274. *Phospholipids. Part III.* The Hydrolysis of the* cis- *and the* trans-*Isomer of Glycerol* 1-(2-*Hydroxy*cyclo*hexyl Phosphate*) *and Related Compounds.*

By D. M. Brown, G. E. Hall, and (Miss) H. M. Higson.

Hydrolysis by acid or alkali of glycerol 1-(*cis*-2-hydroxy*cyclo*hexyl phosphate) yields mainly 2-hydroxy*cyclo*hexyl phosphate (*ca.* 85%), whereas the *trans*-isomer gives mainly glycerol phosphate (*ca.* 75%). Rates of alkaline hydrolysis are, however, similar although appreciably greater than those found for benzyl *cis*- and *trans*-2-hydroxy*cyclo*hexyl phosphate and glycerol 1-(benzyl phosphate). In each case the reaction is of the first order in ester.

The relevance of the experiments to the chemistry of naturally occurring phosphoinositides is briefly discussed.

*myo*Inositol (I) occurs combined in phospholipids isolated from various sources. At least the simpler of such lipids appear [1] to be derivatives of glycerol inositol phosphate (II). We are studying the chemistry of this group and, as one objective, are seeking means of determining the position of esterification of phosphate on the *myo*inositol residue.

In Part I of this series [2] we reported the hydrolysis of the *cis*- and the *trans*-isomer of benzyl 2-hydroxy*cyclo*hexyl phosphate (III; R = CH_2Ph). Each substance gave the corresponding 2-hydroxy*cyclo*hexyl phosphate (IV) only, with acid or base, and rates were in the order *cis*- > *trans*-isomer. Evidence was adduced that hydrolysis involved the corresponding 1 : 2-*cyclo*hexylidene phosphate (V) and that therefore the hydrolytic pathway was the same as that found for other phosphodiesters carrying a vicinal hydroxyl

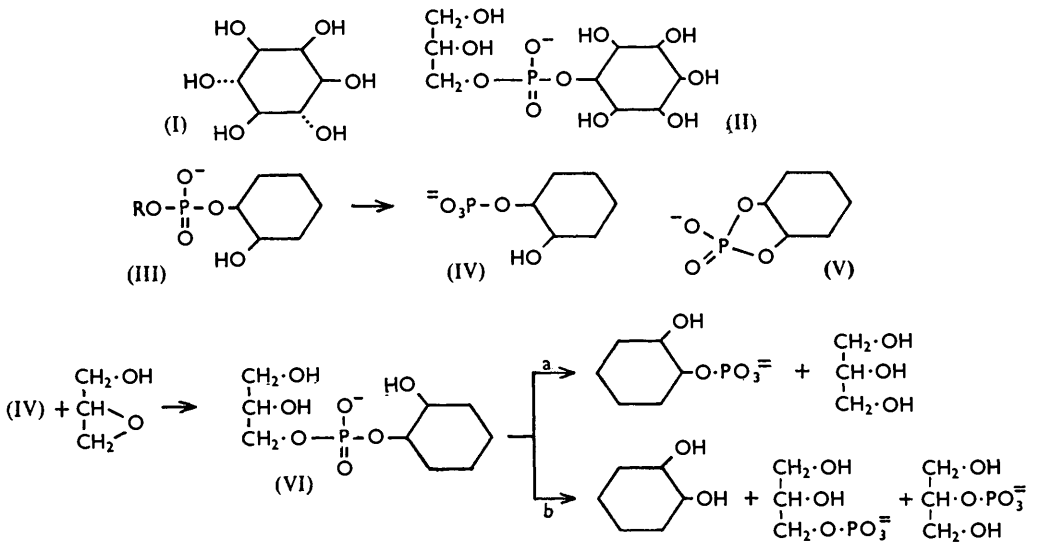
* Part II, *J.*, 1957, 2590.

[1] Folch and LeBaron, *Canad. J. Biochem. Physiol.*, 1956, **34**, 305.
[2] Brown and Higson, *J.*, 1957, 2034.

group.[3] Esters of glycerol phosphate react similarly,[4] hydrolysis *via* glycerol 1 : 2-phosphate being quantitatively the most important mode of fission (cf. refs. 5, 6; further evidence is given in the present paper). In view of this it appeared interesting to examine the hydrolysis of glycerol esters of the 2-hydroxy*cyclo*hexyl phosphates, which might be degraded by either or both of the pathways (a) and (b).

Glycidol reacted with *cyclo*hexylammonium *cis*-2-hydroxy*cyclo*hexyl hydrogen phosphate in boiling aqueous solution, and glycerol 1-(*cis*-2-hydroxy*cyclo*hexyl phosphate) (VI) was isolated as its barium salt after purification by ion-exchange chromatography.

Periodate titration showed that the product was the glycerol-1 ester (VI) containing



10% of the 2-isomer, consistently with the isomer ratio found for the reaction of glycidol with chloride ion.[7] The *trans*-form (VI) was similarly prepared and also found to contain 10% of the glycerol-2 ester. Owing to the solubility characteristics and instability of these esters no attempt was made to obtain the pure 1-isomers; even drying at 80° *in vacuo* caused extensive decomposition of the *trans*-form (VI),[8] the major product being tentatively identified as glycerol cyclic phosphate. It is evident, in theory, that the diester (VI) could react with a further molecule of glycidol to form a triester which would be expected to break down very readily, with phosphate migration.[9] The non-production of diglycerol phosphate, together with the high proportion of 1-isomer in the product (VI), indicates that this reaction did not take place. In initial experiments a considerably larger proportion of the 2-isomer was produced when the free hydroxy*cyclo*hexyl dihydrogen phosphate reacted with glycidol in dry dioxan.

When *cis*- and *trans*-esters (VI) were hydrolysed to completion by sodium hydroxide, paper chromatography showed that glycerol phosphate, 2-hydroxy*cyclo*hexyl phosphate,* glycerol, and *cyclo*hexanediol were produced in each case: thus hydrolysis occurred by

* Presumed to be the *cis*- and the *trans*-isomer respectively by analogy with the products formed on hydrolysis of the corresponding benzyl esters (cf. ref. 2).

[3] Brown, Magrath, Neilson, and Todd, *Nature*, 1956, **177**, 1124.
[4] Bailly and Gaumé, *Bull. Soc. chim. France*, 1935, **2**, 354.
[5] Baer and Kates, *J. Biol. Chem.*, 1948, **175**, 79; 1950, **185**, 615.
[6] Fleury, Lecocq, and Le Dizet, *Bull. Soc. chim. France*, 1956, 1193.
[7] Smith and Skyle, *Acta Chem. Scand.*, 1950, **4**, 39.
[8] A similar experience, with barium di(glycerol phosphate), has been recorded by Stockx and Vandendriessche (*Bull. Soc. chim. belges*, 1956, **65**, 919).
[9] Brown, Magrath, and Todd, *J.*, 1955, 4396.

both paths (a) and (b). A quantitative study of the relative importance of the two pathways was made by estimating total glycerol phosphate in the hydrolysates by Burmaster's method.[10] The results are collected in Table 1. The *cis*-ester (VI) at 100° gave mainly (85%) diol phosphate (VI) and therefore decomposed predominantly by route (a), while with the *trans*-isomer route (b) was favoured (75%). At 60° the difference between the two isomers appeared as the result of one experiment to be accentuated. In the case of

TABLE 1. *Direction of hydrolysis of the glycerol* 1-(2-*hydroxy*cyclo*hexyl phosphates*).

Isomer	Cation of salt	Normality of base or acid	PO_4 (%) as glycerol phosphate	PO_4 (%) as inorg. phosphate	Time of hydrolysis (hr.)
		Hydrolysis with sodium hydroxide (100°)			
cis-	Ba	1·2	14 ± 2	—	2·0
trans-	Ba	1·2	77 ± 5	—	3·0
trans-	Na	1·0	73[a]	—	7·0
		At 60°			
cis-	Ba	1·0	11	—	24
trans-	Ba	1·0	85[b]	—	24
		Hydrolysis with hydrochloric acid (100°)			
cis-	Ba	0·44	17	8	1·5
cis-	Na	0·44	15	2	2·5
trans-	Ba	0·44	94	10	1·5
trans-	Ba	1·33	82	20	7·0
trans-	Na	0·44	89	9	2·0

[a, b] Containing 45 and 44·5% of glycerol 1-phosphate respectively.

the *trans*-isomer glycerol 1-phosphate accounted for 45% of the total glycerol phosphate produced. This confirmed the expectation that glycerol 1 : 2-phosphate was an intermediate in the hydrolysis by path (b), for alkaline hydrolysis of glycerol 1 : 2-phosphate [11] (a convenient synthesis of which is described) led to glycerol phosphate, 42% of which was the 1-isomer.

The results of acid hydrolysis of the glycerol esters (VI) (also in Table 1) were rendered less accurate by the formation of variable amounts of inorganic phosphate but, here again, the *cis*-ester (VI) yielded mainly diol phosphate (IV), whereas the *trans*-ester went almost completely to glycerol phosphate.*

These observations prompted us to study the rates of alkaline hydrolysis of the glycerol diol phosphates (VI) and, for comparison, the benzyl esters (III; R = CH_2Ph). The reactions were followed titrimetrically by determining at intervals the amount of secondary phosphoryl dissociation liberated. In view of the small amounts of the esters available no great accuracy can be claimed for the kinetic results. Hydrolyses were performed with a large excess of sodium hydroxide, and first-order rate constants were calculated with any molecularity due to alkali and water being ignored. These results, including the times of half reaction, are collected in Table 2. In the first place all the compounds are hydrolysed very much faster than dimethyl phosphate,[12] confirming our view that the reaction proceeds essentially through cyclic ester intermediates (cf. Part I). There is a small but definite catalytic effect due to barium ion, although when this ion is present it is only in very small amount relative to sodium ion (cf. refs. 6 and 12).

The benzyl, methyl, and glycerol esters of *cis*-2-hydroxy*cyclo*hexyl phosphate are all hydrolysed principally to the diol phosphate (IV), but at different rates, in the order

* *Added, February 2nd*, 1958.—Ukita, Nagasawa, and Irie [*Pharm. Bull.* (*Japan*), 1957, **5**, 121, 127] describe the synthesis of barium glycerol 1 : 2-phosphate by a method similar to that used here, and find that alkaline hydrolysis of the substance gives glycerol phosphate containing *ca.* 45% of the isomer.

[10] Burmaster, *J. Biol. Chem.*, 1946, **164**, 233.

[11] Ukita, Bates, and Carter, *ibid.*, 1955, **216**, 867; cf. Dekker and Khorana, *J. Amer. Chem. Soc.*, 1954, **76**, 3522; Khorana, Tener, Wright, and Moffatt, *ibid.*, 1957, **79**, 430.

[12] Kumamoto, Cox, and Westheimer, *ibid.*, 1956, **78**, 4858.

benzyl < methyl < glycerol. Since this order is observed in the alkaline hydrolyses of their acetates[13,14] this is consistent with P–O fission in the rate-determining step which must involve the formation, and not the hydrolysis, of the cyclic phosphate (V). For, specifically, the glycerol *cis*-ester (VI) at constant ionic strength the hydrolysis is of the first order in substrate and in hydroxide ion, and this can most simply be rationalised if it is assumed that removal of the proton from the vicinal hydroxyl group, whose pK_a value

TABLE 2. *Rates of hydrolysis of some phosphate esters.*

Compound	Cation of salt	Normality of NaOH	Approx. ionic strength	Temp.	$10^6k'$ (sec.$^{-1}$)	$t_{\frac{1}{2}}$ (hr.)
Glycerol 1-(*cis*-2-hydroxy*cyclo*hexyl phosphate)	Ba	1·0	1·0	60	115	1·9
,, ,, ,,	Ba	0·5	0·51	60	48	4·0
,, ,, ,,	Ba	1·0	1·0	40	23	8·5
,, ,, ,,	Ba	1·0	1·5	40	28	6·9 [a]
,, ,, ,,	Ba	1·5	1·5	40	43	4·5
,, ,, ,,	Na	1·5	1·5	40	36	5·3
Glycerol 1-(*trans*-2-hydroxy*cyclo*hexyl phosphate)	Ba	1·0	1·0	60	89	2·2
Benzyl *cis*-2-hydroxy*cyclo*hexyl phosphate	$C_6H_{11}\cdot NH_3$	1·0	1·0	60	15	12·5
,, ,, ,,	,,	1·0	1·0	60	22	8·6 [b]
Benzyl *trans*-2-hydroxy*cyclo*hexyl phosphate	,,	1·0	1·0	60	2·6	75
Methyl *cis*-2-hydroxy*cyclo*hexyl phosphate	,,	1·0	1·0	60	25	7·7
Glycerol 1-(benzyl phosphate)	,,	1·0	1·0	60	35	5·6

[a] With added NaCl. [b] With 0·5 mol. of $BaCl_2$ added per mol. of substrate.

must then exceed 14, occurs before or simultaneously with the unimolecular rate-determining step in which cyclic phosphate is formed. With $\mu = 1{\cdot}0$, we find $k = 7{\cdot}5 \times 10^6 \exp -16{,}500/RT$. Increasing ionic strength also leads to an increase in k, suggesting reaction between two negative ions in the rate-determining step, but little reliance can be put on this interpretation in view of the high values of μ involved.

Since the *cis*-ester (VI), on complete hydrolysis, gave very little glycerol phosphate, the rate of hydrolysis of glycerol 1-(benzyl phosphate) was determined and, surprisingly, was found to be greater than that of benzyl *cis*-2-hydroxy*cyclo*hexyl phosphate (III; R = CH_2Ph). Moreover, despite their mainly following different pathways, the *cis*- and the *trans*-isomer (VI) were hydrolysed at very similar rates which were also greater than those found for the benzyl esters. These observations are nevertheless self-consistent as can be shown by a calculation based on the following considerations.

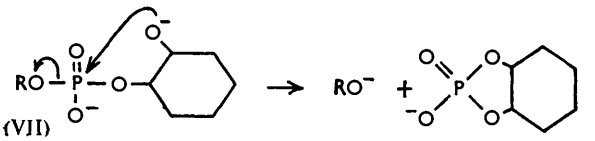
Taking process (VII) as rate-determining, it seems reasonable to assume that the important, although composite, factors influencing the rate will be those concerned with (*a*) the ability of RO^- to be formed, and (*b*) the ability of the vicinal alkoxide ion to attack the phosphorus atom.[15] In comparisons of process (VII) for *cis*- and *trans*-esters (VI), factor (*a*) is constant, whilst relative effects of factor (*b*) are obtained by comparing rates for the benzyl ester of *cis*- and of *trans*-2-hydroxy*cyclo*hexyl phosphate (III; R = CH_2Ph). From the values in the Tables, the partial rate constant ($\times 10^6$) at 60° of process (VII) for *cis*-ester (VI) is $89 \times 115/100 = 102{\cdot}3$; and, when the rates for the two benzyl esters are taken into account, the expected partial rate constant of process (VII) for the *trans*-isomer (VI) is $102{\cdot}3 \times 2{\cdot}6/15 = 17{\cdot}7$. Hence the percentage of glycerol phosphate in the hydrolysate of *trans*-ester (VI) should be $(89 - 17{\cdot}7)/89 \times 100 = 80\%$, in good agreement with the experimental value of 85%.

[13] Newling and Hinshelwood, *J.*, 1936, 1359; Tommila, *Ann. Acad. Sci. Fennicae*, 1941, *A*, **59**, No. 4, 3; *Chem. Abs.*, 1944, **38**, 6172.

[14] Meyer, *Z. phys. Chem.*, 1910, **67**, 257; Jellinek, *Rev. Pure Appl. Chem.*, 1952, **2**, 147.

[15] Cf. Hine, "Physical Organic Chemistry," McGraw-Hill Co. Inc., New York, 1956, p. 274, for a corresponding discussion of the alkaline hydrolysis of carboxylic esters.

It appears that the greater rates of hydrolysis of the glycerol diol phosphates (VI) are dependent on a special factor, *viz.*, the presence of two neighbouring hydroxyl groups. Hydrogen-bonding may then assist reaction by stabilising the ejected anion or by facilitating attack on the phosphorus atom.[16] It may be significant in this connection that



(VII)

glycerol 2-(methyl phosphate) is hydrolysed considerably faster than the 1-isomer.[6] We are investigating in more detail the structural effects which influence rate and direction of cleavage in these and related compounds.

The proportions of the cleavage products from the hydrolysis of the glycerol diol phosphates (VI) might suggest that the only factor influencing the hydrolysis of a phospholipid based on glycerol inositol phosphate (II) would be the *cis*- or *trans*-orientation of the neighbouring hydroxyl groups. If this is so, the predominant direction of breakdown should give information about the position of linkage of the glycerol phosphate residue to the *myo*inositol ring. Certain recorded observations [1,17] could be taken to indicate that some phosphoinositides do behave analogously to the synthetic esters (VI): however, taking our experiments as a whole we do not feel justified in drawing any definite conclusion without further experimental evidence. On the other hand the present work shows that the non-appearance of glycerol phosphate or inositol phosphate should not be assumed to indicate the absence of structure (II) in the parent phosphoinositide. Finally, it must be expected that any inositol phosphate fraction isolated from an acid or alkaline hydrolysate will be a mixture of (positional) isomers.

Experimental

The R_F values, which varied by 0·1 unit in different runs, were taken from chromatograms in which Whatman No. 1 paper and the propan-2-ol–ammonia–water (7 : 1 : 2) solvent system were used.

Barium Glycerol 1-(cis-2-*Hydroxy*cyclo*hexyl Phosphate*).—A solution of *cyclo*hexylammonium *cis*-2-hydroxy*cyclo*hexyl hydrogen phosphate (1·0 g.) and glycidol (1 c.c.; 4·6 mol.) in water (35 c.c.) was boiled under reflux for 6 hr. Paper chromatography indicated 70% conversion of the phosphate into a single phosphorus-containing product (R_F 0·60). After dilution to 80 c.c. and adjustment to pH 8—9, the solution was run on to a column (7·5 × 1·0 cm.) of Dowex-2 resin (formate form). The column was washed with water (500 c.c.), then 0·01N-formic acid (200 c.c.), and the product was eluted with 0·05N-formic acid, fractions of about 200 c.c. being collected. These were separately evaporated very carefully under reduced pressure to 5 c.c., then neutralised with barium hydroxide, and a little barium carbonate was added. Fractions containing only the product were combined and evaporated as above, and the colourless solid residue was dried over calcium chloride. This material was extracted with ethanol (2 × 15 c.c.), the extract evaporated, the residue dissolved in a little ethanol, and the product precipitated by addition of pure dry acetone. Several similar reprecipitations gave the pure *product* as a colourless hygroscopic powder (640 mg.), which gave one spot (R_F 0·55) (Found, in material dried at 50° over P_2O_5 at 1 mm. for 7 hr.: C, 27·7; H, 6·1; P, 7·5. $C_{18}H_{36}O_{14}P_2Ba,6H_2O$ requires C, 27·6; H, 6·1; P, 7·9%).

Periodate titrations showed an uptake of 0·9 ± 0·04 mol. of oxidant per atom of P, indicating the presence in the product of *ca.* 10% of the glycerol-2 isomer.

Barium Glycerol 1-(trans-2-*Hydroxy*cyclo*hexyl Phosphate*).—The preparation was carried out as described for the *cis*-isomer except that *cyclo*hexylammonium *trans*-2-hydroxy*cyclo*hexyl hydrogen phosphate (1·03 g.) was employed. The *product* was obtained as a very hygroscopic

[16] Henbest, *J.*, 1957, 1965.

[17] Hawthorne and Hawthorne in Popjak and LeBreton's "Biochemical Problems of Lipids", Butterworths, London, 1956, p. 104.

colourless, amorphous powder (350 mg.), R_F 0·55 (Found, in material dried *in vacuo* over $CaCl_2$ and SiO_2 gel: C, 31·4; H, 6·0; P, 8·6; Ba, 20·2. $C_{18}H_{36}O_{14}P_2Ba,H_2O$ requires C, 31·2; H, 5·5; P, 8·9; Ba, 19·8%).

Periodate titrations showed an uptake of 0·89 ± 0·04 mol. of oxidant per atom of P, indicating the presence of *ca.* 10% of the glycerol-2 isomer.

Drying the substance at 80°/1 mm. for 12 hr. caused extensive decomposition, the major phosphorus-containing product corresponding to glycerol cyclic phosphate on chromatograms and electrophoretograms.

For certain hydrolytic experiments the above barium salt was converted into the sodium salt. The barium salt (25·05 mg.) in water (2 c.c.) was treated with sodium sulphate decahydrate (12·5 mg., 1·07 mol.) in water (2 c.c.). Precipitated barium sulphate was removed by centrifugation and the aqueous solution used as such. The sodium salt of the *cis*-isomer was prepared similarly.

*Hydrolysis of Above Glycerol Hydroxy*cyclo*hexyl Phosphates.*—(a) *Direction of cleavage.* The appropriate salt (7—10 mg.) was dissolved in the hydrolysis solution (3 c.c.; see Table 1) and boiled under reflux in a Pyrex tube. Blanks, to which no organic phosphate was added, were run concurrently. After cooling, and in the case of alkaline hydrolyses after neutralisation with 10N-sulphuric acid, the solutions were diluted to 25 c.c. Aliquot parts were removed for determination of total phosphate [18] (2 c.c.) and for total glycerol phosphate (5 c.c. and 2 c.c. for the *cis*- and the *trans*-isomer respectively) by the following modification of Burmaster's method.[10] The aliquot parts were put in boiling tubes, 0·05M-sodium metaperiodate (1 c.c.) and 10N-sulphuric acid (1 c.c.) were added, and the solution was heated in boiling water for 1 hr., solution volumes being kept at 5—7 c.c. with distilled water. After cooling and addition of 4% sodium sulphite (1 c.c.) to destroy excess of periodate, amidol (2 c.c.) and molybdate (1 c.c.) were added, the solution was diluted to 25 c.c., and the optical density at 750 mμ determined after exactly 20 min. as in Allen's method for inorganic phosphate.[18] This gave a measure of glycerol phosphate plus inorganic and acid-labile phosphate. The sum of the last two, negligible in the alkaline hydrolyses, was obtained by carrying out the above estimation but without addition or periodate. The results are recorded in Table 1.

In two experiments, *viz.*, the hydrolysis by N-sodium hydroxide at 100° and 60° of barium glycerol 1-(*trans*-2-hydroxy*cyclo*hexyl phosphate), glycerol 1-phosphate was determined in the hydrolysate by diluting an aliquot part (2 c.c.) to 5 c.c., adding 0·05M-sodium periodate (1 c.c.) and 0·1N-sulphuric acid (1 c.c.), and setting the whole aside for 10 min. at room temperature. 4% Sodium sulphite solution (1 c.c.) and 10N-sulphuric acid (1 c.c.) were added and the solution was heated at 100° for 1 hr. Inorganic phosphate was then estimated as above and gave a direct measure of the glycerol 1-phosphate content of the original solution. This was found to be, in each case, 45% of the total glycerol phosphate.

(b) *Rates of hydrolysis.* Solutions of the substances (15—30 mg.) in aqueous sodium hydroxide (6·6 c.c.), together with blanks containing no organic phosphate, were heated in closed Polythene tubes supported in brass cylinders in the thermostat, and aliquot parts (1 c.c.), usually 6 per run, were withdrawn at intervals. These were brought to pH 2—3 with dilute hydrochloric acid and then titrated over the pH range 4·9—8·4 with carbonate-free 0·025N-sodium hydroxide, rigorous conditions being maintained for the exclusion of carbon dioxide. A direct-reading pH meter was used. The volume of alkali required by the blank was subtracted from the corresponding figure for the hydrolysate. For 2-hydroxy*cyclo*hexyl phosphate and glycerol 1-phosphate under similar conditions, it was found that the corrected alkali titre was then proportional to the amount of secondary phosphoryl dissociation, although the absolute values appeared slightly in error.

The first-order rate constants (k') and times of half-hydrolysis ($t_{\frac{1}{2}}$) were calculated and are recorded in Table 2, together with the corresponding values for other related substances.

cyclo*Hexylammonium* cis-2-*Hydroxy*cyclo*hexyl Methyl Phosphate.*—*cyclo*Hexylammonium *cis*-2-hydroxy*cyclo*hexyl hydrogen phosphate (200 mg.) in methanol (10 c.c.) was treated with ethereal diazomethane until the solution remained yellow. Next morning solvent was removed and the *product* crystallised from ethanol–ethyl acetate, as needles (110 mg.), m. p. 188—189·5°, R_F 0·60 (Found, in material dried over P_2O_5 at 70°/0·1 mm.: C, 50·4; H, 9·4; N, 4·75. $C_{13}H_{28}O_5NP$ requires C, 50·5; H, 9·2; N, 4·5%).

cyclo*Hexylammonium* trans-2-*Hydroxy*cyclo*hexyl Methyl Phosphate.*—This was made as

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

above but from *cyclo*hexylammonium *trans*-2-hydroxy*cyclo*hexyl phosphate. The *product* formed needles, m. p. 183—185° after two recrystallisations, R_F 0·63 (Found: C, 50·4; H, 9·3; N, 4·4%).

Di(cyclo*hexylammonium*) *Glycerol* 1-*Phosphate*.—The reaction between glycidol and disodium hydrogen phosphate in water was used but, instead of forming the barium or calcium salt according to Bailly,[19] the reaction mixture was treated first with excess of barium hydroxide to remove phosphate and then with Dowex-50 resin (H form) to remove barium ion. The solution was brought to pH 10 with *cyclo*hexylamine and evaporated under reduced pressure. Recrystallisation of the residue from ethanol–ethyl acetate gave the *product* in fine needles, m. p. 145—150° (Found: C, 48·2; H, 9·6; N, 7·7. $C_{15}H_{35}O_6N_2P$ requires C, 48·6; H, 9·5; N, 7·6%).

The salt consumed 0·98 mol. of periodate per atom of P, on oxidation.

cyclo*Hexylammonium Glycerol* 1-(*Benzyl Phosphate*).—Barium glycerol 1-phosphate (2·3 g.) was shaken with a little Dowex-50 resin (H form) and the solution poured on to a short column of the same resin. Effluent and washings were neutralised with *cyclo*hexylamine (1 c.c., 1 mol.), and the solution was evaporated to dryness. The half-salt was dissolved in ethanol (20 c.c.), and the solution treated with phenyldiazomethane (from 2·4 g. of benzaldehyde hydrazone) in ether. Next morning most of the solvent was evaporated water and chloroform were added, and the aqueous layer (20 c.c.) extracted twice with chloroform. Evaporation of the aqueous solution gave an oil which crystallised from ethanol–ethyl acetate. Two recrystallisations gave the *ester* as fine needles, m. p. 126°, R_F 0·63 (Found: C, 52·8; H, 8·4; N, 4·0. $C_{16}H_{28}O_6NP$ requires C, 53·2; H, 7·8; N, 3·9%).

Brucine Glycerol 1 : 2-*Phosphate*.—An aqueous solution of sodium glycerol 2-phosphate (0·4 g.) was passed through a column of Dowex-50 resin (H form), and the acidic effluent and washings were evaporated to dryness below 40°. The colourless oil was dissolved in dimethylformamide (10 c.c.), and di*cyclo*hexylcarbodi-imide (0·3 g.) in the same solvent (10 c.c.) was added. Di*cyclo*hexylurea separated almost immediately. Paper chromatography showed that, after 12 hr., the required product was present as the major component, contaminated with a little starting material. After the mixture had been brought to pH 10 with dilute ammonia and set aside for 0·5 hr., di*cyclo*hexylurea was filtered off and solvents were removed at 1 mm. The oily residue was purified by chromatography on a cellulose column (30 × 3·5 cm.) with propan-2-ol–ammonia (*d* 0·88)–water (40 : 1 : 14) as developing solvent. The required fractions, located by paper chromatography, were evaporated under reduced pressure, the residue was dissolved in water, and the solution passed through a Zeo-Karb 215 (H form) column. The effluent was neutralised with brucine in methanol, then evaporated to dryness, and the crude product (310 mg.) purified by two recrystallisations from ethanol. *Brucine glycerol* 1 : 2-*phosphate* crystallised as a monohydrate in needles (Found, in material dried at 50°/0·5 mm.: C, 55·1; H, 6·4; N, 5·1. $C_{26}H_{33}O_8N_2P,H_2O$ requires C, 55·1; H, 6·2; N, 5·0%).

The substance gave one phosphate-positive spot (R_F 0·4) on a paper chromatogram. Electrometric titration showed the absence of secondary phosphoryl dissociation. After this experiment had been done, Khorana *et al.*[11] mentioned, without experimental details, that the cyclic phosphate may be prepared by the above method.

Hydrolysis of Glycerol 1 : 2-*Phosphate*.—In a qualitative experiment, heating the brucine salt at 100° in N-sodium hydroxide caused complete conversion into glycerol phosphate in less than 5 min., as shown by paper chromatography. That the glycerol phosphate was a mixture of the 1- and the 2-isomer was shown by periodate titration. The brucine salt (50 mg.) was dissolved in N-sodium hydroxide (20 c.c.) and freed from brucine by chloroform-extraction, and the hydrolysis completed on the water-bath. Periodate titration and phosphorus estimation after 2 and 3 hr. showed an uptake of 0·42 mol. of oxidant per atom of P, corresponding to a 42 : 58 ratio of the 1- and 2-isomers of glycerol phosphate.

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[19] Bailly, *Ann. Chim.* (*France*), 1916, **6**, 123.