The Metabolism of Sulphapyridine Containing Radioactive Sulphur in the Rabbit

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This paper records a further stage in the attempt to draw up a balance sheet for the fate of a single dose of sulphapyridine in the rabbit. Bray, Neale & Thorpe (1946) found that a considerable proportion of a dose of sulphapyridine could not be accounted for in rabbit urine by methods of estimation based upon the diazo reaction, especially at higher dose levels (0.5–1.0 g./kg.). We were unable significantly to improve the recovery by analysis of the faeces or contents of the alimentary canal. An increased excretion of inorganic sulphate in approximately one-third of the animals roughly corresponding to the missing sulphapyridine suggested the possibility of

formation of a metabolite in which the amino group of sulphapyridine has been eliminated, although we have so far failed to isolate such a compound. There is no appreciable retention of the drug in the tissues.

EXPERIMENTAL

Material and Methods

Preparation of radioactive sulphapyridine. ^{35}S was received in the form of a solution of Na_2^{35}S in NaOH (0.2 ml.). The activity of the solution on despatch was stated to be 212 μc . The method of synthesis is summarized as follows, an asterisk indicating the presence of ^{35}S :

complete catabolism, but since the increased excretion was almost within the base-line range we did not feel justified in accepting this interpretation without further evidence. The diazo method upon which we depended for estimation of the metabolites would not have revealed the presence of a metabolite in which the amino group had been modified in such a way that it could not be liberated by ordinary hydrolysis procedures. The estimation of the total sulphur of the urine would include such a metabolite, but since the expected amount of this metabolite was of the same order as that of the neutral sulphur normally excreted by the rabbit, it would be difficult to obtain conclusive information as to the extent of the excretion of a nondiazotizable metabolite owing to fluctuations in the neutral sulphur normally excreted.

In order clearly to distinguish between the excretion of the normal neutral sulphur and that due to sulphapyridine we have carried out experiments with the drug labelled with the radioactive isotope ^{35}S . These experiments indicate that there is no complete catabolism resulting in the elimination of the sulphur as inorganic sulphate. The consistently higher recovery of the drug as estimated by radioactive assay than by the diazo method suggests the

Radioactive sodium sulphate. The NaOH containing Na_2S^* was added to a filtered solution of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (10 g.) in water (50 ml.); with washings the total volume was 120 ml. This was transferred to a large covered beaker and a mixture (160 ml.) of equal volumes of 2N- NH_4OH and H_2O_2 (40 vol.) added. Heat was evolved. After 15 min. the mixture was heated gently until the evolution of O_2 ceased. The solution was then carefully evaporated to dryness. Dry weight 6.6 g.

Radioactive sulphuric acid. The $\text{Na}_2\text{S}^*\text{O}_4$ was mixed with anhydrous Na_2SO_4 and the mixture (32.45 g.) finely ground. H_3PO_4 (18 ml. of 100%) was added and the mixture distilled over a naked flame. Considerable care was necessary to avoid frothing during the early stages of heating. Distillation was continued until the contents of the flask were at a dull red heat. A small amount of water was removed from the distillate by redistillation. Yield 19.3 g. (86% of theory). (We had expected to receive ^{35}S in the form of BaSO_4 . H_2SO_4 can be prepared from BaSO_4 by fusing with excess Na_2CO_3 and K_2CO_3 . After removal of BaCO_3 and neutralization of excess alkali carbonates with H_2SO_4 the dried mixed sulphates are distilled with H_3PO_4 as above.)

Radioactive sulphanilic acid. The method used was essentially that of Erdmann (1894). The above $\text{H}_2\text{S}^*\text{O}_4$ was washed with conc. H_2SO_4 (15 ml.) into a small bolt-head flask and aniline (55 ml.) added. The mixture was heated at 210–230° in an oil bath for 3.5 hr., cooled and dissolved in Na_2CO_3 solution (750 ml., 50 g./l.). The blue solution was

decolorized with charcoal and acidified with conc. HCl. Radioactive sulphanilic acid separated and was collected and washed with water. A second crop was obtained from the filtrate. Total yield 60 g. The acid was then converted to the Na salt by the addition of 2N-NaOH. The solution was evaporated to dryness under reduced pressure and dried for 48 hr. *in vacuo* over P_2O_5 .

Radioactive acetamidodisulphanilyl chloride. This was prepared from the Na sulphanilate by the method of Schroeter (1906). Yield 37.7 g. The mother liquors on keeping deposited radioactive sulphanilic acid which was separated and treated again.

Radioactive sulphapyridine. The condensation of the acetamidodisulphanilyl chloride with 2-aminopyridine and subsequent deacetylation was carried out according to Winterbottom (1940). Total yield of radioactive sulphapyridine 12.4 g. including 3 g. from reworking of the residues from the various stages. Melting point alone or mixed with sulphapyridine (m.p. 189° , 189°). (Found: N, 16.80%. Calc. for $C_{11}H_{11}O_2N_2S$: N, 16.87%.) The intensity of colour in the diazo reaction was the same as that of the standard sulphapyridine. The radioactivity of this material, determined 5 weeks after the assay of the original ^{35}S , was such that it gave, on our apparatus, 15,300 counts/min./g. sulphapyridine or 16.5 counts/min./mg. $BaSO_4$.

Animals, diet and dosage. Rabbits weighing 2 kg., which had not previously been used for experimental purposes were used and maintained for a preliminary period and throughout the experiment on the constant diet previously described (Bray, Ryman & Thorpe, 1947). Sulphapyridine was administered by stomach tube as a suspension in water.

Ashing of organic material for estimation of ^{35}S . The S in all organic substances, and tissues, etc., was converted to $BaSO_4$ by the modification of Pirie's (1932) ashing process used by Bournsnel, Francis & Wormall (1946). Sulphapyridine treated in this way gave a quantitative yield of $BaSO_4$ within 0.7%. The weight of $BaSO_4$ was determined gravimetrically on a separate similar sample in each case.

Preparation of samples for ^{35}S assay. Urine. Inorganic and total sulphate were precipitated from urine as in the Folin (1905-6) method, isolated by centrifuging and then treated by the ashing process referred to above.

Total sulphur. Urine. 20 or 25 ml. were evaporated to 5-10 ml. on a water bath with a few drops of conc. HNO_3 . The concentrate was ashed and converted to $BaSO_4$ as above. Several similar samples gave results within $\pm 1\%$ and with added sulphapyridine the results were within -2% of the theoretical.

Faeces. Specimens (2 g.) were treated with 5 ml. of conc. HNO_3 , warmed gently till frothing ceased and then ashed and converted to $BaSO_4$.

Tissues. These were treated in the same way as faeces. If necessary the ashing process was repeated. The bulk of the $BaSO_4$ precipitate (the ^{35}S assay required approx. 25 mg. of $BaSO_4$) was increased when necessary by adding 0.5 ml. of a solution of K_2SO_4 (37.28 g./l.) to the solutions of the ashed specimens before precipitation. In this way the weight of the $BaSO_4$ was increased by 25 mg.

Assay of ^{35}S . The ^{35}S was determined by the method described by Banks (1946).

Estimation of diazotizable material. The method of Bratton & Marshall (1939) was again used. The quantities of reagents were shown to be sufficient to react with all the diazotizable material in rabbit urine under the conditions used. The maximum error observed in determining known

amounts of sulphapyridine added to normal or metabolic (sulphapyridine) urines was $\pm 5\%$. There was no significant difference in estimations of sulphapyridine in hydrolysed or unhydrolysed urine, so that glucuronic acid liberated from hydroxysulphapyridine glucuronide on hydrolysis does not appear to interfere. Kirch & Bergeim (1943) reported that ascorbic acid and other reducing agents interfered with diazo estimations.

The diazotizable material in faeces was estimated as previously (Bray *et al.* 1946). Tissues were homogenized with up to 10 parts water to 1 part tissue and treated as blood in the Bratton & Marshall (1939) method.

Sampling and collecting errors. Acetylsulphapyridine (about 300 mg.) usually separated from the urine of rabbits heavily dosed with 2-3 g. of sulphapyridine, even when the volume of urine was large. The error in sampling such urines determined on many samples of the same urine was found to be $\pm 1.1\%$.

In spite of daily washing of the metal funnels of the metabolism cages when the day's urine is collected, films of 'phosphate' tend to adhere to the funnels. These have to be removed at intervals with acid, and occasionally a positive diazo reaction can be obtained from the acid extract of the deposit. The significance of this possible error was tested by collecting, through a funnel which had been allowed to accumulate an extensive film of 'phosphate' from normal urine, the 24 hr. urine from a rabbit dosed with 2 g. of sulphapyridine. The film was then dissolved in acid and the solution hydrolysed. It yielded 12.6 mg. of diazotisable material (calc. as sulphapyridine), i.e. 0.6% of the dose. With a freshly cleaned funnel only 6 mg. could be detected.

Fractionation of urine

The urine fractions referred to in Table 3 were obtained as follows: (A) Urine was treated with a saturated solution of basic lead acetate in the usual way. The precipitate was removed and the filtrate treated with hydrogen sulphide to remove lead. (B) The precipitate from A was suspended in water and lead removed as lead sulphide.

Fractions C to H were obtained as shown in Table 1.

Table 1. Fractionation of urine

RESULTS

Table 2 shows the results of diazo and ^{35}S estimations on the excreta and tissues of four rabbits which had each received doses of radioactive sulphapyridine.

bolite. The preparation of urine fractions *A* to *H* has already been described. The distribution of metabolites in these fractions is given in Table 3. In the absence of any obvious and consistent separation in the distribution of radioactive and diazotizable

Table 2. *Recovery of sulphapyridine in the rabbit estimated by ^{35}S assay and diazo method expressed as percentage of dose*

Exp. no. ...	...	...	1		2		3		4	
Rabbit no. ...	...	...	203		205		211		210	
Dose (g.) ...	...	...	2.0		2.5		2.2		3.0	
			^{35}S	Diazo	$^{35}\text{S}^*$	Diazo	$^{35}\text{S}^*$	Diazo	^{35}S	Diazo
Urine										
Inorg. SO_4	1st day		0†	—	0	—	—	—	—	—
Inorg. SO_4	2nd day		0	—	0	—	—	—	—	—
Total SO_4	1st day		0	—	0	—	0	—	—	—
Total SO_4	2nd day		0	—	0	—	—	—	—	—
Total S	1st day		43.2	33.0	25.7	24.0	79.0	61.8	61.4	49.9
Total S	2nd day		10.5	8.4	30.4	26.8	—	(4.1)	19.8	15.0
Total S	3rd day		—	—	33.5	21.6	—	(0.8)	—	—
Bladder urine at death			—	—	0.5	0.4	—	—	—	—
Faeces										
	1st day		6.5	4.0	2.6	2.4	10.6	4.0	—	—
	2nd day		6.1	6.0	0.8	0.7	4.7	1.0	—	—
	3rd day		—	—	—	—	3.5	0	—	—
Alimentary canal‡										
	Stomach contents		—	—	1.7	0	0	0	—	—
	Small intestine contents		—	—	—	0	0	0	—	—
	Caecal contents		—	0	2.4	0.8	0	0	—	—
	Colon contents		—	—	0.3	0.2	0	0	—	—
	Bile		0	0	—	—	—	—	—	—
Tissues										
	Blood		0	0	—	—	—	—	—	—
	Brain		—	—	0	0	—	—	—	—
	Gut wall		—	—	0	0.1	—	—	—	—
	Hair		0	0	—	—	—	—	—	—
	Heart		—	—	0	0	—	—	—	—
	Kidney		0	Tr.	1.8	0.2	—	—	—	—
	Liver		0	0	0	0.1	0.0	0.1	—	—
	Muscle		0	0	0	0	—	—	—	—
	Skin		0	0	—	—	—	—	—	—
	Spleen		0	0	—	—	—	—	—	—
Recovery			66.3	51.4	99.7	77.3	97.8	66.9§ (71.8)	81.2	64.9

* Owing to some doubt as to accuracy of the radioactivity counts of the standard preparations, the results in these two experiments have been calculated from the radioactivity of the nonsulphate fraction of the urinary S compounds which is composed almost entirely of sulphapyridine or its metabolites.

† Values of 0 for ^{35}S assays indicate that the count of the samples was not significantly greater than the background count of the apparatus.

‡ The contents of the alimentary tract were removed 72 hr. after the administration of the sulphapyridine.

§ This total is comparable with that obtained from ^{35}S determinations. That in parenthesis is the true total including the second and third day total S values (given in parenthesis) which were not estimated by the ^{35}S method.

In Exps. 1–3 the rabbit was killed at the end of the third 24 hr. period after the dose, when urinary excretion of the dose was virtually complete. Exps. 1 and 2 were designed to include a large number of tissues, together with urine and faeces. Since these experiments gave no indication of storage in the tissues, a more detailed examination of intestinal contents was made in Exp. 3. In Exp. 4 an attempt was made to fractionate the urine with a view to directing the search for a nondiazotizable meta-

material, it seemed that fraction *D*, the ether extract, would be the most profitable one to examine for a nondiazotizable metabolite.

Search for a nondiazotizable metabolite. Two attempts were made to detect such a metabolite in the solid material obtained by extraction with ether at pH 6 for 48 hr. At this pH very little ether-soluble material is extracted from normal rabbit urine. (1) The ether-soluble material from 24 hr. urines of forty rabbits dosed with a total of 90 g. of sulpha-

Table 3. *Distribution of sulphapyridine and metabolites in urine fractions expressed as percentage of dose*

Exp. no. ...	3		4		4	
	1st day		1st day		2nd day	
	³⁵ S	Diazo	³⁵ S	Diazo	³⁵ S	Diazo
Total S	79.0	61.8	61.4	49.9	19.8	15.0
A	13.9	10.8	8.3	9.7	—	—
B	—	—	32.6	25.2	—	—
C	25.7	9.4	23.3	16.4	6.3	4.2
D	—	—	39.3	31.6	9.2	11.2
E	—	—	9.5	6.2	1.8	1.5
F	—	—	—	—	5.2	2.3
G	—	—	2.9	2.4	—	—
H	—	—	5.2	3.8	—	—

* The preparation of these fractions has been described in the text.

pyridine was dissolved in 50 % aqueous ethanol, decolorized with a small amount of charcoal and progressively concentrated to yield successive crops of crystals. These fractions were mixtures of sulphapyridine and acetylsulphapyridine, which, when hydrolysed, gave only sulphapyridine, m.p. 189°, alone or mixed with an authentic specimen. (2) The ether-soluble material from urine corresponding to a dosage of 60 g. of sulphapyridine was hydrolysed by boiling with 250 ml. of 2*N*-sodium hydroxide. Crude sulphapyridine was precipitated by neutralization with hydrochloric acid and was separated. The filtrate was evaporated to dryness and extracted three times with boiling absolute ethanol, which removed a small amount (0.5 ml.) of phenolic material (S free). The ethanol insoluble material was almost entirely sodium chloride. The crude sulphapyridine was fractionally crystallized. After separation of large amounts of sulphapyridine, m.p. 189°, a final fraction (1 g.), m.p. 182–185° (mixed melting point with sulphapyridine 185–189°), was obtained which gave colours with ferric chloride and with acid nitrite similar to those given by hydroxysulphapyridine (Bray *et al.* 1946). All the other sulphapyridine fractions gave the yellow colour with nitrite, but no ferric chloride colour, although the impurity was not sufficient to depress the melting point or affect quantitative diazo estimation. All fractions identified as sulphapyridine gave the expected values by diazo estimation. No other metabolites were detected and it was concluded that the crude sulphapyridine consisted of sulphapyridine mixed with very small amounts of hydroxysulphapyridine.

DISCUSSION

It is clear from Exps. 1–3 that no significant amount of ³⁵S is excreted in either the inorganic or total sulphate of urine, since in every case the radioactivity of the barium sulphate from these fractions was not

significantly greater than the background count of the apparatus corresponding to less than 0.2 % of the dose. Thus there is no support for the previous suggestion (Bray *et al.* 1946) that some sulphapyridine is completely catabolized. The experiments also indicate that there is no appreciable storage of the drug in the tissues. Only the kidney in Exp. 2 contained significant amounts of ³⁵S and this is, perhaps, not surprising in view of the lag in excretion of the drug by this animal. It is rare to find so high an excretion on the third day. The presence of ³⁵S in the alimentary canal after the third day in this experiment suggests that this animal absorbed the drug more slowly than did the others. Kinnunen (1946) also was unable to find evidence of storage of sulphapyridine. He noted that after administration of aneurin, together with sulphapyridine, there was a decreased excretion of diazotizable material in urine but no storage in the tissues.

The values obtained by the ³⁵S assays were almost always greater than those by diazo estimations. When the urine was fractionated as in Exp. 4, these differences persisted, but there was no indication of the separation of a fraction with a much higher ³⁵S/diazo ratio. With regard to the actual average difference of about 25 % between the ³⁵S and diazo values for material excreted in urine, it has been shown that the errors in the chemical procedures of the two methods are of the order of –2 % and ±5 %, respectively, so that the maximum discrepancy which should have been observed would be of the order of –7 %, leaving nearly 20 % to be accounted for. It was this which prompted our search for a nondiazotizable metabolite. The difficulty of separating such a metabolite from sulphapyridine, as indicated by the similarity of the ³⁵S/diazo ratios of the urine fractions, makes it probable that this metabolite, if it exists, has a structure close to that of sulphapyridine, possibly only the amino group being modified. The attempts at isolation of this metabolite have so far been unsuccessful.

SUMMARY

1. Sulphapyridine containing ^{35}S has been synthesized.

2. Metabolic experiments with this compound in the rabbit have shown that there is no complete catabolism to inorganic sulphate and no significant retention of the drug in the tissues after 3 days.

3. Differences between the values obtained by assays of ^{35}S and diazotizable material suggested the formation of a metabolite which does not diazotize or couple normally but this has not yet been isolated.

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The Fate of Certain Organic Acids and Amides in the Rabbit

10. THE APPLICATION OF PAPER CHROMATOGRAPHY TO METABOLIC STUDIES OF HYDROXYBENZOIC ACIDS AND AMIDES

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Metabolic processes resulting in hydroxylation of aromatic compounds are well known. The proportion of a given compound so changed varies however with the structure and the consequent metabolic alternatives. If the compound already contains conjugable groups or readily convertible 'potential centres for conjugation' (Bray, Ryman & Thorpe, 1948) the phenols obtainable by hydroxylation may only be formed in small amount. In the past it has often not been possible to identify and determine very small quantities of such phenolic compounds in urines containing normal amounts of other phenols. We have now shown, however, that the method of paper chromatography originally developed for identification of small amounts of amino-acids by Consden, Gordon & Martin (1944) can usefully be adapted to the identification of phenolic compounds. This paper shows how the method has enabled us to confirm and to extend some earlier observations on the hydroxylation and glycine conjugation of the hydroxybenzoic acids and their amides.

METHODS

Paper

Whatman filter paper no. 4 was used.

Solvents

(All percentages are v/v.) The two main series of developing solvents used consisted of benzene-acetic acid-water and CHCl_3 -acetic acid-water. By varying the relative proportions of the constituents, R_F values could be varied within wide limits. By increasing the amount of acetic acid (formic acid was equally effective) the R_F value of a compound was increased. By these means R_F values could be adjusted so as to give maximum separation and bring compounds into suitable positions on the chromatogram. The benzene series, especially benzene (40%), acetic acid (40%), water (20%), was suitable for the majority of the compounds. The CHCl_3 series, e.g. CHCl_3 (50%), acetic acid (25%), water (25%), was used for separating compounds having low R_F values in the benzene series. *n*-Butanol-acetic acid-water mixtures gave similar results to the CHCl_3 series. The acetic or formic acid in these mixtures greatly reduced 'trailing'. These solvents have proved suitable for detection of aromatic monohydroxy acids and amides and