712. *Phospholipids. Part V.* The Hydrolysis of the Glycerol 1-Esters of Myoinositol 1- and 2-Phosphate.*

By D. M. Brown, G. E. Hall, and R. Letters.

Myoinisitol 2-phosphate and myoinositol 1-phosphate (isolated after acid rearrangement of the former) each give a glycerol diester on treatment with glycidol. The esters are readily hydrolysed by base, each giving a mixture of inositol 1(and 2)-phosphate and glycerol 1(and 2)-phosphate. The ratios of inositol 1- to 2-phosphate and of glycerol 1- to 2-phosphate are the same for each ester, showing that the corresponding cyclic 1,2-phosphates are intermediates. The ratio of total inositol phosphate to glycerol phosphate, however, differs and serves to distinguish the two esters.

When the glycerol esters are treated with periodate (1 mol.) followed by phenylhydrazine, each is converted, without significant phosphate migration, into its parent inositol phosphate. Two methods are therefore available for establishing the position of linkage of the inositol residue in the natural phosphoinositides.

There is good evidence that many of the simpler naturally occurring phosphoinositides are myoinositol esters of diacyl-L-α-glycerol phosphates.[1,2,3] In these, the major structural problem relates to the position of the phosphate–inositol linkage. There are, in theory, six possible isomers, which include two pairs of optical isomerides. The problem has been rendered difficult by the availability of only one myoinositol phosphate, *viz.*, the 2-isomer (II), as reference compound.[4] The structural problem is further complicated by the fact, not generally recognised until recently, that the inositol phosphate formed on hydrolysis of the lipid must almost certainly consist of a mixture of two or more isomers. If the hydroxyl groups on the inositol residue of a lipid did not take part in this hydrolysis, then glycerol 1- and 2-phosphate alone would be produced (cf., *inter alia*, the hydrolysis under similar conditions of phosphatidyl-choline and -ethanolamine [5]). But inositol phosphate is in general a hydrolytic product, which means that the inositol hydroxyl groups are involved and that therefore an inositol cyclic phosphate is an intermediate. Consequently the inositol phosphate formed must be assumed to be an isomeric mixture.

Our earlier experiments,[6,7] indeed, showed that a vicinal hydroxyl group in a six-membered ring, as in (I; R = $Ph{\cdot}CH_2$ or Me) was effective in the base-catalysed trans-esterification leading to the cyclic phosphate with expulsion of the R group. The hydrolysis of the *cis*- and the *trans*-compound (I; R = glycerol-1) gave both 2-hydroxycyclohexyl phosphate and glycerol phosphate, the former predominating from the *cis*-isomer, the latter from the *trans*-isomer. The kinetics of the hydrolyses showed that the factors which decided the relative importance of the two pathways were multiple, and it

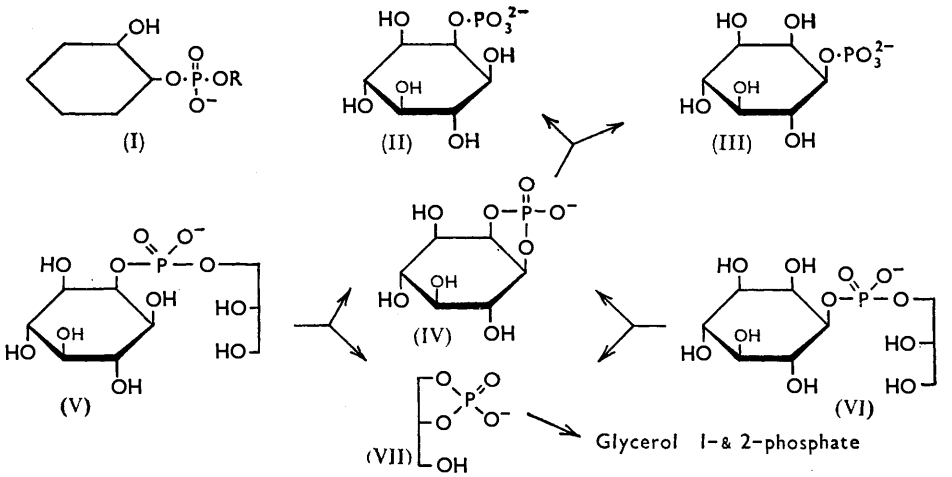
* Part IV, *J.*, 1959, 357.

[1] Hanahan and Olley, *J. Biol. Chem.*, 1958, **231**, 813.
[2] Morelec-Coulon and Faure, *Bull. Soc. Chim. biol.*, 1958, **40**, 1307.
[3] Folch and LeBaron, *Canad. J. Biochem. Physiol.*, 1956, **34**, 305.
[4] Brown and Hall, *J.*, 1959, 357.
[5] Baer and Kates, *J. Biol. Chem.*, 1948, **175**, 79; 1950, **185**, 615.
[6] Brown and Higson, *J.*, 1957, 2034.
[7] Brown, Hall, and Higson, *J.*, 1958, 1360.

was concluded that the stereochemical considerations *per se* could not be applied directly to the structural elucidation of phosphoinositides based only on product ratios.

The present experiments with the glycerol esters of myoinositol 1- and 2-phosphate, *i.e.*, (VI) and (V) respectively, show that hydrolytic evidence can be used to demonstrate, in the instances studied, the position of esterification on the inositol residue. Another method giving the same results is also described, which may be of general applicability and avoids phosphoryl migration. Since completion of the work Pizer and Ballou [8] have shown convincingly that the phosphoinositide derived from soya bean is the diacyl glycerol ester of myoinositol 1-phosphate (III) since alkaline hydrolysis yielded inositol 2-phosphate and an *optically active* inositol 1-phosphate; the intermediate cyclic 1,2-phosphate must therefore have been active, a situation which could not have arisen if the original position of esterification in the inositide had been the 2-hydroxyl group.

When the work was begun, myoinositol 2-phosphate was the only available starting material from which to prepare a glycerol ester, but another isomer was required for comparative degradation studies. Dr. C. E. Ballou informed us that acid-isomerisation of the 2-phosphate led to its mixture with the 1-phosphate (III) and that the latter could be isolated. The cyclohexylammonium salt has since been described.[8,9] but we include a simple preparation of this salt, suitable for its isolation in quantity. This salt differs from that of the 2-phosphate in infrared spectrum, degree of hydration, and paper-chromatographic behaviour. Moreover, when inositol 2-phosphate was treated with dicyclohexylcarbodi-imide in dimethylformamide it gave inositol 1,2-phosphate (IV) isolated in high yield as the crystalline ammonium salt. Posternak [10] and Pizer and Ballou [8] have recently described the corresponding barium and cyclohexylamine salts respectively, and, like these authors, we find that the cyclic phosphate is rapidly hydrolysed by base to inositol 1- and 2-phosphate.



Inositol 2-phosphate reacted under carefully controlled conditions with glycidol, best in aqueous solution at pH 5·4, to give the glycerol 1-ester (V). The product was relatively unstable but could be purified by ion-exchange chromatography and isolated as a barium salt. By similar means inositol 1-phosphate afforded the glycerol ester (VI). Experiments recorded below show that esterification had not been accompanied by appreciable phosphoryl migration and that little, if any, of the glycerol 2-esters had been formed.

When the ester (V) was treated with N-sodium hydroxide at 60°, hydrolysis was complete in 30 minutes and chromatography then showed that glycerol phosphate and

[8] Pizer and Ballou, *J. Amer. Chem. Soc.*, 1959, **81**, 915.
[9] Posternak, *Helv. Chim. Acta*, 1959, **42**, 390.
[10] *Idem, ibid.*, 1958, **41**, 1891.

inositol phosphate were formed in addition to glycerol and inositol, but that orthophosphate was absent. The phosphorylated products were resolved into glycerol 1- and 2-phosphate and inositol 1- and 2-phosphate. The same products were formed from ester (VI) on hydrolysis. The proportions of the phosphorylated products were next examined and because a relatively high degree of accuracy was required two methods of estimation were used. In the first the products were separated on chromatograms, the spots excised, and the phosphorus contents of the eluates determined. In the second method [11,12] the paper chromatograms were bombarded with neutrons, then cut into transverse strips, and the ^{32}P activities were measured. Summation of the activities under each peak allowed the proportions of the phosphorus-containing compounds to be calculated. The results (see Table 1) obtained by the two methods differ by at most $\pm 1\%$. Although there is very close agreement we feel, from our experience, that for this particular application the neutron activation method is the less accurate, but on the other hand when phosphate esters are poorly resolved on chromatograms they can be clearly distinguished by the activation technique.

Earlier work had shown that when glycerol 1,2-phosphate was hydrolysed by alkali the ratio of glycerol 1- to 2-phosphate produced was 45 : 55.[7,13] The cyclic inositol 1,2-phosphate, under the same conditions, gave the 1- and the 2-isomer in a ratio of 69 : 31. It is evident from Table 1 that these product ratios are identical with those obtained in

TABLE 1. *Alkaline hydrolysis of inositol glycerol phosphates.*

	Ratios of phosphate esters					
	Inositol 1- : inositol 2-P		Glycerol 1- : glycerol 2-P		Glycerol-P : inositol-P	
Myoinositol ester	*a*	*b*	*a*	*b*	*a*	*b*
2-(Glycerol 1-phosphate) (V)	69 : 31	70 : 30	44 : 56	45 : 55	61 : 39	60 : 40
1-(Glycerol 1-phosphate) (VI)	70 : 30	70 : 30	44 : 56	45 : 55	65 : 35	66 : 34

[a] Estimated by radioactivation analysis. [b] Estimated chemically after elution from paper chromatograms.

the hydrolyses of the glycerol esters (V) and (VI). This is clear evidence that the cyclic phosphates (IV) and (VII) are obligatory intermediates in the two competing hydrolytic pathways. It also shows, in agreement with the conclusions of Pizer and Ballou,[8] that in the hydrolysis of the 1-isomer (V) little or no attack by the *trans*-6-hydroxyl group in the inositol residue had occurred, consistently with the lack of evidence for the production of a third isomeric inositol phosphate. The esters (V) and (VI), therefore, show the expected close correspondence in their hydrolytic behaviour to the glycerol ester of *cis*-2-hydroxycyclohexyl phosphate; [7] in particular, the high rate of hydrolysis is noted, characteristic of dialkyl phosphates carrying two vicinal hydroxyl groups, but the evidence suggests that there is greater conformational rigidity in the inositol compounds since in these a *trans*-hydroxyl group does not compete effectively whereas it does in the cyclohexane-1,2-diol series. Table 1 shows that the ratio of total glycerol phosphate to total inositol phosphate in the hydrolysate from (V) is not the same as that from (VI). Thus the two esters can be distinguished by this criterion. This is not unexpected since if we consider the pathway by which inositol 1,2-phosphate is formed with expulsion of the glycerol residue, the transition state in the internal displacement [14] cannot be the same for each ester. The present evidence, therefore, strongly suggests that the position of linkage of the inositol residue in phosphoinositides based on (V) or (VI) could be established by a determination of the ratio of alkaline hydrolysis products. One proviso must, however, be made. Compound (V) is a (±)-glycerol ester, but (VI) as here prepared is presumably a mixture of the

[11] Winteringham, Bridges, and Hellyor, *Biochem. J.*, 1955, **59**, 13.
[12] Maruo and Benson, *J. Amer. Chem. Soc.*, 1957, **79**, 4564.
[13] Ukita, Nagasawa and Irie, *Pharm. Bull. (Japan)*, 1957, **5**, 127.
[14] Brown, Magrath, Neilson, and Todd, *Nature*, 1956, **177**, 1124.

(±)-glycerol esters of (±)-inositol 1-phosphate and it is conceivable that the diastereoisomerides in (VI) could give different proportions of products on hydrolysis, the observed value being the mean of these. Further study of this point is needed. The evidence further indicates that inositides based on esters (V) and (VI) could be distinguished from those based on inositol 4- or 5-phosphate (cf. ref. 8) since the latter should give a high ratio of glycerol phosphate to inositol phosphate owing to poor or ineffective competition of the *trans*-vicinal hydroxyl groups.

The value of a degradative method which would proceed with little or no phosphoryl migration is obvious. It is well known that the rate of oxidation of myoinositol by periodate is considerably less than that of glycerol. We found that glycerol 1-phosphate was almost completely oxidised by periodate (1 mol.) in 10—15 minutes whereas less than 5% of inositol 2-phosphate was degraded in the same time. It has been shown, too, that the periodate oxidation product from glycerol phosphate, glycollaldehyde phosphate, is largely broken down to orthophosphate when treated mildly at pH 6 with phenylhydrazine (1 mol.).[15] The glycerol esters (V) and (VI) were each treated with periodate, followed by phenylhydrazine, and the products were chromatographed.

Myoinositol 1-(glycerol 1-phosphate) (VI) yielded orthophosphate and inositol 1-phosphate, no evidence for any of the 2-isomer being found. The ester (V) gave largely inositol 2-phosphate together with small amounts of orthophosphate and inositol 1-phosphate. The product ratios, determined as before, are given in Table 2. They show that specific degradation of the esters can be effected with little or no phosphoryl migration, presumably through the glycollaldehyde ester (inset). The experiments, too, confirm the structures (V) and (VI); since the glycerol residue was oxidised it was evidently esterified at the 1-position and, in addition, the inositol phosphate recovered from the degradation was in each case that used in the preparation of the corresponding diester. When inositol phosphate is oxidised with more than one mol. of periodate much inorganic phosphate is formed, apparently through over-oxidation of the ultimate cleavage product $H_2PO_3{\cdot}O{\cdot}CH(CHO)_2$.[16] This could account for the small amount of inorganic phosphate produced from (V) and a larger amount from (VI) which has a *cis*-diol grouping.

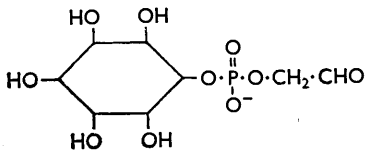


TABLE 2. *Periodate degradation of inositol glycerol phosphates.*

	Phosphates produced (%)					
	Inositol 1-P		Inositol 2-P		ortho-P	
Myoinositol ester	*a*	*b*	*a*	*b*	*a*	*b*
2-(Glycerol 1-phosphate) (V)	5	5	86·5	85	8·5	10
1-(Glycerol 1-phosphate) (VI)	68	67	—	—	32	33

[a] [b] See Table 1.

Methods are now available for converting phosphatidylinositols into the corresponding inositol glycerol phosphates,[2,17,18] so that the value of the procedures described above can now be tested. Their application to the phosphoinositide from horse liver will be reported later.

EXPERIMENTAL

Biscyclohexylammonium Myoinositol 1-Phosphate (with Mr. B. F. C. CLARK).—Myoinositol 2-phosphate (1·0 g.) was boiled under reflux in 80% acetic acid (90 ml.) for 50 min., and the solvent then removed *in vacuo*. The residue was evaporated several times with ethanol to remove remaining acetic acid. It was dissolved in water (7 ml.), cyclohexylamine (1·1 ml.)

[15] Fleury, Courtois, and Desjobert, *Bull. Soc. chim. France*, 1952, **19**, 458.
[16] Courtois and Ramet, *Bull. Soc. Chim. biol.*, 1945, **27**, 610.
[17] Dawson, *Biochim. Biophys. Acta*, 1954, **14**, 374.
[18] Hawthorne and Hübscher, *Biochem. J.*, 1959, **71**, 195.

was added, and the solution was extracted with ether (3 × 7 ml.) to remove excess of cyclohexylamine. After evaporation of the aqueous phase, the residue was dissolved in warm 92% methanol (10 ml.), and the solution filtered. Ether was added to near turbidity, and the solution set aside at room temperature for 12 hr. The crystalline product was collected and dissolved in warm methanol–water (10 : 0·5 ml.), and ether (~12 ml.) was added. After 8 hr. at 0° the product (320 mg.) was collected, washed with methanol–ether, and dried. It had m. p. 184—190° (decomp.) and was distinguished from the salt of the 2-phosphate by infrared spectroscopy and paper chromatography (Found, in material dried at 60° for 7 hr./0·1 mm. over P_2O_5: C, 46·0; H, 8·6; N, 6·0; P, 6·7. Calc. for $C_{18}H_{39}O_9N_2P,0{\cdot}5H_2O$: C, 46·25; H, 8·6; N, 6·0; P, 6·6%).

Ammonium Myoinositol 1,2-*Phosphate* (with Mr. R. Nowack).—Myoinositol 2-phosphate (0·5 g.) was suspended in dry dimethylformamide (30 ml.) in which it was partly soluble. Dicyclohexylcarbodi-imide (0·45 g.) in dimethylformamide (3 ml.) was added and the mixture shaken. After 2 days the solution was filtered from dicyclohexylurea, and the filtrate was treated with concentrated ammonia solution to pH 10. The *ammonium salt* (0·33 g., 66%) which separated was collected by centrifugation, and washed with dry ethanol. It was very hygroscopic and was chromatographically homogeneous (Found, in material dried at 50°/0·1 mm. over P_2O_5: C, 28·0; H, 6·0; N, 5·35. $C_6H_{14}O_8NP$ requires C, 27·8; H, 5·4; N, 5·4%).

On hydrolysis with N-sodium hydroxide at 60° for 30 min. it gave inositol 1- and 2-phosphate, in a ratio of 69 : 31 as estimated by phosphorus analysis or by radioactivation analysis on the chromatographically separated phosphates.

Barium Myoinositol 2-(*Glycerol* 1-*Phosphate*).—A solution of myoinositol 2-phosphate [4] (198 mg.) and glycidol (0·3 ml.) in 0·08N-potassium hydroxide (11 ml., 1·15 equiv.) was boiled for 7 hr. Paper chromatography showed that the major product was inositol glycerol phosphate (together with small amounts of orthophosphate, glycerol phosphate, and inositol). The solution was diluted to 150 ml., adjusted to pH 8 with dilute ammonia solution, and percolated (0·5 ml./min.) through a column (15 × 15 cm.) of Dowex 2 × 8 resin (200—400 mesh; formate form). The effluent was re-percolated through the same column so that all but 12% of the total phosphate was absorbed. The column was washed with water (2 × 25 ml.), then 0·1N-formic acid (250 ml.) was percolated through the column, removing only the desired product. The formic acid eluant was freeze-dried, and the residue dissolved in water (20 ml.), and the solution was neutralised with barium hydroxide and concentrated to small volume. The barium salt was precipitated by addition of ethanol (15 vol.) and, after being set aside overnight at 0°, was collected by centrifugation, washed with ethanol (3 × 10 ml.), and dried (yield, (145 mg., 48%). Several more precipitations from water by ethanol then gave the pure *barium salt*, an amorphous, hygroscopic solid, which was chromatographically pure (Found, in material dried at 40°/6 hr. *in vacuo* over P_2O_5: C, 26·1; H, 5·0; P, 7·6. $C_{18}H_{36}O_{22}P_2Ba,2H_2O$ requires C, 25·8; H, 4·8; P, 7·4%).

Barium Myoinositol 1-(*Glycerol* 1-*Phosphate*).—Myoinositol 1-phosphate was obtained from the cyclohexylamine salt (350 mg.) by treatment with Dowex-50 resin. By the same procedure as above it was converted into the hygroscopic *barium myoinositol* 1-(*glycerol* 1-*phosphate*) (87 mg.) (Found, in material dried at 40° for 6 hr. *in vacuo*: C, 26·7; H, 5·1; P, 7·24. $C_{18}H_{36}O_{22}P_2Ba,H_2O$ requires C, 26·3; H, 4·6; P, 7·6%).

Paper Chromatography.—Whatman No. 1 paper (washed with 2N-acetic acid) was used, with the solvent system propan-2-ol–water–ammonia (*d* 0·880) (7 : 2 : 1 v/v) with descending-front chromatography (2 days). All the phosphorus-containing substances were adequately resolved. The R_F values quoted here are taken relative to inositol 2-(glycerol 1-phosphate) (V) as 1·0; inositol 1-(glycerol 1-phosphate) (VI) 0·91, glycerol 1-phosphate 0·65, glycerol 2-phosphate 0·75, inositol 1-phosphate 0·36, inositol 2-phosphate 0·43, orthophosphate 0·33, and inositol 0·9.

Phosphate esters were detected by the molybdate spray,[19] 1,2-glycols by the periodate–Schiff reagent,[20] and glycerol and inositol by the silver nitrate–alkali method.[21]

Hydrolysis of Inositol Glycerol Phosphates.—The phosphate esters (*ca.* 30 mg.) were hydrolysed in N-sodium hydroxide (0·5 ml.) at 60° for 30 min., and after removal of cations by addition of Dowex-50 (H^+ form) resin the solutions were run on chromatograms (see below). Inositol 1- and 2-phosphate and glycerol 1- and 2-phosphate were identified as the hydrolysis products,

[19] Hanes and Isherwood, *Nature*, 1949, **164**, 1107.
[20] Baddiley, Buchanan, Handschumaker, and Prescott, *J.*, 1956, 2818.
[21] Anet and Reynolds, *Nature*, 1954, **174**, 930.

in addition to inositol and glycerol. The ratios of phosphorylated products were obtained by phosphorus determinations [22] on eluates of excised spots or by radioactivation analysis after neutron activation of the paper chromatogram and are recorded in Table 1.

Radioactivation Analysis.—Chromatograms of the above hydrolysates were run on paper strips 4·5 cm. wide. The strips, after drying, were neutron-activated (pile factor 1) for two days and after a further two days were cut into 1 cm. transverse strips, and the activity in each was counted. The strips were further subdivided when peaks were incompletely resolved. The counts under each peak were summed, after corrections for the resolution of the counter-system and for background; the ratios are recorded in Table 1. The counting was repeated 3—4 times at intervals of about 2 days to ensure the constancy of the ratios and to check the half-life of the β-emitter. In all cases this corresponded to that of ^{32}P.

Periodate Degradation of Inositol Glycerol Phosphates.—Barium myoinositol 2-(glycerol 1-phosphate) (50 mg.) was dissolved in water (1·5 ml.), and sodium metaperiodate (40 mg.) added. The solution was centrifuged to remove precipitated barium metaperiodate, and the supernatant solution was adjusted to pH 6 with dilute formic acid. After 1 hr. a solution of phenylhydrazine (14 mg.), neutralised to pH 6 with formic acid, was added and the mixture left at 37° for 6 hr. Cations were removed by addition of Dowex-50 resin (H^+ form), and the solution was analysed on a chromatogram in the usual way. The phosphorylated products were identified by comparison with standards and were estimated by phosphate analysis on eluates and by neutron-activation. The results, together with those obtained with myo-inositol 1-(glycerol 1-phosphate) are recorded in Table 2. Traces of starting material were sometimes observed on chromatograms of the reaction mixtures, but were ignored in the calculation of the product ratios.

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UNIVERSITY CHEMICAL LABORATORY, CAMBRIDGE. [*Received, May 4th,* 1959.]

[22] Allen, *Biochem. J.*, 1940, **34**, 858.