sulphadimethoxine N^1 -glucuronide 0.1mM). Also, it was shown that no detectable hydrolysis of sulphanilamide N^4 -glucuronide by β -glucuronidase occurred, confirming the result of Bridges and

Williams (1962).

Phenolphthalein glucuronide. The competitive effect of phenolphthalein glucuronide was tested. This is known to have a high affinity for β -glucuronidase (Levy and Marsh, 1959). No detectable effect was shown on the hydrolysis of sulphadimethoxine N^1 -glucuronide. This was almost certainly due to the difficulty of maintaining a sufficient concentration of phenolphthalein glucuronide due to the rapidity with which it is hydrolysed by the enzyme.

Sulphadimethoxine N^1 -glucuronide is the first N -glucuronide shown to be susceptible to β -glucuronidase. It is thus intermediate between the aliphatic and aromatic amine N -glucuronides, which are not hydrolysed, and the various types of O -glucuronide, which are substrates for the enzyme.

As the dimensions of the $-NH-$ and $-O-$ groups are closely similar (Pauling, 1960), it would be expected that most N -glucuronides would fit the enzyme active centre. Since there has been no report that they inhibit β -glucuronidase and that sulphanilamide N^4 -glucuronide did not competitively inhibit the hydrolysis of sulphadimethoxine N^1 -glucuronide, it would appear that β -glucuronidase and amine N -glucuronides do not interact significantly. This must be due to the greater basicity of the imido group compared with the ether link, i.e. a greater electron density. However, in sulphadimethoxine N^1 -glucuronide the nitrogen atom is adjacent to two strongly electron-attracting moieties, the sulphonyl group and the pyrimidine ring. Thus in this type of N -glucuronide the

electron density at the nitrogen atom is greatly reduced, making it resemble an O-glucuronide more closely.

Meprobamate forms an N-glucuronide in vivo which is a conjugate of a primary amide. It is thus similar in structure to sulphadimethoxine N¹-glucuronide, which is derived from a secondary sulphonamide. However, it contains only the weaker electron-attracting carbonyl group and would not be expected to be hydrolysed so rapidly as sulphadimethoxine N¹-glucuronide, which itself is only hydrolysed slowly. Meprobamate N-glucuronide has been reported not to be acted upon by β -glucuronidase (Tsukamoto, Yoshimura and Tatsumi, 1963a).

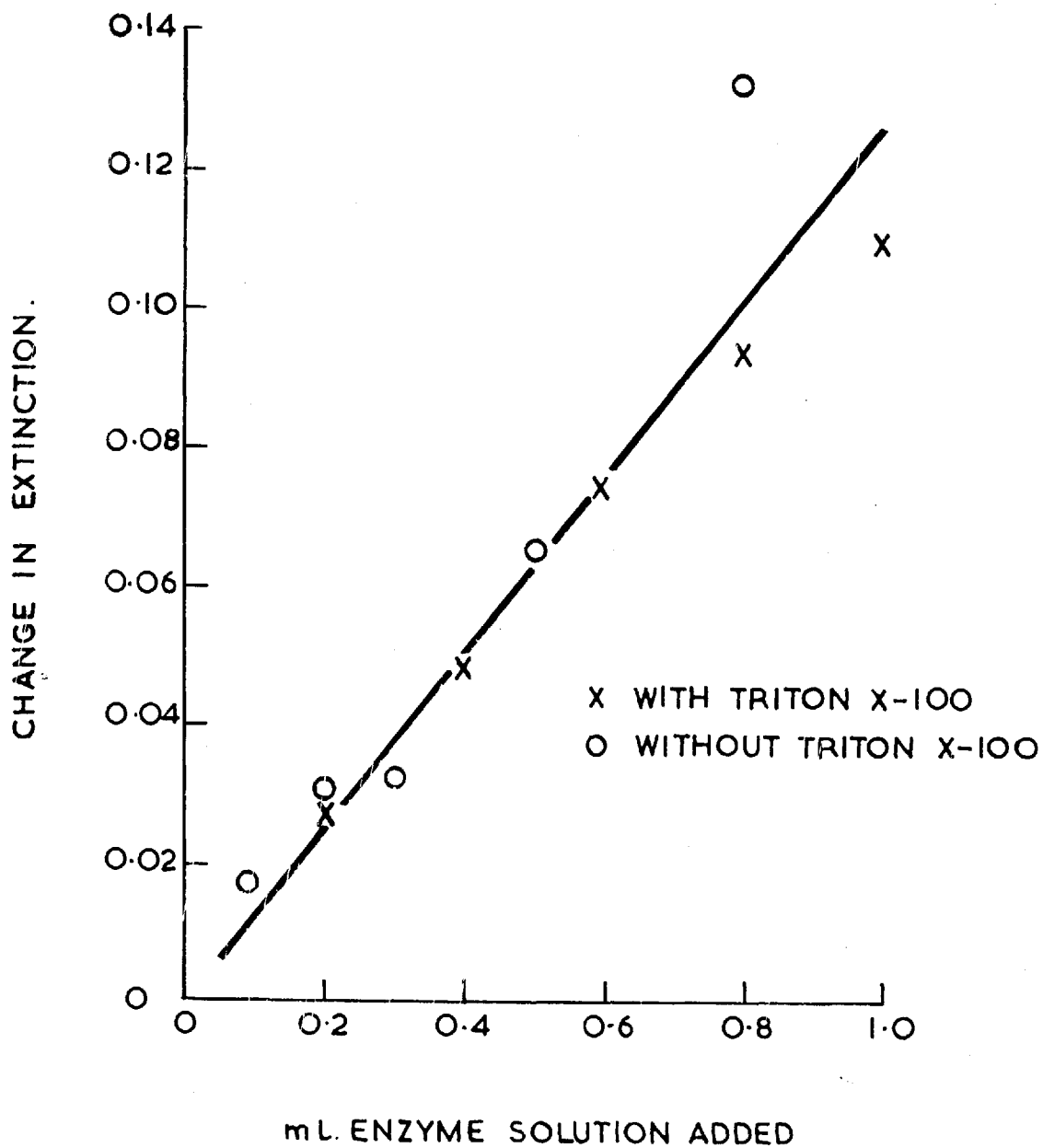


Figure 7.1 Velocity-enzyme concentration curve for the action of β -glucuronidase on sulphadimethoxine N^1 -glucuronide.

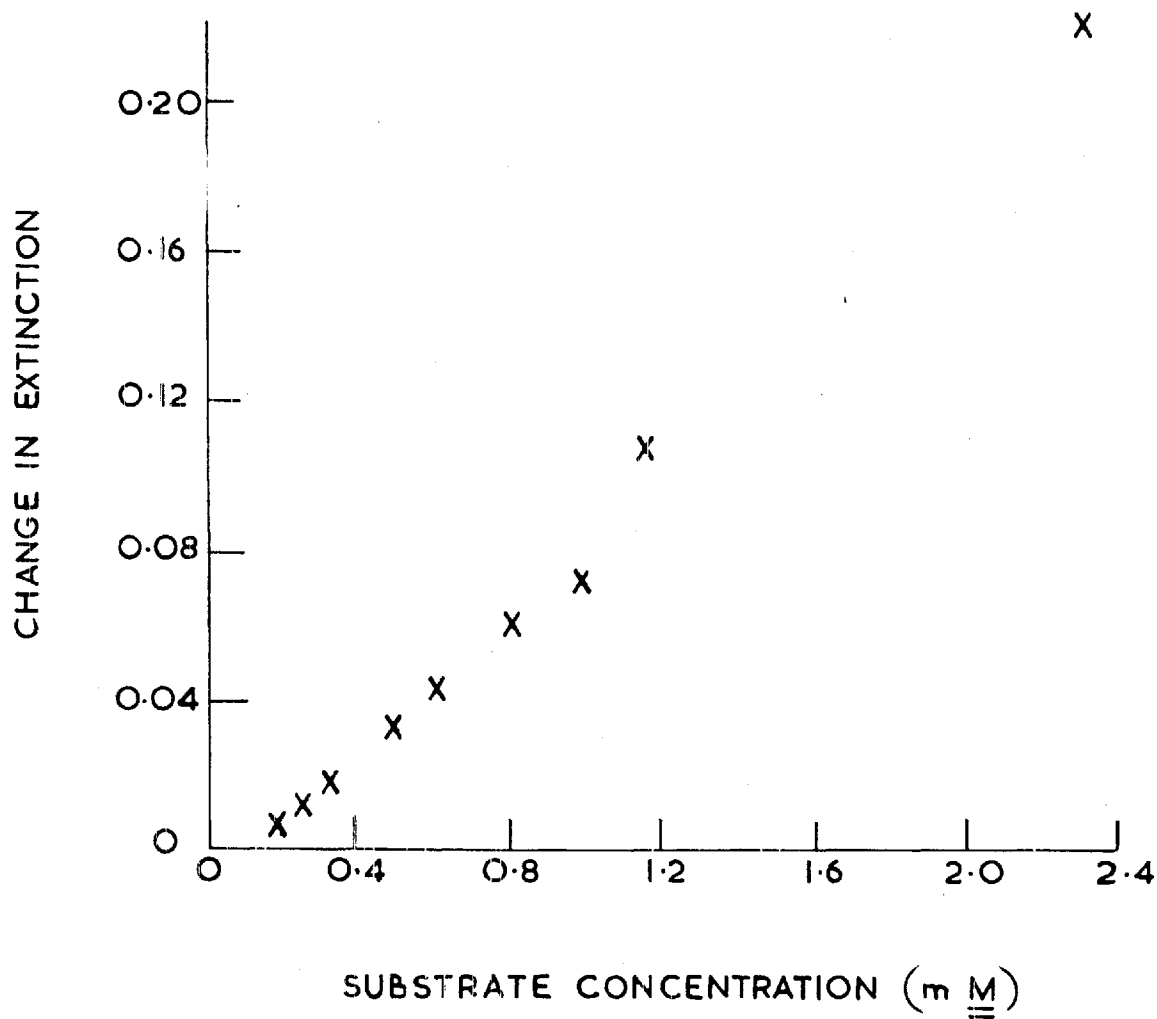


Figure 7.2 Velocity-substrate concentration plot for the action of β -glucuronidase on sulphadimethoxine N¹-glucuronide.

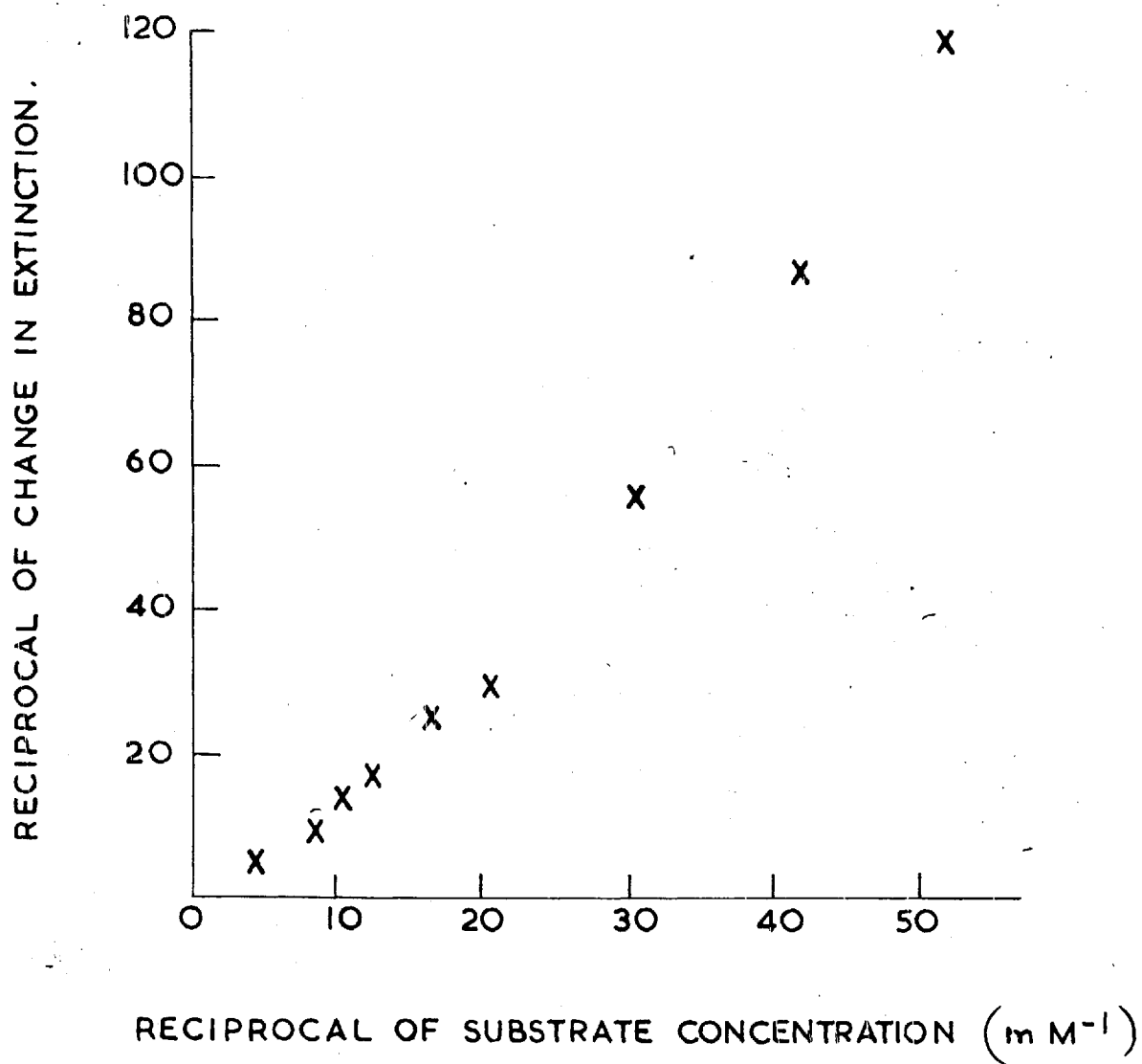


Figure 7.3 Lineweaver-Burke plot of the data shown in Figure 7.2.

CHAPTER 8

DISCUSSION

Recoveries of the methoxy-6-sulphanilamidopyrimidines

Recoveries of the six sulphonamides from the rabbit were uniformly high (71-84% of the dose), but low from the monkey (29-63%) and the rat (29-65%).

Further investigation of the low recovery of sulphadimethoxine from the rat showed that the urine was the only significant route of elimination after oral or parenteral administration. Sulphonamide was not detected in the faeces except after oral administration of high doses (400-800 mg/kg.), which were probably incompletely absorbed. An intraperitoneal dose is unlikely to be excreted even partly in the faeces since excretion in the bile was only a minor route. Poor absorption of sulphadimethoxine from the gastro-intestinal tract at lower doses did not contribute to the low recoveries observed because the recoveries did not differ significantly whether the sulphonamide was given orally or intraperitoneally (Chapter 2). The residual sulphonamide was not detected by qualitative examination of the tissues of a rat six days after administration of sulphadimethoxine (100 mg./kg.).

Low recoveries in the rat have appeared in cumulative recovery curves of sulphadimethoxine (e.g. Randall, Bagdon and Engelberg, 1959) but have not been commented upon.

The unrecovered sulphonamide may have passed undetected by being tightly or irreversibly bound to a component of the excreta or tissues. Urine can be excluded since sulphonamide added to normal urine was completely accounted for. Recovery of added sulphonamide from faeces

was less complete (40-90% according to the concentration). However, the amount which could possibly be overlooked in the faeces would not account for more than a small proportion of the dose.

One possible explanation of the low recoveries lies with the previously unreported observation that part of a dose of sulphadimethoxine is irreversibly bound to plasma protein in man and is probably not released until the protein molecule is broken down by normal catabolism (Kawerau, 1964). The other sulphonamides of the series may be bound similarly since they are structurally closely related.

There remains the possibility that the aromatic amino group, by which the sulphonamide is estimated, is metabolically removed or masked. Early in the biochemical investigation of sulphonamides it was suggested that the aromatic amino group of sulphanilamide might be converted to a hydroxylamino group (Mayer, 1937). This has not been confirmed. However, recently hydroxylamine derivatives of aromatic amines have been implicated in the carcinogenicity of the latter compounds, e.g. 2-acetamidofluorene (Miller, Miller and Hartmann, 1961) and 2-naphthylamine (Boyland, Dukes and Grover, 1963). There is no chromatographic evidence for the occurrence of hydroxylamines as metabolites of the sulphonamides of this series. Aromatic hydroxylamines are easily converted to o-aminophenols (e.g. Booth and Boyland, 1964); o-aminophenolsulphonamides also have not been found as metabolites. There is no other known metabolic reaction by which an aromatic amino group would escape being estimated.

Without the use of isotopically labelled compounds it is not always possible to account for the entire dose of a sulphonamide (e.g. Smith and

Williams, 1948a). The colorimetric estimation agrees with radiometry for determinations performed in blood, but other tissues usually yield much lower values by the former method (e.g. Fingl, Christian and Edwards, 1950). Estimations of ^{35}S in the mouse indicated that levels of sulphaphenazole were higher than those in the blood in the liver, kidney, lung and muscle in contrast with the colorimetric results (Schmid, Tripod and Gross, 1960).

Recoveries of the six sulphonamides investigated in the monkey, rat and rabbit are collected in Table 8.1.

Metabolism of the methoxy-6-sulphanilamidopyrimidines

N⁴-Glucuronides. There is good evidence for the non-enzymic synthesis of the N⁴-glucuronides of sulphonamides, in common with other aromatic amine glucuronides (Bridges and Williams, 1962). Added sulphadimethoxine was recovered from normal human urine as the glucuronide in sufficient quantities to account for the presence of this metabolite in urine after administration of sulphonamide. Combination of the unchanged sulphonamide with endogenous glucuronic acid probably accounts for the presence of N⁴-glucuronides in the urine and bile after administration of the other sulphonamides of the series.

The physiological significance, if any, of this conversion is unknown. It has been suggested that amino acids may be transported as N-glucuronides. However, in general, aliphatic compounds form these conjugates less readily than aromatic amines.

N⁴-Sulphonates. These conjugates appeared as metabolites of all the sulphonamides investigated, although only in trace amounts. Aryl sulphamates are thought to be formed enzymically (Roy, 1958). They undoubtedly occur as minor metabolites of many sulphonamides but there have been few reports of their existence (Uno and Kono, 1960; Uno and Ueda, 1960; Uno and Okazaki, 1960; Uno, Yasuda and Sekine, 1963; Bridges and Williams, 1963b).

N¹-Glucuronides. The significance of this recently discovered metabolic route is unknown. It forms a minor pathway in the metabolism of sulphathiazole (Uno and Ueda, 1962) and sulphamethoxazole (Ueda and Kuribayashi, 1964) and also most of the isomers investigated in this work, with the principal exception of sulphadimethoxine where it forms 70% of the excreted dose in man and the monkey.

In Chapter 6 it was shown that sulphadimethoxine N¹-glucuronide and others were probably synthesised enzymically by a system showing different properties from that conjugating o-aminophenol. The high degree of specificity of the enzyme system for sulphadimethoxine may be due to the latter's close resemblance to some naturally occurring compound (see below), in which the conjugation serves a biochemical function.

Species differences in metabolism

Throughout the series of six sulphonamides investigated, certain trends can be seen in the metabolism by each species. For instance the rabbit was the most active acetylator, followed by the monkey and the rat. The biochemical basis of species differences is largely an unknown field.

TABLE 8.1

Total urinary recovery of sulphonamide in 48 hr. from the monkey,
rat and rabbit. Data expressed as per cent of
the dose. Dose 100 mg./kg. p.o.

Animal	Position of methoxy groups					
	2	4	5	2,4	2,5	4,5
Monkey	59	14 ^a	29	63	44	35 ^a
Rat	30	65	20 ^b	29	58 ^b	46
Rabbit	71	83	84	76 ^c	72	75

* Animals showed oliguria.

^a Dose 17 mg./kg.

^b 24 hr. value.

^c 72 hr. value.

The rabbit appeared unable to form sulphonamide N^1 -glucuronides. Traces of N^1 -glucuronide were also found in the urine of the rat and monkey after administration of most of the sulphonamides of this series.

It appears that man resembles the monkey most closely in the proportion and nature of the metabolites of the methoxy-6-sulphanilamidopyrimidines. The data shown in Chapter 3 indicates that the monkey excretes the sulphonamides and their derivatives at a greater rate than man. Brodie (1964) states that the monkey generally does not treat foreign compounds in a similar way to man. However, he does not produce any examples to support his contention. The metabolism of sulphonamides might prove an exception to the general rule, in which case the monkey could be an appropriate animal in which to study new sulphonamide drugs prior to clinical trial. Their use in this fashion appears not to have been reported.

The rhesus monkey has also been shown to be similar to man in its metabolism of indolylacetic acid (Bridges, King and Williams, 1964). The major metabolite, indolylglutamine, is not a conjugate formed by lower mammals.

Structure-metabolism correlation

The extensive variation of metabolism of the methoxy-substituted 6-sulphanilamidopyrimidines with the location of the methoxy groups on the pyrimidine ring has been reported in detail in Chapters 3 and 5. The major metabolite for each sulphonamide-species combination is shown in the

TABLE 8.2

The principal metabolite of the methoxy-6-sulphanilamidopyrimidines
in the urine of man, the monkey, rat and rabbit.

Animal	2-methoxy	4-methoxy	5-methoxy	2,4-dimethoxy	2,5-dimethoxy	4,5-dimethoxy
Man	-	A	-	G	-	U
Monkey	U	A	A	G	U	A
Rat	U	U	A	A	U	A
Rabbit	A	A	A	A	U	A

A = N⁴-acetyl derivative

G = N¹-glucuronide

U = unchanged sulphonamide.

TABLE 8.3

Proportion (per cent) of the excreted dose as the N⁴-acetyl conjugate
of each sulphonamide in man, the monkey, rat and rabbit

Animal	2-methoxy	4-methoxy	5-methoxy	2,4-dimethoxy	2,5-dimethoxy	4,5-dimethoxy
Man	-	61	-	21	-	28
Monkey	9	89	85	21	3	85
Rat	20	42	63	46	0	67
Rabbit	67	98	92	94	7	87

TABLE 8.4

Proportion (per cent) of the excreted dose as N¹-glucuronide of each
sulphonamide in man, the monkey, rat, rabbit

Animal	2-methoxy	4-methoxy	5-methoxy	2,4-dimethoxy	2,5-dimethoxy	4,5-dimethoxy
Man	-	18	-	62	-	0
Monkey	5	2	2	70	1	2
Rat	2	2	3	7	0	0
Rabbit	0	0	0	0	0	0

It can be seen from the ranking order that the 2-methoxy group appears to strongly depress acetylation. This is confirmed by examination of the detailed results. In all species except the rat the 2,4-dimethoxy sulphonamide is acetylated to a lower degree than the 4-methoxy sulphonamide. In all species the 2,5-dimethoxy sulphonamide is acetylated less than the 5-methoxy analogue. 2-Methoxy-6-sulphanilamidopyrimidine is poorly acetylated in the monkey, rat and rabbit.

2,5-Dimethoxy-6-sulphanilamidopyrimidine is remarkable in that it is scarcely acetylated at all, even in the rabbit, which acetylates all its congeners to 67-98% of the excreted dose.

The compounds need to be examined in vitro with the mitochondrial acetylating system to distinguish whether the observed effects are intrinsic, caused by differing interactions with the enzymes of the acetylating system, or extrinsic, caused by differing ease of access to the mitochondria in the living animal.

There is an interesting correlation with the ultraviolet spectra of the sulphonamides (Appendix 1). Those sulphonamides lacking a 2-methoxy substituent show a single absorption maximum at 272-275 m μ , whereas those which contain a 2-methoxy substituent either show two maxima (2,4-dimethoxy at 261, 273 m μ ; 2,5-dimethoxy at 270, 295 m μ) or a single broad maximum (2-methoxy 268-280 m μ). It is possible that the 2-methoxy substituent affects the delocalisation of electrons within the molecule. By reducing the availability of electrons at the amino nitrogen, so the relative rate of acetylation is reduced (e.g. Perault and Pullman, 1963). In

confirmation, the sulphonamide by far the least acetylated, 2,5-dimethoxy-6-sulphanilamidopyrimidine, has the lowest molar extinction coefficient of the series (16 000 and 16 8000 compared with 19 300-23 000 for the remainder).

Electron delocalisation in the molecule should be reflected by the pK_a values of the amino group of the sulphonamides. An attempt was made to measure these by the ultraviolet absorption method, but the spectra of the ionic forms were not sufficiently differentiated for this to be done. Protonation of the pyrimidine ring was observed at the pH values involved which may render measurement of absolute pK_a values difficult.

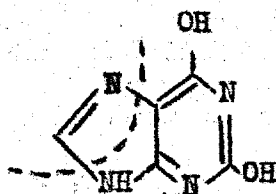
N^1 -Glucuronide conjugation. Data for the degree of conjugation are collected in Table 8.4.

Earlier workers have detected N^1 -glucuronides of sulphathiazole (Uno and Ueda, 1962) and sulphisomezole (Ueda and Kuribayashi, 1964) as minor metabolites in man. Assuming that man and the monkey are similar in their abilities to form these conjugates, as appears from Table 8.4, the N^1 -glucuronide is at best a minor metabolite of most of the sulphonamides investigated in this work. Apart from sulphadimethoxine, sulphamonomethoxine is the only other sulphonamide which may be metabolised to a significant extent along this pathway in man.

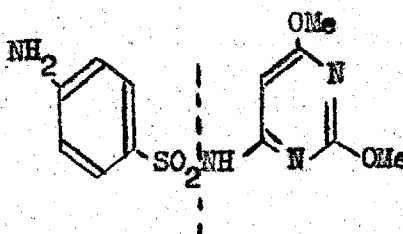
This metabolic route is almost specific for sulphadimethoxine in the series studied; removal of one of the two methoxy groups drastically

reduces the proportion of sulphonamide metabolised in this way by both man and the monkey. Whether it is specific for sulphadimethoxine among sulphonamides in general is unlikely. There are reports of quite unrelated sulphonamides, e.g. sulphaphenazole (Walter, 1963) forming large amounts of unidentified water-soluble metabolites. Some of these may well be N^1 -glucuronides. The more closely related sulphamethomidine (4-methoxy-2-methyl-6-sulphanilamidopyrimidine) has been reported to form a glucuronide in man (DiCarlo, Malament and Phillips, 1963).

It is interesting to speculate whether the specificity of this major route of detoxication for sulphadimethoxine is due to a resemblance to naturally occurring compounds. Sulphadimethoxine contains the pyrimidine ring which occurs in many biologically important compounds. However, none seems to provide a sufficiently close analogy. The purines, in particular xanthine (I), do contain elements in common with sulphadimethoxine (II).



I



II

Apart from the structural similarity, the acidic hydrogen atom of the sulphonamide group is analogous to the hydrogen atom of the imidazole ring of xanthine and other purines. Nucleosides contain a ribose

residue at this position 7. It is known that in intermediary metabolism glucuronic acid can give rise to pentoses, strengthening the analogy. However, as far as is known, purine nucleosides are synthesised by the pathway from ribosylamine derivatives. That is, the sugar residue is already in situ. Only dietary adenine is incorporated into nucleosides (Brown, Roll, Plechl and Cavalieri, 1948). A preliminary experiment designed to test this hypothesis was unsuccessful due to the high toxicity of the purine administered.

Other explanations of the specificity for sulphadimethoxine must rely on differing physico-chemical properties in the series. This data is not yet available. In view of the close structural relationship of the components of the series, it is unlikely that there will be major differences in physico-chemical parameters. Those sulphonamides containing a 5-methoxy group may differ from the remainder since the 5-position is the most truly aromatic in the pyrimidine ring. The 2- and 4-substituents, according to the extent and nature of ring substitution, have most influence on the electronic status of the pyrimidine ring, and hence the properties of the compound.

Long action of sulphonamides

No single convincing explanation has been proposed to account for the long action of sulphonamides. A single hypothesis would appear to be attractive in view of the overall similarities in chemical structure and physical properties of many of the clinically used long-acting sulphonamides.

The principal component of these compounds is a lipophilic substituent on the heterocyclic part of the molecule. It is usually a methoxy group; included in this class are sulphadimethoxine, sulphanethomidine, sulphamethoxy-pyridazine, and sulphamethoxy-pyrazine; sulphaphenazole contains a phenyl substituted imidazole ring. However, the gross similarities in structure of this class of compounds may be partly due to the conservatism of the research chemists employed in the development of sulphonamides and to the technical difficulties of cheaply obtaining heterocyclic compounds with more elaborate substituents.

Enterohepatic recirculation was at one time considered and subsequently rejected as a significant factor in the long action of sulphadimethoxine in the rat. The evidence adduced in Chapter 4 is in agreement with the conclusions of Preziosi, Loscalzo, Marmo and Miele (1963) and Milburn (1965) on different grounds. Most other long-acting sulphonamides for which data exist are excreted less in the bile than sulphadimethoxine (Greif and Seidl, 1960).

Protein binding was considered by Newbould and Kilpatrick (1960). It is true that many long-acting sulphonamides are bound to plasma proteins to a high degree. Certainly protein binding will reduce the amount filterable at the renal glomerulus. However, this cannot be taken as the complete explanation since some shorter acting sulphonamides are also extensively bound (e.g. Rieder, 1963a).

Despopoulos and Callahan (1962) investigated the tubular secretion of sulphonamides, most of which had short or medium duration of action.

Assuming that their conclusions are valid for long-acting compounds, the pK_a values of these sulphonamides, most of which are in the range 6-7, indicate that many should be secreted by the renal tubules. Differences in the rate of tubular secretion do not appear to correlate with the plasma half-lives, eliminating this as a principal factor in the mechanism of long action.

Litchfield (1960) suggested that the long action of sulphamethoxy-pyridazine was due to extensive tubular reabsorption whereas the long action of sulphadimethoxine was due to high protein binding limiting the rate of renal filtration. Such ad hoc explanations for each long-acting sulphonamide do not appear convincing.

Accumulation of sulphonamides in a depot followed by their gradual release could be held to prolong their duration of action. There is no evidence that long-acting sulphonamides are stored in the body in this way. Tissue binding, discussed above, is probably irreversible.

It was tenable that long-acting sulphonamides were metabolised in a specific way, enabling them to be excreted at a slower rate. Many long-acting sulphonamides have been shown to form unidentified metabolites (Walter, 1963). It was shown by us that sulphadimethoxine was metabolised in those animals in which it was excreted most slowly, to an N^1 -glucuronide, a hitherto unknown major metabolite. However, the even longer acting (in man) sulphorthodimethoxine was shown not to be metabolised in this way (Chapter 5).

The one property common to all long-acting sulphonamides is comparatively high solubility in organic solvents, shown by the ease of recovery of the unchanged drug and its N^4 -acetyl derivative from urine by extraction techniques. Pharmacologically, this property has great significance; long-acting sulphonamides are able to penetrate the lipoprotein membranes of the animal more easily than their shorter acting congeners. In particular this property allows them to diffuse from the renal tubules, thereby effecting their reabsorption.

A prolonged stay within the body, combined with increased ease of access to drug-metabolising enzymes, increases the probability that unusual metabolic paths may be taken. Present ideas on the structure of proteins envisage a folding of the amino acid chains and their orientation within the molecule in such a way that the hydrophobic amino acid side chains occur largely in the interior, where Van der Waals forces are dominant. It is understandable that the more lipid soluble a molecule is, the more it will be able to penetrate to the interior of a protein molecule and thus have access to more binding sites. Such factors may explain the extensive protein binding of long-acting sulphonamides; if this is so, then protein binding becomes another effect of the primary cause of long-action and is not per se responsible for it.

It is probable that the half-life of a sulphonamide may be determined by plasma protein binding reducing the filterable concentration, followed by active tubular secretion and passive tubular reabsorption modifying the

the glomerular filtrate. An examination of the data of Rieder (1963_{a,b,c}) shows that a correlation of 0.67 exists for 16 sulphonamides of all types between the ratio of plasma half-life and fraction unbound to plasma protein in the rabbit and the distribution coefficient between aqueous buffer and ethylene dichloride, with $p=0.06$.

This degree of correlation is remarkable since the tubular secretion factor has been ignored in the simple treatment of the data. However, the extent and route of metabolism may be complicating factors in some cases. There is ample scope for research along the lines of these suggestions in investigating the renal behaviour of sulphonamides.

APPENDIX I

APPENDIX I

Ultraviolet spectra of the methoxy-substituted 6-sulphanilamido-pyrimidines and related compounds.

Compound	$\lambda_{\max.}$ (m μ)	$10^{-3} \times \epsilon_{\max.}$
Sulphanilamide	273	17.4
2,4-Dimethoxy-6-sulphanilamidopyrimidine (sulphadimethoxine)	261, 273	29.0, 23.0
Sulphadimethoxine glucuronide ammonium salt (from urine)	269	21.4
Sulphadimethoxine N^1 -glucuronide ammonium salt (synthetic)	269	21.8
2,4-Dimethoxy-6-(N^1 -methylsulphanilamido) pyrimidine	~265, 274	19.4, 21.7
4-Methoxy-6-sulphanilamidopyrimidine	275	19.9
4-Methoxy-6-(N^1 -methylsulphanilamido)pyrimidine	~255, 279	12.9, 22.4
2-Methoxy-6-sulphanilamidopyrimidine	268-280	20.0
5-Methoxy-6-sulphanilamidopyrimidine	272	19.3
2,5-Dimethoxy-6-sulphanilamidopyrimidine	270, 295	16.0, 16.8
4,5-Dimethoxy-6-sulphanilamidopyrimidine	275	22.3
2,4-Dimethyl-6-sulphanilamidopyrimidine	272	20.2
4-Methoxy-2-methyl-6-sulphanilamidopyrimidine	274	19.8
6-Amino-2,4-dimethoxypyrimidine	253	6.7
2,4-Dimethoxy-1- and 2,4-dimethoxy-3-methyl- 6-sulphanilamidopyrimidine	~265, 284	19.6, 24.5
4-Methoxy-1 or 4-methoxy-3-methyl-6- sulphanilamidopyrimidine	~259, 278	14.8, 22.0

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