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Acetylation and Deacetylation of the *p*-Amino Group of Sulphonamide Drugs in Animal Tissues

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The work reported in this paper was originally intended to deal with the mechanism of the acetylation of sulphonamides in animal tissues, with special reference to the source of the acetyl radical and the conditions controlling the rate and degree of acetylation. We thought it would be advantageous to use for this work isolated tissues, sliced or minced, but soon found that the degree of acetylation in isolated tissues was much lower than was to be expected on the basis of observations on the intact organism. Whilst in the intact guinea-pig, for example, 75 % of the sulphamezathine excreted in the urine was conjugated, conjugation by slices of guinea-pig tissues never exceeded 20 % and was usually below 10 %, even when the added amount of drug was small. Such acetylation as did occur in isolated tissues was usually completed within 30 min. of incubation. An investigation of this apparent discrepancy in the metabolic behaviour of tissues *in situ* and *in vitro* occupies the main part of this paper. At least a partial explanation for the discrepancy offered itself when it was found that certain tissues contain an enzyme as already reported by Kohl & Flynn (1940) which hydrolyzes *N*⁴-acetylated (or, generally, *N*⁴-acylated) sulphonamides. It appears that the relative activity of this enzyme is altered by slicing or mincing. The activity is apparently low, perhaps even negligible, in the intact rabbit or guinea-pig, though it is operative in the intact pigeon, dog and cat. The enzyme activity is increased,

either in absolute terms or relative to conjugating systems, by the conditions prevailing in the *in vitro* experiments. *In vitro*, therefore, the process of acetylation appears to be largely counterbalanced by reactions causing deacetylation.

EXPERIMENTAL

Determination of sulphonamides

The methods of Bratton & Marshall (1939) and of Rose & Bevan (1944) were used.

Blood. A suitable volume, usually 0.1 ml., was measured into 0.5 ml. of 0.5 % saponin solution in a 10 ml. measuring tube. Water was added to make up to 9 ml. and 1 ml. 15 % trichloroacetic acid solution was used for deproteinization. One 4 ml. portion of the filtrate was used for the determination of the free sulphonamide and another 4 ml. portion for the determination of total sulphonamide. A visual colorimeter was used; the standard contained 0.02 mg. of the sulphonamide. When total sulphonamide was determined the corresponding acetyl compound, treated in the same way as the unknown, was used as a standard.

Urine. Usually 0.1 ml. of urine, if necessary diluted, was used and treated in the same way as blood, except that the saponin was left out.

Faeces. Faeces were mixed with 10 vol. of water. A sample was deproteinized with trichloroacetic acid and the filtrate treated in the same way as the blood filtrate.

Tissue extracts. Media in which tissues had been suspended or extracts of tissues were treated in the same way

as blood, except that the quantity varied according to the sulphonamide concentration. When practicable the amount of material was so chosen as to contain about 0.02 mg. of sulphonamide.

Blanks. Urines from persons or animals who had not received sulphonamides usually gave a pink-orange colour when total sulphonamide was determined, corresponding to 0.2–0.5 mg./100 ml., suggesting that there are primary aromatic amines or amine-forming substances present in normal urine. Very low concentrations of sulphonamides, therefore, cannot be determined accurately in urine. In faeces, similar difficulties were encountered. In addition, colouring matter present in the faeces interfered with the colorimetry. No satisfactory method for the removal of the interfering substance was found. Charcoal removed the coloured substances in the faeces, but also much of the sulphonamides. Owing to the presence of interfering substances, the results recorded in the paper for faeces tend to be a little too high.

Special points. When trichloroacetic acid was replaced by metaphosphoric acid, satisfactory results were obtained with sulphamezathine and a few other sulphonamides, but in some cases, especially that of sulphathiazole, the dye formed on coupling proved to be unstable; it faded within a few minutes.

*N*⁴-Acylated sulphonamides are slowly hydrolyzed at room temperature in the trichloroacetic acid filtrate (Table 1, see also Poth & Ross, 1944). The free sulphonamides were therefore determined at once after filtration, or, if that was not possible, the filtrate was neutralized for storing. In the latter case, trichloroacetic acid was again added before the determination.

Table 1. *Hydrolysis of N⁴-acetylsulphonamides in trichloroacetic acid solution*

(Acetylsulphonamide (2 mg.) in trichloroacetic solution (10 ml.); room temperature (15–20°).)

Drug	Quantity hydrolyzed (mg.) after	
	18 hr.	76 hr.
<i>N</i> ⁴ -Acetylsulphamezathine	0.12	0.33
<i>N</i> ⁴ -Acetylsulphapyridine	0.13	0.32
<i>N</i> ⁴ -Acetylsulphadiazine	0.12	0.38
<i>N</i> ⁴ -Acetylsulphathiazole	0.12	0.24
<i>N</i> ⁴ -Acetylsulphanilamide	0.13	0.36

The term 'free' sulphonamide in this paper refers to compounds whose *p*-amino group is free; 'conjugated' refers to compounds whose *p*-amino group is set free on acid hydrolysis. The quantities of total or conjugated sulphonamides, when expressed in milligrammes, are given in terms of the free sulphonamides.

RESULTS

Experiments on the intact organism

Man. Two examples of experiments on the conjugation of sulphamezathine in man are recorded in Table 2. After a single intramuscular dose the blood concentration of the 'free' drug quickly reached a

Table 2. *Acetylation of sulphamezathine in the human body*

Blood analysis					Urine analysis							
Time after dosing (hr.) (min.)		Sulphamezathine concentrations (mg./100 ml.)		Ratio: Free/Conjugated	Time after dosing (hr.) (min.)		Volume excreted (ml.)	Sulphamezathine concentrations (mg./100 ml.)		Ratio: Free/Conjugated	Total recovered sulphamezathine (mg.)	
Free	Total	Free/Conjugated	Free	Total	Free	Total	Free	Total	Free	Total		
Exp. 1. Male age 23; 70 kg. 2 g. sulphamezathine intramuscularly. (33% solution of sodium salt.)												
0	40	7.1	7.1	—	3	10	257	34.5	138	0.35	88.7	356
2	10	6.1	8.9	2.2	8	55	285	30.0	287	0.12	174	1173
3	55	4.7	7.5	1.7	10	0	205	4.6	65.5	0.076	184	1308
5	0	3.9	7.3	1.15	11	30	96	8.4	145	0.061	192	1446
6	40	3.1	6.2	1.0	14	40	218	4.9	110	0.047	202	1690
8	10	2.2	5.1	0.76	23	55	443	2.4	55.2	0.045	213	1930
—	—	—	—	—	27	20	305	0.8	15.1	0.056	215	1980
—	—	—	—	—	31	40	338	0.5	6.3	—	217	2000
—	—	—	—	—	33	20	155	0.4	3.8	—	218	2005
—	—	—	—	—	35	20	200	0.4	2.9	—	219	2010
Exp. 2. Male age 24; 70 kg. 1.71 g. sulphamezathine taken orally.												
0	40	1.3	1.7	3	2	30	206	7.0	38.4	0.22	14.4	79.4
1	10	2.8	3.3	6	4	50	250	16.5	105	0.19	55.7	341
1	50	3.8	4.9	3.5	7	20	288	12.5	100	0.14	91.7	631
2	20	4.1	5.3	3.4	9	25	243	8.6	102	0.092	113	898
3	25	4.5	5.5	4.5	11	15	270	5.0	68.6	0.079	126	1083
4	10	3.5	5.4	1.84	15	20	160	8.2	149	0.058	139	1322
5	55	3.0	4.8	1.67	23	05	275	3.7	83.4	0.046	150	1554
7	45	1.6	3.6	0.80	26	40	267	1.1	20.2	0.058	152	1610
9	45	1.3	3.1	0.72	28	20	150	1.0	11.7	0.093	154	1625
12	45	—	2.3	—	30	30	555	0.4	4.3	—	156	1650
—	—	—	—	—	33	20	190	0.5	6.0	—	160	1660
—	—	—	—	—	35	20	145	—	0.9	—	—	—
—	—	—	—	—	36	50	192	0.8	8.1	—	—	—

Table 3. *Fate of acetylsulphamezathine in the human body*

(Both experiments were carried out on the same male person, age 24; 70 kg. 2.30 g. acetylsulphamezathine (equivalent to 2 g. sulphamezathine) taken orally in about 250 ml. water containing one equiv. NaOH.)

Blood analysis				Urine analysis						
Time after dosing		Sulphamezathine concentrations (mg./100 ml.)		Time after dosing		Volume excreted (ml.)	Sulphamezathine concentrations (mg./100 ml.)		Total recovered sulphamezathine (mg.)	
(hr.)	(min.)	Free	Total	(hr.)	(min.)		Free	Total	Free	Total
Exp. 1										
0	25	0	11.2	1	50	284	1.04	193	2.9	550
1	30	0	8.2	4	5	271	2.15	220	8.7	1150
3	5	0	5.8	8	50	308	2.76	195	17.2	1750
5	15	—	3.4	12	35	279	1.47	75	21.3	1960
8	5	0	2.8	24	5	545	0.87	45.3	25.0	2211
—	—	—	—	27	55	330	0.45	6.4	26.5	2230
—	—	—	—	33	35	231	0.60	8.2	27.9	2250
Exp. 2										
—	—	—	—	0	40	276	0.39	59.2	1.1	163
—	—	—	—	2	0	246	1.02	93.0	3.8	392
—	—	—	—	2	5	120	1.06	102	5.1	514
—	—	—	—	7	10	238	5.52	301	18.2	1231
—	—	—	—	13	10	330	3.53	102	28.4	1568
—	—	—	—	21	30	650	1.28	20.9	36.7	1704
—	—	—	—	25	25	930	0.40	2.97	40.4	1732

maximum within the first hour and then gradually fell off. The ratio concentration of free form/concentration of conjugated form decreased with time in blood and urine, indicating that the conjugation proceeded continuously. Virtually all the injected drug was recovered from the urine; the kidneys are thus the main organs of excretion. Over 80% of the excreted drug was present in the conjugated form. On oral intake the maximum concentration in the blood was reached after 3–4 hr. and the proportion of the conjugated form again increased continuously from the start. Recovery from the urine was 97% of the intake. Absorption from the intestine must therefore have been almost quantitative. Over 90% of the excreted drug was conjugated (see also Volini, Engbring & Schorsch, 1945). In the urine the ratio free form/conjugated form fell continuously, and during the later stages of the excretion period 95–97% of the excreted drug was conjugated. These data indicate that as long as free sulphamezathine is present, conjugation progresses. There is no suggestion of an 'equilibrium'

sulphamezathine $\rightleftharpoons$ acetylsulphamezathine.

The reaction virtually goes to completion from left to right. This is borne out by experiments with acetylsulphamezathine. After oral administration of this substance no free sulphamezathine was detected in the blood, and only traces, hardly exceeding the limits of error, appeared in the urine (Table 3).

Sulphamezathine determinations on faeces after oral intake of 2 g. are shown in Table 4. The last three portions, which were collected 5 or more days

after the oral intake and which may therefore be expected not to contain the drug, produced a colour equivalent to 2, 20 and 5 mg. total sulphamezathine respectively. These figures indicate the amounts of substances simulating sulphonamides. The first portion, which contained the non-absorbed drug, gave little more than the 'blank'. The analyses confirm the almost complete absorption of the drug.

Table 4. *Sulphamezathine in faeces after oral intake of 2 g.*

Period of collection of faeces after dosing (days)	'Sulphamezathine' found (mg.)	
	Free	Total
1–4	34	37
5–7	1.6	2
8–10	20	20
11–13	4	5

Pigeon. In the pigeon, in contrast to man, the reaction

sulphamezathine $\rightleftharpoons$ acetylsulphamezathine

proceeds in both directions (Tables 5 and 6). When free sulphamezathine was given the ratio conjugated/free drug fluctuated around unity in the blood and in the excreta. After administration of the acetyl form, considerable amounts of the free drug appeared in the blood and in the excreta. At 4–6 hr. after the injection, about 50% of the drug in the blood and excreta was free.

Rabbit. No significant amounts of free sulphamezathine appeared in the urine of rabbits after injection of acetylsulphamezathine (see Table 7).

Table 5. *Acetylation of sulphamezathine in the pigeon*

(Female pigeon 360 g. Intraperitoneal injection of 100 mg. sulphamezathine in 10 ml. 0.03N-NaOH.)

Blood analysis					Analysis of excreta (urine plus faeces)						
Time after injection (hr.) (min.)		Sulphamezathine concentrations (mg./100 ml.)		Ratio: Free/ Conjugated	Time after injection (hr.) (min.)		Quantities of sulphamezathine excreted (mg.)		Ratio: Free/ Conjugated	Total recovered sulphamezathine (mg.)	
		Free	Total				Free	Total		Free	Total
1	0	14.0	26.6	1.11	2	0	8.3	12.5	1.97	8.3	12.5
2	0	12.0	26.2	0.85	4	0	4.4	7.8	1.29	12.7	20.3
4	5	9.2	21.2	0.66	10	0	21.4	48.5	0.79	34.1	68.8
6	15	5.4	10.7	1.02	22	30	5.3	9.5	1.26	39.4	78.3
8	15	2.7	6.2	0.77	—	—	—	—	—	—	—
11	15	—	—	—	—	—	—	—	—	—	—

Table 6. *Deacetylation of acetylsulphamezathine in pigeons*

Blood analysis					Analysis of excreta (urine plus faeces)						
Time after injection		Sulphamezathine concentrations (mg./100 ml.)		Ratio: Free/Conjugated	Time after injection		Quantities of sulphamezathine excreted (mg.)		Ratio: Free/Conjugated	Total recovered sulphamezathine (mg.)	
(hr.)	(min.)	Free	Total		(hr.)	(min.)	Free	Total		Free	Total
Exp. 1. Female pigeon, 300 g. Intraperitoneal injection of 105 mg. acetylsulphamezathine (expressed as free) dissolved in 5 ml. 0.06N-NaOH.											
—	—	—	—	—	1	50	3.4	47.2	0.078	3.4	47.2
—	—	—	—	—	3	50	6.0	17.2	0.54	9.4	64.4
—	—	—	—	—	5	40	4.7	10.7	0.78	14.1	75.1
—	—	—	—	—	7	15	4.0	6.2	1.82	18.1	81.3
—	—	—	—	—	23	30	2.4	2.9	4.8	20.5	84.2
—	—	—	—	—	32	40	—	2.2	—	—	—
—	—	—	—	—	73	—	—	7.3	—	—	—

Exp. 2. Same pigeon 15 days later. Intraperitoneal injection of 104 mg. acetylsulphamezathine (expressed as free) dissolved in 5 ml. 0.06N-NaOH.

0	40	2.9	17.1	0.204	7	30	2.1	34	0.066	2.1	32
1	35	4.4	18.9	0.304	24	0	5.8	13.8	0.73	7.9	40
3	35	5.1	14.2	0.56	72	0	—	0.2	—	—	40
5	5	3.3	4.5	1.50	—	—	—	—	—	—	—

Table 7. *Deacetylation of acetylsulphamezathine in the rabbit*

(Weight of animal 1250 g., 300 mg. acetylsulphamezathine (equivalent to 261 mg. sulphamezathine) injected subcutaneously as 10% solution of sodium salt.)

Urine analysis

Time after injection		Sulphamezathine excreted/sample (mg.)	
(hr.)	(min.)	Free	Total
5	35	1.29	230
8	15	0.52	12.9
24	20	0.80	16.3
27	5	Nil	1.59
47	20	Nil	3.71
55	30	Nil	1.66
73	30	Nil	0.102
Total		2.6	266

Cat. The cat slowly hydrolyzes acetylsulphamezathine (Table 8). After a single oral dose of 150 mg., 11% of the recovered drug occurred free; after

subcutaneous injection of 500 mg., 15% of the recovered drug was found to be free. The ratio free form/conjugated form rose steadily with time.

Dog. It has been reported that the dog cannot acetylate sulphonamides (Marshall, Emerson & Cutting, 1937). We find (Table 9) that on subcutaneous injection some conjugation occurred whilst the concentration of the drug in the body was relatively high. About 10% of the drug appearing in the urine was conjugated. As in the cat, injected acetylsulphamezathine was hydrolyzed (Table 10).*Enzymic hydrolysis of N⁴-acylsulphonamides**N⁴-Acetylsulphamezathine.* Suspensions of sheep kidney were mixed with acetylsulphamezathine and phosphate buffer of pH 7.7 and kept at 38°C., octanol serving as a preservative. Unless stated otherwise, the suspension consisted of one part of minced tissue suspended in three parts of water. Samples were analyzed for free sulphamezathine

Table 8. *Deacetylation of acetylsulphamezathine in cats*

Urine analysis					
Time after dosing		Sulphamezathine excreted/sample (mg.)			Ratio: Free/ Conjugated
(hr.)	(min.)	Free	Total	Conjugated	
Exp. 1. Weight of cat 1500 g.; 500 mg. acetylsulphamezathine, equivalent to 435 mg. sulphamezathine, injected subcutaneously as 10% solution of sodium salt.					
7	15	5.38	185	180	0.030
21	15	9.25	151	142	0.065
22	30	12.7	47	35	0.36
24	5	6.2	13.1	5.9	1.05
26	15	2.84	4.15	1.3	2.18
46	5	19.8	26.5	6.7	2.96
54	5	7.85	10.1	2.2	3.56
72	5	4.05	4.3	0.25	16
Total 68			441	373	
Exp. 2. Weight of cat 1500 g.; 150 mg. acetylsulphamezathine equivalent to 130 mg. sulphamezathine, dissolved in 0.3 ml. 2N-NaOH plus 5 ml. water taken orally in milk.					
3	5	0.109	58.5	58.4	0.019
4	25	0.061	17.9	17.8	0.034
7	25	2.32	19.5	17.2	0.135
23	20	5.95	21.4	15.45	0.385
27	5	1.37	3.71	2.34	0.585
28	25	2.68	3.84	1.16	2.30
48	—	1.51	1.66	0.16	9.4
Total 14			126.5	112.5	

Table 9. *Acetylation of sulphamezathine in the dog*

(Weight of dog 1190 g.; 100 mg. sulphamezathine injected subcutaneously as 3% solution of sodium salt.)

Time after injection		Urine analysis		
		Sulphamezathine excreted/sample (mg.)		
(hr.)	(min.)	Free	Total	Conjugated
3	20	13.7	16.8	3.1
6	20	16.3	19.2	2.9
22	10	31.3	32.5	1.2
28	—	5.1	4.5	—
30	—	1.9	1.4	—
46	—	1.54	1.54	—
Total 69.8			75.9	7.2

Table 10. *Deacetylation of acetylsulphamezathine in the dog*

(Weight of dog 1190 g.; 100 mg. acetylsulphamezathine, equivalent to 87 mg. of sulphamezathine, injected subcutaneously as 3% solution of sodium salt.)

Time after injection		Urine analysis		
		Sulphamezathine excreted/sample (mg.)		
(hr.)	(min.)	Free	Total	Conjugated
4	—	1.28	17.8	16.5
7	—	1.97	24.6	22.6
24	10	15.6	31.2	15.6
37	20	0.78	1.23	0.45
48	—	3.3	2.6	—
Total 22.9			77.4	55.2

after various intervals. Controls without tissue indicated that acetylsulphamezathine was stable under the experimental conditions. Table 11 shows that the hydrolysis was relatively slow and that the rate fell off with time. About two thirds of the added substrate was split after 9 days when higher concentrations of enzyme were used. Within the range of concentrations tested in this experiment the rate of hydrolysis was approximately proportional to the enzyme concentration, but in more concentrated kidney suspensions the rate did not increase with the tissue concentration (Table 12). The rate was approximately proportional to the substrate concentration (Table 13) when this was below 0.01M. The fall in substrate concentration may in part explain the falling off of the rate of hydrolysis with time. The inhibitory effect of high tissue concentrations was found in every tissue examined (kidney of dog, cat; liver of guinea-pig, sheep, cat, dog). The pH optimum of the enzyme in bicarbonate/CO₂ buffer was found to be between 7.3 and 7.5 (Table 14).

Among the tissues tested (Table 15) kidney cortex showed the highest, liver the second highest rate of activity. All the eight tissues showed some activity, though in some cases, notably brain and pancreas, the enzyme was very weak and ceased to act after a few hours. The enzyme was inactivated by heating the tissue suspension for 5 min. to 100°. Acetone powder of kidney cortex was inactive.

Other N⁴-acetylsulphonamides. Table 16 shows comparative data of the rate of hydrolysis of six

different acetylsulphonamides. Acetyl-4:6-dimethoxy-sulphadiazine was much more rapidly hydrolyzed than acetylsulphamezathine, whilst hydrolysis of the other four compounds was slower than that of acetylsulphamezathine. Rose & Tuey (1946) noted a rapid hydrolysis of acetyl-4:6-dimethoxysulphadiazine *in vivo*. It has been stated that in the intact body sulphanilamide and sulphapyridine are more readily acetylated than sulphathiazole and sulphadiazine (Hawking, 1945). There appears to be no correlation between these differences and the rates of hydrolysis in sheep kidney extracts.

Table 11. *Hydrolysis of acetylsulphamezathine by sheep kidney*

(Each flask contained 4 ml. 0.2M-Na₂HPO₄, 0.5 ml. 0.2M-NaH₂PO₄, final concentration 0.002M-Na acetylsulphamezathine, varying quantities of stock tissue suspension (minced sheep kidney cortex suspended in 2 vol. 0.01N-NaHCO₃), and water to make up to 10 ml. 38°.)

Stock kidney suspension in 10 ml. sample (ml.)	Concentration of free sulphamezathine (mg./100 ml.) after			
	3.5 hr.	56 hr.	6 days	9 days
1.0	6.4	22.7	33.8	37.8
0.5	2.7	19.2	27.8	34.5
0.25	1.8	13.3	22.2	30.1
0.125	0.88	7.3	14.5	19.3
0.0625	0.60	4.0	8.3	12.8

Table 12. *Hydrolysis of acetylsulphamezathine at high concentrations of sheep kidney suspension*

(Conditions as in Table 11.)

Stock kidney suspension in 10 ml. sample (ml.)	Concentration of free sulphamezathine (mg./100 ml.) after	
	4 hr.	22 hr.
4	8.8	14.6
2	8.8	16.4
1	6.2	15.8

Table 14. *Effect of pH on the hydrolysis of acetylsulphamezathine*

(Na₂CO₃/NaHCO₃/CO₂ buffer; pH calculated from pK₁=6.10; pK₂=9.79 (see also Delory & King, 1945). In order to have containers which could conveniently be filled with gas mixtures from cylinders, Warburg vessels were used. They contained 0.5 ml. buffer; 3 ml. 0.005M-Na acetylsulphamezathine and 0.5 ml. sheep kidney cortex suspension (1 part minced kidney, 5 parts water). Incubation 3 hr., 38°.)

Buffer mixture (made up to 0.5 ml. with N-NaCl)			Initial pH (calculated)	Free sulphamezathine found (mg./100 ml.)
M-Na ₂ CO ₃ (ml.)	M-NaHCO ₃ (ml.)	Gas mixtures		
0.25	0.25	Air	9.79	0.32
0.10	0.40	Air	9.20	0.63
0.05	0.45	Air	8.85	0.91
—	0.5	5% CO ₂ in O ₂	8.14	1.83
—	0.25	5% CO ₂ in O ₂	7.84	3.15
—	0.125	5% CO ₂ in O ₂	7.54	3.75
—	0.0625	5% CO ₂ in O ₂	7.34	4.20
—	0.5	100% CO ₂	6.84	1.37
—	0.25	100% CO ₂	6.54	1.26
—	0.125	100% CO ₂	6.24	1.17

Table 13. *Effect of substrate concentration on the rate of hydrolysis of acetylsulphamezathine by minced sheep kidney cortex*

(Each flask contained 1 ml. stock tissue suspension; 4 ml. 0.2M-Na₂HPO₄, 0.5 ml. 0.2M-NaH₂PO₄, varying quantities of Na acetylsulphamezathine, total volume 10 ml.)

Concentration of acetylsulphamezathine (M)	Concentration of free sulphamezathine (mg./100 ml.) after	
	3.5 hr.	24 hr.
0.04	22.5	68.0
0.02	16.8	60.5
0.01	12.3	44.0
0.005	7.9	27.2
0.0025	4.4	11.8
0.00125	2.6	6.4
0.000625	1.1	2.5

Other N⁴-acylsulphamezathines. The rates of enzymic hydrolysis of propionyl- and butyryl-sulphamezathines were roughly two thirds that of acetylsulphamezathine. The hydrolysis of benzoylsulphamezathine was extremely slow (Table 17):

Conjugation of sulphonamides in isolated tissues

Conjugation in different species. The experiments were carried out on sliced or minced material suspended in phosphate saline or bicarbonate saline. When mince was used the ratio of tissue to medium was between 1 : 5 and 1 : 10. The suspensions were shaken in Warburg vessels at 40°. The gas phase of the vessels contained O₂ or 5% CO₂ in O₂.

One of us (Sykes, 1944), and Lipmann (1945) have reported independently that among the tissues of various laboratory animals pigeon liver is best suited for the investigation of conjugation of sulphonamides *in vitro* because this is the only tissue where the degree of conjugation *in vitro* was found to be of the same order as in the intact animal. Tissues

examined by us include the livers and kidneys of rat, guinea-pig, rabbit, cat, dog and sheep. Conjugation never occurred in kidney. In liver it was irregular, varying between 0 and 20% of the added drug. In accordance with the findings of Klein & Harris (1938) rabbit liver was somewhat more effective than the livers of the other species, but yields were always small and irregular. There were no consistent effects with glucose, acetate, pyruvate, lactate, fumarate or tryptic casein digest. In view of the essentially negative nature of the results these experiments are not reported in full. The following experiments were all carried out on pigeons.

Table 15. *Hydrolysis of acetylsulphamezathine by various tissues of the sheep*

(Each test tube contained 4 ml. 0.2M- Na_2HPO_4 ; 0.4 ml. 0.2M- NaH_2PO_4 ; 2.5 ml. 0.04M-Na acetylsulphamezathine; 1 ml. tissue suspension; 2.1 ml. water.)

Tissue	Concentration of free sulphamezathine (mg./100 ml.) after		
	28 hr.	52 hr.	122 hr.
Kidney cortex	32	52	65
Lung	6.5	8.8	10.2
Liver	10.6	16.2	29.2
Brain	3.0	3.4	3.9
Pancreas	2.0	2.0	2.06
Spleen	8.8	9.7	11.0
Mucosa of jejunum	11.4	17.5	27.5
Blood (citratd)	7.0	9.2	11.1

No experiments appear to have been reported in the literature on the nature of sulphonamide 'conjugation' in pigeons. Experiments on the intact pigeon showed that the conjugation of sulphamezathine is essentially an acetylation. Three pigeons received subcutaneously a dose of 200 mg. sulphamezathine, as sodium salt, on three consecutive days and the excreta were collected. The injection caused the animals to empty their crops within about 30 min. and most of the subsequent food was also vomited during the 3-day period. The collected excreta were dried with anhydrous Na_2SO_4 and the dry homogeneous powder was extracted with acetone for 3 hr. in a Soxhlet. The extract contained about 200 mg. each of free and conjugated sulphonamide. The acetone was evaporated and the residue was ground up in a mortar with 2 ml. ice-cold 5% H_2SO_4 to dissolve the free sulphonamide. The insoluble residue was washed twice on the centrifuge with ice-cold water and then dissolved in hot water. On cooling, 54 mg. acetylsulphamezathine crystallized (m.p. 237°). This relatively low yield was mainly due to hydrolysis of the N^4 -acetyl group under the conditions of purification. Acetylsulphamezathine appears to be more readily hydrolyzed in faecal extracts than in pure solutions. After recrystallization from methanol a pure product was obtained which was identified by m.p. (243°), mixed m.p. and elementary analysis.

Time curves of acetylation and deacetylation in pigeon liver. Table 18 shows an example of the time

Table 16. *Hydrolysis of various acetylsulphonamides*

(Each test tube contained 4 ml. 0.2M- Na_2HPO_4 ; 0.4 ml. 0.2M- NaH_2PO_4 ; 1 ml. kidney cortex suspension; 2.5 ml. 0.02M-Na acetylsulphonamide; water to 10 ml.)

Substrate	Concentration of free sulphonamide (mg./100 ml.) after			Concentration of free sulphonamide calculated for complete hydrolysis (mg./100 ml.)
	4 hr.	29 hr.	117 hr.	
Acetylsulphanilamide	0.27	3.8	10.0	88.9
Acetylsulphamezathine	3.78	23.8	45.4	139
Acetylsulphapyridine	1.63	11.4	26.9	125
Acetylsulphathiazole	0.67	5.06	16.5	128
Acetylsulphadiazine	0.73	3.23	7.52	125
Acetyl-4:6-dimethoxysulphadiazine	14.3	66.2	123	147

Table 17. *Hydrolysis of various N^4 -acetylsulphamezathines*

(Conditions as stated in Table 16.)

Substrate	Concentration of free sulphamezathine (mg./100 ml.) after	
	21 hr.	46 hr.
N^4 -Acetylsulphamezathine	11.3	17.3
N^4 -Propionylsulphamezathine	6.8	10.8
N^4 -Butyrylsulphamezathine	8.5	11.6
N^4 -Benzoylsulphamezathine	1.6	1.8

curve of the conjugation of sulphamezathine in suspensions of minced pigeon liver. Conjugation proceeded steadily until about 55% of the added drug had reacted; the reaction then came to a standstill, and under the conditions of the experiment this occurred after about 40 min. Similar degrees of conjugation were obtained irrespective of the initial concentration of sulphamezathine. The standstill was not due to the conjugating system, for addition of further substrate caused again conjugation of about 55% of the added drug. This level is similar to that reached in the intact pigeon.

Table 18. *Conjugation of sulphamezathine in minced pigeon liver*

(Minced pigeon liver suspended in 10 parts phosphate saline containing 0.001 M-sulphamezathine (27.8 mg./100 ml.) shaken in Warburg cups at 40°. Gas phase O₂.)

Time (min.)	Concentration of sulphamezathine (mg./100 ml.)		Conjugation (%)
	Free	Total	
0	27.8	27.8	0
20	21.4	28.1	23
40	12.1	28.2	57
60	12.4	28.1	56
100	12.6	27.3	54

Table 19 gives the rate of deacetylation of sulphamezathine in pigeon liver on the same specimen under the same conditions. Deacetylation was considerably slower than acetylation. After 100 min. 27 % had undergone hydrolysis.

Table 19. *Deacetylation of sulphamezathine in minced pigeon liver*

(Conditions as in Table 16 except that 0.001 M-acetyl-sulphamezathine was the substrate.)

Time (min.)	Concentration of free sulpha- mezathine (mg./100 ml.)		Hydrolysis (%)
	Free	Total	
20	4.7	17	
40	5.6	20	
60	6.4	23	
100	7.6	27	

Effects of added substrates. Under the conditions of our experiments (see Table 18) acetate, pyruvate and acetoacetate had no effects on the rate and degree of conjugation, in contrast to the findings of Lipmann (1945). However, in view of the different experimental conditions, our results are not contradictory to Lipmann's. The tracer work of Bloch & Rittenberg (1945) together with the *in vitro* experiments of Lipmann (1945) leave little doubt that acetate can serve as a source for the acetyl group of acetylsulphonamides.

DISCUSSION

Acetylation in different species. It has often been stated that the dog is not capable of conjugating sulphonamides (Marshall, Emerson & Cutting, 1937). The data reported in this paper indicate that the differences between the dog and other species are quantitative rather than qualitative. All species examined can conjugate sulphonamides as well as hydrolyze the conjugated forms. According to the available data the rate of hydrolysis in extracts of minced tissues is of the same order of magnitude in different species. The degree of acetylation observed

in the intact animal appears to be the resultant of the activities of the acetylating and deacetylating systems. For reasons so far obscure the deacetylating system although present and active in tissue extracts of all species tested seems to be non-operative in the intact body in some species (man, rabbit) but active in others (dog, cat, pigeon).

'*Equilibrium*' between conjugated and free sulphonamide. The data given in Tables 2, 3, 5, 6, 7, 8, 9 and 10 indicate that after a single dose of acetylsulphamezathine the 'equilibrium' or steady state in the system:

appears to be attained in man and rabbit when virtually all the drug is present in the conjugated form, whilst in cat and dog it is reached when virtually all the drug is free. In the pigeon the steady state is reached when the free and acetylated forms are approximately equal in amount. It is very unlikely that these 'equilibria' are true thermodynamic equilibria, though the possibility cannot be excluded. In aqueous solution the thermodynamic equilibrium is attained when almost all the sulphonamide is free. By reducing the concentration of water the thermodynamic equilibrium would shift in favour of conjugation and it is possible that the enzyme is located in a non-aqueous phase of the tissue.

It must be pointed out that the quantitative data in the rates of acetylation and deacetylation in isolated pigeon liver (Tables 18 and 19) are not in full accordance with the conception that the 'equilibrium' is the resultant of the activities of two different enzyme systems. The rate of hydrolysis appears to be too slow to account for an 'equilibrium' at about 55 % acetylation. However, the activity of the deacetylating systems depends on factors which have not yet been studied in detail (such as the inhibitor present in high concentrations of tissue, see Table 12) and the discrepancy in the rates therefore does not necessarily invalidate the above conception.

Previous observations on the hydrolysis of acylaniline derivatives. Kohl & Flynn (1940) were the first to demonstrate the deacetylation of *N*⁴-acylsulphonamides in tissue extracts. They reported that rat liver hydrolyzes *N*⁴-acetyl-, -butyryl-, -valeryl-, -caproyl- and -heptanoylsulphanilamides; the rate increased with the chain length of the acyl group (which according to Table 17 does not apply to acylsulphamezathines). There are several observations recorded in the literature indicating that the hydrolysis occurs in the intact body. Ockerblad & Carlson (1939) found that blood and urine of dogs and human subjects contained free sulphanilamide after oral intake of *N*⁴-acetylsulphanilamide. Rose & Tuey (1946) found 4:6-dimethoxysulphadiazine

in the blood of mice after injection of its acetyl derivative and concluded that the compound is rapidly deacetylated in the body. Michel, Bernheim & Bernheim (1937) showed that acetanilide is hydrolyzed by rat-liver and rat-kidney extracts and Elson, Goulden & Warren (1946) reported that acetanilide and acetylphenetidine ('phenacetine') are deacetylated by the intact rat.

Classification of the enzyme hydrolyzing N⁴-acetylated sulphonamide. The enzyme splitting acylsulphonamides and related compounds belongs to the 'acylases', a term used by Abderhalden & von Ehrenwall (1931) to designate enzymes which hydrolyze substituted amides according to the reaction

Acylases are related to peptidases, differing from them in that the acyl residue of the substrate does not contain an amino group. Examples of acylases are hippuricase (where $R = \text{C}_6\text{H}_5$ and $R' = \text{CH}_2 \cdot \text{CO}_2\text{H}$) and the enzymes described by Kimura (1929) which hydrolyze the *N*-benzyl, acetyl and formyl derivatives of α -amino acids. The substrates used in this paper are all acyl derivatives of substituted anilines. It remains to be investigated whether the enzyme described in this paper is identical with other acylases.

SUMMARY

1. Sulphamezathine and *N*⁴-acetylsulphamezathine were given orally or parenterally to human subjects, rabbits, dogs, cats and pigeons, and the amounts of free and conjugated drug were determined in the blood and in the excreta. In man and rabbit conjugation appears to proceed as long as free sulphamezathine is present and the acetyl compound is not hydrolyzed in significant quantities. In contrast acetylation can be reversed in the pigeon, dog and cat. In these species injected acetylsulphamezathine is hydrolyzed. In pigeons there appears to be an 'equilibrium', or steady state, between free and acetylated sulphamezathine when about 55 %

of the drug is conjugated. In the dog acetylation only takes place when the concentration of the free drug in the body is relatively high and in the steady state the bulk of sulphamezathine is free.

2. Suspensions of minced mammalian tissues contain an 'acylase' first described by Kohl & Flynn (1940) which hydrolyzes *N*⁴-acylsulphonamides. Among sheep tissues, kidney cortex, liver and intestinal mucosa show the highest activity. Some properties of the enzyme are described. The pH optimum is at pH 7.3. The highest rate of hydrolysis of six sulphonamides was given by acetyl-4:6-dimethoxysulphadiazine (Table 16). *N*⁴-Acetylsulphamezathine was hydrolyzed more rapidly than the analogous propionyl, butyryl and benzoyl compounds (Table 17).

3. In sliced or minced liver or kidney of the rat, guinea-pig, rabbit, cat, dog and sheep conjugation of sulphonamides was found to be small and erratic. Minced, sliced or homogenized pigeon liver on the other hand readily conjugated sulphamezathine. The conjugation stopped when about 55 % of the added drug was acetylated. The rate and degree of acetylation were not affected by acetate, pyruvate or acetoacetate. Acetylsulphamezathine was deacetylated in pigeon liver.

4. It is suggested that the position of the steady state in the system: sulphonamide $\rightleftharpoons$ acetylsulphonamide is the resultant of the relative activities of the acetylating and deacetylating system. Though present in all species examined the deacetylating system appears to be inactive in the intact body of some species (man, rabbit). Discrepancies between the acetylating power of sliced or minced tissues and the intact body in rat, guinea-pig and rabbit may be connected with changes in the relative activities of the two enzymes, brought about by slicing and mincing.

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