The Metabolism of Sulphonamides

5. A STUDY OF THE OXIDATION AND ACETYLATION OF SULPHONAMIDE DRUGS AND RELATED COMPOUNDS IN THE RABBIT

By J. N. SMITH AND R. T. WILLIAMS, *Department of Biochemistry, University of Liverpool*

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It has now been established that both the simple and heterocyclic sulphonamides may undergo both acetylation and oxidation in the body (cf. Williams, 1945, 1946, 1947; Dobson & Williams, 1946; Stubbs & Williams, 1947; Bray, Neale & Thorpe, 1946; Gilligan, 1945; Scudi & Silber, 1944; Weber, Lalich & Major, 1943). In the simpler sulphonamides, such as the *o*-, *m*- and *p*-aminobenzenesulphonamides, oxidation occurs in the benzene ring, whereas in the *N'*-substituted sulphonamides, sulphapyridine and sulphathiazine (Bray *et al.* 1946; Weber *et al.* 1943; Scudi & Silber, 1944), oxidation occurs in the heterocyclic ring and acetylation in the benzene ring.

The object of the present work was to find out whether *in vivo* oxidation and acetylation were related. These two processes could occur in four ways: (1) acetylation followed by oxidation of the acetylated molecule, (2) oxidation followed by acetylation of the oxidized molecule, (3) simultaneous oxidation and acetylation of the same molecule, and (4) oxidation and acetylation as separate reactions involving different molecules of the compound:

- (1) $RH.NH_2 \rightarrow RH.NHAc \rightarrow R(OH)NHAc.$
- (2) $RH.NH_2 \rightarrow R(OH)NH_2 \rightarrow R(OH)NHAc.$
- (3) $RH.NH_2 \rightarrow R(OH)NHAc.$
- (4) $2RH.NH_2 \rightarrow RH.NHAc + R(OH)NH_2.$

It was suggested by Dobson & Williams (1946) that, in the case of metanilamide, oxidation does not occur in the molecule undergoing acetylation *in vivo*. Furthermore, orthanilamide is highly oxidized but hardly acetylated (Stubbs & Williams, 1947), whereas sulphanilamide is highly acetylated but only slightly oxidized in the rabbit (Shelswell & Williams, 1940; Williams, 1945, 1946). There are, therefore, suggestions that the fourth of the above mechanisms is plausible.

In the present work evidence that the compound studied has been oxidized is based on glucuronic acid and ethereal-sulphate determinations. If a compound devoid of hydroxyl groups is fed to an animal and gives rise to the excretion of increased amounts of glucuronic acid and/or ethereal sulphate, it is reasonable to conclude that the compound has been oxidized. One has, however, to bear in mind that oxidation could take place without the formation of *O*-conjugates and in such cases proof of oxidation requires the isolation of the oxidation

product (cf. Ambamide; Hartles & Williams, 1947). Such cases, however, are likely to be uncommon in the series of compounds concerned here.

METHODS

Animals

Rabbits (giant chinchilla) were used throughout this investigation. They were maintained on a constant diet of 50 g. of Lever's cubes, with water *ad lib*. All drugs were administered with water by stomach tube.

Analytical methods

Glucuronic acid in urine was determined by the method of Hanson, Mills & Williams (1944). Sanchez (1945) has observed a colour reaction between furfural and sulphadiazine. This fact had to be considered when glucuronic acid was determined in the presence of pyrimidine compounds. Glucuronic acid was therefore estimated in pure glucurone solutions in the presence of sulphadiazine and sulphapyrazine and their derivatives. None of these drugs had any influence on the recovery of glucuronic acid.

Ethereal sulphate was determined by the Folin gravimetric method (cf. Williams, 1938).

Free and combined diazotizable amino groups were determined by the colorimetric method of Bratton & Marshall (1939) using *N*-(1-naphthyl)-ethylenediamine as coupling agent. Colour intensities were measured by means of a Spekker photoelectric absorptiometer with suitable filters (in most cases Ilford no. 604 green or no. 605 yellow green). Standard curves were constructed for each drug studied.

Free amino groups were determined directly on urine appropriately diluted. Total amino groups were determined after acid hydrolysis. The concentration of acid and duration of hydrolysis were varied to suit the compound under investigation. The extent of acetylation was calculated as the difference between the values for free and total diazotizable amino groups. This calculation, however, is not valid in every case, particularly in those cases where oxidation is extensive (see Dobson & Williams, 1946).

RESULTS

Sulphadiazine, sulphapyrazine and their derivatives

The collected results for these compounds are given in Table 1. In Table 2 a complete set of results is given for sulphamezathine in order to illustrate the type of data on which the average figures in Table 1 and other tables are based.

Table 1. *The oxidation and acetylation of sulphadiazine and sulphapyrazine and their derivatives in the rabbit*

Compound fed	Dose (g./kg.)	Drug excreted					
		Total (% of dose)	Free	Acetylated		Oxidized (% of dose)	
				(% of dose)	(% of total)	Glucuro- nide	Ethereal sulphate
Free drugs:							
Sulphadiazine	0.2	74	16	58	78	—	0
	0.5	82	26	56	68	5	0
	1.0	92	21	72	78	0	—
Sulphamerazine	0.2	76	7	69	91	—	0
	0.5	81	9	72	89	8	0
Sulphamezathine	0.2	87	10	77	88	15	0
	0.5	90	16	74	82	16	0
Sulphapyrazine	0.2	74	10	64	86	—	—
	0.5	83	10	73	88	0	—
Acetylated drugs:							
Acetylsulphadiazine	0.45	75	0.5	75	100	0	—
Acetylsulphamerazine	0.4	71	0.5	71	100	0	—
Acetylsulphamezathine	0.5	102	0.5	102	100	0	—
Acetylsulphapyrazine	0.5	79	1.0	78	99	0	—
Oxidized drugs:							
SDOH*	0.2	90	78	12	13	0	—
SM.COOH	0.2	81	73	8	10	0	—
AcSM.COOH	0.2	82	1	81	99	0	—

* SDOH is 2-sulphanilamido-4-hydroxypyrimidine (hydroxysulphadiazine); SM.COOH is 2-sulphanilamido-4-carboxy-6-methylpyrimidine; AcSM.COOH is 2-(*N*⁴-acetylsulphanilamido)-4-carboxy-6-methylpyrimidine.

Table 2. *The excretion of metabolites of sulphamezathine in the rabbit*

(Dose 0.5 g./kg.)

Rabbit no.	Amount of drug recovered (% of dose)				
	Total	Free	Acetylated	As glucuronide*	As ethereal sulphate
72	64	8	56	—	—
68	86	21	65	—	—
71	96	15	81	15	—
78	94	16	78	14	—
76	91	14	76	10	—
67	93	9	84	16	—
78	98	21	77	12	—
81	95	22	72	16	—
60	—	—	—	—	0
66	—	—	—	—	0
67	—	—	—	—	0

* It is assumed that one molecule of the drug gives rise to one molecule of glucuronic acid.

Table 1 shows that neither sulphadiazine (m.p. 252°) nor sulphapyrazine (m.p. 259°) undergoes any oxidation in the rabbit, whereas the substituted sulphadiazines, sulphamerazine (m.p. 236°) and sulphamezathine (m.p. 198°) both give rise to increased outputs of glucuronic acid. None of these compounds gives rise to ethereal sulphates. Similar results were obtained by Gilligan (1945) on hospital patients undergoing treatment with these four drugs. Some of Gilligan's results indicate an oxidation up to 68% with sulphamezathine, but none at all with sulphadiazine and sulphapyrazine.

All four compounds are highly acetylated, although sulphadiazine is less acetylated than the

other three. When the acetylated drugs are fed they are excreted unchanged, being neither deacetylated nor oxidized. Previous work on *N*⁴-acetylsulphonamides had shown that they were not deacetylated in man (e.g. Strauss, Lowell, Taylor & Finland, 1941).

A comparison of the figures (in Table 1) for the amounts of sulphamerazine and sulphamezathine excreted free, with those for the amounts oxidized, shows that they correspond. These figures can be interpreted as meaning that it is the free and not the acetylated drugs that are oxidized. This interpretation is supported by the finding that neither *N*⁴-acetylsulphamerazine nor *N*⁴-acetylsulphamezathine is oxidized. These results indicate that

oxidation and acetylation are separate reactions involving different molecules of the drug. If, therefore, acetylation of these two drugs could be suppressed, the extent of their oxidation would be expected to increase.

Several unsuccessful attempts were made to isolate and identify the glucuronide in sulphamezathine urine. Nevertheless, a consideration of Table 1 does allow us to make a suggestion concerning the possible nature of this oxidation product. The main difference between sulphadiazine on the one hand, and sulphamerazine and sulphamezathine on the other, lies in the presence of substituent methyl groups in the pyrimidine rings of the latter. It is possible that these methyl groups may undergo oxidation. In the case of sulphamezathine (I) oxidation could proceed as follows:

(where $R = \text{NH}_2\text{C}_6\text{H}_4-$).

Compound (III) (2-sulphanilamido-6-methylpyrimidine-4-carboxylic acid, m.p. 253°) was therefore prepared (see below) and fed to rabbits. This compound, however, did not form a glucuronide and was only slightly acetylated (8 %; see Table 1). Its N^4 -acetyl derivative, m.p. 260° (prepared according to Rose & Swain, 1945), like other acetylated sulphonamides, is excreted unchanged. If this acid formed a glucuronide it would be expected to be an ester glucuronide, i.e. a substance which would reduce Fehling's solution (cf. benzoylglucuronide). Sulphamezathine urine, however, does not reduce Fehling's solution, and is therefore unlikely to contain a glucuronide of this type. The carboxylic acid (III) forms a characteristically insoluble copper salt (see below), but such a copper salt could not be isolated from sulphamezathine urine appropriately treated.

Another possible oxidation product of sulphamezathine is 2-(3'-hydroxysulphanilamido)-4:6-dimethylpyrimidine (IV), the analogue of 3-hydroxy-sulphanilamide (cf. Williams, 1946, 1947). Such a compound, however, would be expected to form an

ethereal sulphate. But there is no detectable increase in ethereal sulphate output after feeding sulphame-

zathine. Compound (IV) is therefore a possible but not probable oxidation product. Since sulphamezathine gives rise to a non-reducing glucuronide with no ethereal sulphate, a compound such as 2-sulphanilamido-4-hydroxymethyl-6-methylpyrimidine (II) would be an oxidation product which would fit the evidence obtained so far. We have not been able to synthesize this compound.

Table 2 also includes 4-hydroxysulphadiazine (V). Theoretical considerations of the electron distribution on the pyrimidine ring indicate that position 5

is the most likely point of attack. If sulphadiazine were oxidized *in vivo* then 5-hydroxysulphadiazine (VI) would be expected to be formed. We have not synthesized (VI), but we prepared (V), m.p. 280° , according to Ganapathi, Deliwala & Shirsat (1942). On feeding (V) to rabbits it was largely excreted unchanged although some 12 % was acetylated. It formed no glucuronide. We were unable to detect any hydroxysulphadiazine in sulphadiazine urine. The only compound isolated was N^4 -acetylsulphadiazine (m.p. and mixed m.p. 245°), which was obtained in excellent yields by continuous ether extraction of sulphadiazine urine brought to pH 1 with HCl. N^4 -Acetylsulphamerazine (m.p. and mixed m.p. 242°) and N^4 -acetylsulphamezathine (m.p. and mixed m.p. 241°) were isolated from the corresponding urines in a similar manner.

Sulphapyrazine (VII) undergoes no oxidation in the rabbit, although it is highly acetylated (88 %). Biological oxidation in the pyrazine ring is unlikely on theoretical grounds, for positions 3, 5 and 6 are all deactivated by the ring nitrogens (cf. Krems & Spoerri, 1947).

Synthesis of 2-sulphanilamido-6-methylpyrimidine-4-carboxylic acid. The acetyl derivative of this acid was prepared according to Rose & Swain (1945). The acetyl compound (4.5 g.) was boiled for 1 hr. with 50 ml. 5N-KOH. The mixture was treated with charcoal and filtered. The hot solution was treated with dilute H_2SO_4 till strongly acid to litmus and the carboxylic acid separated as a yellow precipitate. After cooling to 0° the crystalline solid was

filtered off. It was sparingly soluble or insoluble in water and the usual organic solvents, and was purified by dissolving in hot alkali and precipitating with H_2SO_4 . It formed pale-yellow short needles, m.p. 253–256°, and titrated as a dibasic acid. (Found: N, 18.3; equiv. by titration, 157. $\text{C}_{15}\text{H}_{15}\text{O}_4\text{N}_2\text{S}$ requires N, 18.2%; equiv. 154.) (Ultraviolet absorption spectrum: one band at λ_{max} , 255 m μ , with ϵ_{max} , 19,400.) It forms an insoluble copper salt. This salt was prepared by dissolving the acid in dilute NaOH and making just acid with dilute HCl to give a supersaturated solution, and then adding CuSO_4 solution. The salt was precipitated as a pale-blue micro-crystalline powder insoluble in water, dilute HCl and acetic acid, but dissolving in cold KOH and conc. HCl. No precipitates were obtained on adding soluble Zn, Ba, Ca, Hg, Pb and Fe^{+++} salts to a supersaturated solution of the acid. The Ag salt was insoluble in water.

Sulphathiazole, sulphapyridine and sulphaguanidine

The results for these compounds and their N^4 -acetyl derivatives are given in Table 3.

occurring in rabbit urine is likely to be partly acetylated. Considerations of the electron distribution on the pyridine ring suggest that the β -positions are the most likely to be oxidized, and therefore a glucuronide of sulphapyridine can be tentatively formulated as 2-(*p*-acetylsulphanilamido)-3-or-5-glucuronosidopyridine (VIII is the 5-glucuronide).

In the case of sulphaguanidine (m.p. 186°) and its acetyl derivative (m.p. 260°) absorption is so slow that glucuronide formation, if it occurred, could not be detected. Sulphaguanidine itself is highly acetylated and its acetyl derivative appears to undergo a slight deacetylation, possibly by the organisms of the gut.

Table 3. *The oxidation and acetylation of sulphathiazole, sulphapyridine and sulphaguanidine in the rabbit*

Compound fed	Dose (g./kg.)	Total (% of dose)	Free (% of dose)	Acetylated		Oxidized as glucuronide (% of dose)
				(% of dose)	(% of total)	
Free drugs:						
Sulphathiazole	0.3	51	30	21	41	3
	0.45	82	31	51	62	8
	1.0	92	33	59	64	6
Sulphapyridine	0.75	67	19	48	72	29
Sulphaguanidine	0.2	60	7	53	88	?
Acetyl derivatives:						
Acetylsulphathiazole	1.0	8	0.5	7.5	94	0
Acetylsulphapyridine	0.75	62	1	61	98	28
Acetylsulphaguanidine	0.2	63	6	56	89	?

* These drugs are too slowly absorbed to allow glucuronic acid determinations of any significance.

Sulphathiazole (m.p. 195°) is less highly acetylated than the compounds of the sulphadiazine series. It does, however, undergo a small but significant oxidation of about 6% of the dose. Its acetyl compound is very poorly absorbed, and after several days only about 8% of the dose is excreted in the urine. The deacetylation of this compound is not significant.

In the case of sulphapyridine (m.p. 196°) we have an example of a sulphonamide in which oxidation and acetylation may occur in the same molecule. The figures in Table 3 indicate that this may be a case of a compound which is first acetylated and then oxidized, for the oxidation of N^4 -acetylsulphapyridine (m.p. 230°) occurs to the same extent (28%) as with sulphapyridine itself (29%). Although the acetyl compound is oxidized it does not undergo significant deacetylation. Other workers (Weber *et al.* 1943; Bray *et al.* 1946) have shown that sulphapyridine is oxidized somewhere in the heterocyclic ring. The present work indicates that the glucuronide

Sulphanilic and p-aminobenzoic acids

The results for these compounds and their acetyl derivatives are given in Table 4. Sulphanilic acid is excreted unchanged, there being no acetylation and no oxidation. Acetylsulphanilic acid behaves similarly, there being no deacetylation and no oxidation. These results are what would be expected, for both compounds are comparatively strong acids and the body rapidly excretes strong acids unchanged (cf. Quick, 1932b). Hildebrandt (1906) has reported that sulphanilic acid is excreted unchanged by dogs, but Zehender (1943) reports that it is highly acetylated in guinea pigs. *p*-Aminobenzoic acid is partly excreted unchanged, partly acetylated (20%) (cf. Harrow, Mazur & Sherwin, 1933) and partly conjugated with glucuronic acid to form an ester glucuronide (15%). This glucuronide is probably largely *p*-aminobenzoylglucuronide for *p*-acetamidobenzoic acid forms a negligible amount (4%) of glucuronide and is mainly excreted unchanged.

Table 4. *The oxidation and acetylation of sulphanilic and p-aminobenzoic acid in the rabbit*

Compound	Dose (g./kg.)	Total (% of dose)	Free (% of dose)	Acetylated		Oxidized (% of dose)	
				(% of dose)	(% of total)	As glucuronide	Ethereal sulphate
Sulphanilic acid	0.63	97	95	0	0	0	—
p-Aminobenzoic acid	0.4	94	72	22	23	0 (15)*	0
Acetylsulphanilic acid	0.5	99	1	98	99	0	—
p-Acetamidobenzoic acid	0.4	90	0.3	90	100	0 (4)*	—
p-Acetamidophenylacetic acid	0.5	55	0.2	55	100	—	—

* As ester glucuronide (see text).

p-Aminobenzoic acid does not undergo nuclear oxidation for it does not give rise to an ethereal sulphate. It could give rise to a glycine conjugate, but we did not look for it since Quick (1932*a*) has reported that it does not form a glycine conjugate in dogs. The work of Smith, Bayliss, Orgorzalek & McClure (1946), however, suggests that *p*-aminohippuric acid may be formed in man.

p-Acetamidophenylacetic acid (m.p. 168°) was included in this series because we wished to find out whether removal of the carboxyl group from the ring would influence the *in vivo* stability of the *p*-acetamido group. Table 4 shows that this acid is not deacetylated in the rabbit.

DISCUSSION

All the compounds studied here are derivatives of aniline in which there is a para-substituted acidic group (aniline substituted with other types of groups will be the subject of another paper). It is therefore interesting to compare the acid dissociation constants of these compounds with the extent of their acetylation in the rabbit (see Table 5).

Table 5. *The relation between dissociation constant and extent of acetylation of certain p-substituted anilines*(p*K*_a's quoted from Bell & Roblin (1942).)

Compound	p <i>K</i> _a	Drug excreted as acetyl compound (average figures) (%)
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
Sulphamezathine	7.37	85
Sulphapyridine	8.43	72
Sulphanilamide	10.43	50

It appears from this table that there is a rough relationship between p*K*_a and percentage acetylation. When p*K*_a is small, i.e. the compound is a relatively strong acid, acetylation only takes place

to small extent, e.g. sulphanilic acid, p*K*_a = 3.24 and acetylation 0%; *p*-aminobenzoic acid, p*K*_a = 4.68 and acetylation, 23%. Compounds with p*K*_a's varying from about 6 to 9 are all highly acetylated. It appears probable from other work carried out in this laboratory that when p*K*_a is very large, i.e. the compound is not an acid or is an extremely weak acid, then the percentage acetylation again becomes very small (e.g. aniline, unpublished results). These facts suggest that there is an optimum extent of ionization for biological acetylation. If the compound is too highly ionized either as an acid or a base, there is a tendency for acetylation to be low or zero. The alternative explanation is that if a compound is strongly acidic there is a tendency for rapid excretion and there is little time for acetylation. We found, for example, that sulphanilic acid was rapidly excreted by the rabbit.

Another fact which emerges from the present results is that acetylation of the aromatic amino group is a one way reaction. None of the acetamido compounds studied here were deacetylated by the rabbit to any significant extent. Other work in this laboratory indicates that this is true also for such compounds as acetanilide, phenacetin, *p*-acetamidophenol, etc. According to Lipmann (1946) the acetylation of the aromatic amino group in sulphanilamide involves the participation of adenosine triphosphate and acetate, and since the reaction is highly exergonic, the tendency is complete acetylation. Reversal of this reaction resulting in deacetylation is therefore unlikely. However, deacetylation could be brought about by hydrolysis, which is not the reverse of acetylation, by the mechanism suggested by Lipmann. Michel, Bernheim & Bernheim (1937) and Kohl & Flynn (1940) have reported that rat liver and kidney contains an acylase which deacetylates acetylated sulphonamides and acetanilide *in vitro*. In the intact rabbit, if this enzyme system does occur, there appear to be considerable limitations to its activity.

The present work also gives us some information on the relation of oxidation to acetylation. It appears, as far as the compounds studied here are concerned, that there are two types of amino compounds involved, (1) those in which oxidation and

acetylation require separate molecules one for each process, e.g. sulphamezathine, and (2) those in which oxidation and acetylation can occur in the same molecule, e.g. sulphapyridine (cf. also β -naphthylamine, Dobriner, Hofmann & Rhoads, 1941; and 2-acetamidofluorene, Bielschowsky, 1945). Other types of aromatic compounds, such as those which are oxidized but not acetylated, are also known and will be discussed in another communication.

SUMMARY

The extent of acetylation and of oxidation in the rabbit of certain *N*-substituted heterocyclic sulphonamide drugs has been measured. Similar measurements have been made on sulphanilic acid, *p*-aminobenzoic acid and related compounds. The following conclusions were reached:

(1) All sulphonamides with pK_a 's between 6 and 9 are highly acetylated; compounds with a pK_a less than 6 were not highly acetylated.

(2) *p*-Substituted acetanilides are not deacetylated by the rabbit.

(3) In some sulphonamides, e.g. sulphamerazine, sulphamezathine and sulphathiazole, oxidation occurs in the free drug but not with the acetylated drug, whereas in sulphapyridine the acetyl derivative and the free drug are oxidized.

(4) Sulphadiazine and sulphapyrazine are not oxidized.

(5) Sulphanilic acid is neither acetylated nor oxidized whereas *p*-aminobenzoic acid is slightly acetylated (c. 20%) but not oxidized.

(6) The results obtained have been related as far as possible to certain physicochemical concepts.

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