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Preparation of *N*-Acetyl-D-mannosamine (2-Acetamido-2-deoxy-D-mannose) and D-Mannosamine Hydrochloride (2-Amino-2-deoxy-D-mannose)[1]

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A method is reported for the preparation of gram quantities of *N*-acetyl-D-mannosamine and D-mannosamine hydrochloride. The procedure is based on the alkaline epimerization of *N*-acetyl-D-glucosamine to *N*-acetyl-D-mannosamine. The *N*-acetyl-D-mannosamine was obtained as the monohydrate in crystalline form, and converted to D-mannosamine. Some characteristics of an unidentified product of the epimerization reaction were examined.

As previously reported, *N*-acetyl-D-mannosamine (*N*-AcMm) and pyruvic acid are formed by the enzymatic cleavage of *N*-acetylneuraminic acid (sialic acid).[3] The conversion of uridine diphosphoacetylglucosamine to *N*-AcMm by rat liver extracts also has been described.[4] For further studies on the metabolism of D-mannosamine (Mm) and *N*-AcMm, large quantities of the pure compounds were required.

D-Mannosamine hydrochloride (Mm,HCl) has been synthesized[5] by the epimerization of D-glucosaminic acid to D-mannosaminic acid followed by conversion of the D-mannosaminic acid to its lactone and reduction of the lactone to yield Mm,HCl. In a single attempt to repeat this procedure in this Laboratory, a yield of less than 5% was obtained. Recently, another procedure was reported[6] which consisted of treating D- or L-arabinose with cyanide and benzylamine, followed by a partial reduction of the cyanide addition compound to the desired aldehyde. L-Mannosamine hydrochloride was obtained in 1% yield. Some properties of crystalline *N*-AcMm monohydrate have recently been described,[7] but the method of preparation was not given.

In a preliminary report[8] from this Laboratory it was noted that under alkaline conditions *N*-acetyl-D-glucosamine (*N*-AcGm) was epimerized to *N*-AcMm. This reaction is the basis of a convenient method for the large scale preparation of crystalline *N*-AcMm which is then readily converted to Mm,HCl. The isolation of pure *N*-AcMm from the reaction mixture involves the following steps: (1) alkaline epimerization of *N*-AcGm; (2) recovery of most of the *N*-AcGm present in the mixture by fractional crystallization; (3) removal of the remaining *N*-AcGm by incubation of the mixture with *Escherichia coli;* (4) crystallization of *N*-AcMm from the deionized culture fluid.

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About 20% of the *N*-AcGm is epimerized to *N*-AcMm by the alkaline treatment. The over-all yield of crystalline *N*-AcMm is 14% on the basis of the *N*-AcGm originally used, or 37% when corrected for the *N*-AcGm which was recovered. This yield represents 70% of the *N*-AcMm formed in the reaction. The *N*-AcGm recovered in the initial step can be recycled, thus increasing the yield. Pure Mm,HCl was obtained (89% yield) from the crystalline *N*-AcMm by acid hydrolysis.

The mechanism of the epimerization reaction is unknown. However, an unidentified component(s) of the reaction mixture was examined. This material (X) migrated more rapidly on paper chromatography than did *N*-AcGm and *N*-AcMm. Some of the properties of X were examined; it was found to yield *N*-AcGm and *N*-AcM after being subjected to the conditions of the epimerization reaction.

Although the alkaline epimerization reaction has not been applied to *N*-acetylhexosamines other than *N*-AcGm, this procedure may prove useful for the preparation of some of these substances which are difficult to prepare by other methods.

Experimental

N-AcGm was prepared by *N*-acetylation of D-glucosamine hydrochloride.[9]

Paper Chromatography.—Chromatography was routinely performed on borated paper with 1-butanol–pyridine–water, 6:4:3, as the solvent.[10] Relative to the mobility of *N*-AcGm (R_{N-AcGm}), *N*-AcMm exhibited an R_{N-AcGm} of 0.40 to 0.45,[3,8] while X exhibited an R_{N-AcGm} of 2.0 to 2.3. The chromatograms were developed by spraying with 0.5*N* sodium hydroxide in ethanol and heating at 100° for 10 min. When examined under an ultraviolet lamp, the *N*-acetylhexosamines and X exhibited yellow fluorescence. In contrast to the lability of the purple color obtained with the *p*-dimethylaminobenzaldehyde spray reagent,[10,11] the fluorescence was stable; equimolar amounts of the sugars apparently yield equal intensities of fluorescence when examined visually.

Preparation of *N*-Acetyl-D-Mannosamine.—Twenty-five grams of *N*-AcGm was dissolved in 100 ml. of water; the solution was adjusted to *p*H 11.0 by the addition of 10 *N* sodium hydroxide, and left for two days at room temperature. The sodium ions were removed by passing the material through Dowex-50 hydrogen form resin (20–40 mesh)

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(11) J. L. Reissig, J. L. Strominger and L. F. Leloir, *ibid.*, **217**, 959 (1955). NOTE ADDED IN PROOF.—In the procedure of Reissig, *et al.*,[11] N-AcMm yields 53% of the color value obtained with N-AcGm. When this method is modified so that the 100° treatment is extended to 12 min. and the 37° treatment reduced to 10 min., the intensity of the color value obtained with N-AcGm remains unchanged, while that of N-AcMm is doubled, yielding an approximately equivalent molar absorbancy for the two compounds.

and the solution was concentrated to a sirup *in vacuo* (temperature of water-bath maintained at 45°). During concentration of the solution, part of the *N*-AcGm crystallized. After the addition of 100 ml. of absolute ethanol, the mixture was heated on the steam-bath with frequent stirring to dissolve the sirup; only part of the crystalline *N*-AcGm dissolved. The mixture was filtered after standing overnight at 4° and yielded 15.5 g. of *N*-AcGm which was slightly contaminated with *N*-AcMm as determined by paper chromatography. The isolated *N*-AcGm represented 62% of the *N*-AcGm originally used. The filtrate was concentrated to dryness *in vacuo*, yielding a yellow sirup (S). Chromatography of S showed it to contain *N*-AcMm, *N*-AcGm and X. As judged by the relative intensities of the spots, the major component of S was *N*-AcMm.

The following procedure was employed to remove the remaining *N*-AcGm from the mixture: *E. coli* strain B, adapted to grow on *N*-AcGm as the sole carbon source, was grown on a synthetic medium containing the following components in g. per liter: Na_2HPO_4, 6.0; KH_2PO_4, 3.0; $MgSO_4$, 0.20; NaCl, 0.50; NH_4Cl, 1.0; *N*-AcGm, 4.0. One liter of cells was grown in a 2-l. flask on a rotary shaker for 16–20 hr. at 37° (yield 0.2 to 0.4 g., dry weight of cells per liter). The cells obtained from 1 l. of culture medium were used to remove the *N*-AcGm from the sirup S (above) obtained by alkali treatment of 25 g. of *N*-AcGm. The cells were harvested by centrifugation in the cold, washed twice with 0.15 *M* potassium chloride and finally suspended in 50 ml. of 0.15 *M* potassium chloride. The cell suspension was then added to 950 ml. of growth medium which contained S in place of the usual *N*-AcGm; the mixture was incubated in a 2-l. flask for 4 hr. at 37° on a rotary shaker, and finally treated with 150 ml. each of 0.2 *M* zinc sulfate and 0.2 *M* barium hydroxide. In order to maintain the solution below *p*H 7, the zinc sulfate was added first and the final *p*H was adjusted to 6.0 to 6.5 by addition of the barium hydroxide. The heavy precipitate was removed by filtration, washed with a little water, the combined filtrate and washings concentrated *in vacuo* to about 500 ml. (bath temperature 45°), and deionized with ion-exchange resin (Dowex-1 carbonate form and Dowex-50 hydrogen form, both 20–40 mesh). The filtrate and resin washings were concentrated *in vacuo* to a sirup which contained *N*-AcMm and X, but no detectable *N*-AcGm. This sirup was used for the isolation of *N*-AcMm and X. After dissolving the sirup in 10 ml. of 50% ethanol, the solution was heated on the steam-bath, and acetone (approximately 40 ml.) was added slowly until the solution became slightly turbid. *N*-AcMm slowly crystallized from the turbid mixture. As the *N*-AcMm crystallized, the solution cleared, at which point more acetone was added until the solution again became turbid. This procedure was repeated daily for 5 days, during which time the mixture was kept at 4°. Crystalline *N*-AcMm (3 g., m.p. 124–126°) was harvested (60% yield on the basis of the *N*-AcMm formed by the epimerization reaction). The mother liquor was concentrated to a sirup and the process repeated, yielding 0.5 g. of *N*-AcMm. The total yield was therefore 70%, although better yields (up to 80%) were obtained when the procedure was performed on a larger scale. The crystals appeared as large, heavy prisms.

After recrystallization from a water, ethanol and acetone mixture (80% yield), the *N*-AcMm exhibited a m.p. of 128–129° (uncor.). The sugar showed mutarotation. 1.0 g. of the compound was dissolved completely in water in approximately 2 min. and the volume adjusted to 10 ml. The first reading, obtained 2.6 min. after the addition of water, was $[\alpha]^{20}D$ −9.4 ± 0.1°, reaching an equilibrium value in an hour, $[\alpha]^{20}D$ +9.7 ± 0.1° (*c* 10% in water).

Elementary analysis indicates the crystalline *N*-AcMm to be the monohydrate.[12] In the results given, the first set of values was obtained with *N*-AcMm which was dried *in vacuo* at room temperature over phosphorus pentoxide for 3 days; the second set was obtained with a sample which was dried *in vacuo* at 60° over phosphorus pentoxide for 4 hr.

Anal. Calcd. for $C_8H_{15}O_6N\cdot H_2O$: C, 40.17; H, 7.11; N, 5.86; $COCH_3$, 18.0. Found: C, 40.12, 40.13; H, 7.14, 7.21; N, 5.79, 5.89; $COCH_3$,[13] 18.3, 17.5.

In the modified Morgan–Elson reaction,[11] crystalline *N*-AcMm yielded 53% of the molar absorbancy obtained with *N*-AcGm, a figure which is in good agreement with the sirup as indicated in a previous report.[3] A single spot with an $R_{N\text{-AcGm}}$ of 0.45 was obtained on chromatography of the product. Condensation of *N*-AcMm with pyruvate in the presence of NANaldolase yielded *N*-acetylneuraminic acid.[3,14]

Preparation of D-Mannosamine Hydrochloride.—*N*-AcMm was hydrolyzed and isolated as Mm,HCl by a modification of the procedure developed by Anastassiadis and Common,[15] for hydrolysis of hexosaminides; 100 ml. of Dowex-50 hydrogen form resin (200–400 mesh), 200 ml. of 0.05 *N* hydrochloric acid and 5 g. of *N*-AcMm were refluxed for 4 hr. The mixture then was poured into a chromatographic column, the filtrate collected and passed through the resin again to adsorb any free Mm,HCl from the solution. After washing the column with 500 ml. of water, the Mm,HCl was eluted from the column with 2 *N* hydrochloric acid. Portions of the eluate were tested with Benedict solution; the reducing sugar test became negative after 150 ml. of eluate had been collected. The acid eluate from the column was concentrated to dryness *in vacuo* and excess hydrochloric acid was removed by the addition and evaporation of ethanol. After the second addition of ethanol, crystalline Mm,HCl formed in the flask; yield was 4.0 g. (89%), $[\alpha]^{20}D$ −3.9 ± 0.1°. On recrystallization of the Mm,HCl from water, ethanol and acetone, $[\alpha]^{20}D$ −4.6 ± 0.1° (*c* 10% in 5% hydrochloric acid), which is in good agreement with the result reported by Levene.[6]

Anal. Calcd. for $C_6H_{14}O_5NCl$: C, 33.42; H, 6.54; N, 6.50; Cl, 16.44. Found: C, 33.37; H, 6.45; N, 6.45; Cl, 16.61.

X-Ray powder diffraction patterns of the Mm,HCl showed it to be identical with the Mm,HCl prepared by the method of Levene[6] and with the hexosamine hydrochloride obtained from *N*-acetylneuraminic acid after treatment with NANaldolase followed by acid hydrolysis.[3] The acetylacetone reagent[16] yielded a color with the crystalline material which is typical for hexosamines; the relative molar absorbancies obtained with Gm,HCl and Mm,HCl were 1.00 and 0.81, respectively.

Unidentified Components of Epimerization.—In addition to *N*-AcGm and *N*-AcMm, paper chromatography of the sirup (S above) indicated at least 3 other components which were present in small quantities and migrated more rapidly than *N*-AcGm. In this case, the chromatograms were developed using *p*-dimethylaminobenzaldehyde reagent,[10] which is more sensitive than the ultraviolet method. One of these fast moving spots (X above), which seemed to be present in greater amounts than the other two, was isolated by paper chromatography of S followed by elution of the paper. Only X was observed when the sample was immediately rechromatographed, but if X was allowed to stand at room temperature for 2 days, another of the fast moving spots was also observed. Based on the nitrogen content of the solution containing X, the following molar ratios were obtained by colorimetric analysis: N, 1.0; acetylhexosamine,[11] 0.95 (*N*-AcGm as standard); reducing sugar,[17] 1.8 (*N*-AcGm as standard); ketose,[18] 0.1 (fructose as standard). A "direct Ehrlich" color[19] was observed in the cold with 0.1 μmole of X (as determined by N content); the color disappeared upon heating at 100° for 1 min. In these analyses, no correction was made for the presence of an unknown amount of borate in the solution eluted from the paper chromatogram; the borate may have affected some of these analyses. Attempts to remove the borate by distillation of methyl borate were unsuccessful, as the solution turned dark red; X exhibited no absorption peak in the ultraviolet region.

When X was maintained at *p*H 11.0 for 24 hr., paper chromatography indicated the presence of *N*-AcGm and *N*-AcMm. The structural relationship of X to the *N*-acetylhexosamines is not yet known.

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