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ON THE TYPE OF LINKAGE BINDING FATTY ACIDS PRESENT IN BRAIN WHITE MATTER PROTEOLIPID APOPROTEIN

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Summary: Fatty acids (FA) which constitute about 2.5% by weight of exhaustively delipidated proteolipid apoprotein (PLA) have been shown to be bound mainly, if not exclusively, by ester linkages. Indeed, they are progressively released by mild alkaline hydrolysis, and reduction of PLA with $NABH_4$ yields corresponding fatty alcohols. The FA are not constituents of residual contaminating known lipids except possibly in a very small proportion.

It has been shown that PLA delipidated by dialysis against neutral C:M and C:M acidified with HCl (0.04N) to the point where it is free of lipids demonstrable by TLC and contains only 0.01 to 0.03% phosphorus, still retains about 2.5% (2.2 to 3.0%) FA by weight which cannot be removed by any means of physical separation which have been tried.[1,2] These acids consist mainly of 53-60% palmitic, 8-10% stearic, 25-35% oleic and about 4% unidentified. Various samples of PLA contain 0.02-0.08% sialic acid, <0.03% glycerol, <0.1% total carbohydrates and <0.02% ethanolamine. Choline, sphingosine, inositol and hexosamines could not be detected. Hence the FA present cannot be accounted for in terms of any known lipid except to a very small proportion. It was thus of interest to establish the type of linkage(s) by which these FA are retained with the protein. Are they free fatty acids, occluded in or adsorbed on the protein, or are they covalently linked by ester or amide bonds directly or indirectly to the protein?

Experimental part

General - CH_2N_2, prepared from Diazald (R) (Aldrich Chemical Co., Inc.), was carried with a stream of nitrogen bubbling through its ether solution into the ice-cold solution of materials to be esterified until the yellow color persisted. By this procedure no interference due to impurities present in the diazomethane solution was observed in GLC. Ether was peroxide-free and all operations involving this solvent were done in subdued light. Thereby, in control experiments, quantitative recovery of methyl oleate was obtained from oleic acid. Ether extracts were concentrated under vacuum at 30° in a rotary evaporator; remaining water was codistilled with added toluene-ethanol 1:1. GLC was done at 190° on an 8 ft., 1/4" O.D. column packed with 10% LAC 728 (DEGS) on Diatoport S using an F and M 609 chromatograph with hydrogen flame detector and equipped with a Disc chart integrator (Disc Instruments, Inc.). Samples were injected in toluene solution. Quantitation was done by comparison with added known amounts of internal standards. Glycerol was estimated by a procedure involving hydrolysis with 6N HCl, deionization by passage through mixed ion-exchange resins and spectrophotometric determination of consumed periodic acid in the total neutral effluent. Losses of glycerol by evaporation[3] were thus avoided since the solution did not need to be concentrated. The bases were looked for in these hydrolyzates by TLC after passage through Dowex 1-(OH^-). Detection of choline was attempted by an adaptation to TLC (sensitivity 5 µg) of the method of Chargaff et al.[4]

Action of CH_2N_2 on PLA

To 10 ml of an aqueous solution containing 50 mg of PLA, 1 ml of methanol, 5 ml of ether and 1 ml of a solution of 200 µg of arachidic acid in benzene were added. CH_2N_2 was passed, the ether layer was separated and the aqueous layer was extracted two more times with 10 ml of ether. GLC of the combined extracts revealed only the standard methyl arachidate.

Progressive alkaline hydrolysis

Four ml of 0.4N NaOH was added to aqueous solutions containing 22.9 mg of PLA in 4 ml of water. The mixtures were kept respectively for 30 sec, 30 min and 4 h at room temperature. After cooling in ice and acidification with 6 ml of 0.4N HCl, 200 µg of arachidic acid standard was added and the mixtures were extracted 4 times with 10 ml of ether. CH_2N_2 was passed in the combined ether extracts which, after evaporation of the solvent, were examined by GLC. This showed that after 30 sec of contact with alkali, barely detectable amounts of free palmitic, stearic and oleic acids had been liberated and converted into the methyl esters. After 30 min 10% of the FA present in the original PLA had been released in the ratio of 59% palmitic, 9% stearic and 32% oleic, and after 4 h, 38% of FA had been released in the ratio 73% palmitic, 8% stearic and 19% oleic. The aqueous phase remaining after the extraction was evaporated to dryness, 8 ml of 0.2N NaOH was added, and the mixture was kept for an additional 72 h at room temperature. After the same treatment, GLC showed that another 40% of the original FA had been released; their ratio was 78% palmitic, 12% stearic and 10% oleic. By hydrolysis at room temperature but in 0.37N NaOH, 8% of the FA were released after 3 min, and 50% after 1 h. Similar experiments conducted at 37° showed that after 17 h, 80% of the FA had been released. Methanolysis with MeOH-HCl of the residue of the dried aqueous phase followed by GLC showed that 15% of FA were still present in this residue.

Action of $NaBH_4$ on PLA

A solution of 50 mg of $NaBH_4$ (Ventron Corp., Beverly, Mass.) in 1 ml of water was added at 0° to 4 ml of an aqueous solution containing 22.9 mg of PLA and the mixture was kept 5 h at 25° with occasional shaking. It was then acidified with 2 ml of 1N HCl, extracted 6 times with 10 ml portions of ether and CH_2N_2 was passed through the combined ether extracts. After evaporation, GLC showed that fatty alcohols had been released in the ratio of 72.5% palmityl, 4.5% stearyl and 23% oleyl. If fatty acid methyl esters were present it was only in too small proportions to be detectable.

A similar experiment was carried out for 5 days, a second portion of 50 mg of $NaBH_4$ being added after the 3rd day. After acidification, extraction and treatment with CH_2N_2, a standard of 200 μg of methyl behenate (which does not interfere with the shorter chain alcohol peaks on GLC) was added. GLC showed that 51% of the original FA had been reduced to alcohols and that 11% had been liberated as free fatty acids detected as their methyl esters. The aqueous layer was evaporated to dryness and the residue methanolyzed for 30 h at 100° in a sealed tube with 3 ml of MeOH-BF_3. After dilution with 3 ml of water, the solution was extracted 3 times with 5 ml of hexane, and 200 µg of methyl arachidate standard was added. After evaporation, GLC showed that 19% of the original FA were still present in the residue. No fatty alcohols were detected.

Discussion: From these experimental data it can be concluded that there are no free fatty acids associated with PLA since corresponding methyl esters are not formed by direct treatment with CH_2N_2. The progressive and ultimately nearly quantitative release of free fatty acids by mild alkaline hydrolysis as shown, after acidification, by their conversion by CH_2N_2 into methyl esters, suggests that they are bound mainly by ester linkages. This is confirmed by the reduction of the fatty acyl groups with $NaBH_4$ into fatty alcohols in a yield similar to that observed in the reduction of various esters and lactones in aqueous solvents where reduction is limited by the competing alkaline hydrolysis of the ester at the fairly high pH of the medium.[5] Water soluble esters, for example in the carbohydrate series, are reduced in pure water solution.[6,7] So are the ester bonds in water soluble PLA. It is interesting to note that whereas fatty esters like methyl laurate and methyl myristate are not reduced by $NaBH_4$ in pure water, reduction proceeds, however, quite rapidly at room temperature in water-methanol and in water-dioxane solution; free acids and amides are not reduced by $NaBH_4$ under these conditions.[8] For a discussion of reduction of esters with $NaBH_4$ see ref. 9.

It is clear that the major part, if not all, of the FA in PLA are not constituents of contaminating glycerides or phosphatides or other known lipids since other constituents of these lipids are absent or, when detectable, are found in amounts the sum of which could only account for a small proportion of the FA.

It is not possible at present to localize these ester bonds in proteolipids. They may involve the alcoholic groups of hydroxy amino acids, but more complex structures involving undetected constituents are not ruled out. Particularly remarkable is the fact that these alkali labile ester bonds are, on the other hand, relatively resistant to acid. Indeed, they remain intact after prolonged exposure to acidified C:M under conditions which cause relatively rapid methanolysis of isolated phosphatides by transesterification. It should be mentioned that phosphatides and sulfatides present in crude proteolipid preparations are also much more resistant to methanolysis by transesterification than the isolated lipids. Indeed, some are still present in small amounts in proteolipids after weeks of exposure to the acidified solvent. It is conceivable that the protein offers some kind of protection to these ester bonds by steric effect or electrical shielding.

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