The Sialic Acids

II. PREPARATION OF N-GLYCOLYLHEXOSAMINES, N-GLYCOLYLHEXOSAMINE 6-PHOSPHATES, GLYCOLYL COENZYME A, AND GLYCOLYL GLUTATHIONE*

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Few glycolic acid derivatives have been isolated from mammalian tissues. One such compound is the widely distributed sialic acid, N-glycolylneuraminic acid (2). An enzyme, N-acetyleneuraminic aldolase, cleaves this substance in a reversible manner to pyruvate and N-glycolyl-n-mannosamine (3); it is enzymatically formed as the β-phosphate ester by condensation of phosphoenolpyruvate and N-glycolyl-n-mannosamine 6-phosphate (4). The biosynthesis of the glycolylmannosamine derivatives is, therefore, of interest and, by analogy with the pathways established for the N-acetyl derivatives of mannosamine (5–8), N-glycolyl-n-glucosamine and its phosphate esters may be important intermediates in the biosynthesis of this type of sialic acid. N-Glycolylhexosamines have not previously been described. The present report describes a general procedure for N- and S-glycolylation and its application to the preparation of N-glycolyl derivatives of the hexosamines, their 6-phosphate esters, glycolyl coenzyme A, and glycolyl glutathione.

EXPERIMENTS AND RESULTS

Materials—Glycolic acid, CoA, GSH, and n-glucosamine hydrochloride were obtained from commercial sources. The following compounds were prepared by described procedures: n-mannosamine hydrochloride and N-acetyl-n-mannosamine (9), n-galactosamine and N-acetyl-n-galactosamine (10), N-acetyl-n-glucosamine (11), the 6-phosphate esters of n-gluco-"saccharides (25) ; and (c) the product formed by the reaction of glycolic acid with ether chloroformate in anhydrous tetrahydrofuran in the presence of 1.0 equivalent of pyridine, a general procedure for the preparation of mixed anhydrides (26). Each of the products appeared to be an anhydride in that it reacted with the neutral hydroxylamine reagent, giving hydroxamates. On the other hand, none of the reagents reacted satisfactorily with the hexosamines. To evaluate the supposed anhydrides, they were treated under anhydrous conditions with aniline in the presence and absence of pyridine, and at room temperature or under reflux conditions (solvents used included dioxane and tetrahydrofuran). In no case could the formation of glycolic acid be detected. Surprisingly, the reaction mixtures, after aniline treatment, showed no diminution in reaction with the neutral hydroxylamine reagent. These results led to the conclusion that the hydroxamate method is not an adequate test for anhydrides in the case of glycolic acid. As has been reported, glycolic acid readily forms cyclic and linear polyesters (27); some of these may react with hydroxylamine.

Success in the preparation of the desired compounds was achieved with anhydro-O-carboxyglycolic acid (1:3-dioxolan-2:4-dione) as a glycolylating reagent. This compound was prepared as described by Davies (28) and gave a white, crystalline solid that is stable for months when stored in a vacuum over P_2O_5 at −18°C. The anhydro compound reacts instantly with...
aniline at room temperature in anhydrous solvents, giving essentially quantitative yields of the crystalline anilide. Although the anhydro compound also reacts rapidly with water, its rate of reaction with hydroxylamine under neutral conditions is even more rapid, yielding a hydroxamate that appears identical with authentic glycolyhydroxamate (Rf, 0.25) and different from acetylhydroxamate (Rf, 0.47) on paper chromatography in water-saturated butanol. By the colorimetric method of Lipmann and Tuttle (22), glycolyhydroxamate gave 34% of the molar absorbancy obtained with acetylhydroxamate and 43% of that obtained with succinyl hydroxamate.

**N-Glycolylation of Hexosamines and Hexosamine 6-Phosphates**

Owing to the reactivity of the anhydro-O-carboxyglycolic acid with water, the procedures previously used for N-acetylation of these compounds (10, 12) were slightly modified. The hexosamine hydrochloride or hexosamine-6-P ester (5 mmoles) was dissolved in a mixture containing 10 ml of water, 2 ml of methanol, and 1.2 equivalents of KHCO3. The solution was placed in an ice bath, stirred vigorously, and treated with 20 ml of anhydrous, freshly distilled dioxane containing 15 mmoles of anhydro-O-carboxyglycolic acid. The dioxane solution was added dropwise to the center of the aqueous solution from a burette protected with a drying tube. The reaction mixture was maintained between pH 6.5 and 7.5 with KHCO3 while the dioxane solution was added over a period of approximately 30 minutes. After the solution was stirred in the ice bath for an additional 60 minutes, aliquots were assayed; free hexosamine was not detected, whereas the Morgan-Elson color values indicated quantitative N-acetylation.

The glycolyhexosamines were isolated by passage of the reaction mixtures through an excess of mixed bed ion exchange resin (Dowex 50, hydrogen form, and Dowex 1, carbonate form, both 20 to 40 mesh); the respective filtrates and washings were combined and evaporated in a vacuum to clear, colorless sirups. The N-glycolyhexosamines are not crystallized as easily as the corresponding N-acetyl derivaties, which results in substantial losses during this process. N-Glycolyl-D-glucosamine was the most easily crystallized derivative, the crude product being obtained in 78% yield from absolute ethanol; after two subsequent recrystallizations from ethanol-ether mixtures, the product was obtained in a 40% yield. N-Glycolyl-D-galactosamine was initially crystallized from absolute ethanol in 76% yield; after two recrystallizations from a cold mixture of methanol and acetone, the product was obtained in a 44% yield. N-Glycolyl-D-mannosamine was crystallized with difficulty, slowly depositing over a period of 3 months from a solution of methanol and petroleum ether kept at −18°; the yield of the first crop of material was 10 to 15%. The N-glycolyl-D-mannosamine sirup showed all of the properties of a single compound by the techniques of paper chromatography and electrophoresis described below, and it was fully active as a substrate for N-acetyl-neuraminic acid aldolase (9) and for N-acetyl-D-mannosamine kinase (6). The yields of crystalline N-glycolyl-D-mannosamine were substantially increased by allowing the crystals to deposit over a period of a year. In each of the solvent mixtures described above, the second solvent was added to the first until the appearance of a faint but persistent turbidity. The crystalline compounds were dried for analysis in a vacuum over P2O5 for 2 days at room temperature. Table I shows analyses for the N-glycolyhexosamines.

The N-glycolylhexosamine-6-P esters were purified by a different procedure. After treatment with excess Dowex 50 hydrogen form ion exchange resin, the reaction mixtures (5 mmoles of hexosamine phosphates in each) were adsorbed on columns containing 270 ml of Dowex 1 acetate form resin, 200 to 400 mesh, and the resin was washed with 4 column volumes of water. The p-glucosamine and p-galactosamine derivatives were eluted with 4 N acetic acid. Glycolyhexoside was eluted almost immediately, and the p-glucosamine and p-galactosamine derivatives appeared as broad peaks in the fractions between 8.5 and 10.2 liters. N-Glycolyl-D-mannosamine-6-P was prepared on half the scale described for the compounds above, a Dowex 1 column of 135 ml being used; under these conditions, with 6 N rather than 4 N acetic acid used as eluting agent, the product appeared in the eluate between 1.8 and 2.7 liters. The desired compounds were detected by their positive color reactions with the Morgan-Elson reagents and by the presence of organic P. No hexosamine-6-phosphates were detected; these compounds would appear early in the fractionation (12). The peak fractions were pooled, concentrated in a vacuum (external bath temperature, 20°) to approximately 40 ml, and lyophilized. The amorphous products were dissolved in water and lyophilized again to remove traces of acetic acid. The following yields of the amorphous products were obtained: N-glycolyl-D-glucosamine-6-P, 83%; N-glycolyl-D-galactosamine-6-P, 89%; and N-glycolyl-D-mannosamine-6-P, 84%. Neither free hexosamine-6-P nor inorganic P was detectable in the final products. For analysis, the products were dried as described for the glycolyhexosamines; they proved to be extremely hygroscopic. For polarimetric studies, and for storage, the compounds were converted to their potassium salts by passage through excess Dowex 50 potassium form ion exchange resin, 200 to 400 mesh. The analyses obtained on these substances are presented in Table II.

**Preparation of N-Acetyl-p-mannosamine-6-P**—The properties of these compounds were essentially identical with those of the glycolyhexosamines. The compounds were prepared by the method of Lipmann and Tuttle (22) and were purified as described above. The properties of these compounds were essentially identical with those of the glycolyhexosamines. The compounds were prepared by the method of Lipmann and Tuttle (22) and were purified as described above.
of this compound have not previously been recorded. It is easily prepared by the usual method for N-acetylation of the hexosamines and hexosamine-6-P esters (12). A mixture containing 2 mmoles of d-mannosamine-6-P, 2 mmoles of KHCO₃, 5 ml of water, and 0.6 ml of methanol was vigorously stirred in an ice bath while a total of 6 mmoles of acetic anhydride was added in two portions, 30 minutes apart. The mixture was stirred at 0° for an additional 2 hours, with pH maintained between 6.5 and 7.5 by the addition of KHCO₃, and was further treated in the way described for isolating N-glycolyl-n-mannosamine-6-P. The product was obtained in 87% yield and, after drying, showed the following analyses.

\[
\text{C₇H₁₄O₅N₅P} \quad (301.2)
\]

Calculated: C 31.90, H 5.47, N 4.71, P 10.52

Found: C 31.74, H 5.47, N 4.71, P 10.52

Analysis of the amorphous product showed no hexosamine-6-P or inorganic P. For storage, and for optical rotation studies, the compound was converted to its potassium salt as described above. The optical rotation of the potassium salt was \([\alpha]^{24.8}_{D} +24.8\) (c, 4.1% in water).

**Table II**

| N-Glycolyl-6-P derivative | \([\alpha]^{24.8}_{D}\) | C (58) | H (58) | N (58) | P (58) |
|--------------------------|---------------------|--------|--------|--------|--------|
| d-Glucosamine            | +24.8               | 29.44  | 5.08   | 4.10   | 9.64   |
| d-Galactosamine          | +19.5               | 30.49  | 5.06   | 4.37   | 9.84   |
| d-Mannosamine            | +15.0               | 29.37  | 5.33   | 4.19   | 9.99   |

* The specific rotations were obtained on the potassium salts in aqueous solution in concentrations ranging from 4.2 to 9.5%; equilibrium values are given.

† The elementary analyses were performed on the free acids.

### Preparation of Glycolyl-CoA and Glycolyl Glutathione

The method of Ochoa for acetylation of sulfhydryl compounds (33) was modified as follows. A mixture containing 4 μmoles of CoA (approximately 75% purity) or glutathione, 100 μmoles of KHCO₃, and 0.4 ml of distilled water was vigorously stirred in an ice bath during the addition of 10 μmoles of the anhydro-O-carboxyglycolic acid in 1 ml of anhydrous dioxane. The addition of glycolylating reagent took approximately 1 hour, during which time the pH was maintained between 6 and 7 by the addition of KHCO₃. The nitroprusside procedure indicated that 66% of the free —SH groups of the CoA preparation disappeared during the reaction; the glutathione reaction mixture

**Table III**

| Compound                     | Solvent 1* (RONa) | Solvent 2* (RONa) | Paper electrophoretic† |
|------------------------------|-------------------|-------------------|------------------------|
| d-Glucosamine                | 0.51              | 0.00              | 1.8                    |
| d-Galactosamine              | 0.51              | 0.00              | 1.2                    |
| d-Mannosamine                | 0.49              | 0.00              | 6.0                    |
| N-Acetyl-d-galactosamine     | 1.00              | 1.00              | 1.5                    |
| N-Acetyl-d-galactosamine     | 1.00              | 0.43              | 0.3                    |
| N-Acetyl-d-glucosamine       | 1.08              | 0.40              | 3.8                    |
| N-Glycolyl-d-galactosamine   | 0.76              | 0.70              | 1.0                    |
| N-Glycolyl-d-glucosamine     | 0.72              | 0.15              | 0.4                    |
| N-Glycolyl-d-mannosamine     | 0.83              | 0.10              | 3.7                    |

* Solvent 1 was n-butanol-acetic acid-water (50:12.25); Solvent 2 was n-butanol-pyridine-water (6:4:3). Whatman No. 1 paper was used for the paper chromatography; borate-treated Whatman No. 1 paper was used in conjunction with Solvent 2 (29). The N-acetyl derivatives were detected with the alkaline-ethanol spray, followed by the p-dimethylaminobenzaldehyde reagent previously described (9); the N-glycolyl derivatives give fluorescence under ultraviolet light after alkaline treatment in the same manner as the N-acetyl derivatives. The free hexosamines were detected with a silver nitrate reagent (30). The values given are the ratios of the mobilities of the compounds to that of N-acetyl-d-glucosamine.

† Paper electrophoresis was conducted in the apparatus of Markham and Smith (31), with Whatman 3MM paper, carbon tetrachloride as cooling agent, 1% sodium tetraborate, pH 0.5, as buffer, and 22 volts per cm. This method is similar to that used by Maley and Maley for the separation of hexosamines (32). The sugars were visualized as described above. The values given in the table represent the distance (in centimeters) of migration of the compounds from the origin after 30 minutes and indicate the electrode toward which each sugar moved. Migration toward the cathode probably occurs by endosmosis; no correction was made for this phenomenon.
was not analyzed. The products, glycolyl-CoA and glycolyl glutathione, were characterized as follows. (a) Each substance showed an ultraviolet absorption spectrum characteristic for thiol esters, the CoA derivative showing an absorption maximum at 234 μμ, and the glutathione derivative, a maximum at 232 μμ.

(b) Treatment with neutral hydroxylamine gave hydroxamates; (c) the resulting hydroxamates migrated on paper chromatograms at rates identical with authentic glycolylhydroxamate; and (d) paper chromatography of the thiol esters in an ethanol-ammonium acetate solvent (21), followed by treatment with the nitroprusside reagent (21), gave positive results, thus indicating that the products were thiol esters.

FIG. 1. Synthesis of glycolyl derivatives. R—NH₂ = hexoseamines or hexoseamine-P. R—SH = CoA or glutathione.

glycolyl-d-mannosamine or its 6-phosphate ester, i.e. through the UDP derivatives (8) or via the 2-epimerase (5); the activation of the sialic acids by conversion to nucleotides (38, 39); and the polymerization reaction(s).

**SUMMARY**

Hexosamines and hexosamine 6-phosphates were N-glycolylated with anhydro-O-carboxyglycolic acid. The reagent was also applied to the preparation of glycolyl coenzyme A and glycolyl glutathione.

Paper chromatographic and electrophoretic methods have been developed for identifying the individual N-glycolyhexosamines, N-acetyhexosamines, and free hexosamines.

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