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The Fractionation of Ethanolamine Phosphatides of Ox Brain

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In 1961 Hanahan and Watts¹ reported that acylated alkoxy glycerophosphoryl-ethanolamines are deacylated more slowly than phosphatidylethanolamines under mild alkaline conditions. This finding allowed the final purification of the "native alkoxy cephalins"² of bovine erythrocytes. In 1962 Renkonen³ isolated "native alkoxy lecithins" from human serum by removing quite large amounts of phosphatidylcholine with a similar method. In addition, Renkonen³ recently found conditions where the diacyl phosphatides can be eliminated with mild alkaline treatment from native plasmalogens too. This report describes preparative fractionation of highly purified ox brain ethanolamine phosphatides with mild alkaline and mild acid⁴ treatments,

which yielded fairly good concentrates of all the three component lipids of the original mixture, *i.e.* of phosphatidylethanolamines, corresponding native plasmalogens, and "native alkoxy cephalins".

Ox brain cephalins were isolated and fractionated essentially as described by Folch⁵. The fraction V was further purified on DEAE cellulose⁶ and on silicic acid³, which gave a pure preparation of ethanolamine phosphatides (Table 1) containing $57 \pm 8\%$ native plasmalogens, about 4% native alkoxy phosphatides and $37 \pm 8\%$ phosphatidylethanolamines*.

This sample was treated with 0.05 N NaOH in moist chloroform-methanol (3:4, v/v) at 20° for 23 min, and the hydrolysate was fractionated by solvent partition, and by silicic acid chromatography essentially as described previously³. In addition to lysoplasmalogens a phosphatide preparation was thus obtained which contained one third of the original phosphorus and consisted of $83 \pm 8\%$ of native plasmalogens, of about 6% of native alkoxy phosphatides, and of $11 \pm 8\%$ of phosphatidylethanolamines*. Renewed alkaline treatment gave an even more satisfactory preparation of native ethanolamine plasmalogens, which was nearly free of phosphatidylethanolamines. Analytical characterization of this sample (Table 1) showed that it was, however, contaminated by about 8% of alkoxy phosphatides.

Treatment of the pure native ethanolamine plasmalogens (276 $\mu\text{g P}$) with 0.05 N HCl in moist chloroform-methanol (1:1, v/v) at 20° for 60 min, and partitioning of the hydrolysate gave 4% water soluble and 94% lipid soluble phosphorus. Silicic acid chromatography of the lipid soluble fraction gave two phosphatide preparations, one of which (23 $\mu\text{g P}$) contained mainly acylated alkoxy glycerophosphoryl-ethanolamines, *i.e.* native "cephalin B"¹¹, the other (220 $\mu\text{g P}$) was pure lysophosphatidylethanolamine**. Analytical char-

* The quantitative estimation of the different phosphatides was based on analysis of carboxylic esters⁷, enolethers⁸, phosphorus⁹, alkali stable phosphorus³, as well as acid and alkali stable phosphorus¹⁰, and also on results of preparative mild acid hydrolysis.

** The purity of the lysophosphatidylethanolamines was ascertained by thin layer chromatography³, and analysis of enolethers, carboxylic esters, glycerol¹², phosphorus and alkali labile as well as acid stable phosphorus¹⁰.

Table 1. Characterization of the different ethanolamine containing glycerophosphatides isolated from ox brain.

Phosphatide	Appearance on thin layer chromatography ^a	Glycerylethers identified in hydrolysates ^c	Molar ratios				
			Acyl ester	Acid and alkali stable P	Alkali stable P	Vinyl ether	Apparent glycerol
			P	P	P	P	P
Original mixture	Pure spot A ^b		1.32	0.04	0.55 ^d	0.50 ^e	1.02
Native plasmalogens	Pure spot A		0.99	0.08	0.85 ^d	0.76 ^e	0.98
Native "Cephalin B"	Pure spot A	+	1.28	0.67 ^d			
Phosphatidyl ethanolamins	Pure spot A		1.78	0.09	0.09	0.00	0.89

^a Silica Gel G; chloroform-methanol-water (65:25:4); lipid samples containing about 2 μ g P were applied; staining by charring with sulphuric acid.

^b Spot A symbolizes the spot obtained with synthetic L- α -(dipalmitoyl)-phosphatidylethanolamine.

^c Glycerylethers were identified as described elsewhere ¹⁰.

^d We believe that the figure obtained is too low because the "monochain derivatives" of glycerophosphorylethanolamine are partitioned in the neutral Folch-system so that about 7 % of them escape into the aqueous layer ³. This fraction, which appears as labile phosphatide, is probably still greater in the basic Folch-system actually used.

^e Preparative acid hydrolysis (see text) suggests that the figure obtained is too low.

acterization of the native "cephalin B" (Table 1) showed that although the preparation was still contaminated by small amounts of phosphatidylethanolamines it was, nevertheless, pure enough to allow definite establishment of its acylated alkoxy structure. In 1960 Svennerholm and Thorin ¹³ already reported strong, but indirect, evidence for the presence of an acylester group in the native "cephalin B" of ox brain.

Treatment of the original mixture of the ethanolamine phosphatides under the same acidic conditions as above completely destroyed the enolether groupings of the plasmalogens in this sample too, and gave 5 % water soluble and 95 % lipid soluble phosphorus. A sample (470 μ g P) of the lipid soluble fraction yielded fairly pure phosphatidylethanolamines (170 μ g P) and quite pure lysophosphatidylethanolamines ** (310 μ g P) after chromatography on silicic acid. Analytical characterization of the phosphatidylethanolamine fraction (Table 1) showed that the sample contained

15 \pm 6 % native "cephalin B", but that it was free of plasmalogens.

The procedure used for the mild acid hydrolysis converted only about 1–2 % of synthetic L- α -(dipalmitoyl)-phosphatidylethanolamine into lysophosphatidylethanolamine. As the lysophosphatidylethanolamines formed from the plasmalogens were isolated in very pure form, and with a high yield, it seems likely that they, as well as the alkoxy phosphatides, are also fairly stable under the conditions used. Accordingly the procedure seems suitable for the quantitative estimation of the native ethanolamine plasmalogens and also for the analysis of their fatty acids through the lysophosphatides. It thus provides a controlled micro modification of the method of Gray ¹⁴. Therefore it seems also evident that together with mild alkaline hydrolysis the acid treatment used will eventually provide a reliable method for the detection and quantitative estimation of the ether phosphatides.

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Free Radicals in Some Reactions of Ninhydrin

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The similarity between ninhydrin and alloxan has been stressed by many authors¹. Both substances give rise to coloured products in reactions with amino acids. The characteristic product obtained from ninhydrin and amino acids, *i.e.* "Ruhemann's Purple", is considered to have a structure analogous to that of the alloxan derivative murexide. Alloxantin, a substance derived from two molecules of alloxan, has its counterpart in hydrindantin formed by two molecules of ninhydrin.

By the technique of electron spin resonance (ESR) the present authors² have recently shown that free radicals are formed when alloxan is reduced by thiol compounds such as glutathione or cysteine. In view of this result and the analogy between alloxan and ninhydrin, a search has been made for free radicals in the reactions of the latter substance. This note describes some experiments in which free radicals derived from ninhydrin were found.

Experimental. The radical spectra were obtained by a Varian 100 kc spectrometer. All runs were performed at ambient room temperature with the samples contained in a flat aqueous solution cell. The magnetic field was calibrated by the hyperfine splitting field (13.0 gauss) of the radicals derived from peroxyaminodisulphonate. Standard barbiturate and phosphate buffers were used in all experiments. pH of the solutions was measured by standard technique. All reactions with ninhydrin were performed at room temperature.

Results. (i) Free radicals were obtained in buffer solutions of ninhydrin and amino acids. The radical concentration was found to be high, especially in an alkaline medium. No radicals could be detected below pH 7.

(ii) There seems to be no correlation between the radical concentration and the intensity of the purple colour developed in the reaction between ninhydrin and amino acids. No radicals could be detected in an aqueous solution of "Ruhemann's Purple" synthesized as described by MacFadyen³.

(iii) When an equimolar amount of sodium dithionite* was added to a solution of ninhydrin, a high yield of free radicals was obtained. As in the reactions with amino acids, the radical content was higher in an alkaline medium than in a neutral one. No radicals could be detected in a reaction mixture of a pH value lower than 6.5.

* When dithionite is used for the production of free radicals, an excess of this substance should be avoided, as radicals derived from dithionite are sometimes obtained under such a condition. The ESR-spectrum of the dithionite radicals exhibits a very narrow single line. Free radicals from sodium dithionite have been described by Hodgson *et al.*⁴, but the possible occurrence of "false" radicals in reaction mixtures containing dithionite seems not always to have been considered.