RbPP was isolated from the above mixture of flavins by the following procedure. The solution was diluted to 50 ml. with water and was shaken three times with four volumes of $CHCl_3$ in order to remove most of the pyridine. Removal of inorganic impurities was carried out by adsorption of the flavins on a Florisil column. The solution was acidified to *p*H 5 with acetic acid and passed through a washed column of Florisil (*ca.* 10 × 30 cm.). The column was washed with acetic acid, water and pyridine, as described previously,[16] and the flavins removed by elution with 5% pyridine. Pyridine was removed by shaking with $CHCl_3$, giving a clear yellow solution of the flavins.

The solution was concentrated to a small volume by lyophilization. Bulk separation of the flavins was achieved by applying the concentrated flavin solution to the starting line of Munktell's Cremer–Tiselius paper[6] (*ca.* 50 mg. per sheet) and developing the chromatogram in 5% Na_2HPO_4. The separate bands were located by inspection under a Mineralite, cut out, and eluted with water. Inorganic phosphate was removed from each fraction as follows. First, the solutions were stored at 0–5° for several days in order to crystallize out excess phosphate. Silver nitrate was then added in slight excess, with stirring, and the solution was adjusted with dilute acetic acid until the flavin went back into solution as a red complex. The residual silver phosphate was removed by centrifugation and the red supernatant solution passed through a short, chilled column of IR-120(H^+) cation exchange resin to remove silver. The effluent was collected in a tube immersed in a bath of acetone and Dry Ice and was thus frozen immediately. The solution was later thawed and adjusted immediately to *p*H 7 with dilute NaOH. Large-scale paper chromatography was repeated as above but employing *t*-BuOH:water (60:40) as the solvent system.[6] The pure components thus obtained, with the exception of FMN and cyc-FMN, were present in amounts too small to handle conveniently in the dry state, and the analyses and characterization were carried out using concentrated solutions. At this point, all of the flavins including RbPP behaved chromatographically as single components having the R_f values given previously. RbPP was obtained after purification in *ca.* 5% over-all yield, based upon the original NaFMN. The P_f: $P_{tot.}$: flavin ratio was 1.13:2.07:1 and 1.09:2.17:1 in duplicate analyses, and upon gentle acid hydrolysis there was quantitative conversion to FMN.

SEATTLE 5, WASHINGTON

(16) E. Dimant, D. R. Sanadi and F. M. Huennekens, THIS JOURNAL, **74**, 5440 (1952).

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WASHINGTON, AND THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF TEXAS]

Lyxoflavin Nucleotides[1,2]

BY F. M. HUENNEKENS, S. P. FELTON AND ESMOND E. SNELL

RECEIVED OCTOBER 26, 1956

L. lactis cells, grown on lyxoflavin, incorporate the vitamin into lyxoflavin mononucleotide (LMN) and dinucleotide (LAD), which can be isolated by means of chromatography on Florisil and paper columns. LMN and LAD can be distinguished from their riboflavin analogs by means of a differential microbiological assay using *L. lactis* and *L. casei*. They cannot be distinguished, however, by means of paper chromatography. In TPNH cytochrome c apo-reductase from yeast FMN is utilized preferentially to LMN, although the latter exhibits appreciable activity. In a D-amino acid apo-oxidase from pig kidney, FAD is utilized preferentially to LAD.

In 1949 Pallares and Garza[3] reported the isolation of L-lyxoflavin from human cardiac muscle. The proof of its structure, and its non-identity with D-riboflavin, rested principally upon the preparation of the tetraacetyl derivative, whose melting point agreed with that of Lx,[4] but not Rb. Synthetic Lx was first prepared by these authors, using a modification of the Karrer procedure[5] and, subsequently, by other independent routes.

The failure of other workers[6,7] to find Lx in a variety of animal tissues has cast doubt as to the natural occurrence of this substance. In addition, Gardner, *et al.*,[7] have pointed out the difficulty in distinguishing Lx and Rb on the basis of a single derivative.

In spite of the doubtful natural occurrence of Lx, other investigations have been carried out with the synthetic material. The addition of Lx to experimental rations increased the growth of rats,[8,9] chicks[9,10] and pigs.[11] Snell, *et al.*,[6] have shown that Lx does not support growth of *Lactobacillus casei* in the absence of Rb, but that low concentrations of Lx increase the growth response to limiting amounts of Rb. Under the latter conditions it was shown that Lx was not deposited in cells of *L. casei*, and that it actually decreased the concentration of Rb deposited in such cells. In contrast, the similar organism, *Lactobacillus lactis*, grew in the absence of Rb when Lx was supplied to the medium.[6,12] In this case, cells grown with Lx contain no Rb, but do contain Lx; *i.e.*, Lx apparently can fulfill the essential metabolic roles normally played by Rb. The growth requirements of these two organisms made it possible to determine Lx and Rb by means of a differential microbiological assay.[6]

Since it had been established that *L. lactis* utilized Lx directly for growth, it seemed possible that the Lx was being incorporated into the cellular flavin nucleotides. In the present study, the

(1) Paper VI in the series "Flavin Nucleotides and Flavoproteins." For paper V see G. L. Kilgour and F. M. Huennekens, THIS JOURNAL, **79**, 2256 (1956).

(2) Supported by grants from Eli Lilly and Co. and Initiative 171, State of Washington.

(3) E. S. Pallares and H. M. Garza, *Arch. Biochem.*, **22**, 63 (1949).

(4) The following abbreviations will be used: Lx, lyxoflavin; Rb, riboflavin; LMN and FMN, lyxoflavin and riboflavin mononucleotides; LAD and FAD, lyxoflavin- and riboflavin–adenine dinucleotides.

(5) P. Karrer, H. Salmon, K. Schopp, F. Benz and B. Becker, *Helv. Chim. Acta*, **18**, 910 (1935).

(6) E. E. Snell, O. A. Klatt, H. W. Bruins and W. W. Cravens, *Proc. Soc. Exp. Biol. Med.*, **82**, 583 (1953).

(7) T. S. Gardner, E. Wenis and J. Lee, *Arch. Biochem. Biophys.*, **34**, 98 (1951).

(8) G. A. Emerson and K. Folkers, THIS JOURNAL, **73**, 2398, 5383 (1951).

(9) J. M. Cooperman, W. L. Marsuick, J. Scheiner, L. Drekter, E. DeRitter and S. H. Rubin, *Proc. Soc. Exp. Biol. Med.*, **81**, 57 (1952).

(10) H. W. Bruins, M. L. Sunde, W. W. Cravens and E. E. Snell, *ibid.*, **78**, 535 (1951).

(11) R. C. Wahlstrom and B. C. Johnson, *ibid.*, **79**, 636 (1952).

(12) M. S. Shorb, *ibid.*, **79**, 611 (1952).

flavin nucleotides have been isolated from *L. lactis* cells grown on Lx, and shown to be the corresponding counterparts of FMN and FAD. Although the Lx and Rb nucleotides are similar in many chemical aspects, including migration on paper in various solvent systems, they can be distinguished by their behavior as coenzymes in enzymatic assay systems.

Experimental

Materials.—Lx was generously provided by Drs. Karl Folkers and J. M. Cooperman. FAD was prepared by the method of Dimant, *et al.*,[13] FMN was obtained from Hoffmann–LaRoche, Inc., and was recrystallized from an acidic aqueous solution. Cytochrome-c and TPN were obtained from the Sigma Chemical Company. TPNH was prepared by the enzymatic reduction of TPN using the glucose 6-phosphate dehydrogenase system.

Synthetic LMN was prepared as follows[14]: 5 mg. of Lx was added to a mixture of 0.15 ml. of $POCl_3$ and 0.01 ml. of water at room temperature. After 20 minutes the solid had dissolved to yield a dark red-brown solution. Twenty ml. of ether was then added and the resulting precipitate was recovered by centrifugation, washed twice with ether and dried *in vacuo*. The solid was dissolved in 1.5 ml. of water, neutralized with NaOH and subjected to large-scale paper chromatography, using 5% aqueous Na_2HPO_4 as the solvent system. The LMN band, which separated cleanly, was cut out, eluted and standardized spectrophotometrically in solution[15]; yield, *ca.* 25% of theory. The molal extinction coefficient, ϵ, for LMN is assumed to be the same as for FMN,[16] *i.e.*, 12.2×10^6 cm.^{2}/mole.

D-Amino acid apo-oxidase and TPNH-cytochrome-*c* apo-reductase were prepared from pig kidney and baker's yeast, respectively.[17] The growth media for both *L. casei* and *L. lactis* has been described previously by Snell, *et al.*[6]

Methods.—Absorption spectra were determined with a Beckman spectrophotometer, Model DU. Enzymatic assays[17] for FAD and FMN and the differential microbiological assay[6] for Rb and Lx have been described previously.

Using a great variety of solvent systems, or even borate-treated papers, it has not been possible to effect a satisfactory separation of Rb and Lx, or the corresponding nucleotides, by means of paper chromatography.[18]

The isolation of the flavin nucleotide fraction from *L. lactis* cells by means of chromatography on Florisil was carried out as follows: 10 g. of washed, harvested and lyophilized cells were mixed with 30 ml. of water and 4.7 ml. of 50% trichloroacetic acid and homogenized for 1 minute in a Waring blendor. The slurry was centrifuged for 5 minutes at $2300 \times g$ in the cold, and the supernatant fluid was removed by decantation. The residue was homogenized twice with 35 ml. of 6.7% trichloroacetic acid, and the supernatant fluids separated by centrifugation. The combined supernatant fluids (77 ml.) exhibited optical density values of $\log I_0/I = 0.195$ at 450 mμ and 1.095 at 260 mμ. The trichloroacetic acid extract was passed through a Florisil column (3.4 × 14.5 cm.), and, after adsorption of the flavins at the top, the column was washed, in turn, with 1 l. of 2% acetic acid, 2 l. of water and one l. of 0.5% pyridine. The flavins were then eluted with 200 ml. of 5% pyridine. After removal of the pyridine by extraction with chloroform, there remained a volume of 160 ml. which had optical density values of 0.035 at 450 mμ and 0.565 at 260 mμ. This would correspond to 5.0×10^{-4} mmole of flavin, or a recovery of about 38% of that originally placed on the column. The solution was then lyophilized to yield a light yellow powder weighing 35 mg.

Separation of the Lx nucleotides was effected by means of adsorption chromatography on a paper pulp column. Solka-floc[19] was slurried in acetone and packed into a column (1.1 × 23 cm.) under gentle air pressure. The column was washed with 0.05 *M* phosphate buffer (*p*H 8.0) until all of the acetone had been removed, and the column was completely equilibrated with the buffer. The column was taken to incipient dryness and the above lyophilized flavin material, dissolved in 1.5 ml. of water, was added to the top of the column. Separation of the flavins was achieved by developing the column with phosphate buffer. The flow rate was about 2 drops per minute, and the effluent was collected in an automatic fraction collector. The contents of each tube was diluted up to 3 ml. with water and the optical density determined at 450 mμ. The resulting column profile is shown in Fig. 1.

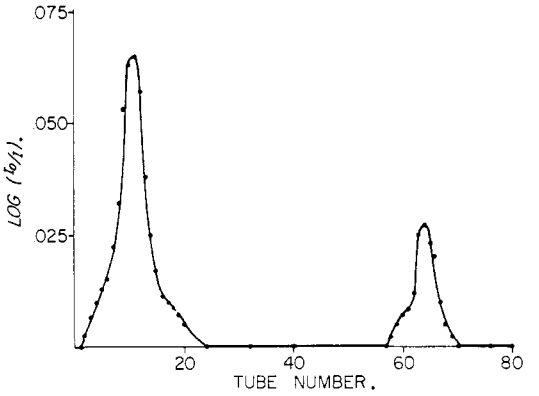

Fig. 1.—Separation of flavins on Solka-floc column. Column effluent was collected in test-tubes using a Technicon Time-Flow Automatic fraction collector, pre-set for 5 drops per tube from 1–56 and 10 drops per tube from 57–80. The contents of each tube was diluted to 3.0 ml. with water and the light absorption log (I_0/I) determined at 450 mμ.

Paper chromatography[15] revealed that tubes 8–13 contained only LMN, whereas tubes 14–19 contained both LMN and LAD. The latter group were pooled, lyophilized to dryness, and the material chromatographed on sheets of No. 1 Whatman paper using 5% $NaHPO_4$ as the solvent system. The separated bands of LMN and LAD were cut out, eluted, and the LMN fraction combined with the contents of tubes 8–13. Assayed spectrophotometrically, the amounts of lyxoflavin-containing materials were: Lx, 0.44×10^{-4} mmole; LMN, 1.21×10^{-4} mmole; and LAD, 0.14×10^{-4} mmole; total, 1.79×10^{-4} mmole; recovery, 36%. About 75% of the flavin is in the mono- or dinucleotide form, the remaining 25% being the free vitamin concentrated within the cell. In animal tissues essentially all of the flavin is distributed between the nucleotide forms.[13]

Results and Discussion

Isolation of Lx Nucleotides.—The total amount of flavin contained in cells of *L. lactis* grown on Lx may be calculated from the data obtained in the Florisil chromatography step. In the pyridine eluate fraction about 230 μg. of flavin was obtained from 10 g. of dried cells, or about 23 mg. of flavin per kg. of dried cells. For pig liver or yeast the value is about 200 mg. per kg.[12] It is probable that the amount of flavin calculated from the pyridine eluate is more accurate than that obtained on the original trichloroacetic acid extract, since the original extract may have contained substances

(13) E. Dimant, D. R. Sanadi and F. M. Huennekens, THIS JOURNAL, **74**, 5440 (1952).

(14) We are indebted to Dr. G. L. Kilgour for carrying out this synthesis.

(15) It has been shown previously that treatment of Rb with (a) $POCl_3$ (H. S. Forrest and A. R. Todd, *J. Chem. Soc.*, 3295 (1950)), or (b) monochlorophosphoric acid (L. A. Flexser and W. G. Farkas, 12th International Congress of Pure and Applied Chemistry, New York, N. Y., Sept. 1951, Abstracts p. 71) results in phosphorylation only at the 5′-position.

(16) L. G. Whitby, *Biochem. J.*, **54**, 437 (1953).

(17) F. M. Huennekens and S. P. Felton in "Methods in Enzymology," Vol. III, ed. by S. P. Colowick and N. O. Kaplan, Academic Press, New York, N. Y., 1957, p. 950.

(18) G. L. Kilgour, S. P. Felton and F. M. Huennekens, THIS JOURNAL, **79**, 2255 (1957).

(19) Solka-floc Paper Pulp, BW 200, was obtained from the Brown Co., 150 Causeway St., Boston 14, Mass.

other than flavin capable of absorbing light at 450 mμ.

Microbiological Assay of Lyxoflavin Nucleotides.—It was of interest to carry out on the isolated Lx nucleotides a differential microbiological assay similar to that which had been done previously on the total Lx-containing compounds in *L. lactis* cells.[6] Table I contains the results of the assay,

TABLE I

FLAVIN CONTENT BY SPECTROPHOTOMETRIC AND MICROBIOLOGICAL ASSAY

Compound	Spectrophotometric assay, μg./ml.	Microbiological assay, μg./ml. *L. casei* Rb Std.	*L. lactis* Rb Std.	*L. lactis* Lx Std.
Rb	19.4	18.4	18.0	
FMN	14.9	14.1	15.9	
FAD	9.5	9.6	8.0	
Lx	0.92	0.0		0.42
LMN	2.22	0.04		2.0
LAD	3.60	0.0		0.0

wherein the isolated Lx, LMN and LAD, and their corresponding Rb analogs, were standardized by means of spectrophotometric determinations at 450 mμ, and then were assayed microbiologically with *L. casei*, using Rb as a standard, and with *L. lactis*, using both Rb and Lx as standards. It can be seen that in the case of the Rb-containing compounds, the spectrophotometric determination and microbiological assay with both organisms were in good agreement. In the case of the Lx analogs, however, there was no apparent response in the *L. casei* assay. Of interest also is the fact that the Lx materials did not function equally well in the microbiological assay with *L. lactis*. Both Lx and LMN showed agreement between the microbiological and spectrophotometric assays, but LAD gave no response in the microbiological assay.

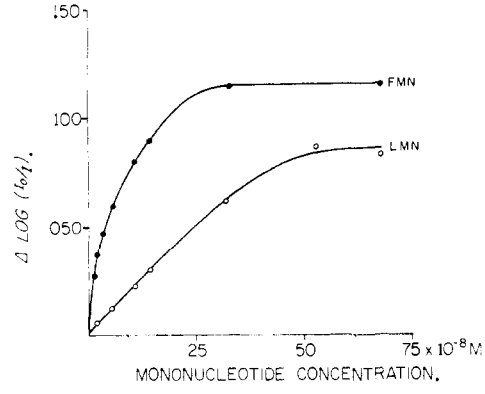

Fig. 2.—Comparison of FMN and LMN as coenzymes for TPNH-cytochrome-C reductase. The following components were added to a Corex cuvette having a light path of 1 cm.: 20 μmoles of phosphate buffer, *p*H 7.3, 0.08 μmole of cytochrome-c, 0.05 μmole of TPNH, the indicated amount of FMN (⊙) or LMN (△) and water to make 2.9 ml. The blank cell was identical except for the omission of cytochrome-c. After taking an initial reading at 550 mμ, 0.1 ml. of the apo-reductase was added to both cuvettes and the readings taken at 1-min. intervals for 6 min. Since the change in optical density was linear over the entire period, the total change, expressed as log (I_0/I) for 6 min., is plotted on the ordinate. All experimental values have been corrected for an additional blank wherein no FMN was added. This value, representing the residual, conjugated enzyme in the preparation, was about one-fourth the experimental value.

Enzymatic Activity of LMN.—The dissociated TPNH-cytochrome-*c* reductase from baker's yeast was chosen as a suitable test system for examining the relative coenzymatic activity of the two mononucleotides. As shown in Fig. 2, FMN restored full activity to the enzyme at a level of about 150 μg. per ml., and has a Michaelis constant K_m, of 5.2×10^{-8} *M*. LMN was less effective than FMN, but at high concentrations it reached a maximum activity about 75% of that shown by FMN. The K_m value for LMN was 6.4×10^{-7} *M*. Further evidence that the isolated LMN is, in fact, the 5′-phosphate derivative of Lx, was afforded by an experiment wherein a synthetic sample of LMN, prepared by the direct phosphorylation of Lx in the 5′-position, was assayed in the above enzymatic system. A K_m value of 8.8×10^{-7} *M* was obtained. The somewhat higher value for the synthetic LMN may have been due to the presence of inhibitory substances carried along from the synthetic reaction mixture.

Enzymatic Activity of LAD.—The D-amino acid oxidase of pig kidney was chosen for a comparison of FAD and LAD. Using the customary manometric assay, the effect of FAD concentration upon activity is shown in Fig. 3. The Michaelis

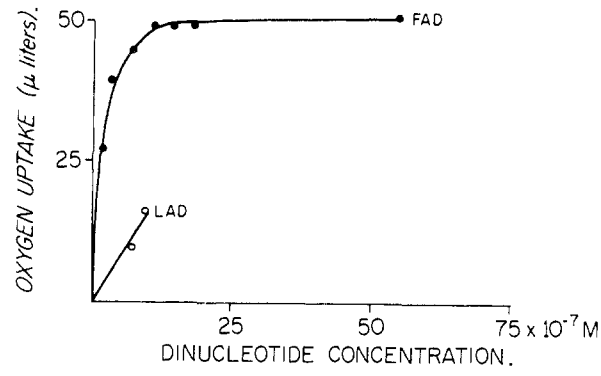

Fig. 3.—Comparison of FAD and LAD as coenzymes for D-amino acid oxidase. The manometer cups contained 25 μmoles of pyrophosphate buffer, *p*H 8.3, 100 μmoles of DL-alanine, *p*H 8.3, 0.5 ml. of D-amino acid apo-oxidase, FAD (⊙) or LAD (△) at the indicated concentration, and water to make 2.8 ml. The center well contained 0.2 ml. of 6 *N* NaOH. The blank cups were identical except for the omission of alanine. The cups were gassed with oxygen and equilibrated for 10 min. with the stopcocks open. Oxygen uptake values are expressed as μliters for the first 10-min. period after the stopcocks were closed. All values are corrected for the blanks without substrate and without added FAD. The blank was about 20% of the experimental values; bath temperature, 30°.

constant, K_m, is calculated to be 1.6×10^{-7} *M*. LAD, on the other hand, was quite inactive in this system at concentrations where FAD had saturated the enzyme, although there was a progressive increase in activity as the concentration was in-

creased. The small amount of LAD available for this study made it impossible to test the activity at higher concentrations.[20]

The results with both enzymatic assay systems give further convincing evidence of the remarkable ability of enzyme systems to distinguish between closely related substances. In this respect, it would be of interest to isolate typical flavoprotein enzymes from *L. lactis* cells, and to determine the relative efficiency of Rb and Lx nucleotides in these systems. Since Lx nucleotides satisfy the requirements of all of the flavoprotein enzymes of this organism that are essential for its growth, they may approach their Rb counterparts more closely in activity. Even in this organism Rb promotes growth at substantially lower concentrations than Lx,[6] and it is unlikely that the Lx coenzymes function as efficiently as the Rb coenzymes.

(20) The present evidence does not constitute unequivocal proof that the material in question is actually lyxoflavin–*adenine* dinucleotide. By all criteria of paper and column chromatography, the material is clearly a lyxoflavin *dinucleotide*. After acid degradation, LMN could be identified by paper chromatography, but the equally minute amount of purine mononucleotide could not be located on the paper chromatograms.

SEATTLE 5, WASHINGTON
AUSTIN, TEXAS

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, LOUISIANA STATE UNIVERSITY SCHOOL OF MEDICINE, AND THE DEPARTMENT OF CHEMISTRY, TULANE UNIVERSITY]

The Shape of Pyranoside Rings. II. The Effect of Sodium Hydroxide upon the Optical Rotation of Glycosides[1]

BY RICHARD E. REEVES AND FLORINE A. BLOUIN

RECEIVED OCTOBER 25, 1956

The optical rotation of many glycosides is essentially the same in neutral aqueous solution or in *N* sodium hydroxide solution. However, there are some glycosides which show relatively large, reversible changes in optical rotation at sodium hydroxide concentrations greater than approximately 0.01 *N*. The observation that these changes are reversible argues against their being due to hydrolysis or to degradation reactions. The possibility is considered that these alkali-sensitive substances might undergo an alteration in the fine structure (conformation) of the molecules arising as a result of a tendency of axially oriented hydroxyl groups to shift toward an equatorial orientation under strongly alkaline conditions.

In a continuing search for an understanding of the effect of alkali upon the optical rotation of amylose,[2] we have been led to a general investigation of the effect of alkali upon the rotation of simple glycosides. The literature contains few observations of an effect of alkali upon the rotation of "alkali-stable" sugar derivatives, and no survey of this effect seems to have been reported. An unusual temperature dependence of optical rotation has been noted for a class of glycosides, the acylated *o*-nitrophenyl glycosides, and this had led to the development of ideas regarding the fine structure of these substances.[3] However, no significance has hitherto been attached to the fact that some glycosides are quite alkali-sensitive while others are not.

Experimental

The glycosides employed in this work possessed melting points and specific rotations in close agreement with the best values reported in the literature.

To avoid the possibility that the different optical rotations observed for neutral and alkaline aqueous solutions might be due to ionic strength differences, we have observed optical rotations in molar sodium chloride and in normal sodium hydroxide. When a substance with different rotations in water and in alkali was encountered, solutions of equal concentration were prepared in molar saline and in normal sodium hydroxide, and the solutions were mixed in various proportions in a sealed-end polarimeter tube made with a side-arm bulb to allow for mixing. Additive volumes were assumed for these solutions. In some instances observations were made at alkali concentrations greater than normal and these solutions were separately prepared in volumetric flasks. The reversibility of the alkali-induced shifts in rotation was checked by neutralizing normal or half-normal sodium hydroxide solutions with hydrochloric acid and observing the rotations of the neutralized solutions.

The observations were made with the sodium D-line at 27.5 ± 2.5° using either a 1- or a 2-dm. polarimeter cell. Because of the fixed volume requirements of the cells and the widely different availability of pure samples of the substances employed, the measurements are not uniformly precise. However, it seems to us that the greatest uncertainty due to experimental error in the difference between specific rotation in saline and in normal sodium hydroxide should not exceed 1°.

In the preparation of a solution of methyl β-D-altropyranoside the crystalline tetraacetate was dissolved in an excess of aqueous sodium hydroxide at room temperature. Back titration with standard hydrochloric acid provided assurance that all four acetate groups had been saponified. The saline and alkaline solutions were prepared from this neutralized stock solution and contained 0.53 *M* sodium acetate in addition to the sodium chloride and sodium hydroxide.

Results

The simple glycopyranosides so far examined in saline and in normal sodium hydroxide solution have fallen into two classes: those which show differences in specific rotations amounting to less than 2.5° and those which show differences greater than 7.0°. These substances are listed in Table I together with the approximate concentrations employed, their observed rotations in molar sodium chloride solution, in normal sodium hydroxide solution and the difference, sp. rot. (NaCl) minus sp. rot. (NaOH). For the first class of substances some of the differences are within the experimental error of the observations, but others, for example that of methyl β-D-glucoside, appear to lie slightly outside the experimental error. Almost an order of magnitude greater are the differences attributed to the second class of substances, described as being alkali-sensitive. These five substances have spe-

(1) Some of the data reported herein were presented before the Division of Carbohydrate Chemistry at the 127th Meeting of the American Chemical Society, Cincinnati, Ohio, March–April, 1955.

(2) R. E. Reeves, THIS JOURNAL, **76**, 4595 (1954).

(3) W. W. Pigman, *J. Research Natl. Bur. Standards*, **33**, 129 (1944).