- Dobriner, K., Kappas, A., Rhoads, C. P. & Gallagher, T. F. (1953). *J. clin. Invest.* **32**, 940.
- Dorfman, R. I. (1954). *Recent Progr. Hormone Res.* **9**, 5.
- Dorfman, R. I. & Ungar, F. (1953). *Metabolism of the Steroid Hormones*, p. 22. Minneapolis: Burgess Publishing Company.
- Edwards, R. W. H., Kellie, A. E. & Wade, A. P. (1953). *Mem. Soc. Endocrin.* No. 2, 53.
- Gibson, G. & Norymberski, J. K. (1954). *Ann. Rheum. Dis.* **12**, 59.
- Jailer, J. W. (1953). *Bull. N.Y. Acad. Med.* **29**, 377.
- Kappas, A. & Gallagher, T. F. (1955). *J. clin. Invest.* **34**, 1566.
- Kellie, A. E. & Smith, E. R. (1956). *Nature, Lond.*, **178**, 323.
- Kellie, A. E. & Wade, A. P. (1956a). *Biochem. J.* **62**, 1 P.
- Kellie, A. E. & Wade, A. P. (1956b). *Acta endocr. Copenhagen*, **23**, 357.
- Lieberman, S., Mond, B. & Smyles, E. (1954). *Recent Progr. Hormone Res.* **9**, 113.
- Mills, G. T. (1948). *Biochem. J.* **43**, 125.
- Norymberski, J. K., Stubbs, R. D. & West, H. F. (1953). *Lancet*, **1**, 1276.
- Pincus, G. & Pearlman, W. H. (1941). *Endocrinology*, **29**, 413.
- Pond, H. M. (1954). *J. Endocrin.* **10**, 202.
- Reddy, W. J., Jenkins, D. & Thorn, G. W. (1952). *Metabolism*, **1**, 511.
- Rubin, B. L., Dorfman, R. I. & Pincus, G. (1954). *Recent Progr. Hormone Res.* **9**, 213.
- Wilkins, L., Bongiovanni, A. M., Clayton, G. W., Grumbach, M. M. & van Wyk, J. (1955). *Ciba Foundation Colloq. Endocrin.* **8**, 460.

The Synthesis of N-Acetylneuraminic Acid

BY J. W. CORNFORTH AND M. E. FIRTH

National Institute for Medical Research, Mill Hill, London, N.W. 7

AND A. GOTTSCHALK

Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

(Received 7 June 1957)

The term neuraminic acid is now reserved for a hypothetical amino sugar whose derivatives are found combined in many animal tissues, secretions and excretions. These derivatives of natural occurrence, which are known as sialic acids (for nomenclature see Blix, Gottschalk & Klenk, 1957), have acyl groups bound to the amino group and sometimes also to a hydroxyl group of the parent neuraminic acid; the commonest substituent is acetyl. The first sialic acid to be crystallized was isolated from bovine-submaxillary-gland mucin by Blix (1936); it is now known to be an *ON*-diacetylneuraminic acid (Blix, Lindberg, Odin & Werner, 1956). Crystalline *N*-acetylneuraminic acid was first prepared from the same mucin by Klenk & Faillard (1954), the *O*-acetyl group having been lost when the mucin's acidity was used to catalyse its own hydrolysis. Later, the influenza-virus enzyme neuraminidase (Gottschalk, 1951, 1957a) was used to release *N*-acetylneuraminic acid from human urinary mucoprotein (Klenk, Faillard & Lempfrid, 1955). Since then, the acid has been isolated from pseudomyxomatous cysts (Odin, 1955a), human-serum proteins (Odin, 1955b; Svennerholm, 1956; Böhm, Ross & Baumeister, 1957), human-cervix-uteri mucus (Odin, 1955c), human meconium (Odin, 1955b), human gangliosides (mucolipids) (Blix & Odin, 1955; Svennerholm, 1956), human

lipid-free brain tissue (Svennerholm, 1956), human liver (Martinsson, Raal & Svennerholm, 1957), stroma of human erythrocytes (Klenk & Lempfrid, 1957), human milk (Svennerholm, 1956), fetuin (Klenk & Faillard, 1957), boar-seminal mucin (Odin, 1955c), ovine-submaxillary-gland mucin (Blix *et al.* 1956), and from ovomucin (Odin, 1955c).

In mucoproteins and mucolipids, the sialic acid forms part of a very large molecule, but Kuhn & Brossmer (1956a) have isolated from cow colostrum a trisaccharide in which *ON*-diacetylneuraminic acid is bound glycosidically to lactose. Gynaminic acid from the non-dialysable fraction of human milk is identical with *N*-acetylneuraminic acid (Zilliken, Braun & György, 1955). Sialic acid of porcine-submaxillary-gland mucin and of the stroma of erythrocytes from horse, ox and swine was shown to be *N*-glycolloylneuraminic acid (Blix *et al.* 1956; Klenk & Uhlenbruck, 1957). Horse-serum lactaminic acid as prepared by Yamakawa & Suzuki (1955) is almost certainly the methyl ester of *N*-acetylneuraminic acid.

When Klenk's (1941) original method of heating at 100° with 5% methanolic hydrogen chloride is used to split sialic acid from mucoids, the methylglycoside of neuraminic acid is obtained, all acyl groups being lost by alcoholysis. This glycoside has been prepared from gangliosides (Klenk, 1941),

amyloid (Klenk & Faillard, 1955), stroma protein of bovine erythrocytes (Klenk & Stoffel, 1956), glycolipid of horse erythrocytes (Yamakawa & Suzuki, 1951, 1952), horse-submaxillary-gland mucin (Klenk & Uhlenbruck, 1956), and from all sialic acids (Blix *et al.* 1956). In other examples where a crystalline product was not isolated, Bial's orcinol reagent and the direct Ehrlich reaction have been used as specific agents for detecting sialic acid (Werner & Odin, 1952; Gottschalk, 1955*a*; Kuhn & Brossmer, 1956*a*; Böhm & Baumeister, 1956; Svennerholm, 1957). Sialic acid is notably absent from mucopolysaccharides containing hexuronic acid (Gottschalk, 1955*a*).

The first degradation product of known structure from a sialic acid was obtained when pyrrole-2-carboxylic acid was isolated from the alkaline hydrolysate of homogeneous mucoprotein of bovine submaxillary gland and from human urinary mucoprotein (Gottschalk, 1953, 1955*a*). The product released from these mucoproteins by the action of neuraminidase from influenza virus or from *Vibrio cholerae* gave the same acid upon very mild alkali treatment (Gottschalk, 1954), and so also did *N*-acetylneuraminic acid (Klenk & Faillard, 1954). These findings, together with a reinterpretation of the data then available from the Laboratories of Blix and Klenk (for review see Gottschalk, 1956) on the properties and composition of sialic acids, and the observation that pyrrole-2-carboxylic acid is obtained by alkaline condensation of D-glucosamine with pyruvic acid, led us to propose the structure (I) for *N*-acetylneuraminic acid (at the time without indication of the stereochemistry) (Gottschalk, 1955*b*).

It was very desirable to prove this structure by synthesis; and since the molecule expressed by formula (I) may be regarded as a product of aldol condensation between an *N*-acetylhexosamine and pyruvic acid, a plan for the work was easily formed. We chose *N*-acetyl-D-glucosamine as the hexosamine component, chiefly because of its availability. When the synthesis was almost completed, the degradation of *N*-acetylneuraminic acid to *N*-acetyl-D-glucosamine, carbon dioxide and an un-

known C₂ fragment by heating with pyridine and nickelous acetate (Kuhn & Brossmer, 1956*b*), and the fission of *N*-acetylneuraminic acid into *N*-acetyl-D-glucosamine and pyruvic acid by alkali (Zilliken & Glick, 1956) and apparently by an enzyme of *Vibrio cholerae* (Heimer & Meyer, 1956), helped to confirm the structure which we were trying to synthesize and showed that the first stereochemical step had been taken in the right direction.

The initial experiments on condensation of pyruvic acid with *N*-acetyl-D-glucosamine gave, however, no product responding to Bial's orcinol test; the condensation of glucosamine with pyruvic acid, which gives pyrrole-2-carboxylic acid in 20 % yield (Gottschalk, 1957*b*), evidently succeeds by initial formation of a Schiff base which makes the aldol condensation an intramolecular reaction. We soon replaced pyruvic acid by oxaloacetic acid in the expectation that reaction would be helped by a more reactive methylene group and that the superfluous carboxyl group could be eliminated even if it survived the aldol condensation. The new approach was successful, as already announced briefly (Cornforth, Daines & Gottschalk, 1957); we now present experimental details and evidence of the product's identity with *N*-acetylneuraminic acid of biological origin.

MATERIALS

N-Acetyl-D-glucosamine was prepared from D-glucosamine hydrochloride according to White (1940), and had m.p. 196° (decomp.); $[\alpha]_D^{25} + 75^\circ \rightarrow +41^\circ$ in water.

Oxaloacetic acid. Commercial ethyl sodio-oxaloacetate (100 g.; previously washed with ether) was added to a stirred mixture of 2*N*-H₂SO₄ (1 l.) and ether (250 ml.) at 0°. After 20 min. no solid remained; the aqueous layer was extracted with ether (3 × 100 ml.) and the combined ether solutions were washed with small quantities of ice-cold *N*-NaHCO₃ until the washings were no longer deeply coloured. The dried (MgSO₄) ether solution was evaporated and the residue distilled at 0.1 mm. to give ethyl oxaloacetate (58–65 g.), b.p. 66–70°. The ester was stored at 0° and hydrolysed as required by dissolving e.g. 30 g. in HCl (120 ml.; sp.gr. 1.18) and leaving for 2–3 days at room temperature. The crystalline oxaloacetic acid was collected, sucked as free as possible from liquid, and dried over KOH; this product (14.8 g.; m.p. 153–155°) was suitable for use. We find this method of preparation more reliable than that given by Heidelberger (1953).

Ion-exchange resins. Zeo-Karb 215 (40–100 mesh) was prepared (or regenerated) by washing with 2*N*-HCl until the effluent gave a negative flame test for Na⁺ ion, then with water until the effluent gave no precipitate with AgNO₃. Dowex 1-X2 (50–100 mesh) was converted into the formate form by Svennerholm's (1956) procedure.

Activated carbon. Sutcliffe Speakman material grade 130 was boiled for 3 hr. with HNO₃ (sp.gr. 1.10), cooled, washed free of acid with water and passed, as aqueous suspension, through a 90-mesh sieve and on to a 280-mesh sieve. The material retained by the finer sieve was washed again with

dilute HNO_3 and with water, dried at 100° and stored. In packing the columns a few millimetres of Celite were first deposited and covered with filter paper; the carbon slurry was then poured on.

EXPERIMENTAL AND RESULTS

Preparation of synthetic *N*-acetylneuraminic acid

Oxaloacetic acid (4.5 g.) was dissolved in water (40 ml.) and brought to pH 9 by addition of $10N$ - NaOH . *N*-Acetyl-D-glucosamine (10 g.) was added and the pH was adjusted potentiometrically to 11.0 by $5N$ - NaOH . During the next 3 hr. frequent additions of alkali were needed to maintain this pH; thereafter, only occasional adjustment was required. After 17 hr. and 45 hr. samples of the mixture, submitted to the quantitative Bial assay (Werner & Odin, 1952), with *N*-acetylneuraminic acid as standard, gave values of 470 and 880 mg. respectively for the total content of *N*-acetylneuraminic acid in the mixture. The latter figure represents a yield of 8.5% based on the limiting reactant (oxaloacetic acid), but this is a maximal figure: other Bial-positive species are present and background absorption is uncertain. After 48 hr. the mixture was brought with acetic acid to pH 6.8 and preserved at 0° for several days before working up. Zeo-Karb 215 was added until evolution of CO_2 had ceased; the mixture was poured on a column of the same resin (35 mm. $\times$ 400 mm.), which was then washed with water until the effluent became neutral. The acidic effluent was added slowly to a Dowex 1-formate column (25 mm. $\times$ 230 mm.) which was afterwards washed with water until the effluent was free from

substances giving a positive direct or indirect (pretreatment with $0.05N$ - Na_2CO_3 at 100° for 3 min.) Ehrlich reaction. The acidic fraction was then eluted with $0.3N$ -formic acid, 1250 ml. being required before the direct Ehrlich test on the effluent became weak. The effluent was concentrated almost to dryness in a rotary evaporator at 25° , redissolved in water and put on a column of activated carbon (40 mm. $\times$ 100 mm.) which was developed with water. The effluent was collected in 66 fractions of 22 ml. each. Fractions 9 to 48, which gave a strong direct Ehrlich reaction, were separately evaporated at about 30° *in vacuo*, yielding a total dry weight of about 840 mg. Spontaneous crystallization was noticed in fractions 13–26. Odd-numbered fractions 9–47 were chromatographed on paper (butanol–pyridine–water, 6:4:3, by vol.; descending). On spraying with orcinol-trichloroacetic acid three series of spots appeared, of R_f values 0.02, 0.08 and 0.13. Spots of R_f 0.02 appeared only in fractions 9 and 11. Spots of R_f 0.08 were observed with all fractions, and these spots coincided in R_f value and colour shade with the spot given by authentic *N*-acetylneuraminic acid. Spots of R_f 0.13 were seen with fractions 13–47, the intensity relative to the spot of R_f 0.08 increasing gradually until in the last fractions it was much the more intense.

Fractions 13–26 were separately crystallized by dissolution in a little water, evaporation *in vacuo* to small bulk and addition of acetic acid to a concentration of 60–70%. After crystallization had proceeded for 2 days at 5 – 10° the crystals were collected and washed with acetic acid and with ether. This product (172 mg.; $[\alpha]_D^{25} - 29^\circ$ in water) was dissolved in water (0.5 ml.) and treated after filtration with acetic acid (2.5 ml.). After 3 days at 10° the white aggregated needles were collected (140 mg.); this product was solvated. A

Fig. 1. Infrared spectrograms of synthetic (broken line) and natural (full line) *N*-acetylneuraminic acid in compressed potassium chloride. The curves for the natural product have been displaced slightly downward.

portion was crystallized from Blix's solvent system (Blix *et al.* 1956) with the proportions given by Kuhn & Brossmer (1956*b*): the acid (67 mg.) was dissolved in water (0.7 ml.), methanol (8 ml.) was added and then dry ether (18 ml.). A slight precipitate was removed by filtration through Celite and light petroleum (b.p. 40°–60°) was added until the clear filtrate became opalescent. Crystallization set in at once; after some hours at 20° it was completed at 0°. The product (42 mg.) was dried at 20° *in vacuo* over P₂O₅ and KOH.

Identification of synthetic *N*-acetylneuraminic acid

The product prepared as above formed snow-white partly aggregated needles. It appeared to be anhydrous, losing no weight on further drying at 56° in high vacuum, and was not hygroscopic (Found: C, 42.6; H, 6.2; N, 4.4%. Calc. for C₁₁H₁₉O₈N: C, 42.7; H, 6.2; N, 4.5%). It melted and decomposed at 185–187° in an evacuated capillary; in air it sometimes blackened without melting. Blix *et al.* (1956) gave m.p. 185–187°, Kuhn & Brossmer (1956*b*) gave m.p. 183–185°, for *N*-acetylneuraminic acid recrystallized in this manner. The product was laevorotatory, with $[\alpha]_D^{25} - 33^\circ$ in water (*c*, 0.8); Blix *et al.* (1956), and Kuhn & Brossmer (1956*b*) gave $[\alpha]_D - 31^\circ$. The X-ray diffraction patterns of natural and synthetic specimens are compared in Pl. 1, and the infrared spectra in Fig. 1; the two specimens show no significant differences. In butanol–pyridine–water the two specimens ran as single spots of *R_f* 0.08.

DISCUSSION

From the data presented there can be no doubt that the synthetic material is chemically and stereochemically identical with *N*-acetylneuraminic acid as obtained from biological sources. The method of synthesis determines the length of the carbon chain in *N*-acetylneuraminic acid, and the nature and position of the functional groups. The presence of a pyranose ring follows from periodate oxidations (Blix *et al.* 1956).

Most of the configuration follows from the known stereochemistry of *N*-acetyl-D-glucosamine, but two details remain obscure: the stereochemistry at C-2 and at C-4. The mode of synthesis is of no assistance here, for several unproved assumptions are needed to deduce the configuration at C-4 from considerations of reaction mechanism; but such weight as can be accorded to negative evidence may be attached to the failure of *N*-acetylneuraminic acid to form lactones (Kuhn & Brossmer, 1956*b*), though it forms a methyl ester with exceptional ease. The pyranose ring in *N*-acetylneuraminic acid does not appear to be completely stable in solution, as is indicated by the ease of oxidation by hypiodite (which presumably involves the α -oxo acid) and by the fact that periodate oxidation continues at an appreciable rate after the initial rapid consumption of 2 mol. prop. of reagent (Blix *et al.* 1956). Thus the configuration at C-2 must be regarded as mobile,

even though mutarotation has not been observed; hence either of the two lactones, 1 → 4 or 1 → 7, might be formed from *N*-acetylneuraminic acid irrespective of configuration at C-4. Now since the acid is derived from *N*-acetyl-D-glucosamine, the trihydroxypropyl and acetamido substituents in the pyranose ring are *cis* to each other. If the hydroxyl group at C-4 is *cis* to these two groups it cannot take up the axial conformation necessary for lactonization without interference from the trihydroxypropyl group, which necessarily becomes axial at the same time. Contrariwise, the trihydroxypropyl group cannot become axial without interference from the hydroxyl group at C-4. This would provide a barrier to lactonization; a similar barrier would oppose pyranose formation from a γ -lactone formed from the open-chain tautomer of *N*-acetylneuraminic acid. The opposite configuration at C-4 presents no such obstacle to lactone formation by either route. Thus there is some indication that in *N*-acetylneuraminic acid the 4-hydroxyl group is *cis* to the trihydroxypropyl and acetamido groups.

The optimum conditions for condensation of *N*-acetyl-D-glucosamine with oxaloacetic acid have not yet been defined, but the yield of 1–2 % obtained in these experiments can probably be improved.

Note added in proof. Kuhn & Brossmer (1957) have deduced from the optical rotation of a monocyclic mercaptal-lactone derived from *N*-acetylneuraminic acid that the hydroxyl group at C-4 is *trans* to the acetamido and trihydroxypropyl groups; and from the newly observed mutarotation of *N*-acetylneuraminic acid in dimethylsulphoxide that the crystalline acid belongs to the β -series.

SUMMARY

1. The occurrence of sialic acids in the animal organism is briefly reviewed.
2. The synthesis of crystalline *N*-acetylneuraminic acid from *N*-acetyl-D-glucosamine and oxaloacetic acid in aqueous solution at 20° and pH 11 is described.
3. The chemical and stereochemical identity of the synthetic product with *N*-acetylneuraminic acid of natural origin is shown by comparison of decomposition points, elementary analyses, optical rotations, infrared spectra and X-ray diffraction patterns.
4. The stereochemistry of *N*-acetylneuraminic acid is discussed.

We are grateful to Professor G. Blix, Uppsala, for a generous gift of authentic *N*-acetylneuraminic acid; to Dr R. K. Callow and Mr M. Dearden for the infrared spectra; to Mrs O. Kennard for the X-ray diffraction photographs; and to Mr J. Grant for willing help with the preparations.

X-ray diffraction patterns of synthetic *N*-acetylneuraminic acid (left half of photograph) and natural *N*-acetylneuraminic acid (right half). Some amorphous low-angle scattering is visible near the origin in the right half.

J. W. CORNFORTH, M. E. FIRTH AND A. GOTTSCHALK—THE SYNTHESIS OF
N-ACETYLNEURAMINIC ACID

(Facing p. 60)

REFERENCES

- Blix, G. (1936). *Hoppe-Seyl. Z.* **240**, 43.
- Blix, G., Gottschalk, A. & Klenk, E. (1957). *Nature, Lond.*, **179**, 1088.
- Blix, G., Lindberg, E., Odin, L. & Werner, I. (1956). *Acta Soc. Med. Upsalien.* **61**, 1.
- Blix, G. & Odin, L. (1955). *Acta chem. scand.* **9**, 1541.
- Böhm, P. & Baumeister, L. (1956). *Hoppe-Seyl. Z.* **305**, 42.
- Böhm, P., Ross, J. & Baumeister, L. (1957). *Hoppe-Seyl. Z.* **307**, 284.
- Cornforth, J. W., Daines, M. E. & Gottschalk, A. (1957). *Proc. chem. Soc., Lond.* p. 25.
- Gottschalk, A. (1951). *Nature, Lond.*, **167**, 845.
- Gottschalk, A. (1953). *Nature, Lond.*, **172**, 808.
- Gottschalk, A. (1954). *Nature, Lond.*, **174**, 652.
- Gottschalk, A. (1955a). *Biochem. J.* **61**, 298.
- Gottschalk, A. (1955b). *Nature, Lond.*, **176**, 881.
- Gottschalk, A. (1956). *Yale J. Biol. Med.* **28**, 525.
- Gottschalk, A. (1957a). *Biochim. biophys. Acta*, **23**, 645.
- Gottschalk, A. (1957b). *Arch. Biochem. Biophys.* **69**, 37.
- Heidelberger, C. (1953). *Biochem. Prep.* **3**, 59.
- Heimer, R. & Meyer, K. (1956). *Proc. nat. Acad. Sci., Wash.*, **42**, 733.
- Klenk, E. (1941). *Hoppe-Seyl. Z.* **268**, 50.
- Klenk, E. & Faillard, H. (1954). *Hoppe-Seyl. Z.* **298**, 230.
- Klenk, E. & Faillard, H. (1955). *Hoppe-Seyl. Z.* **299**, 191.
- Klenk, E. & Faillard, H. (1957). *Dtsch. Z. Verdau.-u. Stoffwechselkr.* **17**, 51.
- Klenk, E., Faillard, H. & Lempfrid, H. (1955). *Hoppe-Seyl. Z.* **301**, 235.
- Klenk, E. & Lempfrid, H. (1957). *Hoppe-Seyl. Z.* **307**, 278.
- Klenk, E. & Stoffel, W. (1956). *Hoppe-Seyl. Z.* **303**, 78.
- Klenk, E. & Uhlenbruck, G. (1956). *Hoppe-Seyl. Z.* **305**, 224.
- Klenk, E. & Uhlenbruck, G. (1957). *Hoppe-Seyl. Z.* **307**, 266.
- Kuhn, R. & Brossmer, R. (1956a). *Chem. Ber.* **89**, 2013.
- Kuhn, R. & Brossmer, R. (1956b). *Chem. Ber.* **89**, 2471.
- Kuhn, R. & Brossmer, R. (1957). *Angew. Chem.* **69**, 534.
- Martinsson, A., Raal, A. & Svennerholm, L. (1957). *Biochim. biophys. Acta*, **23**, 652.
- Odin, L. (1955a). *Acta chem. scand.* **9**, 714.
- Odin, L. (1955b). *Acta chem. scand.* **9**, 862.
- Odin, L. (1955c). *Acta chem. scand.* **9**, 1235.
- Svennerholm, L. (1956). *Acta Soc. Med. Upsalien.* **61**, 75.
- Svennerholm, L. (1957). *Ark. Kemi*, **10**, 577.
- Werner, I. & Odin, L. (1952). *Acta Soc. Med. Upsalien.* **57**, 230.
- White, T. (1940). *J. chem. Soc.* p. 428.
- Yamakawa, T. & Suzuki, S. (1951). *J. Biochem., Tokyo*, **38**, 199.
- Yamakawa, T. & Suzuki, S. (1952). *J. Biochem., Tokyo*, **39**, 175.
- Yamakawa, T. & Suzuki, S. (1955). *J. Biochem., Tokyo*, **42**, 727.
- Zilliken, F., Braun, G. A. & György, P. (1955). *Arch. Biochem. Biophys.* **54**, 564.
- Zilliken, F. & Glick, M. C. (1956). *Naturwissenschaften*, **43**, 536.

Studies in Detoxication

71. THE METABOLISM OF HYDROXYCOUMARINS*

BY J. A. R. MEAD, J. N. SMITH AND R. T. WILLIAMS

Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2

(Received 1 July 1957)

As a preliminary to the study of the fate of coumarin in animals (Mead, Smith & Williams, 1958), it was necessary to study the metabolic behaviour of the six isomeric hydroxycoumarins and to prepare either synthetically or biosynthetically their glucuronides and ethereal sulphates, since any of these compounds could be metabolites of coumarin.

The glucuronides of 3-, 4- and 7-hydroxycoumarin have been previously described (Flatow, 1910; Roseman, Heubner, Pancratz & Link, 1954; Mead, Smith & Williams, 1955), and Sieberg (1921) has reported that umbelliferone (7-hydroxy-), aesculetin (6:7-dihydroxy-) and daphnetin (7:8-dihydroxy-coumarin) after injection into rabbits were excreted in conjugated forms which were not described.

* Part 70: McIsaac & Williams (1957).

MATERIALS AND METHODS

Reference compounds. The following hydroxycoumarins were prepared: 3-, m.p. 153° (Linch, 1912), 4-, m.p. 206° (Boyd & Robertson, 1948), 5-, m.p. 228° (Adams & Bockstahler, 1952), 6-, m.p. 250–252° after recrystallization from dioxan (Bargellini & Monti, 1915) and 8-hydroxycoumarin, m.p. 159–160° (Cingolani, 1954).

Ethereal sulphates of hydroxycoumarins. Potassium salts of the hitherto undescribed ethereal sulphates of 3-, 5- and 8-hydroxycoumarins were synthesized by the general method of Burkhardt & Lapworth (1926) (see Table 1). The sulphate of 6-hydroxycoumarin was prepared in poor yield by the Elb's persulphate oxidation of coumarin. Coumarin (73 g.) and KOH (128 g.) were dissolved in 2 l. of water containing FeSO₄·7H₂O (2 g.). Potassium persulphate (132 g.) was added over 8 hr. to the stirred solution kept below 20°. The solution was then brought to pH 2 with conc. H₂SO₄ and extracted twice with ether to remove coumarin. At this stage a large precipitate, mainly K₂SO₄,