Investigation of the By-products of the Hydrogenation.—The mother liquor that remained after the crystallization of methyl 3-amino-3-deoxy-α-D-mannopyranoside hydrochloride (V) described above was evaporated *in vacuo*. Examination by paper chromatography of the resulting foamy or sirupy material led to the detection of ninhydrin-positive spots with the R_{gm}-values[16]: 2.23, 2.06, 1.92, (1.64), *1.52*, *1.32*, (1.0).

By use of a column (width, 5 cm.; length, 56 cm.) containing 330 g. of powdered cellulose[19] a partial separation of the mother liquor products could be achieved. Two grams of the amorphous product was developed with the Fischer–Dörfel solvent (*cf.* footnote[16]) at a flow rate of 1 drop per 5 seconds. The first 480 ml. of effluent did not contain optically active material and was discarded. Subsequently, fractions of 25 ml. were taken and examined by rotation and paper chromatography. Fractions 1–5 contained strongly dextrorotatory, ninhydrin-negative material (180 mg.).[20] Fractions 6–8 (38 mg.) and 9–14 (129 mg.), both weakly dextrorotatory, contained mixtures which gave spots having R_{gm} 2.23 + 2.06, 2.23 + 2.06 + 1.92, and 2.06 + 1.92. Fractions 15–16 (57 mg.) were mixtures of R_{gm} 2.06, 1.92, 1.64 and 1.52. So far the material was not investigated further.

From fractions 17–18 (126 mg., R_{gm} 1.52) there could be isolated 33 mg. of a crystalline aminoglycoside hydrochloride. Although it had the same R_{gm}-value as the aminomannoside IV, its rotation $[\alpha]^{25}D$ +83° (*c* 1, water), as well as its X-ray diffracton pattern and its infrared spectrum were different from those of IV.

Fractions 19–22 (469 mg., strongly dextrorotatory) were mixtures of the products of R_{gm} 1.52 and 1.32; they were discarded. In fractions 23–26 (645 mg., maximum of dextrorotation) mainly the substance of R_{gm} 1.32 was present, along with traces of that of R_{gm} 1.52. Fractions 27–36 (700 mg.) contained the R_{gm} 1.32 product only.

Aminoglycoside R_{gm} 1.32.—The combined fractions containing the amino-glycoside R_{gm} 1.32 from an analogous column afforded, upon evaporation and drying *in vacuo* (14 days at 25°), a sirupy residue (924 mg.) which still contained acetic acid and pyridine; $[\alpha]^{28}D$ +56.2° (*c* 1, water). For purification the substance was stirred with Dowex-1 (OH^-) in aqueous solution, which rapidly became alkaline. Evaporation at reduced pressure, at last under repeated addition of absolute ethanol until the pyridine smell had disappeared, afforded a colorless sirup that was dissolved in water and carefully neutralized to *p*H 5 with dilute hydrochloric acid. The solution was then brought to dryness again, thus yielding 443 mg. of a colorless foam, $[\alpha]^{27}D$ +110° (*c* 1, in water). Analysis suggested the material to be a methyl aminodeoxy-hexoside hydrochloride: Calcd.: N, 6.10; OCH_3, 13.51. Found: N, 6.03; OCH_3, 14.54. For acetylation, 400 mg. of the above aminoglycoside was refluxed for 5 minutes with 2 g. of anhydrous sodium acetate and 10 ml. of acetic anhydride. The excess anhydride was distilled off *in vacuo*, and the residue was then dissolved in 50 ml. of water. Extraction with five 50-ml. portions of chloroform, washing the combined extracts with two 25-ml. portions of saturated sodium bicarbonate solution and with 25 ml. of water, and finally drying the chloroform with anhydrous sodium sulfate afforded an amorphous residue of $[\alpha]^{27}D$ +99° (in chloroform). Crystallization took place from 95% ethanol giving diamond-shaped prisms of m.p. 181.5° and $[\alpha]^{26}D$ +111.5° (*c* 0.75, chloroform). In another experiment crystallization was first achieved from acetone–ether–pentane. The product was recrystallized from carbon tetrachloride (m.p. 177–180° after preliminary softening) and subsequently from 95% ethanol (m.p. 180–181°); $[\alpha]^{26}D$ +111° (*c* 1.2, chloroform).

Anal. Calcd. for a tetraacetate, $C_{15}H_{23}O_9N$ (361.3): C, 49.85; H, 6.42; OCH_3, 8.59. Found: C, 49.92; H, 6.23; OCH_3, 8.69.

(18) M. L. Wolfrom and Z. Yosizawa, THIS JOURNAL, **81**, 3477 (1959), report m.p. 154–156° and $[\alpha]D$ +147.5° → +94° (in water) for the L-enantiomorph.

(19) Linterspulver, Schleicher and Schüll No. 124.

(20) Weights are approximate only; they were determined after drying the evaporation residues in a vacuum desiccator for several days. There were small amounts of solvent still present, however.

[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL AND BIOLOGICAL CHEMISTRY AND THE DEPARTMENT OF CHEMISTRY OF THE PENNSYLVANIA STATE UNIVERSITY, UNIVERSITY PARK, PENNSYLVANIA]

Plant Phospholipids. II. Isolation and Structure of Glycerophosphoryl Inositol[1,2]

BY M. LEPAGE, R. MUMMA AND A. A. BENSON

RECEIVED NOVEMBER 9, 1959

The location of the phosphate diester linkage in phosphatidyl inositol of plant origin has been examined. Glycerophosphoryl inositol was isolated from deacylated corn phosphatides by column chromatography on Dowex 2-acetate. Each of the other anionic derivatives of plant lipids was separated using gradient elution technique and labeled compounds for qualitative and quantitative analysis. Proton magnetic resonance spectrometry of glycerophosphoryl inositol heptaacetate revealed acetoxy hydrogen absorption corresponding to one axial acetoxy group. These results and hydrolysis product studies indicate that the plant phosphatide is 1-phosphatidyl-*myo*-inositol.

Phosphatidyl glycerol[3] and inositol are the most actively metabolized of the plant phospholipids.[4] The rapid transfer of phosphatidyl groups and the structural singularity of phosphatidyl inositol suggests function of enzyme systems for phosphatidylation of the *myo*-inositol at a specific hydroxyl group.

Of the six possible positions for the phosphate diester linkage, four are readily subjected to experimental approach. 2- and 5-glycerophosphoryl-*myo*-inositol may be expected to possess no optical rotatory contribution from the inositol moiety. Concurrent studies by Hawthorne and Hübscher[5] and by Brockerhoff and Hanahan[6] with phosphatidyl inositol of animal origin revealed asymmetry in the inositol ester. Ballou and Pizer[7] have established by synthesis the structure of the inositol monophosphate derived from hydrolysis of soy bean phosphoinositide as L-*myo*-inositol 1-phosphate. Studies by Brown, *et al.*,[8] of hydrolysis mechanisms of synthetic *cis* and *trans* analogs

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(2) Presented at the 136th meeting of the American Chemical Society, Atlantic City, N. J., September 13–18, 1959, Abstracts of Papers, p. 28C, and is excerpted from the thesis submitted by M. L. to the Graduate School of The Pennsylvania State University in partial fulfillment of the requirements for the Master of Science degree.

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(4) R. A. Ferrari and A. A. Benson, to be published.

(5) J. N. Hawthorne and G. Hübscher, *Biochem. J.*, **71**, 195 (1959).

(6) H. Brockerhoff and D. J. Hanahan, THIS JOURNAL, **81**, 2591 (1959).

(7) C. E. Ballou and L. I. Pizer, *ibid.*, **81**, 4745 (1959).

(8) D. M. Brown, G. E. Hall and H. M. Higson, *J. Chem. Soc.*, **360** (1958).

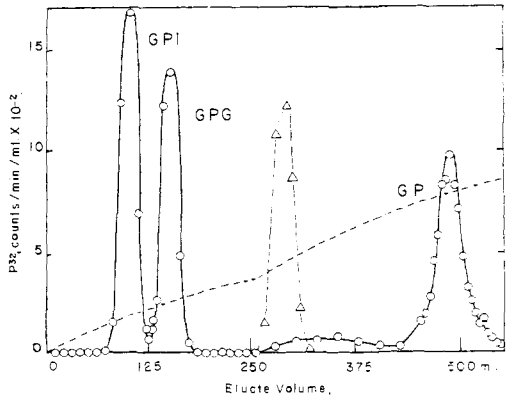

Fig. 1.—Elution of deacylated plant lipids from Dowex 2-acetate. O, P^{32} measurements; △, S^{35} measurements in relative units: – – – –, concentration of *p*H 4 eluent buffer during gradient elution.

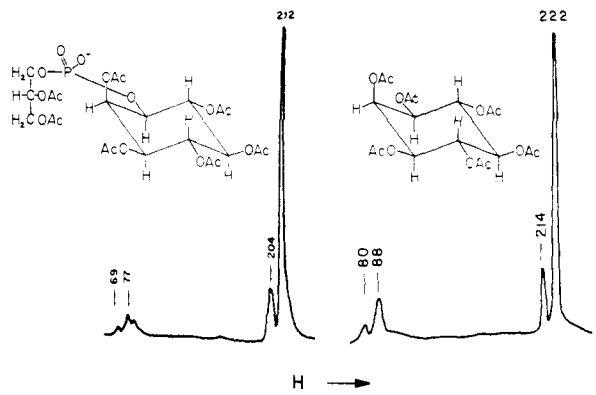

Fig. 2.—The conformations and proton magnetic resonance spectra of glycerophosphoryl *myo*-inositol heptaacetate in deuterochloroform and of *myo*-inositol hexaacetate in chloroform (Lemieux, *et al.*[10]). Frequencies of signals at 40 Mc.p.s. are given in c.p.s.

demonstrated that adjacent *cis* hydroxyl groups are requisite for rapid cleavage *via* the cyclic triester intermediate. Therefore, only phosphate diesters at positions 1, 2 or 3 should allow production of appreciable amounts of inositol phosphate upon hydrolysis.

Results and Discussion

The phosphatides of photosynthetic tissues contain 15–25% of phosphatidyl inositol. In the presence of lipids with similar physical properties, separation of this plant phosphatide is experimentally difficult.[9] The deacylated lipids, however, were readily prepared and separated. The phosphate diester, glycerophosphoryl inositol, was obtained by base-catalyzed transesterification. Anion exchange resin chromatography using methods similar to those of Hawthorne and Hübscher[5] and paper chromatography separated glycerophosphoryl inositol from the three other anionic products glycerophosphate, diglycerophosphate and glyceryl glycoside sulfonate. Elution curves from Dowex 2-acetate resin for these compounds are shown in Fig. 1. Glycerophosphoryl inositol was isolated from deacylated crude corn phosphatides by similar elution using P^{32}-labeled tracer and converted to its cyclohexylammonium salt. Its optical activity, $[\alpha]^{25}D$ $-14°$, which exceeded by several fold those of naturally occurring glycerophosphoryl esters ($[\alpha]^{20}D$ $-2.9°$) precluded location of the diester group on C-2 or C-5 of the inositol ring.

The C-2 hydroxyl, being axial, was subjected to proton spin resonance spectrometric investigation. The heptaacetate of glycerophosphoryl inositol exhibited characteristic absorptions separated by 8 cycles for hydrogen atoms of axial and equatorial acetoxy groups (Fig. 2) with integrated intensities in the ratio of 1 to 6 ± 1. These correspond almost exactly with those reported for *myo*-inositol hexaacetate by Lemieux, *et al.*,[10] and shown in Fig. 2. Thus, the axial hydroxyl group on C-2 of *myo*-inositol is not involved in the phosphate diester linkage. It is clear that glyceryl and equatorial inosityl acetoxy groups absorb in the same region. Complementary absorptions of axial and equatorial hydrogen atoms on the ring, separated by 8 c.p.s., were also observed.

Diesters at positions 4, 5 and 6, having adjacent *trans* hydroxyl groups would be expected neither to allow rapid hydrolysis of glycerophosphoryl inositol nor to yield inositol monophosphate. The axial 2-hydroxyl group, being most nearly in the direction of the equatorial 1-phosphate ester, appears most susceptible to cyclic phosphate formation and subsequent hydrolysis. The acid hydrolysis rate was observed to be comparable to that of diglycerophosphate. The ratio of inositol phosphate to glycerophosphate after hydrolysis was 1.8:1. These observations are consistent with the location of the phosphate diester on C-1 of *myo*-inositol in the plant phosphatide.

Experimental

Preparation of Glycerophosphoryl Inositol.—An aqueous solution of P^{32}-labeled glycerophosphoryl esters derived from *Chlorella*-P^{32} phosphatides[32] and decylated sulfolipid-S^{25} derived from *Scenedesmus*-S^{35} extracts were fractionated on a 12 cm. × 0.8 cm.^{2} Dowex-2 ×8 acetate (200–400) mesh column at *p*H 4. Gradient elution with eluent concentration changing from 0.05 *N* acetic acid–0.0125 *N* ammonium acetate to 0.19 *N* acetic acid–0.0478 *N* ammonium acetate at the inflection and then to 0.389 *N* acetic acid–0.10 *N* ammonium acetate (Fig. 1) separated the radioactive components. Collected fractions were assayed for radioactivity and the products identified by two-dimensional chromatography. These radioactive compounds chromatographed on Whatman No. 4 paper with the major deacylation products of crude corn phosphatides.

Larger scale separation of deacylated lipids from 20 g. of crude corn phosphatides was accomplished on a 15 cm. ×16 cm.^{2} column using the same elution conditions. The collected fractions were assayed for their P^{31} content and also for P^{32} activity of added glycerophosphoryl inositol-P^{32}. The eluted fractions containing the coinciding peaks of inorganic phosphate and P^{32} activity were decationized with Dowex-50-H^+. The solution was concentrated *in vacuo* and neutralized with cyclohexylamine. The salt was twice recrystallized from alcohol, $[\alpha]^{25}D$ $-14.0°$ in water (*c* 6, *p*H 3.5).

Anal. Calcd. for $C_{18}H_{32}O_{11}NP$: P, 7.14. Found: P, 7.17.

Glycerophosphoryl Inositol Heptaacetate.—A solution of 100 mg. of glycerophosphoryl inositol free acid in 2 ml. of pyridine was treated for 24 hours at 25° with 1 ml. of acetic anhydride. The volatile compounds were evaporated *in vacuo* and the product was crystallized from ethanol. Base-catalyzed deacylation of the heptaacetate yielded chromatographically-pure glycerophosphoryl inositol.

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(10) R. U. Lemieux, R. K. Kullnig, H. J. Bernstein and W. G. Schneider, THIS JOURNAL, **80**, 6098 (1958).

Anal. Calcd. for $C_{23}H_{33}O_{18}P$: P, 4.93. Found: P, 4.50.

Proton Magnetic Resonance Spectrum of Glycerophosphoryl Inositol Heptaacetate.—A sample of 20 mg. of the heptaacetate in 0.2 ml. of deuteriochloroform was examined in the 40 mc. Varian V-4300 n.m.r. spectrometer. Two absorptions, separated by 8 cycles, appeared (Fig. 2). Positions of the signals were measured by the side band technique using chloroform as an internal standard.

Acid Hydrolysis Products of Glycerophosphoryl Inositol.—A sample of the P^{32}-labeled diester (R_f = 0.12 in phenol–water) from *Chlorella* hydrolyzed at 100° in 0.1 *N* hydrochloric acid with a half time of approximately twenty minutes. The products were glycerophosphate, 35%, and inositol monophosphate, 65%, as determined by radioactivity measured on one-dimensional paper chromatograms developed in phenol–water solvent.

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[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, UNIVERSITY OF PITTSBURGH, SCHOOL OF MEDICINE]

Studies on Polypeptides. XIV. The Synthesis of Peptides Related to the N-Terminus of α-MSH and of the Corticotropins[1,2]

BY KLAUS HOFMANN, THOMAS A. THOMPSON, HARUAKI YAJIMA, ELEANORE T. SCHWARTZ AND (IN PART) HIROYUKI INOUYE

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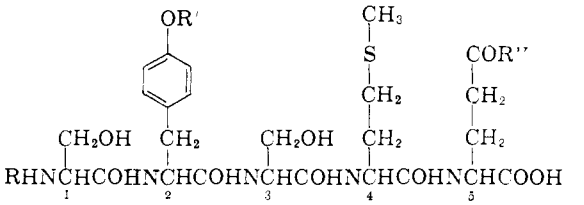
Carbobenzoxyseryltyrosylserylmethionylglutamine was prepared by treating the azide of carbobenzoxyseryltyrosine with the triethylammonium salt of serylmethionylglutamine. Optimum yields were obtained when the solid azide was added to a solution of the tripeptide salt in dimethylformamide. When the coupling was performed in the solvent system ethyl acetate–water, a rearranged product was obtained. A plausible structure for this substance is postulated Decarbobenzoxylation of the carbobenzoxypentapeptide with sodium in liquid ammonia gave seryltyrosylserylmethionylglutamine which was shown by paper chromatography and enzymatic studies to be homogeneous and of the all-L-variety. Acetylation of the pentapeptide with acetic anhydride in sodium bicarbonate solution gave a mixture of N-acetylseryltyrosylserylmethionylglutamine and of N-acetylseryl-O-acetyltyrosylserylmethionylglutamine. The position of the second acetyl group in the latter substance followed from its spectroscopic properties. The crystalline N-acetylpentapeptide was completely digestible by carboxypeptidase. Thus, acetylation was not accompanied by racemization. The preparation of the hydrazides of the carbobenzoxy-, and of the N-acetylpentapeptide is described.

The sequence corresponding to the pentapeptide seryltyrosylserylmethionylglutamic acid (I) represents the N-terminus of the corticotropins,[3–6] and its N-acetyl derivative II occupies positions 1 to 5 in the peptide chain of the melanocyte-expanding hormone α-MSH.[7]

In 1955, we reported the first synthesis of this pentapeptide and (in collaboration with W. F. White) established its identity with a peptic fragment derived from the N-terminus of corticotropin-A.[8,9]

Recently we have prepared the amides of the tridecapeptides carbobenzoxyseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycyl-N^{ϵ}-tosyllysylprolylvaline[10] and of acetylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycyl-N^{ϵ}-tosyllysylprolylvaline[11] and have recorded their ability to expand melanocytes *in vitro*. These compounds, which embody within their structures the entire amino acid sequence of α-MSH, were prepared by treating the azides of carbobenzoxyseryltyrosylserylmethionylglutamine (III) and of acetylseryltyrosylserylmethionylglutamine (IV), respectively, with histidylphenylalanylarginyltryptophylglycyl-N^{ϵ}-tosyllysylprolylvaline amide. The azide method was employed to establish the final peptide bond of these tridecapeptide derivatives, since, in our opinion, this procedure is still the method of choice for linking large peptide fragments without risk of racemization.



	R	R′	R″
I	H	H	OH
II	Ac	H	OH
III	Cbzo	H	NH_2
IV	Ac	H	NH_2
V	H	H	NH_2
VI	Ac	Ac	NH_2

Holley and Sondheimer[12] convincingly demonstrated the utility of carbobenzoxyglutamine azide as a reagent for introducing a glutamine moiety into peptides and, using their procedure, we have shown[13] that this azide reacts with simple peptide

(1) The authors wish to express their appreciation to the U. S. Public Health Service, the National Science Foundation, the National Cancer Society, Armour and Co. and Eli Lilly and Co. for generous support of this investigation.

(2) All the amino acid and peptide derivatives mentioned in this communication are of the L-configuration. In the interest of space conservation we have eliminated the customary L-designation for individual amino acid residues.

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