Selective Reduction of Phosphatides with Lithium Aluminum Hydride. I. Ovolecithin

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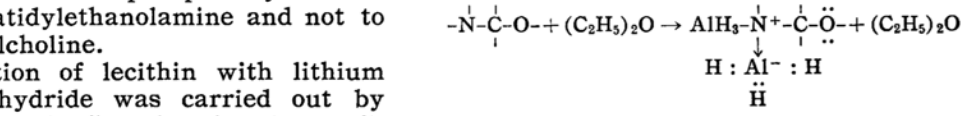
For determination of the structures and the configurations of phosphatides, the following two methods were available. Long and Magure[1] carried out the reactions to derive L-α-glycerylphosphoric acid as described below:

L-α-Lecithin —(Phospholipase C)→ 1,2-Diglyceride —(Phosphorylation)→ L-α-Phosphatidic acid —(Hydrolysis)→ L-α-Glycerylphosphoric acid

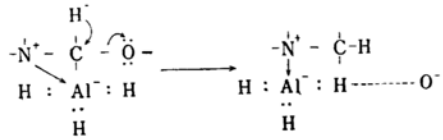
Baer and Maurukas[2] devised the so-called "diazometholysis" giving the phosphatidic acid dimethyl ester. In both methods, unsaturated fatty acids, if present, should be hydrogenated before carrying out these reactions. These two methods are, however, not always convenient for general application. The former is far more complicated and would require a sufficient amount of purified phosphatides for analysis of the final product. The latter is, on the other hand, simpler, but it is applicable to those containing the free amino group such as phosphatidyl serine and phosphatidylethanolamine and not to phosphatidylcholine.

The reaction of lecithin with lithium aluminum hydride was carried out by Karrer and Jucker[3] under drastic conditions, at the boiling point of ether. Inorganic phosphorus, phosphine, glycerol and a substance with an amine-like odor were reported to be present in the reaction mixture. Apparently, all ester linkages of the lecithin molecule were cleaved. A number of instances of selective reduction by this reagent were reported, e. g. methyl 4-nitrovalerate was reduced to the corresponding alcohol at −35°C in 20 min., the nitro group being unattacked[4]. A selective reduction similar to this example may possibly be achieved with lecithin under carefully controlled conditions.

The authors' attention was focused on an ionic character of the coordinate covalences of the sulfonyl, nitro and phosphoryl groups. Under mild conditions approach of a hydride ion of lithium aluminum hydride present in ether solution to these groups may encounter some difficulty due to the presence of negatively charged oxygen atoms which are stabilized by resonance. If this plays a part in reduction of the lecithin molecule, the cleavage of the two fatty acid ester linkages would be easier than that of the two ester linkages of the phosphoryl group. However, the nitrogen atom of the choline may also play a role since Trevoy and Brown[5] showed the reaction to be a bimolecular nucleophilic substitution by a hydride ion and Gaylord[6] proposed a mechanism for the reduction of an –N–C–O– group, utilizing the ionic species, $AlH_4^- \rightleftharpoons AlH_3 + H^-$, and the concept of ether participation which have been proposed by Paddock[7], as shown below:

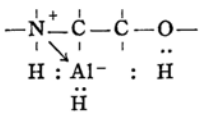


The coordination of AlH_3 with the lone pair on nitrogen can occur in the initial step. By analogy with Grignard reaction, the formation of a five-membered quasi-ring is suggested. As the result, nitrogen would become deficient in electron and tend to withdraw electron from the adjacent carbon giving the latter a positive character. The hydride ion could then displace the ethereal oxygen resulting in cleavage of the C–O bond:



1) C. Long and M. F. Magure, *Biochem. J.*, **57**, 223 (1954).
2) E. Baer and J. Maurukas, *J. Biol. Chem.*, **212**, 39 (1955).
3) P. Karrer and E. Jucker, *Helv. Chim. Acta*, **35**, 1586 (1952).
4) H. Feuer and T. J. Kucera, *J. Am. Chem. Soc.*, **77**, 5740 (1955).
5) L. W. Trevoy and W. G. Brown, ibid., **71**, 1675 (1949).
6) N. G. Gaylord, *Experientia*, **10**, 351 (1954).
7) N. L. Paddock, *Nature*, **167**, 1070 (1951).

This mechanism can possibly be applied to the choline moiety of the lecithin molecule, as far as the formation of a six-membered quasi-ring is concerned:



However, an electrostatic effect on the β-carbon atom would be not as strong as that on the α-carbon. The nitrogen atom in this case is already in the cationic form and has no lone pair available for coordination with AlH_3 but approach of AlH_4^- would be possible, resulting perhaps in a complex formation*. This may also encounter some difficulty, particularly at low temperatures at which the molecule exists in its ground state, because a hydride ion must overcome zwitterionic and possibly steric effects of the trimethyl group in its approach to the cationic nitrogen. Thus, a higher amount of energy would be required for the cleavage of the phosphoryl-choline linkage than that for the cleavage of the fatty acid ester linkage where no such obstacle exists.

By similar qualitative examination the following speculations may be made on relative ease of cleavage of the two phosphoryl ester linkages. The cleavage of the phosphoryl-glyceryl linkage depends on an attack by a hydride ion on the phosphoryl group only. On the other hand, in the cleavage of the phosphoryl-choline linkage a mechanism involving nitrogen may also participate; this may operate independently of the mechanism involving the phosphoryl group or assist the latter by making oxygen somewhat deficient in electron as a result of the formation of a six-membered quasi-ring. Accordingly the phosphoryl-choline linkage is considered to be cleaved somewhat more readily than the linkage between the phosphoryl and glycerol.

Thus, a step-wise cleavage of the lecithin molecule may be possible, i. e. the cleavage of the fatty acid ester linkages, giving the corresponding alcohols and glycerylphosphorylcholine (with retention of the configuration); or the cleavage of the choline moiety, following the removal of the fatty acids, giving choline and a reduced form of glycerylphosphoric acid**. Accordingly, attempts were made to find suitable reaction conditions to achieve either one of the two possibilities by controlling temperature, time and amounts of lithium aluminum hydride.

* The authors used "AlH_3" in a preliminary report on this subject, This Bulletin, **31**, 779 (1958), but presently consider that it must be reserved as such.

Experimental

Materials.—*Lecithin.*—Crude egg yolk lecithin was purified according to Hanahan's method[8], (P : N, 1 : 1.10 and 15.8% choline).

DL-α-Glycerylphosphorylcholine.—This compound was synthesized according to the method of Baer and Kates[9], (α-glyceryl ester: choline, 1.03 : 1.0).

Analytical Methods. — *Choline.* — Choline in glycerylphosphorylcholine was determined by Schmidt's method[10] and in lecithin by Glick's method[11].

Phosphorus.—The conventional method of wet incineration, with 10 N sulfuric acid and hydrogen peroxide, was used to convert organic phosphorus to the orthophosphate. The inorganic phosphate was then determined spectrophotometrically according to the method described by Burmaster[12] or by Ernester et al.[13] Concentrations of the reagents were altered, however, as circumstances required.

Ester.—The method described by Bauer and Hirsch[14] was employed.

α-Glycol.—The periodate oxidation method of Voris et al.[15] was used.

Reaction of Lecithin with Lithium Aluminum Hydride.—One hundred milligrams of lithium aluminum hydride was dissolved in 50~60 ml. of dry ether (0.1~0.2% solution). The solution was filtered first through glass wool packed in a glass tubing of about 6 mm. in diameter and then through a sintered glass filter sealed in a glass tubing of about the same diameter and transferred into a specially constructed burette. The filtration and transfer were carried out by applying pressure with dry nitrogen gas. Calcium chloride tubes were provided to keep the entire system in a dry state. A flask of about 100 ml. capacity containing a cooled ether solution of lecithin was attached to the burette and a calculated amount of the reducing agent*** was added in small portions so that the temperature

** A trivalent phosphorus compound may be formed as a result of an attack of AlH_4^- on the positively charged phosphorus atom. This sort of interaction was shown to occur under drastic conditions (the formation of phosphine was recognized)[3] and it may be possible that even under mild conditions this may occur to a certain extent.

8) D. J. Hanahan et al., *J. Biol. Chem.*, **192**, 623 (1951).
9) E. Baer and M. Kates, *J. Am. Chem. Soc.*, **70**, 1394 (1948).
10) G. Schmidt et al., *J. Biol. Chem.*, **197**, 601 (1952).
11) D. Glick, ibid., **156**, 643 (1944).
12) C. F. Burmaster, ibid., **164**, 233 (1946).
13) L. Ernester et al., *Acta Chem. Scand.*, **4**, 942 (1950).
14) F. C. Bauer, Jr. and E. F. Hirsch, *Arch. Biochem.*, **20**, 242 (1949).
15) L. Voris et al., *J. Biol. Chem.*, **133**, 491 (1940).

*** Concentrations of the ether solution were determined from time to time by the conventional method of hydrogen gas liberation. Aliquots were delivered from the burette.

of the reaction mixture could be maintained constant. Stirring with the magnetic stirrer was continued for a desired length of time. The reaction mixture was cooled and decomposed with wet ether. A sufficient amount of water was added to separate the organic layer. The aqueous phase was shaken twice with a small portion of fresh ether and the ether extracts were combined. The aqueous layer was then washed with chloroform to remove lysolecithin (this step was omitted in later experiments since no lysolecithin was found to be present in the chloroform extract). The ether extract was made up to a known volume, usually 25 ml., and aliquots were taken for analyses of organic phosphorus and esters. The aqueous phase was made acid with 10 N sulfuric acid to dissolve the precipitate, the solution filtered and made up to a known volume, usually 50 ml., for analyses of inorganic phosphorus, total phosphorus, free choline and total choline.

Isolation of L-α-Glycerylphosphorylcholine. — Lecithin (about 400 mg.) was reduced under the optimum conditions (14°C, Table V). After decomposing the reaction mixture with water, the precipitate formed was removed by centrifugation. The clear solution was passed through a column of IRC-50 and the eluate was analyzed for choline and α-glycol (α-glycol: choline, 1.02: 1.00). The eluate was then concentrated under reduced pressure in the atmosphere of nitrogen and the amorphous cadmium chloride addition complex was prepared according to the procedure described by Baer[9] (Found: P, 5.24%). A solution of the complex was passed through a mixture of 4 ml. of Dowex 3 and 2 ml. of IRC-50 and the solution analyzed for α-glycol, total P and choline (Found: α-glycol : P : choline, 0.97 : 1.00 : 1.02). α-Glycerylphosphorylcholine recovered after passing the resin column was found to be 94%. The crystalline cadmium chloride complex was prepared and analyzed (Found: C, 21.56; H, 4.80) after drying over phosphorus pentoxide at 56°C for 8 hr. under 3 mmHg. The infrared spectrum of this compound was found to be identical with that of the synthetic DL-α-glycerylphosphorylcholine. Its optical rotation was determined on the concentrate of the aqueous solution freshly prepared by repeating the reduction and purification in the same manner as described above [Found: $[\alpha]_D^{24}-2.96\pm0.2°$ (c, 2.2 water); $[\alpha]_D^{23}-2.85\pm0.1°$ (Baer)].

Preliminary experiments with α- and β-glycerylphosphoric acids in the same manner as described for the reduction of lecithin showed that no migration of the phosphoryl group occurred. Similar experiments with glycerylphosphorylcholine was not possible due to technical dfficulties. The migration of the phosphorylcholine groups is, however, unlikely to occur under such mild reaction conditions.

Results and Discussion

The results shown in Tables I and II indicate that the reaction proceeds through

TABLE I. REDUCTIVE CLEAVAGE OF OVOLECITHIN WITH LITHIUM ALUMINUM HYDRIDE AT 20°C: 15 mol. OF $LiAlH_4$ PER MOLE OF LECITHIN*

Reaction time (hr.)	3	5
Sample wt. (mg.)	201	86
Ch. in sample (mg.)	31.8±0.3	13.9±0.0
Total Ch. (mg.) in aq. phase	31.8±0.6	11.7±0.01
Free Ch. (mg.) in aq. phase	27.7±0.3 (87)	11.3±0.2 (81)
Inorganic P (mg.) in aq. phase	0.72±0.00 (9)	0.56±0.04 (16)
Organic P (mg.) in ether phase	0.00	0.00
Organic P (mg.) in chloroform extract	0.00	0.00

* For the calculation of the mole ratio 778 was used as an average molecular weight of lecithin. Ch. stands for choline. The figures in parentheses are percentages based on an amount of choline or phosphorus present in each sample weight.

TABLE II. REDUCTIVE CLEAVAGE OF OVOLECITHIN WITH LITHIUM ALUMINUM HYDRIDE AT 20°C: 3 mol. OF $LiAlH_4$ PER MOLE OF LECITHIN

Reaction time (hr.)	3	5	10
Sample wt. (mg.)	86	113	113
Ch. in sample (mg.)	13.9 ±0.0	17.8[a)]	17.8[a)]
Total Ch. (mg.) in aq. phase	13.9 ±0.0	19.6±0.5	15.7±0.3
Free Ch. (mg.) in aq. phase	5.65±0.2 (41)	11.3±0.2 (64)	12.3±0·3 (69)
P in sample (mg.)[a)]	3.42	4.52	4.52
Inorganic P (mg.) in aq. phase	0.00	0.13 (3)	0.13 (3)
Total P (mg.) in aq. phase	2.12±0.01 (62)	2.88±0.11 (64)	3.06±0.1 (68)
Total P in ether phase	0.00	0.00	0.00
Total P in chloroform extract	0.00	0.00	0.00

Ch. stands for choline. The figures in parentheses are percentages based on an amount of choline or phosphorus present in each sample weight.

a) Calculated values.

TABLE III. REDUCTIVE CLEAVAGE OF OVOLECITHIN WITH LITHIUM ALUMINUM HYDRIDE AT 18°C: 2 mol. OF $LiAlH_4$ PER MOLE OF LECITHIN

Reaction time (min.)	5	5	10
Sample wt. (mg.)	100	120	120
Ch. in sample (mg.)	15.3 ±0.3	19.3±0.3	19.3±0.3
Total Ch. (mg.) in aq. phase	13.1 ±0.1	12.1±0.4	20.4±0.0
Free Ch. (mg.) in aq. phase	3.01±0.1 (19)	2.1±0.2 (11)	6.4±0.2 (33)

Ch. stands for choline. Those figures in parentheses are percentages based on the choline content of the sample.

TABLE IV. REDUCTIVE CLEAVAGE OF OVOLECITHIN WITH LITHIUM ALUMINUM HYDRIDE AT 16°C: 2 mol. OF $LiAlH_4$ PER MOLE OF LECITHIN

Reaction time (min.)	30	60
Sample wt. (mg.)	113	113
Ch. in sample (mg.), calcd.	17.8	17.8
Total Ch. (mg.) in aq. phase	19.1±0.9	19.6±0.5
Free Ch. (mg.) in aq. phase	4.1±0.2 (23)	4.4±0.2 (25)
P in sample (mg.), calcd.	4.52	4.52
Total P (mg.) in aq. phase	3.56±0.0 (79)	3.31±0.2 (73)
Total P (mg.) in ether phase	0.02	0.00
Total P (mg.) in chloroform extract	0.02	0.00

Ch. stands for choline. Those figures in parentheses are percentages based on an amount of choline or phosphorus present in each sample taken.

TABLE V. REDUCTIVE CLEAVAGE OF OVOLECITHIN WITH LITHIUM ALUMINUM HYDRIDE 2 mol. OF $LiAlH_4$ PER MOLE OF LECITHIN

Temperature (°C)	14	10	10
Reaction time (min.)	10	20	20
Sample wt. (mg.)	120	160.9*	128.6*
Ch. in sample (mg.)	19.1±0.3	22.7[a)]	18.1[a)]
Total Ch. (mg.) in aq. phase	18.0±0.1	23.6±0.1	18.3±0.0
Free Ch. (mg.) in aq. phase	0.8±0.1 (4)	0.0	0.0
P in sample (mg.), calcd.	4.78	6.82	5.45
Total P (mg.) in aq. phase	4.00±0.06	7.03±0.07	5.40±0.03
Total P (mg.) in ether phase	0.02±0.00	0.04±0.00	0.05±0.00
Ester (m. e.), calcd.		0.444	0.355
Ester (m. e.) in ether phase		0.004±0.002 (0.9)	0.004±0.001 (1.12)

* The sample of lecithin employed was prepared by the method of Rhodes and Lea, *Biochem. J.*, **65**, 526 (1957), and its analytical results were; choline : P : ester, 1 : 1.17 : 2.50.

a) Calculated values based on the analytical results.

Ch. stands for choline and m. e. for milliequivalent. The figures in parentheses are percentages based on choline or ester content of each sample taken.

the formation of glycerylphosphorylcholine and not phosphorylcholine, since choline containing substances in the aqueous phase release the total amounts of choline present in the samples on acid hydrolysis. Therefore, the experiments under much milder conditions were carried out so that only glycerylphosphorylcholine would be produced. These results are shown in Tables III—V.

It was of interest to see if ether insoluble glycerylphosphorylcholine itself would release the choline moiety by lithium aluminum hydride in ether, the synthetic glycerylphosphorylcholine was, therefore, reacted at 20°C for 3 hr. with 15 mol. of lithium aluminum hydride per mole of the compound. It was found that about 21% free choline was released.

From the results shown in Tables III—V,

the optimum conditions for the reductive cleavage of lecithin giving a 100% yield of glycerylphosphorylcholine appears to be 20 min. at 10°C with a mole ratio of lecithin to lithium aluminum hydride 1 : 2 (Table V). Some phosphorus and ester were found to be present in the ether phase, but this is considered to be due to some impurities present in the sample employed as indicated by its analytical results. According to its optical rotation observed, the glycerylphosphorylcholine formed in the reaction has the L-α-configuration. This method is, therefore, not only simple for the determination of configurations of lecithin, but also a useful method for the preparation of L-α-glycerylphosphorylcholine.

Tables III and V show that the reaction involving the release of the choline moiety is quite sensitive to temperature. With the temperature difference of only four degrees, 18, 14 and 10°C, amounts of choline released decrease from 30 to 4% and finally to 0%, respectively. On the other hand, it is not affected much by reaction time as shown in Tables III, IV and II. It appears then that temperature must be raised higher than 20°C for a complete release of the choline moiety as shown in Table I. The fact that the reaction is sensitive to temperature would mean that an amount of energy required for the cleavage of the phosphoryl-choline linkage is significantly higher than that required for the cleavage of the fatty acid ester linkage. It is quite in accord with what the authors have anticipated.

In those cases where considerable amounts of choline are formed, the total amounts of phosphorus recovered in the aqueous phase are only 62~68% of the calculated amounts (Table II), despite the absence of phosphorus in the organic phase. It is also noticed in the same table that the amounts of the inorganic phosphorus in the aqueous phase are not equivalent to that of the choline moiety released; only 0~3% of the inorganic phosphorus are detected where 41~69% free choline are formed. These results may indicate that some complex mechanisms are involved in the cleavage of the choline moiety.

If a mechanism similar to that proposed by Gaylord[5] operates in the cleavage of the choline moiety, trimethylethylamine should be formed. If, on the other hand, a mechanism involving the phosphoryl moiety participates, choline and a reduced form of α-glycerylphosphoric acid, which may not be detected by the method of analysis employed must be produced. In Table IV, 79 and 73% of phosphorus found in the aqueous phase can be considered to have been derived from 77 and 75% of glycerylphosphorylcholine, respectively. Therefore, 21 and 27% of undetected phosphorus may be ascribed to a reduced form of glycerylphosphoric acid, indicating that the latter mechanism may have played a role. Under such mild conditions, liberation of phosphine would not be possible, for an amount of lithium aluminum hydride used is far less than that required for reduction of the phosphoryl group to its ultimate stage. When three moles of the reducing agent is used (Table II), of 62% total phosphorus detected in the aqueous phase after three hours of reaction time 59% can be accounted for glycerylphosphorylcholine and the remaining 3% may have come from glycerylphosphoric acid (since no inorganic phosphorus is found to be present). Similarly, with 5 and 10 hr. of reaction time 25 and 34%, respectively, may have been derived from glycerylphosphoric acid. Undetected phosphorus would then amount to 38, 36 and 32% for 3, 5 and 10 hr., respectively, of the reaction time in these instances. Therefore, under these conditions, the two mechanisms mentioned above appear to have participated simultaneously, though participation of the latter mechanism seems to predominate in the three hour run. This may indicate that energy requirements for the formation of an intermediary complex with the cationic nitrogen are somewhat higher than that with the phosphoryl group.

Suppose that the formation of the inorganic phosphate is the only indication available for the cleavage of the glycerylphosphoryl linkage, this ester linkage is considered to be the most stable one in the lecithin molecule against lithium aluminum hydride. It may also be pointed out that "free choline" given in the tables constitutes of a mixture of choline itself and the quarternary amine according to the foregoing discussions.

Summary

The optimum conditions for cleavage of the fatty acid ester linkages in the lecithin molecule were found to be 20 min. at 10°C with the use of two moles of lithium aluminum hydride per mole of lecithin.

The product formed under these conditions was identified to be L-α-glycerylphosphorylcholine when ovolecithin which was shown to be L-α-lecithin[8] was used.

Discussion has been presented as to two mechanisms possibly participating in the cleavage of glycerylphosphorylcholine, one involving a complex formation with the phosphoryl group and the other with the cationic nitrogen atom of the choline base.

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