Selective Reduction of Phosphatides with Lithium Aluminum Hydride. II. Reductive Cleavage of the Glycerylphosphorylcholine Linkage

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Reduction of egg yolk lecithin with lithium aluminum hydride under mild conditions, using one-molar excess of the hydride at 10°C for 20 min., gave rise to L, α-glycerylphosphorylcholine,[1] the optical purity of which was confirmed later by van Deenen et al.[2] During the course of this study it was observed that the choline moiety of the lecithin and of glycerylphosphorylcholine molecules was released, to an extent of 20% in the case of the latter compound, on either raising the temperature or prolonging the reaction time. It would be of interest, therefore, to investigate this reaction further to see whether optically pure L, α-glycerylphosphoric acid is produced under appropriate reaction conditions.

The difficulty of reducing the phosphoryl group with lithium aluminum hydride has been reported by Freeman and Doak.[3] Furthermore, in the literature are found the observations that neither the sulfonyl group of 2-(ethylsulfonyl)ethanol[4] nor the nitro group of methyl 4-nitropentanoate[5] are reduced with this reagent under mild conditions. It may be assumed, therefore, that these phosphorus, sulfur, and nitrogen atoms, which are shielded with oxygen atoms of higher electron densities, form complexes less readily with AlH_4^- or AlH_3.[6],* In the lecithin molecule, however, the choline moiety may offer a site for such a coordination with either species, as we have suggested earlier[1] (Fig. 1). This suggestion was based on the fact that at least one extra mole of lithium aluminum hydride, over that required theoretically, had to be used for reductive cleavage of the two fatty acid ester groups of the lecithin molecule; the extra amount of the reagent was assumed to be consumed for complexing with the choline nitrogen. This may be supported in part by the fact that amines form complexes quite readily with aluminum hydride at low temperatures.[7,8]

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* Paddock suggested the following equilibrium in an ether solution of lithium aluminum hydride: $AlH_4^- \rightleftarrows AlH_3 + H^-$.

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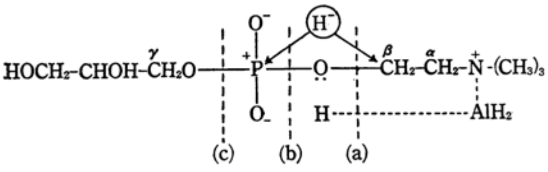

Fig. 1

There are three possible sites for cleavage by the reducing agents; (a), (b) and (c) as shown in Fig. 1. A carbon-oxygen fission (a) has been suggested previously1) as the most favored one since the hydride ion may readiy attack the β-carbon which is more electropositive than the γ-carbon atom of the glyceryl moiety owing to the inductive effects of both the phosphoryl and quaternary ammonium groups. The products of such a cleavage would be ethyltrimethylammonium ion and α-glycerylphosphoric acid. A fission at (b), on the other hand, would give choline and a reduced form (or forms) of glycerylphosphoric acid. This cleavage may be brought about either by complexing of the hydride reagent with the ammonium nitrogen as just mentioned, or with the phosphorus atom, followed by attack of hydride ion on the phosphorus atom. The latter mechanism may also bring about a cleavage at (c), giving rise to glycerol and reduced forms of phosphorylcholine. It is less likely, however, that the cleavages proceed through a complex formation with the phosphorus atom under the mild conditions being considered in the present study.

Since this reaction had been found to be quite sensitive to reaction temperature and mole ratios of the reactants, care was taken in controlling these two factors; the temperature was kept in a range of 10—15°C and mole ratios were raised from four to fifteen moles of the reducing reagent per mole of lecithin depending on the extent of cleavage of the choline moiety.

However, the results obtained were contrary to those expected. Instead of a carbon-oxygen fission, a phosphorus-oxygen fission occurred with the formation of choline and reduced forms of the phosphate group.

Experimental

Lecithin.—The sample of hydrogenated egg yolk lecithin was prepared according to Hanahan's method9) after purifying the crude material through a column of alumina with the solvent system of Rhodes and Lea.10) The product was recrystallized twice from warm dioxane. This sample showed an R_f value of 0.67 according to the method of Lea,11) phosphorus: choline = 1.0 : 0.95 (calculated as distearoyllecithin). Another sample without hydrogenation was prepared according to the method described by Nelson.12)

DL-α-Glycerylphosphorylcholine.—This compound was synthesized by the method of Baer and Kates.13) The free base was liberated from its cadmium chloride complex by passing its aqueous solution through columns of Amberlite IRC-50 and Dowex-3.

Barium α-Glycerylphosphate.—The calcium salt procured from Delta Chemical Works, Inc. (New York) was purified by adsorption chromatography on a small column of alumina and converted to the barium salt.14) The sample was thoroughly dried for infrared analysis.

Choline Chloride Reineckate.—This was prepared for infrared analysis, by a modification of the method used for estimation of choline, and dried over phosphorus pentoxide in a vacuum tube for 24 hr. at the boiling temperture of acetone. The free base released by the reduction was treated similarly.

Estimation of Choline.—The base released on reductive cleavage and the choline in glycerylphosphorylcholine were determined according to Schmidt's method,15) and that in lecithin by Glick's method.16)

Determination of Phosphorus.—Organic samples were digested by the conventional method using 10 N sulfuric acid solution and a few drops of hydrogen peroxide and the inorganic phosphorus released was determined according to the method of Ernester and his co-workers.17) Phosphorus in the products formed on cleavage was also determined by Burmaster's method.18)

α-Glycol. — The periodate oxidation method described by Voris19) was used.

Paper Chromatography.—Paper chromatography for deacylated glycerylphosphatides and the cleaved *phosphorus-containing product(s)* was carried out according to Wheeldon's method,20) using acid-washed Toyo No. 51 paper and water-saturated phenol containing 1% ammonia as the developing solvent system. The phosphatide samples prepared were examined by both Lea's method11) and Marinetti's,21) using Toyo No. 51 paper impregnated with silicic acid in conjunction with the solvent systems of chloroform-methanol (4 : 1, v/v) and diisobutylketone - acetic acid - water (40 : 20 : 3, v/v/v).

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TABLE I. RECOVERY OF THE WATER-SOLUBLE PRODUCTS SEPARATED BY AMBERLITE IR-112 (H)

	Total P		Free Ch*		CPC Ch*		Total Ch*	
	mg.	%	mg.	%	mg.	%	mg.	%
Lecithin	9.99	100					39.06	100
Aq. phase	3.82	38	20.8	53	20.7	53	41.5	106
Eluate a	3.80	38	0.0	0	19.0	49	39.0	100
Eluate b			20.0	51	0.0	0		

* Ch stands for choline and GPC for glycerylphosphorylcholine.

Reaction with Lithium Aluminum Hydride.—The procedure employed for the reaction was essentially the same as that described previously,1) except that a small amount of chloroform was used to dissolve the sample of hydrogenated lecithin. The reaction mixture was made acidic with 2—3 ml. of a 10 N sulfuric acid solution, the organic phase separated from the aqueous phase, and each made up to a known volume. The former was analyzed for total phosphorus and the latter for total phosphorus and choline, both in free and ester-bound form. For separation of the individual components of the water-soluble products, the aqueous phase separated without the acid treatment was passed through a column of Amberlite IR-112 (H), and collected eluate a was made up to a known volume for analysis of phosphorus and choline. The column was then washed with a sufficient amount of a normal hydrochloric acid solution to give eluate b, which was made up to a known volume and analyzed for free choline.

Since good separation with a minimum loss of each component was achieved as shown in Table I, this procedure was employed for isolation of the products. The free base in eluate b was converted to the reineckate salt as mentioned earlier. Eluate a (pH 2.4) contaning both glycerylphosphorylcholine and the cleaved *phosphorus-containing product* was passed through a column of Amterlite IR-4B (OH). The glycerylphosphorylcholine which came through was converted to its cadmium chloride complex and recrystallized. The average recovery of glycerylphosphorylcholine, based on analysis for phosphorus, was 95%. The column was then washed with a sufficient amount of a 0.28% solution of ammonia to elute the *phosphorus-containing product*. The last procedure, when tested with the sodium salt of glycerylphosphoric acid, gave an almost quantitative recovery of phosphorus. The eluate was concentrated under mild conditions and the residue treated with barium hydroxide. The barium salt was recrystallized several times from water and alcohol. These isolated products were dried over phosphorus pentoxide in a vacuum desiccator.

Results

The Cadmium Chloride Complex of the Isolated Glycerylphosphorylcholine.—Sample wt. 29 mg. Found: total choline 8.0 mg. (0.066 mmol.); total phosphorus 1.4 mg. (0.045 mmol.); α-glycol 0.067 mmol. Calcd.: choline, phosphorus and α-glycol 0.063 mmol. R_f value was 0.84 for the isolated sample; 0.84 for the synthetic sample of DL-glycerylphosphorylcholine.

The Reineckate Salt of the Free Base.—Found: C, 25.88; H, 4.80; N, 24.57; Cr, 14.60. Calcd. for choline reineckate, $C_5H_{14}N[(NH_3)_2Cr(CNS)_4]$, mol. wt. 422.58: C, 25.60; H, 4.76, N, 23.20; Cr, 14.00%. The infrared absorption spectrum of the salt was found to be identical with that of the reineckate salt of choline, both showing an absorption band at 1080—1075 cm^{-1} for the C–OH stretching vibration of the primary alcohol group.

The Phosphorus-containing Products. — See Table III for the analytical results and Table IV for the characteristic infrared absorption bands.

Discussion

The maximum extent of cleavage of the choline ester linkage appeared to be about 50% as shown in Table II and beyond this extent a loss of the water-soluble phosphorus was observed in separate experiments, perhaps due to further degradation to phosphine.23) Since it is reasonably certain that this cleavage proceeds via L, α-glycerylphosphorylchloline,1)

TABLE II. ANALYTICAL RESULTS OF THE REACTION PRODUCTS OF EGG YOLK LECITHIN WITH $LiAlH_4$ AT 10°C

L : Hyd.* mole ratio	Time min.	Aq. phase		Org. phase P, %
		P, %	Free Ch, %	
1 : 4	20	63	32	20
1 : 4	40	70	27	22
1 : 6	60	76	18	20
1 : 8	60	65	43	—
1 : 10	60	87	56	0
1 : 15	120	97	49	0
1 : 15**	60	89	38	0

* L : Hyd. denotes lecithin : lithium aluminum hydride.

** At 20°C

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TABLE III. ANALYSIS OF THE PHOSPHORUS-CONTAINING PRODUCTS ISOLATED FROM THE REACTION MIXTURE

Product	R_f	mmol.				$[\alpha]_D$
		Total P[a]	Total P[b]	α-GP P	Glycol	
A	0.21	0.022	0.023	0.014	0.016	
E		0.032	0.024	0.016	0.027	+0.12±0.5
C	0.28					
D	0.53					
α-GP	0.25					−1.0[c]

A was prepared under the reaction conditions—lecithin : the hydride reagent 1 : 10, 10°C, 60 min. B 1 : 15, 15°C, 120 min. C and D were separated from B by the paper chromatographic technique of Wheeldon.[20] a) Ernester's method.[17] b) Burmaster's method.[18]
c) The reported value for a saturated solution of the silver salt.[22]
The optical activity of B is so small that it is considered to have no optical activity (measured with a Rudolph polarimeter).

TABLE IV. INFRARED ABSORPTION BANDS OF α-GLYCERYLPHOSPHORIC ACID AND THE PHOSPHORUS-CONTAINING PRODUCTS, cm^{-1}

No.	α-GP	A	B	C
	3419—3119	3443—3320	3430	3480(sh)
			3310	3340
			3145 3120	
			3035	
I	2945	2920	2910	2940
	2865 (sh)	2860 (sh)	2850 (sh)	2880 (sh)
	2355—2312 (w)	2320 (br, w)	2370 (br, w)	
			2180 (w)	2180 (w)
			2100 (w)	2100 (w)
II		1620	1755 (w)	1643
			1629 (sh)	1605
			1615	
			1597 (sh)	
III	1463	1460—1455	1452	1460
	1418 (w)		1408	
IV	1379	1375	1377 (sh)	1380
V	1251			1255—1260
	1210			
VI	1120—1104	1095	1151	1099—1090
	1069		1085 (w)	
			1049 (w)	
VII	970 954 (d)	956		995
VIII	957	853 (w)		875 (w)
		820 (w)	822— 813	820 (w)
	780 (w)	710 (w)	720 (w, br)	750 (w)
				690

α-GP means the barium salt of α-glycerylphosphoric acid.
(d) Stands for doublet, (sh) shoulder, (w) weak, (br) broad. For A, B and C refer to Table III. The absorption bands of D were not very distinct due to some impurities and thus omitted.

its insolubility in anhydrous ether propably prevented the reaction from going to completion.

The isolation of choline evidently indicates that a phosphorus-oxygen fission occurred in this cleavage reaction. Such a fission, therefore, should be accompanied by reduction of the phosphate moiety. This is supported in part by the following observations; the *phosphorus-containing products* isolated differ from α-glycerylphosphoric acid with respect to optical activity, behavior on periodate oxidation (Table III), and infrared absorption bands (Table IV).

Since the interpretations given for the shift in the absorption bands of the P→O and P-O-C groups (pentavalent and trivalent phosphorus) due to modification of the alkyl moiety are still open to discussion, only the absorption bands of interest will be pointed out here to show differences between the spectrum of α-glycerylphosphoric acid and those of the *cleaved products*. In the region indicated by I, Table IV, glycerylphosphorylethanolamine shows an absorption band at 2910—2860 cm^{-1} and glycerylphosphorylcholine at 2865 cm^{-1} [24] but these have not been correlated so far with the groups attached to these molecules. One of the characteristic differences between α-glycerylphosphoric acid and the *products* is shown in the region II. Daasch and Smith[25] have suggested that the absorption band at 1665 cm^{-1} is associate with phosphonic acid and thus the bands in II may indicate the presence of reduced forms of the phosphate group. The absorption bands in the regions III and IV are common to all the compounds under consideration and the former bands have been reported to be associated with the CH_2 group.[26] The characteristic P→O stretching vibration in the region V is absent in samples A and B but present in C; its presence in the last sample annot be explained but the P→O group might have been formed on exposure to air during its separation and isolation from the paper chromatogram. Daasch and Smith,[25] however, have pointed out that these bands are so broadened in the case of phosphonic and phosphinic acids that it is difficult to distinguish them from each other, particularly in the spectra of the solid samples. Accordingly, A and B may be considered here again to be reduced forms of the phosphate. The P-O-C (alkyl) group is reported to show absorption bands in the regions 1163 to 1156 cm^{-1} and 1050 to 995 cm^{-1} [26] but the bands (VI and VII) appear to be shifted in α-glycerylphosphoric acid and the *products*. Recently, Chapman and Harper[27] have attributed absorption bands at 990—980 cm^{-1} and 875—855 cm^{-1} to the P-O-C (alkyl) group in phosphites, and Ackerman and his co-workers[28] report that a band near 794 cm^{-1} is due to the same group in acylphosphonates. Since α-glycerylphosphoric acid shows some of these bands (VIII), no comments will be made on the similar band observed in these products.

The fact that the characteristic absorption bands of A differ from those of B and C may indiate that B (or C) is in a more reduced state than A, since the reaction conditions employed for preparation of B were somewhat more drastic than those employed for A (see also Table III). The loss of phosphorus observed on raising the temperature might have resulted from fission at both (b) and (c), Fig. 1, giving rise to phosphine as has been observed by Karrer[23] during the use of drastic conditions.

Brockerhoff[29] attributed the ease of removal of choline in the alkaline hydrolysis of glycerylphosphorylcholine to the following two factors; (1) the influence of the quaternary ammonium group which can balance in part the negative charges on the phosphate oxygens and thus facilitates approach of a nucleophilic reagent, and (2) the initial attack on the phosphorus by the nucleophilic oxygen of the secondary hydroxyl group of the glyceryl moiety. In the present case, however, ionization of the secondary alcohol would be less likely in such a medium as anhydrous ether. Instead, intramolecular hydrogen bonding between the hydroxyl hydrogen of the β-carbon of the glyceryl moiety and the P→O oxygen atom plus an ionic effect of the quarternary ammonium group on the $P-O^-$, or the latter alone, probably distort the normal tetrahedral bond angles of the phosphorus atom[30] in such a manner that the central phosphorus atom, of large radius, might readily be attacked by hydride ion. This may bring about fission at the phosphorus-oxygen linkage, (b) in Fig. 1. The stronger cationic nature of the phosphorus atom, compared with that of the β-carbon of the choline moiety, may also play a role in this cleavage.

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Summary

Reduction of egg yolk lecithins with lithium aluminum hydride under the following reaction conditions resulted in release of choline in about 50% based on the total water-soluble phosphorus; mole ratios of lecithin : lithium aluminum hydride of 1 : 10 or 1 : 15 at 10—15°C for 60—120 min. were used. Unexpectedly, the reaction did not give any glycerylphosphoric acid. Instead, the analytical results indicated that a phosphorus-oxygen fission of the cholin-phosphate linkage is accompanied by reduction of the phosphate moiety.

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