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Synthesis of Artificial Group A and B Substances*

By

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Regarding blood group substances from various kinds of body fluid and visceral organ, Masamune[1] one of us claimed in 1947 that certain hexosamine-galactosides or disaccharides of structures related to them act as the unit determinant group to which α- or β-agglutinin is adapted complementarily. He also announced,[2),2a)] being based on the phenomenon of interconversion in vitro of Group A and B in a group lipoid, that the foremost structural difference existing between Group A and B substances exists in which mode of union, α- or β-glycosidic, the carbohydrate moity, which constitutes the determinant group, is linked with aglycon portion in the molecule. According to him, the aglycon most probably shields the right or wrong side of the plain carbohydrate molecule or one of the cut ends of the coiled carbohydrate molecule, laying the other side or the other cut end bare to combine with the agglutinin. Our later structural study[3)4)4a)] indicated that blood group lipoids are N-glycosides of polysaccharides consisting of chondrosamine, galactose and mannose or of hexosamines and galactose, and that the aglycon is a lipid-polypeptide complex whose glutamyl residue participates with its amino group in linkage with the polysaccharide. On the other hand Yamakawa[5)] discovered, apart from our studies, remarkable group potency in "globosides"[6)] which comprise an acylsphingosine and a carbohydrate made up of 1 molecule of N-acetylchondrosamine, 3 molecules of galactose and a trace of glucose. After these considerations, it appeared to us that group active substances might be prepared only by *adequately* fixing N-glycosidically a hexosamine-galactoside to different amphipatic structures, that form large micelles, such as phosphatidylpeptide, sphingosine and proteinic materials. Hence we synthesized two series of N-glycosides of hexosamine-4-β-galactoside and of its dimer, employing firstly various phosphatidyl-

* 175th report of "Biochemical Studies on Carbohydrates"; 19th report of "Chemistry and Biology of Lipids"; 10th report of "On Proteins and Amino Acids."

peptides and secondly sphingosine and a 1-O-acylsphingosine, and found most of the former N-glycosides Group B potent and most of the latter Group A potent. We further witnessed that one of the latter group of compounds partially changed into a Group B substance on standing in physiol. saline in harmony with mutarotation from right to left, and also that one of the former neither mutarotated nor showed group alteration. This correlation of group conversion and mutarotation suggests that the carbohydrate moiety is generally bound α-glycosidically in Group A substances and β-glycosidically in Group B substances.

Experimental

Materials and Synthesis of Typical Group-active Substances

1) (N-Acetyl)-glucosamine-4-β-galactoside and (N-acetyl)-glucosamine-4-β-galactosido-2-β-(N-acetyl)-glucosaminido-4-β-galactoside[7]

Preparation procedure. Yosizawa's procedure[7a] for separating N-acetylglucosamine-4-β-galactoside was modified. Namely, 3 g. of a product at the third stage of his procedure for preparing the group mucopolysaccharide (the main part) from pig stomach mucus were subjected to acetolysis by treatment with 25 cc. of acetic anhydride and 3.5 cc. of 99.5% sulfuric acid at 15–20°C for 2 days and subsequently at 52–53°C for 12 hours. The centrifuged supernatant was poured into 120 cc. of ice-water containing 20 g. of crystalline sodium acetate under agitation, whereby a red-brown oily deposit appeared. After addition of Na_2CO_3 and $NaHCO_3$ powders to neutral, whereby the oily deposit changed thick-pasty, the solution containing the deposit was extracted with two 70 cc. portions of chloroform. The chloroform solutions were combined, washed with three 100 cc. portions of water and dried with 10 g. of $CaCl_2$. They were then distilled in vacuo (30°C) to a syrup, and after taking up in 5 cc. of dry methanol, placed in a refrigerator overnight. The precipitate was filtered off with suction and washed with dry methanol, and the filtrate and washing were distilled together under diminished pressure and dried in vacuo over H_2SO_4.

Next, the product (2.5 g.) was shaken with 113 cc. of 0.2 *N* KOH for 4 hours (15–20°C) and the dark brown solution was neutralized with dilute sulfuric acid and distilled under diminished pressure to a syrup. This was further distilled with changes of dry methanol to eliminate water as far as possible, then refluxed with 50 cc. of dry methanol for 5 minutes and filtered hot with suction. The filtrate was again condensed to a syrup, boiled with 50 cc. of ethanol and filtered similar to above. The ethanol-insoluble part on the filter was washed with three 10 cc. portions of hot ethanol and dried—Crude product of the tetrasaccharide. This material weighing 250 mg. was dissolved in 3 cc. of water and added to with 40 cc. of methanol. Some flocculent deposit was rejected by centrifugation and the supernatant was precipitated with 200 cc. of ether and dried. Followingly, it was dissolved in 100 volumes of water and passed through columns of cation exchange (Amberite IR-120) and anion ex-

change (Amberite IR-4B) resins (10 mm. ×27 cm.) three times each, followed by distillation. The still-residue was washed with an ethanol-ether mixture (2:1 by volume) and dried to a constant weight in vacuo over H_2SO_4—(N-Acetyl)-glucosamine-4-β-galactosido-2-β-(N-acetyl)-glucosaminido-4-β-galactoside. Yield 180 mg. It was a very hygroscopic, brownish powder.

On the other hand, the filtrate of the crude tetrasaccharide and washings were cooled together and about 50 cc. of ethanol were added until no more precipitate occurred. The centrifuged supernatant was distilled to a syrup, and exhausted with 50 cc. of cold abs. ethanol. The solution here was inspissated as far as possible. The non-colored powder obtained was taken up in 20 cc. of butanol, and a flocculent precipitate occurring on additional addition of butanol to 200 cc. was centrifuged and dissolved in 30 cc. of 70% ethanol. The insoluble was discarded and the solution was distilled in vacuo. The syrupy residue here was dissolved in 10 cc. of 70% ehtanol and added to with 50 cc. of abs. ethanol and 20 cc. of ether. Some deposit was removed by means of a centrifuge, and the supernatant was precipitated by the aid of 500 cc. of ether and dried. The substance given was treated with the resins, recovered and dried as above. 410 mg. of a non-colored powder were yielded—N-Acetylglucosamine-4-β-galactoside.

Properties. a) N-Acetylglucosamine-4-β-galactoside. It melted under decomposition at 174–179°C. Indirect-Osaki-Turumi and Molisch reactions positive, direct-Osaki-Turumi negative. Analysis: N (micro Kjeldahl) 3.62% (Calc. 3.65%); glucosamine (Blix) 46.6% (Calc. 46.7%); acetyl (a modification of Friedrich, Rapoport and Sternberg method) 11.2% (Calc. 11.2%); iodine use as glucose (Macleod & Robison) 48.2% (Calc. 47.0%), ash (Pregl without use of H_2SO_4) 0.67%. Rotation: $[\alpha]_D^{\infty}$ in water +65.2° (4 min. after dissolution)→+58.1°. Sugar-paper-chromatographically, it gave only one spot by either of a basic (a butanol-pyridine-water (5:3:2 by volume) mixture) and an acidic (a *n*-butylacetate-acetic acid-water (4:1:2 by volume) mixture) solvent, and its hydrolysate two spots corresponding to glucosamine and galactose by the former solvent (Fig. 1).

The chromatographic procedure of the monosaccharide components. 5 mg. of the substance dissolved in 0.5 cc. of 1 *N* HCl were heated in an ampoule at 100°C for 5 hours, and freed from HCl by repeated condensations with water in a vacuum desiccator containing solid caustic soda, without evaporating to dryness until a condensate of pH 2.0 was given. It was freed from the insoluble matter in a centrifuge, the evaporated dry residue was dissolved in 0.1 cc. of water and neutralized with NaOH and 0.02 cc. was sampled. Irrigation descending. Solvent butanol-pyridine-water (5:3:2 by volume). Temperature and duration of test 20–22°C and 24 hours respectively.

It was found inactive with respect to all of groups A, B and O (Normal goat- and anti-O saliva chicken-serum were used for Group O assay) even at a dilution of 1:50. The substance thus proved identical with the preparation of Yosizawa[7a].

b) (N-Acetyl)-glucosamine-4-β-galactosido-2-β-(N-acetyl)-glucosaminido-4-β-galactoside. It was Molisch and indirect-Osaki-Turumi positive but biuret

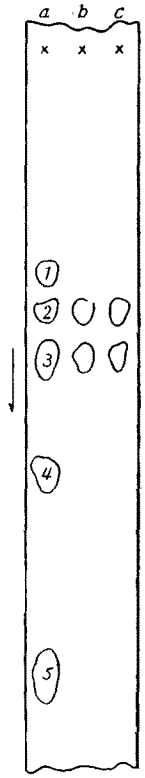

Fig. 1. Butanol-pyridine-water chromatogram of the monosaccharide components of the disaccharide and tetrasaccharide (Regarding the procedure, see the text).
a: reference run, b: the disaccharide, c: the tetrasaccharide. 1: chondrosamine hydrochloride, 2: glucosamine hydrochloride, 3: galactose, 4: mannose, 5: rhamnose.

and direct-Osaki-Turumi negative, and analyzed: N 3.80% (Calc. 3.74%); glucosamine 47.8 % (Calc. 47.9%); acetyl 11.4% (Calc. 11.5%); iodine use as glucose 23.8% (Calc. 23.8%); ash 0.73%. The rotation was measured after treatment of the substance with activated charcoal. $[\alpha]_D^5$ in water +28.9°(4 min. after dissolution)→+24.6°. Paper chromatography of a hydrolysate effected similar to above demonstrated the presence of glucosamine and galactose but no other sugar (Fig. 1). It had no group potency like the disaccharide.

2) Phosphatidyl-(α-glutamyl)-ethanolamine-N-β-(N-acetyl)-glucosaminido-4-β-galactosido-2-β-(N-acetyl)-glucosaminido-4-β-galactoside (**I**)

Phosphatidylethanolamine. The phosphatidylethanolamine employed as a material was isolated according to Folch[8] from a 3 g. portion of a batch of the cephalin fraction of brains from Group O persons. Its emulsion was dialyzed against running water for 1 week, and precipitated with a large excess of acetone. The deposit was washed with the same solvent repeatedly until it took fluffy appearance, and dried over P_2O_5 in vacuo to a nearly constant weight. Yield 230 mg. N 1.79%; P (modified Plimmer) 3.70%; Molisch negative. (Calc. for the stearyl-oleyl $C_{41}H_{80}O_8NP$: N 1.88%, P 4.16%).

N-Carbobenzoxyglutamyl-(phosphatidyl)-ethanolamine. 200 mg. of phosphatidylethanolamine above were dissolved in 5 cc. of dry chloroform[9] and a solution of 70 mg. of carbobenzoxyglutamic anhydride[10] in 2 cc. of the same solvent

was added, and the mixture was refluxed for 30 minutes, followed by standing overnight at room temperature. Then, the chloroform was evaporated off, and the remainder was dissolved in 30 cc. of dry ether. Some crystalline precipitate (carbobenzoxyglutamic acid) that occurred on standing for a short time was filtered off. The filtrate and washings were distilled in vacuo as far as possible and extracted twice with 10 cc. portions of warm acetone, and the insoluble (probable phosphatidylethanolamine which had not reacted) was rejected. The combined acetone solutions were chilled at $-10°$ and the yellowish precipitate was quickly centrifuged (30 sec. at 3000 r.p.m.), washed with chilled acetone and dried in vacuo over H_2SO_4. Yield 110 mg. Since the substance obtained here showed slight positive ninhydrin reaction,[11] it was purified by many reprecipitations from acetone, when it was almost non-colored and ninhydrin-negative. Yield 90 mg. N 2.85%; P 3.15% (Calc. for $C_{54}H_{93}O_{13}N_2P$: N 2.77%; P 3.07%).

Splitting off of N-carbobenzoxy group. The balance (70 mg.) of carbobenzoxyglutamyl-(phosphatidyl)-ethanolamine was dissolved in a mixture of 15 cc. of ethanol and 5 cc. of glacial acetic acid and hydrogenated by the aid of Pd-black of Willstätter & Waldschmidt-Leitz[12] freshly prepared. About 80 cc.[13] of hydrogen were consumed in 3 hours. After the catalyst was centrifuged off, the transparent brownish supernatant was distilled in vacuo. The still-residue was extracted with warm acetone, and a bulky precipitate occurring when the solution was chilled at 0° was centrifuged and reprecipitated. The product with yellowish hue exhibited marked ninhydrin reaction. Yield 55 mg. N 3.12% (Calc. for $C_{46}H_{87}O_{13}N_2P$ 3.07%).

Condensation with the tetrasaccharide. 40 mg. of the phosphatidyl-(glutamyl)-ethanolamine and 15 mg. of the tetrasaccharide above were taken up in a mixture of 3 cc. of absolute methanol and 0.01 cc. of glacial acetic acid and refluxed, avoiding moisture, in a glycerol bath at about 80–90° for 12 hours, whereby the tetrasaccharide went gradually into solution. Cooled. A portion still remaining undissolved was filtered off. The filtrate and washings were distilled in vacuo to about 0.5 cc. and a part of the polysaccharide, which had not reacted but was in solution, was carried down by addition of 3 cc. of chloroform and filtered off. The filtrate was condensed to a syrup by distillation under reduced pressure and completely dried over P_2O_5 in a vacuum desiccator. It was then stirred up in 3 cc. of dry ether and the opalescent solution given was cooled at 0°. The deposit here was quickly centrifuged and dried as above. Yield 20 mg. This end product was very soluble in chloroform, moderately soluble in methanol, less soluble in alcohol and benzene, and insoluble in acetone, ether (dry) and petrol ether. N 3.3% (Calc. for $C_{74}H_{134}O_{31}N_4P$ 3.48%).

3) Hydrophosphatidyl-(γ-carbomethoxy-α-aminobutyryl-glycyl-glycyl)-ethanolamine-N-β-(N-acetyl)-glucosaminido-4-β-galactosido-2-β-(N-acetyl)-glucosaminido-4-β-galactoside (**II**)

Catalytic hydrogenation of phosphatidylethanolamine. 350 mg. of phosphatidylethanolamine prepared as in 2) were dissolved in a mixture of 30 cc. of ether and 8 cc. of glacial acetic acid and shaken with hydrogen under atmospheric

pressure using 0.5 g. of active Raney nickel[14] as a catalyst, whereby turbidity appeared. 150 cc. of hydrogen were absorbed in 3.5 hours. The fluid was clarified by addition of chloroform and then the catalyst was centrifuged off. The supernatant was distilled under reduced pressure to syrupy consistency, and after precipitation with a large excess of methanol, washed twice with acetone and dried in a desiccator ($CaCl_2$). The fluffy powder obtained was soluble readily in chloroform and sparingly in ether and glacial acetic acid, but not in methanol, ethanol and acetone. Yield 320 mg.

Acylation of the amino group of hydrophosphatidylethanolamine with carbobenzoxyglycylglycine chloride. This acylation was carried out by Yamashita and Yashiro's method[15] in stead of Schotten and Baumann's, because browning of the reaction mixture happens in the alkaline medium if the latter method is applied. 5.2 g. of crude carbobenzoxyglycylglycine were prepared after Bergmann & Zervas[10] with 4.5 g. of glycinanhydride and 7.6 g. of carbobenzoxychloride. It was recrystallized from methanol and 4.8 g. of a pure product with melting point of 171° were given. 0.1 g. of it was dissolved in 2 cc. of chloroform, and warmed gently together with 0.5 cc. of purified thionylchloride[16] for 30 minutes at 60°C, disregarding slight browning. The reaction mixture was then distilled in vacuo until the offensive smell of thionylchloride disappeared (It took several hours), taken up in 5 cc. of benzene and cooled. In the next, 300 mg. of hydrophosphatidylethanolamine were dissolved in 10 cc. of glacial acetic acid containing 40 mg. of anhydrous sodium acetate, added to with the solution of carbobenzoxyglycylglycine chloride obtained above, under cooling in water, and stood at room temperature protecting from moisture. Within 1 hour NaCl crystals began to precipitate. After 24 hours the deposit was centrifuged off, and the clear supernatant was distilled at below 30°C. The still-residue was dissolved in 10 cc. of chloroform, and the solution was washed twice with water, dried with $CaCl_2$ and distilled in vacuo. To the remainder here was added hot alcohol, some insoluble matter was filtered off while hot, and the precipitate occurring on standing the solution at 0°C was separated in a centrifuge. It was reprecipitated from 10 cc. portions of alcohol and a yellowish-brownish, ninhydrin-negative powder was obtained. This was dissolved in benzene and treated with charcoal, but no perfect decolorization was achieved. Hence the solution was distilled, and the still-residue was dissolved in hot alcohol and after addition of acetone, stood at −10°C. A bulky yellowish-tinged precipitate was quickly centrifuged cold and dried. Yield 240 mg. N 4.1%, P 3.05% (Calc. for $C_{53}H_{91}O_{12}N_3P$: N 4.2%, P 3.1%).

Decarbobenzoxylation. Carbobenzoxyglycylglycyl-(hydrophosphatidyl)-ethanolamine weighing 220 mg. was dissolved in a mixture of 10 cc. of glacial acetic acid and 10 cc. of ether, and shaken with hydrogen in the presence of 0.2 g. of Pd-black of Willstätter & Waldschmidt-Leitz. 48 cc. of hydrogen were consumed in 3 hours but no more on further shaking. The centrifuged supernatant was distilled to dryness and washed with acetone. The tan powder yielded weighed 180 mg. It gave strong ninhydrin reaction.

Further acylation with carbobenzoxyglutamic anhydride. The product above was

completely dried over P_2O_5. And 150 mg. of it and 40 mg. of carbobenzoxy-glutamic anhydride were each dissolved in 5 cc. of dry chloroform, mixed together and after addition of a few drops of absolute pyridine, heated over an oven under reflux for 1 hour, followed by standing overnight at room temperature. The mixture was washed with 1 *N* HCl, 1 *N* Na_2CO_3 and water in turn, dried with $CaCl_2$ and distilled in vacuo to a sticky syrup, which became fluffy when kneaded with several changes of cold acetone. It was next taken up in a hot alcohol-acetone (1:1 by volume) mixture, the insoluble part was removed and the solution was precipitated by cooling at 0°C, spun while cold and dried in vacuo over H_2SO_4. The product was soluble in ether, ethanol and methanol, though sparingly, but not in acetone and petrol ether. Ninhydrin negative. Yield 118 mg. N 5.38%, P 3.11% (Calc. for $C_{58}H_{102}O_{15}N_4P$: N 5.45%, P 3.11%).

Methylation with diazomethane and decarbobenzoxylation. All the balance (100 mg.) of the substance above was dissolved in 100 cc. of dry ether containing diazomethane, which had been derived from 1 g. of nitrosomethylurea,[17] and a few cc. of methanol were added as a catalyst. The mixture was stood at room temperature for 2 hours (Mild evolution of nitrogen lasted for about 1 hour) and vacuum-distilled to dryness, then, the residue was taken up in 12 cc. of a mixture of chloroform, glacial acetic acid and ether (1:1:1 by volume) and hydrogenated in the presence of 0.2 g. of Pd-black. About 50 cc. of hydrogen were used in the course of 1.5 hours. The centrifuged supernatant was distilled at low temperature to dryness, washed well with abs. alcohol and placed in a vacuum-desiccator (H_2SO_4). Ninhydrin strongly positive. Yield 95 mg. N 5.60%, P 3.20% (Calc. for $C_{51}H_{98}O_{13}N_4P$[18]: N 5.56%, P 3.17%). A hydrolysate by 6 *N* HCl gave on paper chromatograms (Solvs. 80% phenol and butanol sat. w. water-phenol sat. w. water-glacial acetic acid (5:3:1)) three ninhydrin-reacting spots corresponding to glutamic acid, glycine and ethanolamine.

Condensation with the tetrasaccharide. 50 mg. of the product and 40 mg. of the tetrasaccharide were taken up in a mixture of 3 cc. of dry chloroform and 1 cc. of absolute methanol, and 0.01 cc. of glacial acetic acid was added. The reaction mixture was boiled under reflux, avoiding moisture, on a boiling water bath for 12 hours, until most of the tetrasaccharide dissolved. A small undissolved part of the carbohydrate was filtered off, and the filtrate was distilled in vacuo to perfect dryness and exhausted with two 2 cc. portions of chloroform. The chloroform solutions were concentrated together to 1 cc., shaken well with 2 cc. of dry ether and cooled at 0°C. An almost non-colored, uniformly shaped, half-crystalline deposit occurred, which was quickly centrifuged, washed once with ether and dried in a vacuum-desiccator ($CaCl_2$). Yield 35 mg. The substance was soluble easily in chloroform and methanol, less in ethanol and glacial acetic acid and hardly in benzene, but insoluble in ether, petrol ether and acetone. N 4.90%, glucosamine 20.0% (Calc. for $C_{79}H_{144}O_{33}N_6P$: N 4.83%, glucosamine 20.52%).

4) Hydrophosphatidyl-(γ-carbomethoxy-α-aminobutyryl-glycyl-glycyl)-ethanolamine-N-β-(N-acetyl)-glucosaminido-4-β-galactoside (**III**)

N-Acetylglucosamine-4-β-galactoside was replaced for the tetrasaccharide at the last stage of preparing **II**, and 23 mg. of a product aimed at were yielded from 15 mg. of the disaccharide and 35 mg. of the other component. It showed almost the same solubilities as **II**. N 5.21%, glucosamine 12.8% (Calc. for $C_{65}H_{121}O_{23}N_5P$: N 5.10%, glucosamine 13.0%).

5) α-Glutamyl-(α,α'-dipalmitylglycero-β-phosphoryl)-ethanolamine-N-β-(N-acetyl)-glucosaminido-4-β-galactoside (**IV**)

α,α'-Dipalmitylglycerophosphoryl-(N-carbobenzoxy)-ethanolamine. Rose[20] was followed essentially. Into a mixture of 3.06 g. of phosphorus oxychloride,[19] 25 cc. of dry pyridine and 25 cc. of chloroform (under vigorous agitation in a two-necked flask), 50 cc. of chloroform containing 11.4 g. of α,α'-dipalmitin[20] were poured in small portions, and after 30 minutes, 10 cc. of a chloroform solution containing 3.90 g. of N-carbobenzoxy-ethanolamine[21] were added under the conditions of Rose. The mixture was stirred up well and then after further addition of a mixture of 0.3 cc. of water and 4 cc. of pyridine and repeated agitation, freed from chloroform by distillation in vacuo. To the remaining solution were added 100 cc. of water, 50 g. of snow and 200 cc. of ether in sequence. The mixture here was stirred up for a few minutes and transferred to a separatory funnel to further shake energetically. Since the emulsion formed did not separate into layers on standing, it was acidified with HCl (Sometimes sodium chloride was added instead) and centrifuged in a large tube for 30 minutes (2000 r.p.m.). The upper ethereal layer was siphoned out, and the lower emulsion layer was shaken again with ether and centrifuged. The ethereal solutions were washed with water and after drying with anhydrous sodium sulfate, distilled to half the volume to place in a refrigerator (0°C) overnight. A crystalline deposit that occurred was filtered off. The filtrate was vacuum-distilled to dryness and extracted with three 100 cc. portions of warm methanol, whereby the most part dissolved and the sticky insoluble adhered to the bottom of the vessel. The methanolic solutions were united and cooled at 5° and the bulky deposit was filtered off with suction, which took a long time. The filtrate hereby was concentrated to 100 cc. and cooled at 0°C. Turbidity occurred but was only slight so that the fluid was cooled at −20° for 15 minutes and the appearing abundant white lustrous crystals were quickly separated by centrifugation (30 sec. at 4000 r.p.m.). Dissolution in methanol and precipitation by strong chilling was effected twice more and snow-white lustrous crystals given were dried in vacuo over $CaCl_2$. Yield 2.2 g. The substance melted at 44°, sintering at 43°. N 1.65%, P 3.50% (Calc. for $C_{45}H_{80}O_{10}NP$: N 1.70%, P 3.75%). 50.91 mg. of it were dissolved in 10 cc. of warm alcohol, and titrated with 0.05 *N* NaOH (Solv. 50% alcohol), taking phenolphthalein as an indicator, and 1.35 cc. of the NaOH solution were found used (Calc. 1.26 cc.).

Decarbobenzoxylation.[22] 1.8 g. of the product above were dissolved in 30 cc. of glacial acetic acid, and shaken for 2 hours together with hydrogen and 0.2 g. of freshly prepared Pd-black of Tausz & Putnocky[23] in a 300 cc. reduction flask. Hydrogen absorbed amounted to 110 cc. The solution, which became turbid, was clarified by addition of chloroform and filtered. The filtrate was

distilled in vacuo and the sticky residue was taken up in 50 cc. of hot acetone and cooled at 0°C. The voluminous flocculent precipitate hereby was quickly spun, reprecipitated likewise three times, and after washing with ether, dried in vacuo over P_2O_5. A fluffy almost non-colored powder given sintered at about 120° and melted at 168° and exhibited markedly positive ninhydrin reaction. It was insoluble in cold methanol, acetone and ether, slightly soluble in hot acetone and cold glacial acetic acid, and readily soluble in chloroform. Yield 1.2 g. N 2.09% (Calc. for $C_{37}H_{74}O_8NP$ 2.06%).

Acylation with carbobenzoxyglutamic anhydride. The substance obtained above was thoroughly dried in an Abderhalden apparatus (35°C), and 1.0 g. of it in 15 cc. of dry chloroform and 320 mg. of N-carbobenzoxyglutamic anhydride in 5 cc. of the chloroform and 1 cc. of dry pyridine were refluxed together for 30 minutes, and stood at room temperature for 10 hours. Then, the mixture was washed with several changes of 1 *N* HCl, with 5% sodium carbonate until it became almost neutral and with water in turn and dried with anhydrous sodium sulfate, followed by distillation at low temperature to dryness. The still-residue was stirred up with 15 cc. of an acetone-methanol (1 : 1 by volume) mixture and the centrifuged clear supernatant was cooled at −10°. A bulky flocculent precipitate that appeared (tinted brownish) was centrifuged, dissolved in acetone and after agitation with activated alumina, filtered. And the filtrate and washings were distilled together to dryness as above, taken up in hot methanol and centrifuged while warm to liberate from some insoluble. The supernatant was cooled at −10°, and a white half-crystalline precipitate was centrifuged, washed with ice-cold methanol and dried in vacuo over $CaCl_2$. Yield 0.8 g. It sintered at 45° and melted at 51°. N 2.4%, P 3.23% (Calc. for $C_{50}H_{87}O_{13}N_2P$: N 2.90%, P 3.34%). The water-soluble components after hydrolysis gave spots of glutamic acid and ethanolamine on a paper-chromatogram (Solv. 80% phenol) which analyzed equimolar.

Decarbobenzoxylation. Processed as above, 0.55 g. of a fluffy, almost non-colored, ninhydrin positive powder was given from 0.7 g. of the carbobenzoxy derivative. It exhibited birefringence in polarized light, and melted at 85°, having sintered at 80°. 2.08 cc. of 0.05 *N* alcoholic NaOH were used to neutralize 45 mg. of it in 10 cc. of warm ethanol (The quantity of the NaOH solution calculated 2.21 cc.).

Condensation with the disaccharide. 180 mg. of α,α'-dipalmitylglycerophosphoryl-(α-glutamyl)-ethanolamine obtained above and 80 mg. of the disaccharide were boiled with 6 cc. of an abs. ethanol-abs. methanol (1 : 1 by volume) mixture under a return condensor fitted with a $CaCl_2$-tubelet (A glycerol bath at 90–100° was used). Boiling was continued for 12 hours. The sugar dissolved entirely within the first 1 hour. The reaction mixture colored light yellow was stood in a refrigerator (0°) overnight. A voluminous crystalline deposit was given, which was centrifuged and washed with three 3 cc. portions of dry ether. It was next dissolved in 5 cc. of dry chloroform, and filtered and the filtrate was subjected to vacuum-distillation. The dry remainder was taken up in 3 cc. of the ethanol-methanol mixture by heating and cooled

slowly to room temperature. On scratching the vessel wall with a glass rod a half-crystalline deposit appeared. This was centrifuged and dried in vacuo over H_2SO_4. Yield 45 mg. Molisch strongly positive. N 3.30%, P 2.51%, glucosamine 15.0% (Calc. for $C_{55}H_{104}O_{21}N_3P$: N 3.58%, P 2.81%, glucosamine 15.26%).

6) γ-Glutamyl-(α,α'-dipalmitylglycerophosphoryl)-ethanolamine-N-β-(N-acetyl)-glucosaminido-4-β-galactoside (**V**)

γ-Phthalylglutamyl-(α,α'-dipalmitylglycerophosphoryl)-ethanolamine. In general, acylation with N-phthalylglutamic anhydride gives a γ-glutamyl derivative[24] contrary to the case with N-carbobenzoxyglutamic anhydride, where an α-glutamyl derivative is produced. Therefore, the synthesis was carried out as follows. 0.5 g. of α,α'-dipalmitylglycerophosphoryl-ethanolamine and 0.18 g. of phthalylglutamic anhydride[25] were dissolved together in 3 cc. of dry chloroform, and after addition of 0.2 cc. of dry pyridine, refluxed for 1 hour over an oven, with subsequent standing at room temperature for 10 hours. This mixture was then distilled in vacuo. The still-residue was taken up in dry ether by slight warming, and stood at room temperature, whereby crystals began to appear. The solution containing some deposit was further chilled in an ice-salt mixture at −10° so as to complete crystallization. The product after recrystallization from dry ether weighed 0.41 g. It sintered at 75° and melted at 91°. N 3.02%, P 3.18% (Calc. for $C_{50}H_{89}O_{13}N_2P$: N 2.92%, P 3.24%).

Cleavage of phthalyl group. 0.40 g. of the phthalylglutamylcephalin was dissolved in 12 cc. of ethylcellosolve and 0.03 cc. of 90% hydrazine hydrate was added in drops under agitation. The mixture was heated for 30 minutes under reflux in a boiling water bath, whereby it became markedly cloudy, and cooled. Thereafter it was neutralized with 1 *N* HCl, poured into 100 cc. of ice-water, and without separating off the flocculent precipitate, exhausted with changes of chloroform. The combined chloroform solutions were washed well with water, dried with $CaCl_2$ and distilled. The remainder was dissolved in the smallest possible volume of an acetone-ether (1 :1 by volume) mixture and stood overnight in an ice chest. A half-crystalline deposit given was centrifuged and dried in a desiccator ($CaCl_2$). Yield 0.35 g. It contracted at 135° and decomposed gradually on further raising of the temperature.

Condensation with N-acetylglucosamine-4-β-galactoside. In a manner similar to above 150 mg. of the γ-glutamyl-cephalin and 65 mg. of the disaccharide were condensed. The purified product, which looked like crystals and showed birefringence in polarized light, amounted to 35 mg. Molisch strongly positive. The indirect-Osaki-Turumi reaction positive, but not the direct. N 3.27%, P 2.50%, glucosamine 15.09% (Calc. for $C_{55}H_{104}O_{21}N_3P$: N 3.58%, P 2.81%, glucosamine 15.26%). It contracted with slight browning at 82–85° and decomposed at 187°.

7) Sphingosine-N-α-(N-acetyl)-glucosaminido-4-β-galactosido-2-β-(N-acetyl)-glucosaminido-4-β-galactoside (**VI**)

Sphingosine sulfate was prepared according to Klenk & Diebold[26] from human brain. It broke up under browning at 235–245°. Free sphingosine was derived from the sulfate, and 20 mg. of it and 100 mg. of the tetrasaccharide were

boiled with 2 cc. of absolute methanol under a return condensor (ground joint), avoiding moisture, in a current of dry CO_2 gas for 12 hours (glycerol bath) and then allowed to cool in air. The precipitate was centrifuged off. The supernatant was distilled in vacuo to a syrup, washed with a large excess of dry ether and dried in vacuo over $CaCl_2$. It was next taken up in 5 cc. of warm chloroform, some insoluble was eliminated by rapid filtration while warm, and the filtrate was condensed to a thick syrup as above, washed with 5 cc. of dry ether and dried. This was further purified as follows:— It was taken up in the smallest possible quantity of warm absolute methanol (1 cc.). A small insoluble part appearing on cooling was discarded after centrifugation and the supernatant was precipitated by addition of 5 cc. of dry ether and centrifuged. The centrifugate here was dissolved in 1 cc. of absolute methanol, and after cautious addition of dry ether to persistent turbidity, placed in an ice chest. Crystal-like granules came out, which were centrifuged, washed with dry ether and dried in vacuo over $CaCl_2$. Yield 42 mg. The product turned light brown at about 80°, then contracted more and more and broke up at 180–190°. It was ninhydrin negative, but became ninhydrin positive when it was warmed (50°, 5 hours) in advance with 0.2 *N* acetic acid. Fehling reaction occurred, though very slowly. Molisch test resulted in strong positive. N 4.09%, glucosamine 32.5% (Calc. for $C_{46}H_{82}O_{22}N_3$: N 4.18%, glucosamine 34.8%).

8) Sphingosine-N-*α*-(N-acetyl)-glucosaminido-4-*β*-galactoside (**VII**)

This N-glycoside was prepared similar to above by heating the components together in absolute ethanol for 5 hours. In the subsequent fractionation, cold chloroform and absolute ethanol were employed instead of hot chloroform and absolute methanol. Starting with 50 mg. of sphingosine and 80 mg. of the disaccharide, 35 mg. of the product were given. It turned brown at about 115° and decomposed at 130°. N 4.18%, glucosamine 25.5% (Calc. for $C_{32}H_{59}O_{12}N_2$: N 4.22%, glucosamine 26.9%).

9) *α*-Sphingosine (Niemann)-N-*α*-(N-acetyl)-glucosaminido-4-*β*-galactoside (**VIII**)

From 2 g. of sphingosine sulfate prepared according to Klenk & Diebold were obtained 1.4 and 0.4 g. respectively of the *α* and *β* isomers (sulfates) after Niemann[27]. Each of them was converted into free base at 0°C by the routine procedure and immediately condensed with the disaccharide by boiling with absolute ethanol for 2 hours. The condensation product with 60 mg. of *α*-sphingosine and 90 mg. of the disaccharide amounted to 30 mg. N 4.20% (Calc. 4.22%).

10) 1-O-Palmitylsphingosine-N-*α*-(N-acetyl)-glucosaminido-4-*β*-galactoside (**IX**)

N-Anisal(p-methoxybenzylidene)-sphingosine. 650 mg. of free sphingosine were dissolved in 4 cc. of ethanol and shaken well with 440 mg. of anisaldehyde. Water was added drop by drop under vigorous stirring till appearance of faint persistent turbidity (12 cc. of water were requested). The mixture was chilled at about −10° in an ice-salt mixture, scratching the vessel-wall frequently. Fine oily droplets occurred and turned soon into colorless needles. They were

filtered cold, washed with an ice-cold mixture of alcohol and water (1 : 4 by volume) and dried in vacuo over $CaCl_2$. Yield 0.725 g. The product melted at 40° with charring. N 3.18% (Calc. for $C_{26}H_{44}O_3N$ 3.33%). It became tinted orange on standing even in a vacuum-desiccator containing $CaCl_2$.

1-*O-Palmityl-(N-anisal)-sphingosine.* 700 mg. of a fresh preparation of the compound above were dissolved in a mixture of 5 cc. of dry, alcohol-free chloroform and 1 cc. of dry quinoline, and a solution of the equivalent amount (630 mg.) of palmitylchloride in 3 cc. of the chloroform were added in small portions in the course of 20 minutes, under agitation. The reaction mixture was stood for 2 hours at room temperature (5°), washed three times with watery 1 *N* HCl and four times with water and made clear by means of anhydrous calcium chloride, followed by vacuum distillation to thorough dryness. The remainder partially changed into crystals on addition of 3 cc. of dry ether. This mixture was stood at −10° in order to complete the change. The crystals with yellow hue thus given were filtered off, and after washing once with cold ether, dried in vacuo over $CaCl_2$. Yield 210 mg. The product melted at 68° under charring. N 1.95 % (Calc. for $C_{42}H_{73}O_6N$ 1.98%).

1-*O-Palmitylsphingosine hydrochloride* (*Cleavage of anisal group*). 180 mg. of 1-O-palmityl-(N-anisal)-sphingosine were suspended in 6 cc. of an acetone-ether (1 : 1 by volume) mixture and after addition of 0.15 cc. of watery 2 *N* HCl, boiled for a few minutes until a perfectly transparent solution was given. Thereafter the solution was cooled at −10°. Long non-colored prisms that precipitated were quickly centrifuged (30 sec. at 3000 r.p.m.), washed with ice-cooled ether and dried in vacuo over H_2SO_4—Cryst. I. The yellowish mother fluid of Cryst. I was distilled in vacuo to dryness, taken up in 2 cc. of ether and chilled at −10°. A deposit of crystals resembling the ones above but with yellowish tint was obtained—Cryst. II. Yield : Cryst. I 48 mg.; Cryst. II 10 mg. F.P. : Cryst. I 185° (decomp.); Cryst. II 182° (decomp.). N : Cryst. I 2.30% (Calc. for $C_{34}H_{67}O_3N{\cdot}HCl$ 2.44%).

1-*O-Palmitylsphingosine-N-α-(N-acetyl)-glucosaminido*-4-*β-galactoside.* 1-O-Palmitylsphingosine hydrochloride weighing 40 mg. was dissolved in 4 cc. of absolute ethanol, and shaken for 1 hour with a small amount of fine silver oxide powder to free the base. Filtered. The filtrate was added to with 70 mg. of the disaccharide and 0.015 cc. of glacial acetic acid and boiled under reflux (glycerol bath) for 5 hours. The condensation product was isolated as in 7) and reprecipitated from ethanol by the aid of ether, and 24 mg. of a white amorphous substance were yielded. Ninhydrin negative; positive Fehling reaction occurred but very slowly; Molisch strongly positive. Soluble in ethanol, methanol and chloroform; insoluble in ether and acetone. N 3.50%, glucosamine 21.92% (Calc. for $C_{48}H_{89}O_{13}N_2$: N 3.62%, glucosamine 22.3%).

Group Potencies of Compounds **I–IX**

Method of assay

0.4 cc. of neutral 1% solutions or emulsions of the substances in physiological saline were progressively-doubled soon after preparation with the saline

in a range of test tubes, taking 0.2 cc. from the preceding tubes, and immediately thereupon 0.2 cc. of the sera, which had been diluted to titre 16, were added. The mixtures were stood at 37°C for 2.5 hours with occasional shaking at first and afterwards at 0–5°C for 4 hours. The subsequent hemagglutination stage was processed in test tubes excepting for the compounds **VI–IX**. Namely, the mixtures were added to with 2 drops of a 5% erythrocyte suspension and placed in a water-thermostat at 37°C. Controls were carried out in parralel, by standing in the thermostat the sera, once diluted to titre 16 as above, after further progressive dilution and addition of erythrocytes. The group potencies of the specimens were judged by determining by means of a magnifier their dilutions wehre they inhibited hemagglutination, just when the sera diluted to titre 1, i. e., those at a dilution of 1 : 16 of the sera once diluted to titre 16, in the control runs began the agglutination. The corresponding stage regarding **VI–IX** was made on a glass plate, employing 1 drop of the absorbed sera and 1 drop of erythrocyte suspensions. The mixtures here were maintained at room temperature, and the results were judged under conditions similar to those above.

Results

The results of the assay are illustrated in Table I.

Relationship between Mutarotation and Group Specificity

1) On one hand, 15.0 mg. of Compound **VII** were dissolved in 0.75 cc. of absolute ethanol and filled up to 1.5 cc. by addition in portions of *M*/15 phosphate buffer pH 7.0 (Sörensen). A tiny precipitate (phosphate) was quickly centrifuged off (4000 r.p.m.), and the supernatant was examined at 15°C regarding the rotation change with time. On the other hand, a 1% solution of the compound in *M*/30 phosphate buffer pH 6.8 was prepared and stood at 15°C for a series of assays of group potency at intervals (plate method for the hemagglutination test). One and the same solvent was not used unavoidably for the both examinations, because a clear solution which allows measurement of optical rotation can not be given by using the aqueous phosphate buffer alone whereas ethanol interferes with the serological assay. The results are shown in Table II and Fig. 2. Both the dextrorotation and Group A potency decreased quickly in parallel after dissolution of the N-glycoside, the former solution becoming levorotatory and the latter Group A as well as B potent, followed by a repeated reversal of rotation due to hydrolysis of the substance (The free sugar is dextrorotatory) and diminution of both Group A and B potencies.

2) Next, 1% solutions of Compound **IV** were prepared and examined as above, replacing a mixture of pyridine and *M*/15 phosphate buffer pH 7.0 (1 : 1 by volume) for the alcohol-phosphate buffer mixture to prepare the solution for optical measurement (Table II, Fig. 3). Here neither the rotation nor the group activity changed noticeably.

The findings reveal that the Group A active synthetical compounds are α-N-glycosides and the Group B active β-N-glycosides (Cf. Tsuiki[28])[29]), and further suggest that, in natural Group A and B substances, the carbohydrate

TABLE I

Anti-hemagglutinative Potency of Synthetical Compounds

h: hemolysis. —: no agglutination, ±: faint aggl., +: slight aggl., ++: marked aggl., +++: strong aggl.

Compound	Serum	Group of red cells	Dilution of the substances (1:)								Dilution of the sera of titre 16 in control runs (1:)				
			10^2	2×10^2	4×10^2	8×10^2	1.6×10^3	3.2×10^3	6.4×10^3	1.28×10^4	2	4	8	16	32
I	α-	A	—	—	—	+	+	+	++	++	+++	++	+	±	—
	β-	B	—	—	—	—	—	—	±	+	+++	++	+	±	—
	Eel-	O	+	+	++	++	++	++	+++	+++	+++	++	++	+	—
II	α-	A	±	±	+	+	+	++	++	++	+++	++	+	±	—
	β-	B	—	—	—	—	—	—	+	++	+++	++	+	+	—
	Eel-	O	+	+	+	+	++	++	++	++	+++	++	+	+	—
	Goat-	O	±	±	+	+	++	++	++	++	+++	++	+	±	—
III	α-	A	±	±	+	+	+	+	+		+++	++	+	±	—
	β-	B	—	—	—	—	—	±	+		++	+	+	±	—
	Eel-	O	+	+	++	++	++	++	++		+++	++	+	+	±
	Goat-	O	±	±	+	++	++	++	++		+++	++	+	±	—
	Anti-O saliva chicken-	O	±	±	+	++	++	++	++		+++	++	+	±	—
IV	α-	A	—	±	+	+	+	+	+	++	++	+	+	±	—
	β-	B	—	—	—	—	—	—	+	++	++	+	+	+	—
	Eel-	O	+	++	++	++	++	++	++	++	++	+	+	+	—
	Goat-	O	±	+	+	++	++	++	++	++	++	+	+	+	—
V	α-	A	—	±	+	+	+	+	+		++	+	+	+	—
	β-	B	—	—	—	—	—	±	+		++	+	+	±	—
	Eel-	O	+	+	++	++	++	++	++		++	+	+	+	—
	Goat-	O	±	+	+	++	++	++	++		++	+	+	+	—
VI	α-	A	h—	h—	—	—	—	—	+		++	+	+	±	—
	β-	B	h—	h±	±	+	+	+	++		++	+	+	±	—
	Eel-	O	h+	h+	++	++	++	++	++		+++	++	+	+	—
	Goat-	O	h+	h+	+	+	++	++	++		+++	++	+	+	—
	Anti-O saliva chicken-	O	h±	h+	+	+	++	++	++		+++	++	+	+	—
VII	α-	A	h—	—	—	—	—	±	+		++	+	+	±	—
	β-	B	h—	±	+	+	+	+	++		++	+	±	±	—
	Eel-	O	h+	h+	++	++	++	+++	+++		+++	++	+	+	—
	Goat-	O	h±	+	+	++	++	++	++		+++	++	+	+	—
	Anti-O saliva chicken-	O	h±	h+	++	++	++	++	+++		+++	++	+	+	—
VIII	α-	A	h—	—	—	—	—	±	+		++	+	±	±	—
	β-	B	h—	±	±	+	+	+	+		++	+	+	±	—
	Goat-	O	h±	±	+	++	++	++	++		++	++	+	±	—
	Anti-O saliva chicken-	O	h±	+	++	++	++	++	++		++	++	+	±	—
IX	α-	A	h—	—	—	—	—	±	+		++	+	+	±	—
	β-	B	h±	h±	+	+	+	+	+		++	+	+	±	—
	Eel-	O	h+	h+	++	++	++	++	++		++	++	+	+	—
	Goat-	O	h±	h+	+	++	++	++	++		++	++	+	+	—

TABLE II

Changes of Group Potency and of $[\alpha]_D$ with Time of Compounds VII (Sphingosine-N-α-(N-acetyl)-glucosaminido-4-β-galactoside) and IV (α-Glutamyl-(α,α'-dipalmitylglycero-β-phosphoryl)-ethanolamine-N-β-(N-acetyl)-glucosaminido-4-β-galactoside)

h, $-\sim\pm\pm$: signify the same as in Table I.

Time of standing (hrs.)	Assay of group specificity: Serum used	Group of red cells	Dilution of the substances (1 :) 2× 10^2	2× 10^2	4× 10^2	8× 10^2	1.6 ×10^3	3.2 ×10^3	6.4 ×10^3	Dilution of the sera of titre 16 in control runs (1 :) 2	4	8	16	$[\alpha]_D$ (°)
A. Compound VII														
1/4	α	A	h−	−	−	−	−	±	++	++	+	+	±	+17.5
	β	B	h−	−	±	+	+	++	++	++	+	+	±	
1	α	A	h−	−	−	−	+	+	++	++	+	+	±	−2.0
	β	B	h−	−	−	±	+	++	++	++	+	+	±	
2	α	A	h−	−	−	+	+	+	++	++	++	+	±	−6.5
	β	B	h−	−	−	−	±	+	++	++	++	+	±	
3														−10.0
4														−10.0
5	α	A	h−	±	±	+	+	++	++	++	+	±	±	−7.5
	β	B	h−	−	±	±	+	++	++	++	+	±	±	
10														0.0
12	α	A	h±	±	+	+	++	++	++	++	+	±	±	+2.5
	β	B	h−	±	+	+	++	++	++	++	+	±	±	
15														+3.5
24														+3.0
B. Compound IV														
immediately after dissolution	α	A	±	+	+	+	++	++	++	++	+	±	±	+4.5
	β	B	−	−	−	−	−	−	+	++	+	+	±	
1	α	A	±	±	+	+	++	++	++	++	+	+	±	+4.0
	β	B	−	−	−	−	−	−	+	++	+	+	±	
2	α	A	±	±	+	+	++	++	++	++	+	+	±	+5.5
	β	B	−	−	−	−	−	−	+	++	+	±	±	
3														+5.0
5														+6.3
12	α	A	+	+	+	+	++	++	++	++	+	+	±	+6.0
	β	B	−	−	−	−	−	−	+	++	+	+	±	
24														+6.0

moiety is linked to the aglycon moiety of non-carbohydrate nature α- and β-glycosidically in general.

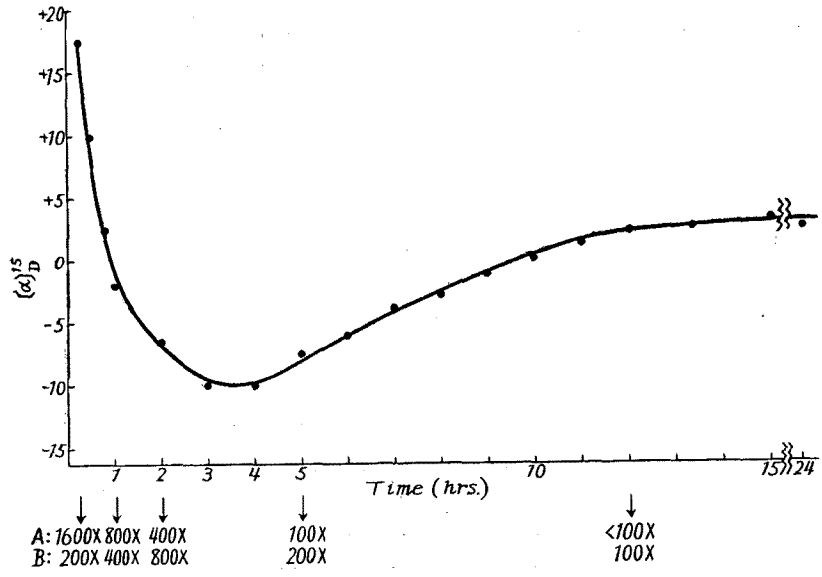

Fig. 2. Mutarotation and change of group activity with time of Compound **VII** in solution (Regarding details, see the text and Table II).

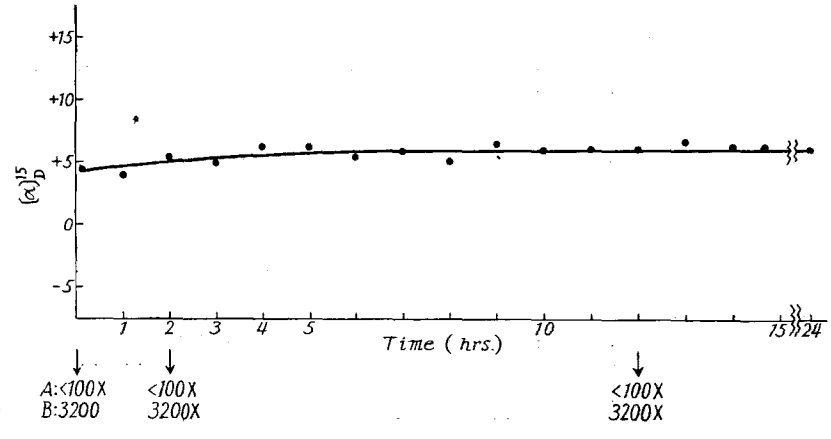

Fig. 3. Behaviours of optical rotation and group activity of Compound **IV** stood in solution (Regarding details, see the text and Table II).

Other Compounds Synthesized and Serologically Assayed

Besides the compounds above, we have synthesized not a few N-glycosides, some of which exhibited group activity though not typical. Their names will be enumerated, appending group active dilutions in parentheses with respect to the group active : phosphatidylserine-N-(N-acetyl)-glucosaminido-4-β-galactoside (A $1:10^2$, B $1:4\times10^2$), α-D-glutamyl-(α,α'-dioleylglycerophosphoryl)-ethanolamine-N-(N-acetyl)-glucosaminido-4-β-galactosido-2-β-(N-acetyl)-glucosaminido-

4-β-galactoside (A $1:8\times10^2$, B $1:3.2\times10^3$), α-D-glutamyl-(α,α'-dioleylglycerophosphoryl)-ethanolamine-N-(N-acetyl)-glucosaminido-4-β-galactoside (A $1:4\times10^2$, B $1:3.2\times10^3$), sphinogosine-N-L-fucoside (E $1:4\times10^2$), sphingosine-N-galactoside, sphingosine-N-(N-acetyl)-glucosaminide (A $1:4\times10^2$, B $1:10^2$), sphingosine-N-lactoside, β-sphingosine(Niemann)[27]-N-(N-acetyl)-glucosaminido-4-β-galactoside (A $1:4\times10^2$, B $1:2\times10^2$), dihydrosphingosine-N-(N-acetyl)-glucosaminido-4-β-galactoside (A $1:4\times10^2$, B $1:4\times10^2$, O (goat serum) $1:10^2$, O (anti-O saliva chicken-serum) $1:10^2$), mugirin(Hirohata)-N-(N-acetyl)-glucosaminido-4-β-galactoside, zein-N-(N-acetyl)-glucosaminido-4-β-galactoside, α-glutamylglutamic diethylester-N-(N-acetyl)-glucosaminido-4-β-galactoside (E $1:10^2$), glutamic diethylester-N-lactoside.

Summary

1. To verify our hypothesis regarding the configuration requisite for developing A and B group specificity, we have prepared a number of N-glycosides, of which the following proved typically group active: phosphatidyl-(α-glutamyl)-ethanolamine-N-β-(N-acetyl)-glucosaminido-4-β-galactosido-2-β-(N-acetyl)-glucosaminido-4-β-galactoside (**I**), 2) hydrophosphatidyl-(γ-carbomethoxy-α-aminobutyryl-glycyl-glycyl)-ethanolamine-N-β-(N-acetyl)-glucosaminido-4-β-galactosido-2-β-(N-acetyl)-glucosaminido-4-β-galactoside (**II**), hydrophosphatidyl-(γ-carbomethoxy-α-aminobutyryl-glycyl-glycyl)-ethanolamine-N-β-(N-acetyl)-glucosaminido-4-β-galactoside (**III**), α-glutamyl-(α,α'-dipalmitylglycero-β-phosphoryl)-ethanolamine-N-β-(N-acetyl)-glucosaminido-4-β-galactoside (**IV**), γ-glutamyl-(α,α'-dipalmitylglycero-β-phosphoryl)-ethanolamine-N-β-(N-acetyl)-glucosaminido-4-β-galactoside (**V**), sphingosine-N-α-(N-acetyl)-glucosaminido-4-β-galactosido-2-β-(N-acetyl)-glucosaminido-4-β-galactoside (**VI**), sphingosine-N-α-(N-acetyl)-glucosaminido-4-β-galactoside (**VII**), α-sphingosine (Niemann)-N-α-(N-acetyl)-glucosaminido-4-β-galactoside (**VIII**), 1-O-palmitylsphingosine-N-α-(N-acetyl)-glucosaminido-4-β-galactoside (**IX**).

2. **I–V** were Group B active at dilutions of $1:3.2\times10^3$, $1:3.2\times10^3$, $1:1.6\times10^3$, $1:3.2\times10^3$ and $1:1.6\times10^3$ respectively and **VI–IX** Group A active at dilutions of $1:3.2\times10^3$, $1:1.6\times10^3$, $1:1.6\times10^3$ and $1:1.6\times10^3$ respectively.

3. Behaviours of **VII** and **IV** examined regarding the relationship between optical rotation and group potency showed that Group A synthetical substances are α-N-glycosides and Group B substances β-N-glycosides, suggesting in turn that in natural Group A and B substances the carbohydrate moiety is bound to the non-carbohydrate moiety α- and β-glycosidically respectively in the molecule.

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8) Folch, J. Biol. Chem., 1942, **146**, 35.
9) Alcohol free. Commercial chloroform was washed with a dilute NaOH, a dilute HCl and water in turn, dried with $CaCl_2$ and further with P_2O_5 and distilled.
10) Prepared according to Bergmann & Zervas (Ber. deut. chem. Ges., 1932, **65**, 1192).
11) The ninhydrin reagent used in the present work was Stein & Moore's modified by Masamune, Hakomori & Masamune (Tohoku J. Exp. Med., 1954, **60**, 347). By this reagent, not only the purified natural phosphatidylethanolamine but also synthetical dipalmitylglycerophosphorylethanolamine (See 5) in the same chapter) exhibited strong positive reaction, although colaminecephalins have been considered inert in this respect.
12) Willstätter & Waldschmidt-Leitz, Ber. deut. chem. Ges., 1921, **54**, 123.
13) Much larger than the theoretical volume. When phosphatidylethanolamine itself was treated in a similar manner, the consumption of hydrogen reached only 1/3 of the calculated for 1 double bond.
14) Very active pyrophoric Raney Ni was prepared by digesting the Ni-Al-alloy according to Billica & Adkins (Cope & Nace, Org. Syntheses, Vol. 29, p. 24). Levene & West, Salisbury & Anderson and Gray hydrogenated cephalin and lecithin using nobel metal catalysts. However, these catalysts are often poisoned by a trace of contaminant of the phosphatides in contrast to the active Raney Ni.
15) Yamashita & Yashiro, Nippon Nogeikagaku Kaishi (J. Agr. Chem. Soc. Jap.), 1954, **28**, 674.
16) Purified according to the description of Fieser (Experiments in Organic Chemistry, 2nd ed., Reinhold Publ. Corp. New York, 1942, p. 381).
17) Kindly furnished from the Organic Chemical Laboratory of Prof. Nozoe, Faculty of Science. It was converted into diazomethane by the method described in Backmann & Struve, Organic Reaction, Vol. 1, 1935, p. 50, and Arndt, Organic Syntheses, Vol. 15, 1935, p. 4.
18) Methyl, which was attached to the phosphoryl residue when the compound was treated with diazomethane, was proved by alkaline titration to have been cleft off during decarbobenzoxylation.
19) Fresh preparation after distillation avoiding moisture.
20) Rose, J. Amer. Chem. Soc., 1947, **69**, 1384.
21) Ethanolamine hydrochloride, generously supplied by Dr. S. Takizawa in the Applied Chemistry Department, Faculty of Technology, was changed into free base by dissolving 10 g. of it in a calculated volume of 20% NaOH followed by distillation. The distillate was dropped alternatively with 15% NaOH (27 cc. in total) onto the calculated amount of carbobenzoxychloride (17 g.) during 1 hour, under vigorous stirring and ice-cooling, and the mixture was further shaken at room temperature for 30 minutes. A lot of needles which came out were

separated by filtration and recrystallized from ether. F.P. 62°.

22) Bear *et al.* (J. Amer. Chem. Soc., 1952, **14**, 154) succeeded to cleave off the carbobenzoxy group from carbobenzoxycephalin by hydrogenolysis, contrary to Rose (20)), who claimed it impossible and recommended reduction with phosphonium iodide according to Harington & Mead.

23) Tausz & Putnocky, Ber. deut. chem. Ges., 1919, **52**, 1573.

24) King, Jackson & Kidd, J. Chem. Soc., 1951, 243.

25) Kidd & King, J. Chem. Soc., 1949, 3315.

26) Klenk & Diebold, Z. physiol. Chem., 1931, **198**, 25.

27) Niemann, J. Amer. Chem. Soc., 1941, **63**, 1763. Niemann pronounced that his α- and β-sphingosine take cis and trans configurations respectively, being based on their difference in hydrogen absorption and fluorescence. Our results that a Group A substance was produced with α-sphingosine like with natural sphingosine and the scarcely group-active with β-sphingosine suggest the natural sphingosine to be the α-isomer mostly at least whether it is cis or trans form (Cf. Mislow, J. Amer. Chem. Soc., 1952, **74**, 5155).

28) Tsuiki, Tohoku J. Exp. Med., this volume, p. 267.

29) The N-glycosidic form α or β has been assigned to the synthetical compounds **I–IX** on this basis.

Note at proof-reading: This article has been read before the 26th Annual Meeting of the Japanese Biochemical Society, Sendai, April 25～27, 1954.