with continued heating, the reducing power after 0, 40 and 50 min. of heating being 100, 91 and 89 % respectively.

The findings with filter paper and glucose suggest that the conditions of the analysis, as adapted to bacterial cellulose, can be used with cellulose polymers of widely varying degree of polymerization. Although some loss of reducing power due to the instability of a free reducing group may be expected when the analysis is applied to the lower homologues, it is evident that, even in the extreme case of glucose, the overall loss of reducing power is not greater than 20 %.

Influence of salt concentration on colour intensity

The neutralized cellulose hydrolysate contains approximately 1.7 m-moles/ml. sodium acetate and 0.3 m-mole/ml. sodium sulphate. The colour formed with the arsenomolybdate reagent was found to be unaffected by the presence of these salts, provided that the buffering action of the sodium acetate was overcome by an appropriate addition of acid.

SUMMARY

1. A technique for the purification and quantitative recovery of small samples of bacterial cellulose has been detailed.

2. A micromethod for cellulose has been based on the dissolution of the purified polysaccharide by acetolytic degradation, the conversion of the product into reducing hexose by acid hydrolysis, and the colorimetric estimation of the formed sugar with the aid of a copper reagent.

3. The applicability of the technique to bacterial celluloses and cotton cellulose has been verified.

4. The method is relatively rapid and specific and is appropriate to serial analysis. The lower threshold is 20 μ g.; the error range is ± 4 %.

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Biochemical Studies of Toxic Agents

5. OBSERVATIONS ON THE FATE OF ^{35}S -LABELLED ARYLSULPHURIC ACIDS FOLLOWING THEIR ADMINISTRATION TO THE RAT

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A number of workers (for example, Rhode, 1923; Sperber, 1948; Garton & Williams, 1949) have studied the fate of arylsulphuric acids in the animal body, and their results suggest that when these compounds are administered to animals almost the whole of the dose is excreted unchanged in the urine. These findings were based on the determination of the increase in ethereal sulphate excretion over the 'normal level' following the administration of the sulphuric acid derivative. As ethereal sulphate excretion fluctuates somewhat under normal conditions, it is clear that experiments of this type cannot be expected to reveal with certainty a small degree of hydrolysis of an arylsulphuric acid *in vivo*. A more satisfactory method for studying this problem is provided by the measurement of the excretion of ^{35}S -labelled inorganic sulphate following the administration of an arylsulphuric acid labelled with ^{35}S . This method has been used in the present investigation. The potassium salts of [^{35}S]phenylsulphuric acid, and [^{35}S]1- and [^{35}S]2-naphthyl-

sulphuric acid were synthesized and administered to rats by stomach tube and by intraperitoneal injection. The urinary excretions of inorganic sulphate- ^{35}S and total sulphate- ^{35}S were determined at various times during the 96 hr. after dosing. Similar results were obtained with the three compounds, namely, that although most of the administered arylsulphuric acid was quickly excreted unchanged, some breakdown of the compound did occur in the organism.

EXPERIMENTAL

Synthesis of the potassium salts of phenylsulphuric acid and 1- and 2-naphthylsulphuric acid labelled with ^{35}S

The method used for the synthesis of the radioactive arylsulphuric acids was based on that described by Burkhardt & Lapworth (1926). Except in the final stages, the same procedure was used for the preparation of all three compounds. The [^{35}S]chlorosulphonic acid used in the syntheses was obtained from the Radiochemical Centre, Amersham. It was

packaged in glass vials each containing 1 ml., and at the time of its receipt its specific activity was 1.06 mc/ml. The hazardous stages of the synthesis were carried out in glove boxes.

CS₂ (20 ml.) and dimethylaniline (14.5 ml.) were stirred together in a wide tube cooled in ice water. Radioactive chlorosulphonic acid (2.0 ml.), containing about 1 mc ³⁵S/ml., was added slowly to the mixture. The reaction vessel was then removed from the ice water and the theoretical quantity of the phenolic compound (2.8 g. phenol, 4.3 g. 1- or 2-naphthol) was added with stirring. Stirring was continued for 1 hr. and the mixture was then allowed to stand overnight. The potassium salt of the arylsulphuric acid was formed by slowly adding 50% (w/v) aqueous KOH with continuous stirring and cooling until all the dimethylaniline had been liberated and the mixture was alkaline (about 12 ml. of the KOH soln. were required). The potassium salt separated and as much as possible of the liberated dimethylaniline was poured off. The remainder was removed by washing the potassium salt with three 100 ml. portions of ether. The potassium salt was then dissolved in the minimum quantity of water at 70–80° and the hot solution was filtered rapidly. The filtrate was allowed to cool to room temperature and then transferred to the refrigerator. The crystals which separated were filtered off, sucked dry on the filter and then washed with ether.

Potassium phenylsulphate and potassium 2-naphthylsulphate were readily purified by repeated crystallization from water. The potassium 1-naphthylsulphate was contaminated with 1-naphthol and it was purified by converting it into the *p*-toluidine salt by the procedure described by Barton & Young (1943). The *p*-toluidine salt was dissolved in hot water and treated with charcoal. The hot solution was filtered and the filtrate was treated with 20% (w/v) aqueous KOH to decompose the *p*-toluidine salt. The crystals of potassium 1-naphthylsulphate which separated on cooling were recrystallized from water.

After three crystallizations from water each of the three compounds was found to be free from inorganic sulphate, chloride and phenol (or naphthol). Further recrystallization of the compounds had no significant effect on their specific activity. Analysis of the compounds for S by the Carius method gave: potassium phenylsulphate, 15.4; calc. for C₆H₅O₄SK, 15.1%; potassium 1-naphthylsulphate, 11.8; potassium 2-naphthylsulphate, 12.2; calc. for C₁₀H₇O₄SK, 12.2%.

Measurement of ³⁵S

Radioactive sulphate was separated in the form of benzidine sulphate preparatory to the measurement of its ³⁵S content. Although detailed studies of this method of measuring ³⁵S have already been reported (Henriques, Kistiakowsky, Margnetti & Schneider, 1946; Young, Edson & McCarter, 1949), it was necessary to modify these procedures in order to meet the requirements of the present investigation.

³⁵S content of solutions containing radioactive inorganic sulphate. The inorganic sulphate content of the solution was adjusted by dilution or by the addition of non-radioactive Na₂SO₄ solution so that 6.0 ml. of the solution contained not less than 2 mg. of inorganic sulphate-S, and preferably not more than 3 mg. To this solution, in a lipped boiling tube, were added 4.0 ml. of 95% (v/v) ethanol followed by 2.0 ml. of benzidine hydrochloride reagent (prepared as described by Young *et al.* 1949). The contents of the tube were then gently

mixed and allowed to stand for 30 min. before filtration. The precipitate of benzidine sulphate was separated using an apparatus of the type described by Henriques *et al.* (1946) in which the filter paper rests on a sintered-glass plate. Whatman no. 30 filter papers of 2.3 cm. diameter were used for the filtration. The boiling tube, filter and precipitate were washed with three 5 ml. portions of 95% (v/v) ethanol. As soon as filtration was complete the disk of filter paper carrying the precipitate was mounted on a carrier which consisted of two Perspex plates held together by three brass bolts. The dimensions of the lower plate were such that it fitted the rack of the chamber of the lead castle. The upper plate was somewhat smaller. The filter-paper disk carrying the radioactive benzidine sulphate was placed in the centre of the lower plate. The upper plate, in the centre of which was a circular hole 2.1 cm. in diameter, was then bolted to the lower plate so that the filter paper was held around its edge and the precipitate was left exposed. After it had been mounted on the carrier, the benzidine sulphate was dried in a desiccator over anhydrous CaCl₂ for at least 1 hr. before counting. The thickness of the window of the Geiger-Müller tube employed in the counting equipment was 1.8 mg./sq.cm. (EHM2, General Electric Co., Ltd., London).

After each sample of radioactive benzidine sulphate had been counted, it was transferred to a boiling tube with 5.0 ml. of water and titrated with 0.02 N-NaOH using phenol red as indicator. By this means the sulphate-S content of the sample was determined, thus enabling a correction for self-absorption to be applied to the radioactivity measurement.

³⁵S contents of the inorganic sulphate and total sulphate fractions of urine. When the benzidine precipitation procedure was applied to a solution containing Na₂SO₄ and a radioactive arylsulphate, a precipitate was obtained which contained a significant proportion of the radioactivity originally present in the solution. It appeared, therefore, that ethereal sulphate had been precipitated together with the inorganic sulphate on the addition of the benzidine reagent. This was not unexpected in view of the known low solubility of organic base salts of arylsulphuric acids (cf. Barton & Young, 1943; Laughland & Young, 1944; Berenbom & Young, 1951). The radioactive material in the benzidine sulphate precipitate could not be removed by various washing procedures. Experiment showed, however, that when the benzidine sulphate precipitate was separated, dissolved and then reprecipitated, the amount of radioactive material contaminating the precipitate was greatly reduced and there was no significant loss of inorganic sulphate. This finding led to the development of the following reprecipitation procedure for the measurement of the ³⁵S content of the inorganic sulphate fraction of a solution containing radioactive ethereal sulphate.

A measured volume (less than 6.0 ml.) of the solution to be analysed was transferred to a 15 ml. tapered centrifuge tube and its inorganic sulphate-S content was adjusted to 2–3 mg. The volume of the solution was brought to 6.0 ml. with water, and 4.0 ml. of 95% (v/v) ethanol were added followed by 2.0 ml. of benzidine reagent. The mixture was allowed to stand for 10 min. and was then centrifuged for 10 min. The supernatant liquid was discarded and the precipitate was suspended in 5 ml. of 95% (v/v) ethanol. It was centrifuged and the supernatant was again discarded. The precipitate was decomposed by warming it with 1.0 ml. of 0.2 N-NaOH. The volume of the solution was brought to 5.9 ml. by the addition of water, and 4.0 ml. of 95% (v/v) ethanol were added followed by 0.1 ml. of 2 N-HCl and 2.0 ml.

of benzidine reagent. Where only two precipitations of the benzidine sulphate were required, the second precipitate was filtered, washed, dried, and counted by the procedure already described. Where three precipitations of the benzidine sulphate were required, the second precipitate was washed in the centrifuge tube, dissolved in NaOH sol., and reprecipitated. The benzidine sulphate was then filtered, washed, and counted in the usual way.

The effect of this reprecipitation procedure in reducing the amount of arylsulphate carried down with the benzidine sulphate was studied quantitatively. A series of experiments was carried out in which 2.0 mg. of inorganic sulphate-S were precipitated once in the presence of 0.01, 0.05, 0.10, 0.15, 0.20 and 0.25 mg. radioactive ethereal sulphate-S (as phenylsulphate, 1-naphthylsulphate or 2-naphthylsulphate) and the radioactivity of the precipitates was measured in the usual way. This series of experiments was then repeated using the same amounts of inorganic sulphate and radioactive ethereal sulphate, but instead of measuring the radioactivity of the first precipitate of benzidine sulphate, this precipitate was dissolved and precipitated a second time (phenylsulphate) and a third time (1- and 2-naphthylsulphate) by the methods already described. All determinations were carried out in duplicate, and Table 1 shows the mean values for experiments with solutions of inorganic sulphate and radioactive arylsulphate in water and in normal rat urine (2 ml.). It will be noted that two precipitations in the case of solutions containing phenylsulphate and three precipitations in the case of solutions containing 1- or 2-naphthylsulphate reduced the radioactivity of the final benzidine sulphate precipitate to less than 2% of the total radioactivity originally present as arylsulphate.

In the measurement of the ^{35}S content of the inorganic sulphate fraction of the urine the volume of urine used was between 1.0 and 5.0 ml., the amount used being dependent on its radioactivity. In each determination 2.0 mg. of sulphate-S (as Na_2SO_4) were added so that the inorganic sulphate-S was between 2.0 and 3.0 mg. Reprecipitation of the benzidine sulphate was carried out as already described.

The following method was used for the measurement of the ^{35}S content of the total sulphate fraction of the urine. A measured volume of urine was made $\approx$ with respect to its content of HCl and 2.0 mg. of sulphate-S (as Na_2SO_4) were added. The urine was evaporated to dryness and the residue

was taken up in 6.0 ml. of water, filtered if necessary, and 5.0 ml. of the solution was analysed for its ^{35}S content.

Total ^{35}S content of faeces. The faeces were ground in a mortar, and a weighed sample was oxidized by the Carius method as described by Young *et al.* (1949). The sulphuric acid produced by this procedure was precipitated as the benzidine salt, the radioactivity of which was then measured.

Total ^{35}S content of compounds. The Carius oxidation procedure was used.

Observations on the hydrolysis of arylsulphates in urine

The presence of radioactive inorganic sulphate in the urine of rats dosed with ^{35}S arylsulphates can only be used as evidence of the breakdown of ethereal sulphates in the organism if it can be shown that no significant amount of hydrolysis of these compounds occurs in the urine during the period between its excretion and the time it is analysed. This was studied as follows.

To 20 ml. of rat urine was added 0.05 m-mole of radioactive ethereal sulphate (phenylsulphate, 1- or 2-naphthylsulphate). Two 1.0 ml. portions were withdrawn immediately and analysed for the ^{35}S content of the inorganic sulphate fraction using the reprecipitation procedure already described. The urine was allowed to stand at room temperature and samples were taken for ^{35}S analysis at 24, 48 and 72 hr. No evidence was found of breakdown of ethereal sulphate during the first 24 hr. From 24 to 72 hr. some breakdown did occur, but this was quite small and was not significant in the present investigation since no collection period exceeded 24 hr. and the urines were analysed immediately after they had been collected. It is possible that the small liberation of sulphate which did occur in the urine on standing was the result of bacterial action, for a number of bacteria have been shown to possess arylsulphatase activity (Young, Morrison & Whitehead, 1952; Whitehead, Morrison & Young, 1952).

Table 1. *The influence of reprecipitation of benzidine sulphate on the amount of arylsulphate- ^{35}S precipitated together with 2 mg. inorganic sulphate-S*

Arylsulphate-S (mg.)	Percentage of arylsulphate- ^{35}S precipitated											
	Potassium phenylsulphate				Potassium 1-naphthylsulphate				Potassium 2-naphthylsulphate			
	In water		In urine		In water		In urine		In water		In urine	
	1st	2nd	1st	2nd	1st	3rd	1st	3rd	1st	3rd	1st	3rd
	ppt.	ppt.	ppt.	ppt.	ppt.	ppt.	ppt.	ppt.	ppt.	ppt.	ppt.	ppt.
0.01	3.5	0.8	2.8	1.0	12.9	1.2	8.4	1.0	25.6	2.0	28.2	1.6
0.05	3.5	0.7	3.1	0.8	8.6	1.2	7.8	1.1	19.9	0.8	19.0	0.9
0.10	3.1	0.7	2.9	0.7	6.8	1.1	7.0	1.1	15.4	0.6	15.6	0.8
0.15	2.7	0.7	3.1	0.6	6.4	1.4	5.9	1.2	14.7	0.5	15.3	0.7
0.20	2.5	0.7	2.5	0.7	5.8	1.2	5.3	1.1	11.3	0.4	12.4	0.6
0.25	2.5	0.7	2.5	0.7	5.5	1.2	5.7	1.1	11.5	0.3	13.0	0.4

(For details, see text.)

Metabolic experiments

Two metabolic experiments were performed with each of the three ^{35}S -labelled compounds: potassium phenylsulphate, potassium 1-naphthylsulphate, and potassium 2-naphthylsulphate. In the first experiment the compound was given by intraperitoneal injection, and in the second it was given by stomach tube. Two male rats weighing between 140 and 200 g. were used in each experiment. They were housed in a metabolism cage which permitted the collection of urine separate from faeces and they received food and water *ad lib*. Each rat received a single dose of 0.05 m-mole of the potassium salt of the radioactive arylsulphate dissolved in water (0.5 ml. in the case of phenylsulphate and 1-naphthylsulphate, and 1.0 ml. in the case of 2-naphthylsulphate). The urine of the dosed animals was collected for the periods 0-6, 6-24, 24-48, 48-72 and 72-96 hr. After each collection the cages were washed down with distilled water, and the urine and washings were filtered through a cotton-wool plug and made up to 100 ml. The urine was analysed for inorganic sulphate- ^{35}S and total sulphate- ^{35}S . The results obtained are shown in Table 2. The faeces were collected and stored in the refrigerator. The total collection of faeces for each experiment was sampled and analysed for total ^{35}S .

In every experiment over 80 % of the administered ^{35}S was excreted in the urine as total sulphate during the 96 hr. period after dosing. The amount of ^{35}S which appeared in the faeces during this period was small. After intraperitoneal injection of the

compounds, the total ^{35}S content of the faeces corresponded to the following percentages of the ^{35}S administered: phenylsulphate, 0.2; 1-naphthylsulphate, 0.3; 2-naphthylsulphate, 2.1; the amounts found after administration of the compounds by stomach tube were 0.2, 5.6 and 3.1, respectively.

Irrespective of whether the arylsulphates were given by intraperitoneal injection or by stomach tube, there was a rapid excretion of ^{35}S in the ethereal sulphate fraction of the urine, indicating that the administered compounds were being excreted unchanged. Some hydrolysis of the compounds did occur in the organism, however, for in the 96 hr. after their intraperitoneal injection the ^{35}S excreted as inorganic sulphate in the urine corresponded to 3.5 % of the phenylsulphate, 11.4 % of the 1-naphthylsulphate and 8.1 % of the 2-naphthylsulphate, and after administration of the compounds by stomach tube the values were 5.5, 11.8 and 8.4 %, respectively.

DISCUSSION

The presence of radioactive inorganic sulphate in the urine following the administration of the salt of a [^{35}S]arylsulphuric acid can be accepted as evidence that breakdown of the arylsulphuric acid has occurred in the organism provided certain conditions obtain. One of these is that the administered compound should be free from radioactive inorganic sulphate. This was ensured in the present work by recrystallizing each compound just prior to its use in the metabolic experiments and by testing it for the

Table 2. *The distribution of ^{35}S in the inorganic, ethereal, and total sulphate fractions of the urine ($I^{35}\text{S}$, $E^{35}\text{S}$ and $T^{35}\text{S}$) of pairs of rats following the administration of potassium phenylsulphate, potassium 1-naphthylsulphate, and potassium 2-naphthylsulphate (0.10 m-mole per pair of rats) by intraperitoneal injection and by stomach tube*

Period after dosing (hr.)	Excretion of ^{35}S in urine (% of ^{35}S administered)*								
	Potassium phenylsulphate			Potassium 1-naphthylsulphate			Potassium 2-naphthylsulphate		
	$I^{35}\text{S}$	$E^{35}\text{S}$	$T^{35}\text{S}$	$I^{35}\text{S}$	$E^{35}\text{S}$	$T^{35}\text{S}$	$I^{35}\text{S}$	$E^{35}\text{S}$	$T^{35}\text{S}$
Administration by intraperitoneal injection									
0-6	1.1	83.3	84.4	3.7	66.0	69.7	2.5	67.8	70.3
6-24	1.8	5.1	6.9	6.5	9.3	15.8	4.5	12.0	16.5
24-48	0.3	0.9	1.2	0.9	0.7	1.6	0.8	1.4	2.2
48-72	0.2	0.3	0.5	0.2	0.2	0.4	0.2	0.4	0.6
72-96	0.1	0.4	0.5	0.1	0.1	0.2	0.1	0.2	0.3
0-96	3.5	90.0	93.5	11.4	76.3	87.7	8.1	81.8	89.9
Administration by stomach tube									
0-6	3.2	79.0	82.2	1.7	41.5	43.2	2.1	55.2	57.3
6-24	1.9	9.3	11.2	8.0	26.0	34.0	5.0	23.6	28.6
24-48	0.3	0.4	0.7	1.5	3.7	5.2	0.9	1.8	2.7
48-72	0.1	0.1	0.2	0.4	0.5	0.9	0.3	0.5	0.8
72-96	<0.1	<0.1	<0.1	0.2	0.3	0.5	0.1	0.2	0.3
0-96	5.5	88.8	94.3	11.8	72.0	83.8	8.4	81.3	89.7

* Counts/min. administered by intraperitoneal injection: potassium phenylsulphate, 221990; potassium 1-naphthylsulphate, 162400; potassium 2-naphthylsulphate, 262000. Counts/min. administered by stomach tube: potassium phenylsulphate, 221990; potassium 1-naphthylsulphate, 148900; potassium 2-naphthylsulphate, 248900.

presence of inorganic sulphate. It is also necessary to avoid hydrolysis of urinary radioactive ethereal sulphate during the period which elapses between the excretion of the urine and the taking of samples for analysis. Experiments in which each of the radioactive arylsulphates used in the metabolic studies was added to rat urine showed that liberation of inorganic sulphate from these ethereal sulphates in the urine could be disregarded as a source of error provided the collection period did not exceed 24 hr. and the urine was analysed immediately afterwards.

In the present work it was found that when non-radioactive inorganic sulphate is precipitated by the benzidine method from a solution containing ethereal [^{35}S]sulphate, the precipitate which forms is radioactive, and the radioactivity cannot be removed by ordinary washing procedures. This difficulty was largely overcome by separating the precipitate, dissolving it in alkali, and reprecipitating it with benzidine hydrochloride. As Table 1 shows, the reprecipitation procedure always reduced the error to the point at which not more than 2% of the radioactivity present in the urine as arylsulphate appeared in the inorganic sulphate fraction. In general the error introduced from this source was considerably less than 2%.

The amounts of radioactive inorganic sulphate found in the urine corresponded to 4–12% of the administered arylsulphates and it appears, therefore, that the urine contained inorganic sulphate arising from a breakdown of the arylsulphates within the organism. The results in Table 2 also show that, except in one experiment, over half the dose of each arylsulphate appeared unchanged in the urine in the 6 hr. period after dosing, and that in every case more than two-thirds of the dose was excreted unchanged in the urine in the first 24 hr. period after dosing. In view of the small amount of arylsulphate which underwent hydrolysis in the organism, it is noteworthy that Hanahan & Everett (1950) found that in the rat a large proportion of a dose of sodium oestrone [^{35}S]sulphate was hydrolysed. They also found that after the administration of radioactive sodium oestrone sulphate to rats by the intravenous, subcutaneous, and gastric routes, the amounts of ^{35}S which appeared in the faeces were not greatly different from those excreted in the urine. In the present work the amounts of ^{35}S in the faeces were small compared with those in the urine and none exceeded 6% of the ^{35}S administered as arylsulphate. These observations show that the arylsulphates studied were well absorbed when given by stomach tube. The amounts of radioactive inorganic sulphate excreted in the urine following the administration of the arylsulphates by stomach tube were similar to those present after these compounds had been given by intraperitoneal injection and it

appears likely that little hydrolysis of the arylsulphates occurred in the gastro-intestinal tract.

SUMMARY

1. Potassium phenylsulphate, potassium 1-naphthylsulphate and potassium 2-naphthylsulphate labelled with radioactive sulphur (^{35}S) have been synthesized using [^{35}S]chlorosulphonic acid.
2. A study has been made of the conditions necessary for the accurate measurement of the amount of ^{35}S present as inorganic sulphate in urine containing [^{35}S]arylsulphate.
3. Experiments have been carried out in which the potassium salts of [^{35}S]phenylsulphuric acid, and [^{35}S]1- and [^{35}S]2-naphthylsulphuric acid have been administered to rats (0.05 m-mole per rat) by intraperitoneal injection and by stomach tube.
4. Under these conditions little hydrolysis of these arylsulphates occurred in the rat, for the total excretion of radioactive inorganic sulphate in the urine in the 96 hr. after dosing was only 4–12% of the ^{35}S administered as arylsulphate.
5. Most of the administered arylsulphate was excreted unchanged in the urine within a few hours of dosing. Less than 6% of the ^{35}S administered as arylsulphate appeared in the faeces.

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