732. *Phospholipids. Part VII.*[1] *The Structure of a Monophosphoinositide.*

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The monophosphoinositide isolated from horse liver has been shown to be 2,3-di-*O*-acyl-D-glycerol 1-(L-myoinositol 1-phosphate). The nature of the long-chain fatty acids has not been investigated.

Papers in this series have described studies on a variety of phosphate esters with the object of extending knowledge of the chemistry and structure of phospholipids, particularly those containing inositol. Phosphoinositides are very widely distributed.[2] The lipid from horse liver was chosen for an initial application of the degradative methods previously [3] examined on model substances, as it was reported to be present in relatively high concentration (~0·2%).[4,5] A brief account of part of our work on this inositide has already been published.[6]

McKibbin [7] isolated a phosphoinositide, almost nitrogen-free, from horse liver and showed that on hydrolysis it gave myoinositol, glycerol, fatty acids, and orthophosphate in the ratio 1 : 1 : 2 : 1. It was a secondary phosphate and gave myoinositol phosphate on alkaline hydrolysis. In the present work the liver phospholipids were extracted by a modification of a method due to Folch *et al.*[8] and the pure monophosphoinositide was separated by chromatography on silicic acid with the elution procedure of Hanahan and his co-workers.[5,9] The product was a colourless, amorphous sodium salt with analytical values corresponding to those of a diacylglycerol inositol phosphate,* the structure suggested by McKibbin.[7] The substance is shown to have the structure (I; R = acyl), in which the stereochemistry is completely defined. The composition of the fatty acid mixture obtained on hydrolysis has not been studied.

Hydrolysis by acetic [11] or hydrochloric [9] acid, applied variously to the inositides from wheat germ, cardiac muscle, beef and rat liver, and yeast, leads to diglyceride. The reaction is undoubtedly dependent [12] on an acid-catalysed displacement of the diglyceride residue by the neighbouring hydroxyl group on the myoinositol. Applied to the horse-liver lipid, hydrolysis by acetic acid led to diglyceride and myoinositol phosphates; neither monoglyceride nor glycerol phosphate was detected. Thus both fatty acids are esterified to the glycerol residue. This was confirmed by treating the phospholipid with periodate: the inositol residue was destroyed and a product indistinguishable chromatographically from a synthetic phosphatidic acid [1] (II; R = acyl) was formed. This substance, without isolation, was hydrolysed by alkali which removed the fatty acids, to yield a glycerol phosphate isolated as its cyclohexylamine salt. Periodate oxidation and chromatographic comparisons showed it to be the 1-phosphate, uncontaminated by the 2-isomer. Muscle glycerophosphate-dehydrogenase has been shown [13] to be specific for the D-1

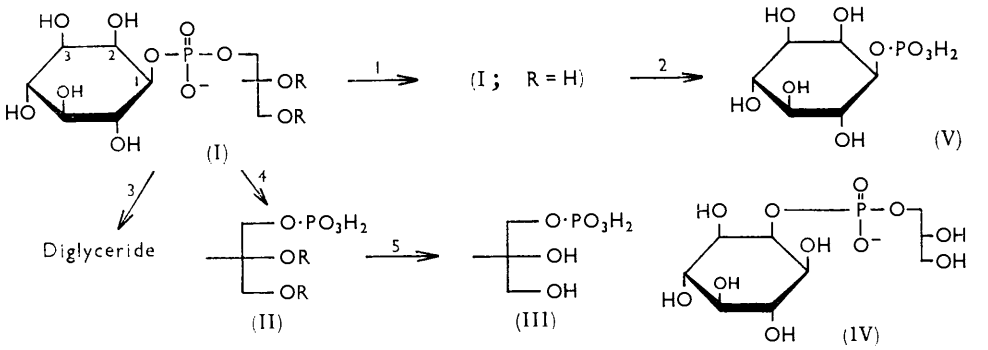
* Calculated as the distearate. Liver phosphoinositides appear to contain a high proportion of C_{18} acids.[10]

[1] Part VI, Brown and Hammond, *J.*, 1960, 4232.
[2] Hawthorne, *J. Lipid Res.*, 1960, **1**, 255.
[3] Brown, Hall, and Letters, *J.*, 1959, 3547.
[4] Bloor, *J. Biol. Chem.*, 1928, **80**, 443.
[5] Hanahan, Dittmer, and Warashina, *J. Biol. Chem.*, 1957, **228**, 685.
[6] Brown, Clark, Hall, and Letters, *Proc. Chem. Soc.*, 1960, 212.
[7] McKibbin, *J. Biol. Chem.*, 1956, **220**, 537.
[8] Folch, Lees, and Sloane-Stanley, *J. Biol. Chem.*, 1957, **226**, 497.
[9] Hanahan and Olley, *J. Biol. Chem.*, 1958, **231**, 813.
[10] Hanahan, "Lipide Chemistry," John Wiley and Sons, Inc., New York, 1960, p. 118; Gray and Macfarlane, *Biochem. J.*, 1958, **70**, 409; Macfarlane, *Biochem. J.*, 1961, **78**, 44.
[11] Faure, Coulon-Morelec, and Lecocq, *Compt. rend.*, 1959, **248**, 2252.
[12] Coulon-Morelec, Faure, and Maréchal, *Bull. Soc. Chim. biol.*, 1960, **42**, 867.
[13] Baer and Fischer, *J. Biol. Chem.*, 1940, **135**, 321; Meyerhoff and Kiessling, *Biochem. Z.*, 1933, **264**, 62; 1933, **267**, 330.

(L-α) isomer of glycerol phosphate. The isolated compound was completely oxidised by coenzyme I in presence of the dehydrogenase and is therefore D-glycerol 1-phosphate (III).*

Thus the glycerol phosphate residue in this inositide falls into the same configurational class as in the lecithins and other cephalins.[16] We are not aware of any characterisation of this feature in other monophosphoinositides, but its generality together with that of the fatty acid linkage points is rendered very probable by recent biosynthetic studies.[17]

The question of the position of linkage of phosphate to the myoinositol residue was our main concern since it was apparent that the early degradative methods involving hydrolysis could lead to ambiguous structural conclusions owing to phosphate migration.[18] This was confirmed in model experiments and a method was devised to overcome the difficulty.[3] For its application to a phosphoinositide it was necessary, first, to remove the fatty acids. Mildly alkaline conditions [19] have been used successfully with many phospholipids, but it was necessary to terminate the reaction before complete hydrolysis in order to avoid degradation of the sensitive diester. An alkaline hydroxylamine solution,[20] on the other hand, allowed complete removal of fatty acids without significant breakdown of the phosphodiester. In this way the inositide was converted into an optically active glycerol myoinositol phosphate, isolated in good yield as its crystalline cyclohexylammonium salt. On alkaline hydrolysis it yielded a chromatographically resolvable mixture of myoinositol



Reagents: 1, NH_2·OH–MeOH. 2, (*a*) IO_4^-, (*b*) NH_2·NHPh. 3, H^+. 4, IO_4^-. 5, OH^-.

1- and 2-phosphate and glycerol 1- and 2-phosphate, showing that the position of esterification of phosphate was at the 1- or the 2-position. Thus the substance was similar in its hydrolytic behaviour to the synthetic racemic diesters, glycerol 1-(myoinositol 1-phosphate) (I; R = H) and glycerol 1-(myoinositol 2-phosphate) (IV).[3] Chromatographically it was identical with the former and distinguishable from the latter. When the synthetic racemates (I; R = H) and (IV) were treated under controlled conditions with periodate followed by phenylhydrazine at pH 6, the glycerol residue was eliminated, yielding the corresponding myoinositol phosphate with little or no phosphate migration.[3] The natural

* We prefer, following Benson and Maruo,[14] to describe this enantiomorph as D-glycerol 1-phosphate, showing its direct relation to D-glyceraldehyde,[15, 16] and adhering to the normal conventions of nomenclature. The term L-α-glycerophosphate does not, of itself, define the configuration unambiguously since confusion can, and does, arise from whether α refers to the 1- or the 3-position; actually, α refers to the 3-position in the glycerophosphate series.

[14] Benson and Maruo, *Biochim. Biophys. Acta*, 1958, **27**, 189; see also Baddiley, Buchanan, and Carss, *J.*, 1957, 1869.

[15] Mills and Klyne in " Progress in Stereochemistry," ed. Klyne, Butterworths Scientific Publications, London, 1954, Vol. I, p. 191.

[16] Baer and Buchnea, *J. Amer. Chem. Soc.*, 1959, **81**, 1758.

[17] Paulus and Kennedy, *J. Biol. Chem.*, 1960, **235**, 1303; Agranoff, Bradley, and Brady, *J. Biol. Chem.*, 1958, **233**, 1077.

[18] Brown and Higson, *J.*, 1957, 2034.

[19] Dawson, *Biochim. Biophys. Acta*, 1954, **14**, 374; *Biochem. J.*, 1960, **75**, 45.

[20] Cf. Hübscher, Hawthorne, and Kemp, *J. Lipid Res.*, 1960, **1**, 433.

substance was treated likewise and gave myoinositol 1-phosphate characterised as its crystalline biscyclohexylammonium salt, $[\alpha]_D^{20}$ +2·25°; no 2-phosphate was detected. Optical-rotation measurements at several wavelengths showed it to be identical with the myoinositol phosphate isolated by Pizer and Ballou [21] from a soya-bean monophosphoinositide and characterised [22] as the L-1-enantiomorph (V). The monophosphoinositide is therefore the phosphatidylinositol, 2,3-di-*O*-acyl-D-glycerol 1-(L-myoinositol 1-phosphate) (I; R = acyl).

Thus the phosphate is linked to myoinositol in the liver compound identically with that in the soya-bean inositide since it was shown [21] that the latter on alkaline hydrolysis gave myoinositol 2-phosphate and optically active myoinositol 1-phosphate (*via* the 1,2-phosphate).

More recently the inositides from wheat germ and cardiac muscle [11,23] have been shown by a related process involving acid hydrolysis to have the same linkage to the L-1-position. Enzymic evidence shows that the 1-position is also involved in the ox-liver lipid.[24] Our degradative method appears advantageous in that the position of esterification of the phosphate to the myoinositol is uniquely determined by the myoinositol phosphate isolated, so that fractionation of isomers is unnecessary. Consequently much smaller quantities of lipid may be used. Indeed it can be carried out on a microscale, with paper-chromatographic analysis by which all four isomeric myoinositol phosphates can now be differentiated.[25] This technique may simplify the study of difficultly accessible phosphoinositides and would leave only the configuration of the myoinositol phosphate in doubt.

Since the glycerol myoinositol phosphate (I; R = H), isolated during the degradation, is structurally defined, it can now be used for purposes of comparison. The corresponding substances isolated from pig- and beef-liver [26,27] and maize [28] phosphoinositides have specific rotations closely similar to our value and it seems clear that they are all structurally identical.

The alkaline hydrolysis of glycerol myoinositol phosphate (I; R = H) gave glycerol phosphate and myoinositol phosphate in the ratio 60 : 40 in several determinations. The corresponding ratios [3] for racemic (I; R = H) and (IV) were 65 : 35 and 60 : 40 respectively. The discrepancy between the natural and the synthetic compound (I; R = H) may be due to the fact that the latter is a mixture of diastereoisomerides, and therefore not a satisfactory model, but in any event the variation in recorded values [29] indicates that this ratio cannot readily be used for structural definition.

Experimental

Paper-chromatographic Methods.—Phospholipids were chromatographed by the ascending technique normally with a running time of 12 hr. on formaldehyde-treated [30] Whatman No. 1 paper. As solvent, the upper phase of the butan-1-ol–water–acetic acid (4 : 5 : 1 v/v) solvent system mixed with peroxide-free ether (4 : 1) was used. Detection was effected by dipping in aqueous Nile Blue solution.[30] Primary amino-functions were detected by ninhydrin before the Nile Blue treatment.

Water-soluble phosphate esters were separated on acid-washed Whatman No. 1 paper by the descending technique in the propan-2-ol–water–ammonia (7 : 2 : 1 v/v) system. Development for 60 hr. was necessary to separate the isomeric glycerol myoinositol phosphates and

[21] Pizer and Ballou, *J. Amer. Chem. Soc.*, 1959, **81**, 915.
[22] Ballou and Pizer, *J. Amer. Chem. Soc.*, 1959, **81**, 4745.
[23] Coulon-Morelec and Lecocq, *Compt. rend.*, 1960, **251**, 1831.
[24] Hawthorne, Kemp, and Ellis, *Biochem. J.*, 1960, **75**, 501.
[25] Angyal, Murdoch, and Tate, *Proc. Chem. Soc.*, 1960, 416; cf. ref. 21.
[26] Hawthorne and Hübscher, *Biochem. J.*, 1959, **71**, 195.
[27] Brockerhoff and Hanahan, *J. Amer. Chem. Soc.*, 1959, **81**, 2591.
[28] Lepage, Mumma, and Benson, *J. Amer. Chem. Soc.*, 1960, **82**, 3713.
[29] Hawthorne, *Biochem. J.*, 1960, **75**, 495.
[30] Hörhammer, Wagner, and Richter, *Biochem. Z.*, 1959, **331**, 155.

myoinositol phosphates, and 24 hr. for the glycerol phosphates. Comparative R_F values of the substances studied have already been recorded.[3]

Extraction of Horse-liver Phospholipids.—The mixed phospholipid fraction was isolated by a modification of the technique due to Folch *et al.*[8] Fresh horse liver (5 kg.) was minced and extracted with chloroform–methanol (2 : 1; 10 l., 2 × 5 l.). The combined filtrates were shaken with 0·73% sodium chloride solution (0·2 vol.) and allowed to settle for 12 hr. at −15°. The organic layer was shaken with chloroform–methanol–water (3 : 48 : 47 v/v; 0·2 vol.) containing sodium chloride (0·29% w/v) and allowed to settle as before. This washing was once repeated and the lower, organic, layer then concentrated *in vacuo* at 30° to a thick yellow syrup which was triturated with 95% ethanol (2 × 3·5 l.). The solid product was collected by centrifugation and dried *in vacuo*. It was shaken with light petroleum (b. p. 40—60°; 4 × 2·5 l.) and set aside for 12 hr. at 0° and the solution after centrifugation was concentrated at room temperature to 200 ml. Acetone (2 l.) was added and after 4 hr. at −30° the yellow precipitate was collected by centrifugation and then dissolved in light petroleum (b. p. 40—60°)–chloroform (10 : 1; 200 ml.). The phospholipid fraction was precipitated by addition of acetone (10 vol.) and this reprecipitation again repeated. The product, after drying, was extracted with 95% ethanol (650 ml., then 800 ml.) for 4 hr. at room temperature and 0° respectively to remove lecithin. The solid residue was collected, dried *in vacuo*, and thereafter manipulated under nitrogen. It was dissolved in chloroform (190 ml.) and contained 1·07 g. of phosphorus (from a P analysis on an aliquot part). This solution was stored as such and chromatography showed it to contain, as the major components, phosphatidylserine, phosphatidylethanolamine, and monophosphoinositide. Paper chromatography of fractions throughout the extraction showed that only small amounts of inositide had been lost.

Isolation of Monophosphoinositide.—The monophosphoinositide was isolated as described by Hanahan *et al.*[5] by chromatography on a silicic acid (Mallinckrodt AR)–Hyflo Supercel (2 : 1 w/w; 420 g.) column (4·2 cm. diameter). A sample of the mixed phospholipids (equiv. to 230 mg. of P) in chloroform (62 ml.) was applied to the column and elution was followed by phosphorus analysis and paper chromatography of the fractions. An elution curve similar to that recorded by Hanahan *et al.* for beef liver was obtained. The chloroform–methanol (3 : 2) fractions containing inositide (equiv. to 37·5 mg. of P) were combined, concentrated, and rechromatographed [9] on a column (2·5 cm.) of silicic acid–Hyflo Supercel (37·5 g.) with a loading factor of 0·5 mg. of P per g. of silicic acid. Alumina chromatography [31] led to poor recovery of product. The colourless amorphous monophosphoinositide (10 mg. of P) obtained by precipitation from its chloroform solution by acetone (10 vol.) was chromatographically homogeneous and gave a negative reaction to the ninhydrin reagent. Microtests [32] for N and S were negative [Found, in material dried at 0·1 mm. at room temperature: P, 2·9; Na, 2·2 (flame spectrophotometry); P : Na : fatty acids,[33] 1 : 1·05 : 1·9. Calc. for $C_{45}H_{86}NaO_{13}P$: P, 3·5; Na 2·6%; P : Na : fatty acids = 1 : 1 : 2].

Cyclohexylammonium D-*Glycerol* 1-(L-*Myoinositol* 1-*Phosphate*).—The hydroxylamine reagent was prepared by mixing an equal volume of methanolic 5% hydroxylamine hydrochloride with methanolic 12·5% sodium hydroxide and removing sodium chloride by filtration. The freshly prepared reagent (4 ml.) was added to monophosphoinositide (equiv. to 10 mg. of P) dissolved in chloroform (16 ml.), and the solution was kept at 40° for 20 min. The colourless precipitate was collected by centrifugation, washed with chloroform (3 ml.), dissolved in water (10 ml.), and acidified to pH 3 by addition of Dowex-50 (H^+) resin. Further small quantities of product were removed by stirring the organic phases with water (10 ml.) and Dowex-50 (H^+) resin. The aqueous solutions were combined and shaken sucessively with portions (25 ml.) of carbon tetrachloride, light petroleum, and isobutyl alcohol. The aqueous phase was adjusted to pH 9 with dilute cyclohexylamine solution, extracted with ether (3 × 20 ml.), and concentrated *in vacuo* to 2 ml. Adding acetone (2 ml.) and keeping the mixture for 3 hr. at 0° gave a very small precipitate (myoinositol phosphate) which was removed, and the solution was concentrated to 0·5 ml. Ethanol (10 ml.) was added; the product separated and was collected after 12 hr. at −30°. This material was chromatographically homogeneous and was recrystallised from ethanol–water–acetone (yield 100 mg., 71%); it had m. p. 124—127° (decomp.), $[\alpha]_{5891}^{20}$ −13·2° (*c* 5·5 in water) (Found, in material dried at 20°/0·1 mm. for 6 hr. over

[31] Dawson, *Biochem. J.*, 1958, **68**, 352.
[32] Feigl, "Spot Tests in Organic Analysis," Elsevier, Amsterdam, 1956, p. 95.
[33] Stern and Shapiro, *J. Clin. Path.*, 1953, **6**, 158.

P_2O_5: C, 40·7; H, 7·5; P, 7·05. Calc. for $C_{15}H_{32}NO_{11}P,0{\cdot}5H_2O$: C, 40·7; H, 7·5; P, 7·0%), $\nu_{max.}$ (Nujol mull) 3400b, 3270b, 2920, 1700sh, 1640, 1543b, 1453w, 1391w, 1370w, 1197s, 1123s, 1080sh, 1055s, 1021sh, 1003s, 936, 883b, 845sh, 819w cm.$^{-1}$.

The organic solutions and extracts (above) were shown by paper chromatography to contain fatty acids and their hydroxamates but no unchanged monophosphoinositide.

Hydrolysis of Above Cyclohexylammonium Salt.—The salt was purified by preparative, descending chromatography in order to remove possible, although undetected, traces of glycerol phosphate or myoinositol phosphate. It was heated at 60° for 30 min. in N-sodium hydroxide, which was then acidified by addition of Dowex-50 (H^+) resin, and the solution was transferred to chromatograms. The sole products were glycerol 1- and 2-phosphate and myoinositol 1- and 2-phosphate. The ratio glycerol : myoinositol phosphate was 60 : 40 (chemical analysis; 2 expts.) and 61 : 39, 58 : 42 (radioactivation analysis).[3]

Isolation of L-*Myoinositol* 1-*Phosphate.*—The above cyclohexylammonium salt (80 mg.) and sodium metaperiodate (47·5 mg., 1·2 mol.) were dissolved in water (3 ml.) and set aside for 1 hr. at room temperature and then glycerol (0·02 ml.) was added. After 15 min. a solution (1·7 ml.) of phenylhydrazine (28 mg., 1·4 mol.) adjusted to pH 6 with formic acid was added and the solution was kept at 40° for 6 hr. The solution was filtered from a brown precipitate and the filtrate and washings were acidified with Dowex-50 (H^+) resin. After filtration, the solution was brought to pH 9 with aqueous cyclohexylamine and, after extraction with ether (3 × 5 ml.) and concentration to 1 ml., acetone was added to turbidity. The solution was set aside for 12 hr. at 0° and the product collected. The biscyclohexylammonium salt was recrystallised from ethanol–water–acetone (yield 25 mg.). A further quantity of pure material (15 mg.) was obtained by working up mother-liquors by paper chromatography on Whatman 3 MM paper (total yield 40 mg., 48%). It had m. p. 180—195° (decomp.) (Found, in material dried at 20°/0·1 mm. for 12 hr. over P_2O_5: C, 45·4; H, 8·3; P, 6·75. Calc. for $C_{18}H_{39}N_2O_9P,1H_2O$: C, 45·4; H, 8·6; P, 6·5%). Chromatographic properties were identical with those of biscyclohexylammonium myoinositol 1-phosphate prepared synthetically [3] or isolated from soya-bean monophosphoinositide.[21]

Optical rotatory dispersion measurements (Rudolph polarimeter) at pH 7 gave a positive plain dispersion curve over the range 589—400 mμ; $[\alpha]^{20}$ values (*c* 2·2 in water) calculated on the weight of anhydrous salt were +2·25° (589 mμ), +2·65° (540), +2·88° (500), +3·87° (450), +4·85° (435), +9·66° (400). After adjustment to pH 2 [Dowex-50(H^+)], a negative plain dispersion curve was obtained; $[\alpha]^{20}$ −8·45° (589 mμ), −10·2° (540), −12·1° (500), −15·5° (450), −16·6° (435), −21·5° (400).[34]

The infrared spectrum (Nujol mull) had bands at 3300b, 2930, 2850, 2220w, 1631, 1550, 1527sh, 1455, 1393b, 1340b, 1324w, 1264w, 1230w, 1187, 1150sh, 1105s, 1046s, 994, 962s, 943sh, 925sh, 899, 830s, 750sh, 730 cm.$^{-1}$.

Heating a solution of the salt in 75% acetic acid for 50 min. at 100° or in N-hydrochloric acid for 2 hr. at 80° yielded a chromatographically identified mixture of myoinositol 1- and 2-phosphate.

Degradation of Inositide to Diglyceride.—The monophosphoinositide (10 mg.) was heated in 97% acetic acid (1 ml.) for 30 min. at 100° and, after cooling to 0°, chloroform (1 ml.) was added. The solution was set aside at −30° for 3 hr. and then centrifuged to remove precipitated myoinositol phosphate. The solution was shaken with water (5 ml.) and Dowex-50 (NH_4^+), and then ethanol (0·5 ml.) was added to the chloroform layer before evaporation *in vacuo*. The waxy solid was identified chromatographically as diglyceride by a method [35] which clearly distinguished it from monoglyceride.

Degradation of Inositide to D-*Glycerol* 1-*Phosphate.*—The monophosphoinositide (105 mg.), dissolved in ethanol–chloroform–water (5 : 2 : 2; 10 ml.), was shaken in darkness at room temperature with sodium metaperiodate (214 mg.) dissolved in the same solvent (15 ml.). After 22 hr. glycerol (0·1 ml.) was added and the solution clarified by centrifugation. It contained phosphatidic acid, identified by chromatographic comparison with synthetic distearoylglycerol 1-phosphate.[1] The solution was evaporated at room temperature to 0·5 ml., and chloroform (10 ml.) and 0·02N-hydrochloric acid (5 ml.) were added. After shaking, the aqueous layer was extracted with portions (4 ml.) of ether, light petroleum, and isobutyl alcohol,

[34] Similar results were obtained by Dr. C. E. Ballou for the compound from soya-bean inositide (personal communication).

[35] B. F. C. Clark, *J. Chromatog.*, 1961, **5**, 368.

and all the organic phases were combined and taken to dryness *in vacuo*. The residue was dissolved in 0·5N-sodium hydroxide (5 ml.), the gel was diluted with water (15 ml.), and the solid fatty acids were filtered off. The solution was acidified (Dowex-50) and extracted with ether (10 ml.), and the aqueous phase neutralised with cyclohexylamine in the usual way. Paper chromatography showed the presence of glycerol 1-phosphate (but none of the 2-isomer) and some orthophosphate. These were separated by preparative paper chromatography, and the glycerol 1-phosphate was reconverted into its biscyclohexylammonium salt which crystallised from ethanol–ethyl acetate (12 mg., 28%). It was identical with the synthetic racemic salt [36] on chromatography and electrophoresis and gave a positive periodate–Schiff reaction.

Configuration of Above Glycerol 1-Phosphate.—The method was essentially that of Bublitz and Kennedy.[37] The above cyclohexylammonium salt (0·5 mg.) was incubated with coenzyme I (3·6 mg.) and an aqueous solution of muscle α-glycerophosphate dehydrogenase (L. Light and Co. Ltd.) (0·2 mg.) in hydrazine hydrochloride buffer (pH 9; 2 millimoles in 4 ml.) at 37° for 45 min. A blank, without enzyme, was also run. Formation of reduced coenzyme I ($\lambda_{max.}$ 338 mμ) was observed in presence, but not in absence, of the enzyme. The reaction mixture was concentrated to 0·2 ml. and N-sodium hydroxide added to pH 14. After 3 hr. at room temperature electrophoresis in a pyridine acetate buffer (pH 4·25) showed that no glycerol phosphate remained and that orthophosphate had arisen from the alkali-labile dihydroxyacetone phosphate.

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[36] Brown, Hall, and Higson, *J.*, 1958, 1360.
[37] Bublitz and Kennedy, *J. Biol. Chem.*, 1954, **211**, 951.