5. After treatment with leucocidin, leucocytes and macrophages swell, lose potassium and gain sodium. The permeability to amino acids, nucleotides and phosphate esters is only slightly changed. There is a release of inorganic phosphorus from the leucocidin-treated leucocyte.

6. There is an accumulation of inorganic phosphorus in the leucocidin-treated leucocyte and a corresponding decrease in the acid-soluble organic phosphorus content. There is a decrease in the adenosine triphosphate content. These effects are not inhibited by fluoride.

7. Protein is released from the leucocidin-treated leucocyte but is not derived from the soluble protein of the cell. Lysozyme and phagocytin activities have been found in the protein released by leucocidin.

8. The protein released by leucocidin from the leucocyte can be readsorbed by the insoluble fraction of homogenized leucocytes.

I wish to thank Mr L. Taylor for valuable technical assistance. This work was assisted in part by a grant from the United States Office of Naval Research (Project 103-474).

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The Metabolism of Exogenous N-Acetyl-D-glucosamine 6-O^[35S]-Sulphate in the Normal Rat

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(Received 6 February 1961)

Several workers have observed the excretion of inorganic and ester [³⁵S]sulphate after the administration of chemically synthesized polysaccharide [³⁵S]sulphates to laboratory animals (Morrow *et al.* 1952; Huseman, Hoffman, Lotterle & Widersheim, 1952; Ricketts, Walton & Sadding-

ton, 1954). Similar observations were made after the administration of ³⁵S-labelled biosynthetic preparations of chondroitin sulphate to rat (Dohlman, 1956; Dziewiatkowski, 1956) and of heparin to dog (Danishefsky & Eiber, 1959). None of the polysaccharide sulphates used so far is

suitable for specific examination of the ester sulphate group *in vivo*. The structures of both chemically synthesized and biosynthetic preparations are still incompletely defined, and in no instance have the positions of the ester sulphate groups on the carbohydrate moieties been established with certainty. Further, from the methods of preparation reported, the samples of chondroitin sulphate administered by Dohlman (1956) and Dziewiatkowski (1956) almost certainly consist of a mixture of the two isomeric forms, chondroitin sulphates A and C. Available evidence (summarized by Meyer, 1960) suggests that the sulphate group occupies position 4 of the *N*-acetyl-D-galactosamine residues in the A isomer, but is esterified to position 6 of these units in the C isomer. In α -heparin, as used by Danishefsky & Eiber (1959), the structure proposed by Wolfrom, Montgomery, Karabinos & Rathgeb (1950) suggests polysulphation of the polysaccharide chain with sulphate esterified to the amino group and position 4 of the D-glucosamine residues and to position 2 or 3 of some of the D-glucuronic acid moieties. Obviously, the use in metabolic studies of materials possessing [^{35}S]sulphate groups esterified to several chemically different positions makes subsequent interpretation of experimental data excessively complicated.

Previous work (Lloyd, 1959*a*, 1960) has been concerned with the development of methods for the preparation of monosaccharide sulphates of defined constitution suitable for metabolic examination. The present paper describes observations on the metabolism of potassium *N*-acetyl-D-glucosamine 6-*O*-[^{35}S]sulphate in the rat. A preliminary account of part of this work has already been given (Lloyd, 1961).

MATERIALS AND METHODS

Potassium N-acetyl-D-glucosamine 6-*O*-[^{35}S]sulphate. This was prepared by a modification of the methods described by Lloyd (1959*a*, 1960). *N*-Acetyl-D-glucosamine (1.0 g.; Roseman & Ludowieg, 1954) was dissolved in 9 ml. of dry pyridine over 200 mg. of Drierite (L. Light and Co. Ltd.) at 50°, and the whole cooled to 5° before the dropwise addition of a mixture of 0.22 ml. of chloro[^{35}S]sulphonic acid (total activity 21 mc) and 0.13 ml. of unlabelled chlorosulphonic acid in 0.9 ml. of dry CHCl_3 . After the mixture had been stirred at 5° for 30 min. its temperature was raised to 20° and stirring continued for a further 2 hr. On standing two layers were formed; the upper pyridine layer was removed by decantation and the lower oily layer was dissolved in water with vigorous stirring. Removal of inorganic [^{35}S]sulphate ions, pyridine and CHCl_3 was achieved as described by Lloyd (1960). The clear aqueous solution containing the crude [^{35}S]sulphate ester as the barium salt was concentrated to small volume, and the ester precipitated by the addition of 5 vol. of ethanol. Precipitated material was separated by centrifuging, and then recrystallized three times via the brucine salt before conversion into the potassium salt (Lloyd, 1960).

The homogeneity of the crude product was examined by electrophoresis on Whatman no. 1 filter paper in 0.1M-ammonium acetate-acetic acid buffer (pH 4.0) for 2 hr. at 400 v. The strip was dried, sprayed with Morgan-Elson reagents (Partridge, 1948) and scanned for radioactivity. Three components were detected, two of which were radioactive and identical in electrophoretic mobility with authentic *N*-acetyl-D-glucosamine 6-*O*-sulphate and *N*-acetyl-D-glucosamine disulphate respectively. The third component was not radioactive and corresponded to *N*-acetyl-D-glucosamine (cf. Lloyd, 1959*a*, 1960).

The *N*-acetyl-D-glucosamine mono[^{35}S]sulphate was separated from the contaminating di[^{35}S]sulphate and parent compounds by zone electrophoresis on a cellulose column (80 cm. $\times$ 2 cm.) in 0.1M-ammonium acetate-acetic acid buffer (pH 4.0) at 500 v. After 28 hr. the electrophoresis was stopped and the column eluted at 12 ml./hr. Fractions were monitored for radioactivity and where positive were examined by paper electrophoresis as above. Fractions containing a single component identical in electrophoretic mobility with the authentic 6-*O*-sulphate ester were pooled and concentrated to dryness by rotary evaporation at 35° followed by freeze-drying over KOH. The residue was dissolved in the minimum amount of water, adjusted to pH 7.6 with 0.05N-KOH and precipitated by the addition of 10 vol. of ethanol. Precipitated material was separated by centrifuging, washed by suspension in ethanol (five times) and ether (three times) with intermediate centrifuging, and finally dried *in vacuo* over CaCl_2 . The yield of dry product was 488 mg. The radioactivity of the product immediately after preparation corresponded to 2.1×10^4 counts/min./mg. when measured after hydrolysis as an 'infinitely thick' plate of $\text{Ba}^{35}\text{SO}_4$ (see below).

Filter-paper electrophoresis showed the material to be homogeneous, identical in mobility with the authentic 6-*O*-sulphate ester and free from inorganic [^{35}S]sulphate. Descending chromatography on Whatman no. 1 filter paper, with butan-1-ol-acetic acid-water (50:12:25, by vol.) or isobutyric acid-aq. 0.5N- NH_3 soln. (5:3, v/v) as solvent, showed only a single component, identical in chromatographic mobility with the authentic 6-*O*-sulphate ester.

Experimental animals. Male and female M.R.C. hooded rats (aged 2-3 months) maintained on a standard diet were used throughout. Injections were performed under light ether anaesthesia. In preliminary experiments animals were housed in the funnel type of metabolism cage, but in later experiments animals were placed in restraining cages modified from the design of Peacock & Harris (1950) in which urine and faeces could be collected strictly separately. The animals were allowed water without restriction and were removed from the cages for 30 min. in each 24 hr. when they were able to feed. Collected urines were filtered through a glass-wool plug, and the filtrate and washings used immediately or stored at -12°. Faeces were dried at 50° *in vacuo* before use.

Preparation of samples for the determination of radioactivity. The inorganic [^{35}S]sulphate fraction of rat urine was precipitated as follows. The collected urine and washings as above were diluted to 100 ml. A portion of the diluted urine (15 ml.) was transferred to a modified 50 ml. centrifuge tube (Walkenstein & Knebel, 1957) and 3 ml. of aq. 0.15M- K_2SO_4 added. To the mixture was added 5 ml. of

4N-HCl followed by 4 ml. of aq. 10% (w/v) BaCl₂. After careful mixing the tube was capped with Parafilm and kept at 2° for 6 hr. The Ba³⁵SO₄ precipitate was separated by centrifuging and washed three times by suspending in 40 ml. portions of water with intermediate centrifuging. After washing with 40 ml. of acetone and recentrifuging, the precipitate was dried at 110°.

For the determination of the combined inorganic and ester [³⁵S]sulphate fraction a sample (15 ml.) of the diluted urine was mixed with 5 ml. of 4N-HCl and heated at 100° for 4 hr. After cooling, Ba³⁵SO₄ was precipitated as above. Preliminary experiments with these methods showed that (a) there was no co-precipitation of the ³⁵S-labelled ester in the presence of inorganic sulphate in rat urine, (b) the ester was not hydrolysed during the precipitation of the inorganic sulphate fraction, and (c) inorganic [³⁵S]sulphate could be recovered quantitatively in the presence of the labelled ester.

For the determination of the total ³⁵S content of urine a sample (15 ml.) was evaporated to dryness, the residue oxidized with fuming HNO₃ according to Young, Edson & McCarter (1949), and Ba³⁵SO₄ then precipitated as above. The same procedure was used in the determination of the total ³⁵S content of faeces or the whole carcass after digestion of solid with hot fuming HNO₃.

Determination of radioactivity. Precipitated Ba³⁵SO₄ was plated at 'infinite thickness' in plastic pans giving a surface area of 1 cm.² The Ba³⁵SO₄ layer was then compressed with firm pressure with an accurately machined stainless-steel plunger. Measurements of radioactivity were made with a thin (1.4 mg./cm.²) mica end-window Geiger-Müller tube in automatic scaling equipment (Nuclear-Chicago Corp., Ill., U.S.A.). Corrections were made for background, decay and coincidence, and sufficient counts were recorded to give a standard error of less than 2%.

Detection of radioactivity on paper chromatograms and paper electrophoresis strips. When material of sufficient activity was available components were detected on chromatograms and electrophoresis strips with a thin (1.4 mg./cm.²) mica end-window Geiger-Müller tube in the C. 100 Actigraph chromatogram strip-scanner (Nuclear-Chicago Corp.). Alternatively radioautographs were obtained from chromatograms and electrophoresis strips kept in contact with Ilford Industrial B X-ray film for periods up to 21 days.

RESULTS

Rats were injected intraperitoneally with doses of 10 mg. or 20 mg. of potassium *N*-acetyl-D-glucosamine 6-O-[³⁵S]-sulphate in 1.0 ml. of water.

In initial experiments urine and faeces were collected at 24 hr. intervals to a total of 96 hr. However, the amount of total [³⁵S]sulphur appearing in the urine after 48 hr. was negligible (see Table 1) and in subsequent experiments urine and faeces were collected at 12 hr. intervals for 48 hr. only. After 48 hr. animals were killed by a blow on the back of the head and the carcass was used for the determination of residual ³⁵S. The results of these experiments are recorded in Table 2. Total ³⁵S values for faeces did not exceed 0.45% of the injected dose during the 48 hr. period and are not detailed.

Dohlman (1956) showed that organisms in the faecal flora of the rat were able to hydrolyse chondroitin [³⁵S]sulphate *in vitro*, 26% of the available radioactivity appearing as inorganic [³⁵S]sulphate overnight at 38°. The ability of faecal organisms of rat to hydrolyse *N*-acetyl-D-glucosamine 6-O-[³⁵S]-sulphate was tested in the following way. A solution of the ester (10 ml. of 0.2M-sodium acetate-acetic acid, pH 6.2, containing 2 mg. of the ester/ml.) was mixed with an equal volume of a 2% (w/v) homogenate of freshly voided rat faeces, and the whole incubated at 21° or 37°. Samples (2 ml.) were withdrawn at various time intervals up to 48 hr., and diluted to 15 ml. with water. Solid material was removed by centrifuging, inorganic [³⁵S]sulphate precipitated from the clear supernatant as Ba³⁵SO₄, and the radioactivity determined as described. Control determinations were made in which a solution of the ester was incubated for the same time and at the same temperature, but to which the incubated faeces suspension was added only immediately before precipitating the inorganic sulphate fraction. Over 48 hr. inorganic [³⁵S]sulphate precipitated from the test incubated suspensions at 21° and 37° corresponded to 18 and 39% respectively of the total ester [³⁵S]sulphate available for hydrolysis (Fig. 1). When the ester was incubated at 37° for 36 hr. with a 2% (w/v) faeces suspension in 0.2M-sodium acetate-acetic acid in the pH range 5.0-7.2 and inorganic [³⁵S]sulphate determined as above, a pH-activity curve with a maximum at pH 6.2 was obtained (Fig. 2).

Table 1. *Excretion of total urinary ³⁵S after the administration of potassium N-acetyl-D-glucosamine 6-O-[³⁵S]-sulphate*

Results are expressed as a percentage of the injected dose. Mean values are given with ranges in parentheses. The radioactivity of the doses corresponded to 1.1×10^5 counts/min. (10 mg.) and 2.2×10^5 counts/min. (20 mg.) measured after hydrolysis as an infinitely thick plate of Ba³⁵SO₄.

Sex	Dose (mg.)	No. of animals	Body wt. range (g.)	Total urinary ³⁵ S			
				24 hr.	48 hr.	72 hr.	96 hr.
Male	10	3	117-152	89.4 (87.3-92.1)	2.1 (0.7-2.5)	0.7 (0.6-0.8)	0.4 (0.3-0.6)
				87.0 (85.6-89.5)	3.1 (2.8-3.3)	0.8 (0.7-0.9)	0.6 (0.5-0.8)
Male	20	3	120-132				

Table 2. Distribution of radioactivity in rat urine and carcass after injection of *N*-acetyl-D-glucosamine 6-O-[³⁵S]-sulphate

Results are expressed as percentage of the injected dose. Mean values are given with ranges in parentheses. The radioactivity of the doses corresponded to 1.1×10^6 counts/min. (10 mg.) and 2.2×10^6 counts/min. (20 mg.) measured after hydrolysis as an infinitely thick plate of Ba³⁵SO₄.

Sex	Dose (mg.)	No. of animals	Body wt. range (g.)	Distribution of ³⁵ S in urine												Residual ³⁵ S in carcass after 48 hr.
				Inorganic sulphate fraction				Combined inorganic and ester sulphate fraction								
				12 hr.	24 hr.	36 hr.	48 hr.	12 hr.	24 hr.	36 hr.	48 hr.					
Male	10	4	112-140	4.6 (3.2-5.7)	2.1 (1.2-2.4)	1.3 (0.6-1.8)	0.6 (0.2-0.9)	82.4 (78.9-88.4)	2.7 (2.1-3.2)	1.6 (1.4-1.9)	1.1 (0.9-1.2)	3.6 (2.4-5.2)				
Male	20	4	150-180	3.5 (2.1-4.2)	1.9 (1.8-2.1)	1.2 (0.9-1.4)	0.5 (0.3-0.8)	84.8 (80.5-89.4)	2.4 (2.2-2.6)	1.5 (1.2-1.8)	0.9 (0.7-1.1)	5.2 (4.1-7.4)				
Female	20	4	131-167	4.2 (3.8-5.3)	2.7 (1.9-3.2)	1.9 (1.5-2.7)	0.8 (0.6-1.1)	85.1 (79.4-88.6)	3.1 (2.1-3.7)	2.7 (1.9-3.2)	1.2 (0.8-1.6)	6.1 (5.9-7.2)				
Male*	20	3	127-148	2.23 (1.4-3.1)	0.74 (0.5-1.1)	0.31 (0.2-0.4)	0.30 (0.17-0.38)	88.3 (84.2-91.1)	1.8 (1.6-2.3)	0.56 (0.48-0.64)	0.42 (0.36-0.49)	6.8 (5.1-7.9)				
Male†	20	3	130-159	2.52 (1.9-2.8)	0.87 (0.7-1.4)	0.45 (0.3-0.6)	0.37 (0.28-0.49)	89.1 (85.4-93.6)	2.1 (1.8-2.5)	0.82 (0.72-0.91)	0.64 (0.52-0.73)	5.9 (4.9-7.2)				

* Animals housed in restraining cages for collection of excreta.

† Animals pretreated with a total of 300 mg. of Terramycin and 1.5 g. of Sulphasuxidine by mouth during 70 hr. before injection and then housed in restraining cages for the collection of excreta.

In view of these findings urinary inorganic [³⁵S]sulphate observed in the initial studies *in vivo* could have arisen from the degradation of excreted ³⁵S-labelled esters by faecal organisms contaminating the urine during collection in the funnel type of metabolism cage. Consequently, a further series of experiments were performed *in vivo* in which injected animals were kept in restraining cages allowing the separate collection of urine and faeces. Results are recorded in Table 2.

Fig. 1. Liberation of inorganic [³⁵S]sulphate from potassium *N*-acetyl-D-glucosamine 6-O-[³⁵S]-sulphate by the faecal flora of the rat. □, Incubated at 37°; △, incubated at 21°; ○, incubated at 37° with faeces from rats pretreated by mouth for 70 hr. with Sulphasuxidine and Terramycin (see text).

Fig. 2. Effect of pH on the liberation of inorganic [³⁵S]-sulphate from potassium *N*-acetyl-D-glucosamine 6-O-[³⁵S]-sulphate by the faecal flora of the rat.

Alternatively, urinary inorganic [^{35}S]sulphate could have arisen after the excretion of ester [^{35}S]sulphate compounds into the intestinal tract via the bile, followed by degradation by the intestinal flora and subsequent reabsorption of inorganic [^{35}S]sulphate from the large intestine (cf. Dohlman, 1956). In this context, Dodgson, Powell & Tudball (1960) have reported an appreciable reduction in the number of viable intestinal organisms when rats are treated by mouth with 300 mg. of Terramycin (Pfizer Ltd.) and 1.5 g. of Sulphasuxidine (Merck, Sharp and Dohme Ltd.) over a period of 70 hr. The effect of such treatment on organisms involved in the hydrolysis of *N*-acetyl-D-glucosamine 6-*O*-[^{35}S]sulphate *in vitro* at 37° by a 2% (w/v) suspension of faeces obtained after the 70 hr. period is shown in Fig. 1. Animals pretreated according to the method of Dodgson *et al.* (1960) were injected with the labelled ester and housed in restraining cages for the collection of excreta. The results of these experiments are recorded in Table 2.

Analysis of urine by paper chromatography and paper electrophoresis. Samples of urine collected at 12 hr. and 24 hr. intervals after the injection of 20 mg. of potassium *N*-acetyl-D-glucosamine 6-*O*-[^{35}S]sulphate were freeze-dried and the residue was dissolved in the minimum amount of water. Portions (2 $\mu\text{l.}$) of the resulting solution were examined by electrophoresis on Whatman no. 1 filter paper in 0.1M-ammonium acetate-acetic acid buffer (pH 4.0) at 400 v for periods of 2–4 hr. The paper strips were dried and scanned for radioactivity. The same qualitative pattern was apparent in all samples examined. A faster-moving component of low activity corresponded to inorganic [^{35}S]sulphate, and a slower-moving band accounted for the greater part of the radioactivity applied to the strip. Scanner diagrams and radioautographs of 12–24 hr. urines revealed the same pattern as the 0–12 hr. urines, but of very much lower intensity. In no instance were electrophoretically-immobile components observed, suggesting the absence of neutral ^{35}S -labelled compounds from the urines collected at both time intervals.

For paper chromatography, samples of urine as above were freeze-dried, redissolved in the minimum amount of water and then dialysed twice against 100 ml. portions of water for 4 hr. on a shaking dialyser. Preliminary experiment showed that 98–99% of the total ^{35}S content of 12-hr. urines was dialysable during this treatment. The diffusates were combined and after they had been concentrated to small volume inorganic [^{35}S]sulphate was precipitated by the cautious addition of 5 mM-BaCl₂. The mixture was clarified by centrifuging and the clear supernatant concentrated to small volume by freeze-drying. Portions

(2 $\mu\text{l.}$) of the concentrated supernatant were examined by downward chromatography on Whatman no. 1 filter paper with butan-1-ol-acetic acid-water (50:12:25, by vol.) as solvent for periods up to 72 hr. The chromatograms were then dried and scanned for radioactivity. In each case chromatography revealed that the major constituent of the urines was identical in mobility with authentic *N*-acetyl-D-glucosamine 6-*O*-sulphate. By computing the area under the curve in scanner diagrams this component was found to account for an average of 97% (range 95–98%) of the treated 12 hr. urine applied to the chromatogram. Examination of radioautographs of the same chromatograms revealed the presence of three further components with mobilities relative to the 6-*O*-sulphate ester of 0.35, 0.74 and 1.25 respectively. However, from the scanner diagrams each of these components corresponded to only 1% or less of the radioactivity originally applied to the chromatogram. Scanner diagrams and radioautographs of treated 12–24 hr. urines revealed the same pattern, but once again of very much lower intensity.

DISCUSSION

When potassium *N*-acetyl-D-glucosamine 6-*O*-[^{35}S]sulphate was administered intraperitoneally to rats, between 82 and 89% of the radioactivity was excreted during the first 12 hr. after the injection. This rate of excretion is higher than that observed after the administration of polysaccharides containing hexosamine [^{35}S]sulphate residues (Dohlman, 1956; Dziewiatkowski, 1956; Danishefsky & Eiber, 1959). However, it has been shown that the excretion of radioactivity in the urine after the administration of xylan [^{35}S]sulphate was related to the average chain length of the preparation and that the higher the degree of polymerization the lower was the rate of excretion (Huseman *et al.* 1952). There can be little doubt that the relatively low molecular weight of the ester used in the present study contributes to the observed high rate of excretion.

Although the greater part of the radioactivity excreted during the 48 hr. after injection consisted of *N*-acetyl-D-glucosamine 6-*O*-[^{35}S]sulphate, significant quantities of inorganic [^{35}S]sulphate also appeared in the urine during this period. When excreta were collected in the funnel type of metabolism cage a total of 7.1–9.6% of the radioactivity excreted in 48 hr. corresponded to inorganic [^{35}S]sulphate. However, when restraining cages were used and cross-contamination of urine and faeces could not have occurred, the inorganic [^{35}S]sulphate fraction was reduced to 3.5–4.3% in 48 hr. This suggests that inorganic [^{35}S]sulphate observed in initial experiments could have arisen

at least in part from metabolism of the excreted ester by faecal organisms contaminating the urine during collection. On the other hand, depression of the gut flora has little effect on the amount of inorganic [^{35}S]sulphate excreted, suggesting that the intestinal organisms are unlikely to play a major part in the metabolism of the ester *in vivo*. The observed desulphation of the ester by organisms from the intestinal tract is of interest in that liberation of sulphate from monosaccharide sulphate esters by bacterial enzymes has been reported previously only by Tanko (1932).

The consistent appearance of 3.5–4.3 % of the injected dose as inorganic [^{35}S]sulphate provides additional evidence that mammalian tissues are in some way able to separate the ester sulphate bond of hexosamine sulphate esters (cf. Dohlman, 1956; Dziewiatkowski, 1956; Danishefsky & Eiber, 1959). Of the possible routes that exist it appears unlikely that a specific carbohydrate sulphatase is involved in this process. Dodgson & Lloyd (1961) tested a wide variety of tissue preparations from rat, pig and human against the 6-*O*-sulphate esters of D-glucose, D-galactose, *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine. In no instance could the liberation of inorganic sulphate be detected with either the chloranilate (Lloyd, 1959*b*) or benzidine (Dodgson & Spencer, 1953) micromethods, although each of these esters is known to function as a substrate for the glycosulphatase of *Littorina littorea* (Lloyd, 1959*c*; Dodgson, 1961; Dodgson & Lloyd, 1961). Alternatively, inorganic sulphate might appear as a result of the complete degradation of the *N*-acetyl-D-glucosamine sulphate molecule. Lutwak-Mann (1941) examined the degradation of hexosamines and their *N*-acetyl derivatives by mammalian tissues and recorded the appearance of ammonia and unknown compounds. Since it is proposed (see Roseman, 1959) that the first step in the degradation of D-glucosamine and *N*-acetyl-D-glucosamine is the formation of the corresponding 6-*O*-phosphate ester it is improbable that *N*-acetyl-D-glucosamine 6-*O*-sulphate can be metabolized by this route. Finally, in the presence of an appropriate sulphokinase the sulphate group of *N*-acetyl-D-glucosamine 6-*O*-sulphate might be transferred to adenosine 3':5'-diphosphate to give adenosine 3'-phosphate 5'-sulphatophosphate. Subsequently, inorganic sulphate might appear directly from the breakdown of the sulphatophosphate or after the transfer of the sulphate group from it to yield a sulphate ester labile in the presence of mammalian tissues. Precedents exist for each of these possibilities (see Gregory & Robbins, 1960).

The earlier synthetic (Lloyd, 1959*a*, 1960) and infrared (Lloyd & Dodgson, 1959, 1961) studies, on

hexose and *N*-acetylhexosamine sulphate esters of defined constitution in relation to the structure of naturally occurring polysaccharide sulphates (Lloyd, Dodgson, Price & Rose, 1961), and the present metabolic study assume new significance in the light of observations on the structure of keratosulphate. This sulphated polymer, reported as a constituent of cornea (Meyer, Linker, Davidson & Weissmann, 1953), cartilage (Meyer, Hoffman & Linker, 1958) and nucleus pulposus (Gardell, 1955), has been shown to contain *N*-acetyl-D-glucosamine, D-galactose and ester sulphate together with 1–2 % of a methyl pentose (Rosen, Hoffman & Meyer, 1960). Results of methylation studies (Hirano, Hoffman & Meyer, 1960) suggest that the main structure of keratosulphate contains the repeating unit β -D-galactopyranosyl-*O*-(*N*-acetyl- β -D-glucosamine 6-*O*-sulphate). After the injection of *N*-acetyl-D-glucosamine 6-*O* [^{35}S]sulphate into rats 3.6–6.8 % of the radioactivity is retained in the carcass after 48 hr. However, because inorganic [^{35}S]sulphate is released from the ester *in vivo* it is not possible to decide whether this retention is due to a direct incorporation of the intact radioactive ester into the keratosulphate fractions of rat or to the general incorporation of inorganic [^{35}S]sulphate into all the sulphate esters of rat tissues.

SUMMARY

1. Potassium *N*-acetyl-D-glucosamine 6-*O* [^{35}S]sulphate was administered intraperitoneally to male and female rats. The main feature of the metabolism of the ester was the rapid elimination of radioactivity, 82–89 % of the dose appearing in the urine in the first 12 hr.

2. Although the greater part of the radioactivity was excreted as ester sulphate, inorganic [^{35}S]sulphate also appeared in the urine in the 48 hr. period after injection.

3. Organisms from the faecal flora of rat were shown to metabolize the labelled ester *in vitro*, with the liberation of inorganic [^{35}S]sulphate, but available evidence suggests that the intestinal flora are not primarily responsible for the metabolism of the ester observed *in vivo*.

4. Paper-chromatographic examination of 12 hr. and 24 hr. urine samples showed that the radioactivity associated with the ester sulphate fraction was due largely to unchanged *N*-acetyl-D-glucosamine 6-*O* [^{35}S]sulphate; three additional compounds of unknown structure and low radioactivity were also detected.

This work has been supported by grants from the Empire Rheumatism Council and the Nuffield Foundation, and in part by a grant (A 1982) from the Arthritis and Metabolic Diseases Division of the U.S. Public Health Service.

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The Isolation of the α -Toxin of *Clostridium welchii* Type A by Zone Electrophoresis in Vertical Columns

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(Received 16 January 1961)

The α -toxin of *Clostridium welchii* type A is believed to be the most important single factor involved in clostridial toxæmia (van Heyningen, 1950, p. 25). It has been extensively studied biochemically and serologically, as a result of which it has been recognized as a lecithinase capable of splitting phosphorylcholine from lecithin (Macfarlane & Knight, 1941). Such enzymic activity is believed sufficient (Macfarlane, 1950) to explain the haemolytic, necrotizing and lethal activities assigned to it by Glenny, Barr, Llewellyn-Jones, Dalling & Ross (1933). Previous attempts have been made by van Heyningen (1941a, b), Adams & Hendee (1945), van Heyningen & Bidwell (1948) and Roth & Pillemer (1953) to isolate it from the other *C. welchii* antigens such as hyaluronidase, collagenase and θ -toxin (a reversibly oxidizable

haemolysin), with which it usually occurs in culture filtrates. The techniques employed by these workers were mainly fractional precipitation with salt and organic solvents.

A series of experiments was planned in which the newer techniques applicable to such a problem were to be applied, and the first attempt was by zone electrophoresis in vertical columns. At the conclusion of the experimental stage of this part of the work, a paper was published by Habermann (1959), who described the results of a similar approach to the same problem. He found that the α -toxin could be purified to a considerable degree by a combination of either fractional precipitation and chromatographic techniques or electrophoretic and chromatographic techniques. The yield from the chromatographic stage was high but that from