opposite direction; the decrease in rate (which may be 1000-fold) at moderate substrate concentration can be accounted for only partly by decreased affinity of substrate for enzyme.

5. The effect of increasing the *N*-acyl chain length from formyl to hexanoyl has been tested. Hydrolysis is fastest at acetyl or propionyl, depending upon the nature of the *para* substituent. Of all the substrates tested *p*-acetamidophenyl-acetic acid has the lowest apparent K_m (78 μ M). Chloroacetylated compounds are hydrolysed most rapidly; *p*-chloroacetamidoanisole, for instance, is deacylated at a maximal rate corresponding to 6.1 m-moles/g. of fresh tissue/hr. Some *N*-benzoyl derivatives of *p*-aminobenzoic acid are slowly hydrolysed.

6. On the basis particularly of distribution, sub-cellular localization, sensitivity to metal inhibition and the effect on rate of ring substitution and *N*-acyl chain length the enzyme is seen to have properties very different from those of kynurenine formamidase.

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REFERENCES

- Birnbaum, S. M., Levintow, L., Kingsley, R. B. & Greenstein, J. P. (1952). *J. biol. Chem.* **194**, 455.
Bratton, A. C. & Marshall, E. K. (1939). *J. biol. Chem.* **128**, 537.
Dalglish, C. E. (1952). *J. chem. Soc.* p. 141.
Gomori, G. (1954). *Proc. Soc. exp. Biol., N.Y.*, **85**, 570.
Hogeboom, G. H., Schneider, W. C. & Palade, G. E. (1948). *J. biol. Chem.* **172**, 619.
Jakoby, W. B. (1954). *J. biol. Chem.* **207**, 657.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Lipmann, F. (1945). *J. biol. Chem.* **160**, 173.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, F. J. (1951). *J. biol. Chem.* **193**, 265.
Mehler, A. H. & Knox, W. E. (1950). *J. biol. Chem.* **187**, 431.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Potter, V. R., Recknagel, R. O. & Hurlbert, R. B. (1951). *Fed. Proc.* **10**, 646.
Schneider, W. C. (1959). *Advanc. Enzymol.* **21**, 1.
Shaffer, J. M. & Bieter, R. N. (1950). *J. Pharmacol.* **100**, 192.
Tabor, H., Mehler, A. H. & Stadtman, E. R. (1953). *J. biol. Chem.* **204**, 127.
Venkataraman, A., Venkataraman, P. R. & Lewis, H. B. (1948). *J. biol. Chem.* **173**, 641.

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Purification of a Glycoprotein from Bovine-Submaxillary Glands

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Investigations of the composition of purified mucins from salivary glands have been made by Tanabe (1939), Blix, Svennerholm & Werner (1952) and Blix, Lindberg, Odin & Werner (1956). Degradative procedures were employed in the preparations, since the purpose was to study the composition of the carbohydrate or of the sialic acid. A purified material was prepared by Heimer & Meyer (1956) from mucin clots obtained from extracts of bovine-submaxillary glands. A 6*M*-urea solution adjusted to pH 9.0 was used for the extraction of acetone-extracted material.

In earlier work from this Laboratory (Nisizawa & Pigman, 1959), mucin clots from bovine-submaxillary glands were freeze-dried as sodium salts.

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The materials retained the viscosity of the extracts and were soluble in water. The procedure was a modification of the method of Hammarsten (1888). These clots, composed of an anionic glycoprotein combined with a protein cation, precipitated between pH 3.5 and 4.0.

The present work deals with the purification and further investigation of the physical characteristics and chemical composition of the material mainly responsible for the viscous nature of the mucin clot. Two preparations obtained at intermediate stages of purification have also been analysed.

EXPERIMENTAL

Source material. In the previous work (Nisizawa & Pigman, 1959) the sodium salt of the bovine-submaxillary mucin clot was obtained as follows. Bovine-submaxillary

glands which had been kept frozen were extracted with the dilute phosphate buffer (pH 7.0). The extract was adjusted to pH 3.5 with dilute hydrochloric acid and the resulting mucin clot was dissolved in water by the addition of a small amount of sodium hydroxide. The solution was freeze-dried after extensive dialysis against water. It contained 12.8% of sialic acid (as *N*:O-diacetylneuraminic acid), 8.1% of galactosamine and 3.4% of glucosamine (as *N*-acetylhexosamines) and nearly 74% of protein. All work was done at 2–4°. This freeze-dried mucin was the starting material for the present study.

Chemical analyses. For qualitative determination of the sugars, paper chromatography was used after hydrolysis by 1 *N*-hydrochloric acid or -sulphuric acid in a boiling-water bath for 5 hr. When hydrochloric acid was used the hydrolysates were filtered and dried in a vacuum desiccator over sodium hydroxide and calcium chloride. Barium carbonate was used to neutralize sulphuric acid. The system butanol-pyridine-water (5:3:2) (Yosizawa, 1953) was used as developer, and aniline hydrogen phthalate was used as the colouring reagent (Partridge, 1949). The paper was Whatman no. 1.

Nitrogen was determined by the micro-Kjeldahl method (Clark, 1943). Sialic acid was measured by the modified direct Ehrlich method (Pigman, Hawkins, Blair & Holley, 1958) with *N*-acetylneuraminic acid used as the standard. Hexosamine was analysed by the Elson-Morgan method modified by Boas (1953). For the measurement of glucosamine and galactosamine separately, the method of Gardell (1953) was utilized. Protein was determined by both the biuret reagent (Rosenthal & Cundiff, 1956) and the phenol reagent (Lowry, Rosebrough, Farr & Randall, 1951), crystalline bovine-serum albumin being used as the standard. Fucose (6-deoxyhexose) was measured by the cysteine-sulphuric acid method of Dische (1955). Hexose was determined as galactose by the anthrone method (Hanson, Schwartz & Barker, 1960) after correction for fucose. Uronic acid was measured as glucuronic acid by the Dische carbazole method (Dische, 1955).

Physical measurements. Viscosities were determined in Cannon-Manning Ostwald-type viscometers of about 0.2 ml. capacity at 30°. The flow time for water in the three viscometers was no. 100, 90 sec.; no. 200, 9.9 sec.; no. 300, 3.8 sec. Electrophoretic analyses were conducted at 1° in a Perkin-Elmer model 38 Tiselius-type apparatus with 6 ml. cell. The ascending boundaries were utilized for the calculations of mobility. Ultracentrifugal analyses were made at room temperature by use of the Spinco model E analytical ultracentrifuge at 259 700 *g* with a 12 mm. cell.

pH. All pH values were measured with a Beckman model G pH meter and glass electrode.

Purification with ethanol. To 100 ml. of aqueous 2% solution of mucin clot, 11 ml. of 50% calcium chloride solution was added at 2–4°; absolute ethanol was then added slowly with mechanical stirring in an ice-water bath. The precipitate produced at 30% (v/v) ethanol concentration was removed by centrifuging at 26 360 *g* for 15 min. and discarded. Additional ethanol was added to the supernatant to give a final concentration of 60%. After similar centrifuging, this precipitate was suspended in 30 ml. of water. Insoluble portions were nearly completely dissolved by the addition of a 4% solution of sodium ethylenediaminetetra-acetate, pH 9.5. The solution was then dialysed against two changes of 2 l. of water for 3 days with stirring.

A small precipitate produced during dialysis was centrifuged off. The mucin solution contained 247 mg. of protein/100 ml. (biuret method) and 110 mg. of sialic acid/100 ml. The yields were 19 and 50% respectively. As the solution was diluted during dialysis it was concentrated to half its volume by dialysis against a polyvinylpyrrolidone solution. This product was called F1.

Purification by Sevag (1934) procedure and with ethanol. The mucin clot (0.4 g.) was dissolved in 30 ml. of water with 3.16 g. of calcium acetate at 2–4°, and 20 ml. of 0.5 *M*-acetic acid was added. The resulting slightly turbid solution was shaken mechanically with 100 ml. of amyl alcohol-chloroform (1:3, v/v) for several minutes and then was separated from the solvent by centrifuging. The procedure was repeated many times until no interfacial precipitate formed. Ethanol was added to the deproteinized solution in an ice-salt bath with stirring to give a final concentration of 40% (v/v) of ethanol. The resulting precipitate was removed by centrifuging at 26 360 *g* for 15 min. and was suspended in 3–4 ml. of water and centrifuged.

A number of solutions obtained in this way from a total of 3.5 g. of mucin clot were combined to give 50 ml. To the combined solution was added a little 5% sodium ethylenediaminetetra-acetate, pH 6.7, and the solution was dialysed for 3 days against water. The solution was adjusted to pH 8.5 by adding a little 2% sodium carbonate solution and the solutions were centrifuged at 26 360 *g* for 20 min. The resulting solution was product F2. The yield was 3%, based on the original dry weight of mucin clot.

The supernatant of a 40% ethanol solution from the preparation of product F2 was diluted with ethanol to 60% ethanol (v/v). The precipitate was separated and redissolved in water, and the solution was treated with sodium ethylenediaminetetra-acetate and dialysed as for product F2. The resulting 50 ml. from 3.5 g. of mucin clot contained product F3. Its yield was 10% of the original dry mucin clot.

RESULTS

Mucin clot of the previous work from the neutral cold extracts of bovine-submaxillary glands was purified by precipitation with ethanol in the presence of calcium chloride or acetate and by the Sevag procedure. The physical properties and chemical analyses of these products are given below. Three products were isolated at different stages of the purification. These are designated as F1, F2 and F3, in increasing order of purification.

Mucin-clot formation. Aqueous solutions of F1 and F2 became turbid when adjusted to pH 3.5 with dilute hydrochloric acid, and formed a typical clot only if serum albumin had been added previously. When albumin was added to a very dilute solution of F3 before acidification, only a turbidity appeared at pH 3.5. However, the turbid solution deposited a viscous mass at the bottom of the test tube after 18 hr. at room temperature.

Viscometry. The viscosity of product F1 was measured in a phosphate buffer, pH 7.4, *I* 0.1, at 30°. The viscosity was 4.64 centipoises for a 1.2% solution (dry-weight basis). This corresponded to

53% of the viscosity of the original mucin clot when the products were compared on the basis of an equal amount of sialic acid.

The viscosity of an aqueous 0.72% solution of

Fig. 1. Intrinsic viscosity of mucin A in different solvents at 30°: A, water; B, phosphate-NaCl buffer, pH 7.4, *I* 0.1; C, phosphate-NaCl buffer, pH 7.4, *I* 0.2.

Fig. 2. Intrinsic viscosity of product F3 in different solvents at 30°: A, water; B, phosphate-NaCl buffer, pH 7.4, *I* 0.1; C, phosphate-NaCl buffer, *I* 0.2.

mucin F3 at 30° was measured in three Cannon-Manning capillary viscometers of quite different rates of flow (nos. 100, 200 and 300). The viscosity was 18.8 centipoises (viscosity, relative to water, 22.1), and no effect of flow rate was noticeable. This product (F3) was degraded by drying at 100° for several hours, and the viscosity dropped from 18.8 to 12.1 centipoises.

The viscosity of F3 was measured at different concentrations in water and in phosphate buffers of pH 7.4 (*I* 0.1 and 0.2). The viscosity of the original mucin clot was also measured under the same conditions. These results are shown in Figs. 1 and 2. The intrinsic viscosity of mucin clot was 26.3 in water and 2.0 and 1.7 at *I* 0.1 and 0.2 respectively. It was 64.6, 3.9 and 3.6 respectively for product F3.

Electrophoretic behaviour. In the work by Nisizawa & Pigman (1959) the mucin clot from bovine-submaxillary glands separated into several components during electrophoresis at pH 10.0, whereas the resolution was poorer at pH 7.0–8.0. Therefore the electrophoretic patterns were studied at pH 10.0–11.0 in the present work. Fig. 3 shows the electrophoretic pattern of F1 at pH 10.0. The fast-moving component with a mobility about $-16 \times 10^{-5} \text{ cm}^2 \text{ v}^{-1} \text{ sec}^{-1}$, present in the original mucin clot (Nisizawa & Pigman, 1959), was not detectable, but the material still showed several components. In the electrophoretic pattern of product F2, two components were still evident. Their mobilities were -6.7 and $-16.5 \times 10^{-5} \text{ cm}^2 \text{ v}^{-1} \text{ sec}^{-1}$. These mobilities corresponded to those of the most slowly moving and the most rapidly moving components in the original mucin clots.

In Fig. 3, only a single peak can be seen for product F3, and its mobility was $-6.9 \times 10^{-5} \text{ cm}^2 \text{ v}^{-1} \text{ sec}^{-1}$.

Fig. 3. Electrophoretic patterns: F1 in phosphate-NaCl buffer, pH 10.0, *I* 0.1, at 70 min. Concentration: 1.23%. Mobilities of components: -6.6 , -7.6 , $-8.7 (\times 10^{-5} \text{ cm}^2 \text{ v}^{-1} \text{ sec}^{-1})$. F2 in phosphate-NaCl buffer, pH 10.0, *I* 0.1, at 40 min. Concentration: 0.2%. Mobilities of components: -6.7 , $-16.5 (\times 10^{-5} \text{ cm}^2 \text{ v}^{-1} \text{ sec}^{-1})$. F3 in phosphate-NaCl buffer, pH 11.0, *I* 0.1, at 120 min. Concentration: 0.5%. Mobility $-6.9 (\times 10^{-5} \text{ cm}^2 \text{ v}^{-1} \text{ sec}^{-1})$.

sec.⁻¹ under the same conditions. This component corresponds to one of the principal ones observed in the patterns of the original clot (-7.4) and seems to be mainly responsible for the viscosity of the extracts of the gland. The single fractionation with ethanol mainly removed the fastest-moving component, and the Sevag procedure seemed to remove the protein components with intermediate mobilities.

Optical rotation. The specific optical rotation of F3 was measured in a 2 dm. tube as $[\alpha]_D^{24} -3.5$ (1.44% in water).

Ultracentrifugal characteristics. The ultracentrifugal patterns of products F1 and F3 are shown in Fig. 4. In the pattern of F1, two major peaks and one minor peak were still present, as in that of the original mucin clots. The pattern of F3 also

Fig. 4. Ultracentrifugal patterns of products F1 and F3 in phosphate buffer, pH 10.0, I 0.1. F1: concentration, 1.2%; time, 120 min. F3: concentration, 0.5%; time, 160 min.

showed two peaks, whereas its electrophoretic pattern showed a single peak. However, the slower peak in the pattern of F3 represented less than 10% of the area. The $S_{20,w}$ for the three peaks in F1 are 2.15, 2.41 and 3.07 respectively and 1.88 and 2.28 for the two components in F3. The $S_{20,w}$ of the corresponding peaks are sufficiently close to assume that they represent the same components in both preparations. However, four components were found in the electrophoretic pattern of F1, whereas F3 showed a single peak. From the area of the peaks, however, the faster (2.3s) component in Fig. 4 seems to represent the major substance in product F3.

Chemical analyses. The results of the chemical analysis of the three products are given in Table 1. The ascending order of purity was F1, F2, F3.

The purified preparation F3 contained about 38% of protein and 62% of carbohydrate (comprising 32.3% of sialic acid and 27.3% of hexosamine). Minor components were galactose, fucose and hexuronic acid.

The less-pure product F2 consisted of almost equal amounts of protein and carbohydrates. The amount of hexosamine was larger than that of sialic acid, and galactosamine and glucosamine were found in almost equal amount.

The molar ratio of galactosamine to sialic acid was almost exactly unity for both F2 and F3. Some of the glucosamine in F2 seemed therefore to originate from a component other than that which was composed of an equal amount of galactosamine and sialic acid. F2 contained 6% of hexuronic acid and about 10% of glucosamine. Since sulphate was absent, this composition suggested the presence of some hyaluronic acid.

Table 1. Chemical analyses of submaxillary mucin at different stages of purification

Compositions are given on a dry-weight basis without correction for ash. The ester sulphate test on F2 was negative for a sample of 17 mg. dry wt. No ash was detected when a sample of F3 was ignited in a platinum crucible. On paper chromatograms of hydrolysates of mucin A and F3, definite spots for galactose, galactosamine and glucosamine were found. Spots corresponding to glucose and mannose were not detected, but those of fucose were found in traces when sulphuric acid was used for hydrolysis.

Constituent	Composition (%)		
	F1	F2	F3
Total nitrogen	11.9	9.6	9.4
Total N minus non-protein N (corr. total N)	10.1	8.7	6.0
Corr. total N $\times 6.25^*$	62.5	47.7	37.5
Biuret protein (as serum albumin)	43.1	20.9	34.1
Phenol-reagent protein (as serum albumin)	—	38.5	32.5
Sialic acid [as <i>O</i> : <i>N</i> -diacetyl, 'direct' Ehrlich reagent method (see Whelan, 1957)]*	17.3	15.4	32.3
Hexose (as galactose, corr. for fucose)*	3.2	2.5	1.7
6-Deoxyhexose (cysteine- H_2SO_4 method, as fucose)*	—	1.2	0.7
Hexuronic acid (as glucurone)*	0.1	6.0	0.7
Hexosamine (as <i>N</i> -acetylglucosamine)*	16.2	21.0	27.3
<i>N</i> -Acetylgalactosamine	—	10.7	21.6
<i>N</i> -Acetylglucosamine	—	10.3	5.7
Total (of items marked *)	99.3	93.8	100.2

DISCUSSION

The mucin clot obtained from the extracts of bovine-submaxillary glands and which contained the viscous material of the extracts was further fractionated. By removing more than half of the protein from the clot by the Sevag procedure and a subsequent ethanol fractionation, a material (F3) principally responsible for the viscous nature of the clot has been prepared in a purified state.

This material (F3) had an intrinsic viscosity of 3.6 in a phosphate buffer of pH 7.4, I 0.2 (Fig. 2). The intrinsic viscosity of the original mucin clot was 1.70 under the same conditions (Fig. 1). These intrinsic viscosities were much lower than those for the aqueous solutions and apparently are an example of the well-known electroviscous effect (Overbeek & Bungenberg de Jong, 1949). Although the intrinsic viscosity was doubled as a result of purification, the purity of F3 was approximately three times that of the starting mucin on the basis of the sialic acid or the galactosamine content.

The intrinsic viscosity of solutions of F3 in pure water was nearly three times that of a similar solution of the mucin clot, and the plots of $\log(\eta_{sp}/c)/\text{concentration}$ were quite different (Figs. 1 and 2). The line for the clot was straight and seemed virtually independent of concentration, but that for the purified material was a more normal curve rising with a decrease in concentration (Overbeek & Bungenberg de Jong, 1949). These effects may have arisen in part from a change in the pH with concentration, since an aqueous 0.72% solution was pH 4.5 and a 0.1% solution was pH 6.0. In contrast, the solution of mucin clot in water remained in the range pH 6.0–6.8 upon dilution. However, the additional protein of the mucin clot may be a factor in the unusual constant viscosity of mucin clot.

The purified material (F3) showed one main peak in the ultracentrifugal pattern (Fig. 4), and a single peak in the electrophoretic pattern (Fig. 3). The small peak in the ultracentrifugal pattern and the small amounts of glucosamine, hexose and fucose suggest that the material still is not completely homogeneous.

A principal component of the submaxillary mucin is thus a viscous substance apparently composed of about one-third of peptide units and equimolar proportions of galactosamine and sialic acid. Since the material showed a single peak in the electrophoretic pattern, even at pH 11.0, the linkage between carbohydrate and protein moieties seems to be firm, probably covalent. The product appears to belong to the glycoprotein group (Pigman, 1957).

This material is similar to that obtained by Heimer & Meyer (1956) and has a similar optical

rotation. However, the earlier product contained slightly less sialic acid and galactosamine although described as electrophoretically and ultracentrifugally homogeneous. The molar ratio of galactosamine to sialic acid was close to unity in their product also.

A less-purified material (F2) was richer in glucosamine, galactose and fucose. The molar ratio of glucosamine to the galactosamine was as great as 0.4, even after the glucosamine content was corrected for the contribution by a presumed hyaluronic acid (based on the hexuronic acid content). The same ratio obtained for the more purified product (F3) was nearly 0.25. Nevertheless, the molar ratios of galactosamine to sialic acid were exactly unity in both F2 and F3. These facts suggest the presence of additional glycoproteins in addition to hyaluronic acid and the principal one comprised of equimolar amounts of galactosamine and sialic acid.

SUMMARY

1. The mucin clots obtained from the extracts of bovine-submaxillary glands have been fractionated and purified. The material mainly responsible for the viscous nature of the extract has been obtained purified by the Sevag procedure and by fractionation with ethanol. It showed a single component in the electrophoretic pattern, but a small component separated from the major one in the ultracentrifugal run. The electrophoretic mobility of the product was $-6.9 \times 10^{-5} \text{ cm}^2 \text{ v}^{-1} \text{ sec}^{-1}$ in phosphate buffer, pH 11.0, I 0.1. The sedimentation constant of the major component was 2.28 in phosphate buffer, pH 10.0, I 0.1.

2. This material appeared to be a glycoprotein composed of about 38% of protein and 62% of carbohydrate. Of the latter, sialic acid was 32.3% (as the diacetyl compound) and the hexosamines were 27.3% (as the acetyl compound). The hexosamines contained 21.6% of galactosamine and 5.7% of glucosamine. The molar ratio of galactosamine to sialic acid was unity, as in the original mucin clots.

3. A less-purified product obtained from the mucin clots after the treatment with the Sevag procedure appeared to contain some hyaluronic acid. Although the glucosamine content was nearly equal to the galactosamine, the molar ratio of galactosamine to sialic acid still was unity.

4. In view of the electrophoretic and chemical analyses, it was established that the most slowly moving component in the electrophoretic patterns at pH 10.0 represented the principal component responsible for the viscous nature of the mucin and that the product is probably a glycoprotein.

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REFERENCES

- Blix, G., Lindberg, E., Odin, L. & Werner, I. (1956). *Acta Soc. Med. Upsalien.* **61**, 1.
- Blix, G., Svennerholm, L. & Werner, I. (1952). *Acta chem. scand.* **6**, 358.
- Boas, N. F. (1953). *J. biol. Chem.* **204**, 553.
- Clark, E. P. (1943). *Semimicro Quantitative Analysis*, 1st ed., p. 37. New York: Academic Press Inc.
- Dische, Z. (1955). *Method of Biochemical Analysis*, 1st ed., vol. 2, p. 343. New York: Interscience Publishers Inc.
- Gardell, S. (1953). *Acta chem. scand.* **7**, 207.
- Hammarsten, O. (1888). *Hoppe-Seyl. Z.* **12**, 163.
- Hanson, R. W., Schwartz, H. & Barker, S. B. (1960). *Amer. J. Physiol.* (in the Press).
- Heimer, R. & Meyer, K. (1956). *Proc. nat. Acad. Sci., Wash.*, **42**, 728.
- Lowry, O. M., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Nisizawa, K. & Pigman, W. (1959). *Arch. oral. Biol.* **1**, 161.
- Overbeek, J. Th. G. & Bungenberg de Jong, H. G. (1949). *Colloid Science*, vol. 2, p. 184. Ed. by Kruyt, H. R. New York: Elsevier Publishing Co.
- Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
- Pigman, W. (1957). *The Carbohydrates*. New York: Academic Press Inc.
- Pigman, W., Hawkins, W. L., Blair, M. G. & Holley, H. L. (1958). *Arthritis and Rheumatism*, **1**, 151.
- Rosenthal, H. L. & Cundiff, H. I. (1956). *Clin. Chem.* **2**, 394.
- Sevag, M. G. (1934). *Biochem. Z.* **273**, 419.
- Tanabe, Y. (1939). *J. Biochem., Tokyo*, **30**, 181.
- Whelan, W. J. (1957). *Rep. Progr. Chem.* **54**, 321.
- Yosizawa, Z. (1953). *Tohoku J. exp. Med.* **59**, 1.

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The Glycogenolytic Activity of some Substituted Salicylic Acids

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Sodium salicylate in moderate or large dosage has been shown to decrease glycogen concentration in the liver of fed and starved mice 2 hr. after injection (Sproull, 1954) and in rats 4–7 hr. after administration (Lutwak-Mann, 1942; Smith, Meade & Bornstein, 1952; Feeney, Carló & Smith, 1955). Lutwak-Mann (1942) studied the effect of large doses of certain substituted benzoic acids on rat-liver glycogen, and found that although acetylsalicylate, benzoate, anthranilate, *o*-cresotate (2-hydroxy-3-methylbenzoate) and *m*-hydroxybenzoate all caused some decrease in liver-glycogen concentration 7 hr. after injection, only acetylsalicylate and *o*-cresotate showed an effect similar to salicylate; with *o*-cresotate (0.6 mg./g.) there was virtually no glycogen present over the 24 hr. period of the experiment. Feeney *et al.* (1955) reported that salicylate, acetylsalicylate and *m*-hydroxybenzoate all decreased rat-liver-glycogen concentration significantly although the reduction after *m*-hydroxybenzoate was not as pronounced as that obtained with salicylate.

The present investigation was to determine which of ten substituted benzoic acids related to salicylic acid and of therapeutic interest in the treatment of rheumatic fever decreased the liver-glycogen concentration of fed mice 2 hr. after injection.

METHODS

Swiss albino mice, were used in all experiments and the drugs were all given by intraperitoneal injection. The mice were killed by cervical dislocation 2 hr. after injection and 300–400 mg. of liver was removed for the estimation of liver glycogen. Glycogen was separated and hydrolysed by the Good, Kramer & Somogyi (1933) modification of Pflüger's method, and finally it was estimated as glucose by ceric sulphate titration (Miller & van Slyke, 1936). The glycogen concentration was expressed as mg./g. wet wt. of liver.

In the first experiments mice (age 3–5 months) were dealt with in batches of nine of the same sex per day, and were allowed food and water freely until the start of the experiment. Three animals from each batch were killed without injection at zero time, another three were injected with 0.2 ml. of 0.9% NaCl soln. and the remaining three received the same volume of the test drug, these two last-named trios being killed 2 hr. after injection. Four such batches, two for each sex, were used for each drug, which was given as a solution of the sodium salt (pH 7.0–9.0).

Dose-response curve for salicylic acid. Mice (age 2–4 months) of weight range 30–50 g. were allowed food and water until the start of the experiment. The mice were treated in batches of seven mice of the same weight and sex; each of six mice received a dose of 2, 4, 6, 8, 10 or 12 mg. of salicylic acid as a solution of the sodium salt (pH 6.0–8.0) and the seventh mouse received 0.05 ml. of 0.9% NaCl soln. The order in which the doses were given was determined from a table of random numbers. Sixteen estimates (eight males, eight females) of ΔG , i.e. the differ-