Browning in Phosphatide-Containing Fat Emulsions.* (22852)

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Soybean phosphatides are used as primary emulsifier in many fat emulsion formulations developed for intravenous alimentation. These emulsions, in widespread clinical testing, have produced adverse physiological effects(1) of considerable concern. One of the most obvious possible faults of these emulsions is formation of brownish material, mostly colloidal in nature, some of which occasionally settles after several months of storage. This material should be distinguished from filterable foreign material which may enter inadvertently during homogenization process. Various emulsifiers have been investigated in this laboratory, and the brown material has been observed only in those emulsions containing both dextrose and phosphatides. Since nitrogenous systems undergo a browning reaction in presence of glucose, phosphatides present in fat emulsions may be reacting with glucose to form brown material. The browning which occurs in sugar solutions was described by Maillard(2), as reaction between an amino acid and a reducing sugar. Recently, Schroeder *et al.*(3,4) found that the Maillard reaction takes place only in the dry state or at alkaline pH values in aqueous solutions, since only under these conditions is there a decrease in free amino groups. These same investigators found that another browning reaction in acidic solutions occurs without a decrease in concentration of free amino groups but is accompanied by a decrease in aldehyde groups.

The present investigation was undertaken to determine if the nitrogenous groups found in soybean phosphatides were involved in the formation of the brown material. The technic employed to characterize this brown material was paper chromatography.

Materials. A sample of crude, unbleached soybean phosphatides, obtained from commercial source, was freed of oil by repeated precipitation from acetone and of other impurities by filtration through Hyflo Supercel. Another batch of soybean phosphatides, fractionated in a similar manner by a pharmaceutical company for use in emulsions for clinical testing, was compared with those fractionated in this laboratory. The yeast lecithin was prepared by Dr. D. J. Hanahan(5). Choline chloride and animal phosphatides were obtained from Nutritional Biochemical Corp.‡ Dextrose solutions were prepared with Baker's analytical grade dextrose or were 5% dextrose solutions from the Cutter Laboratories.‡

Methods. Chromatograms were obtained by the method of Hanahan(6) and by procedure similar to that used by Dieckert. In both methods ascending technic was used. Ether solutions as well as water slurries of the various substances were employed. The ninhydrin reagent in buffered methyl cellosolve used by Moore and Stein(7) for determination of amino acids and later by Lea and Rhodes(8) for determination of unhydrolyzed phospholipids, was applied to estimation of amino groups present in dextrose solutions containing phosphatides and choline chloride before and after autoclaving. Autoclaving of various materials was at 121°C for 15 minutes at 15 psi. In the Hanahan method, the solvent was a solution of n-propanol, acetic acid, and water (8:1:1). The paper strips were Whatman's No. 1. In the procedure similar to the Dieckert method, the

* This investigation was supported in part by funds from Office of Surgeon General.

† One of the laboratories of Southern Utilization Research Branch, Agric. Research Service, U. S. Department of Agriculture.

‡ Trade names have been used only for the purpose of identifying equipment or materials actually used in conducting the work, and such use does not imply endorsement or recommendation by the U. S. Dept. of Agric. over other firms or similar products not mentioned.

solvent was a solution of absolute methanol and absolute diethyl ether (1:1 by volume). Glass fiber paper was obtained from H. Reeve Angel and Co.‡ The glass fiber strips impregnated with silicic acid were prepared as follows: 100 g silicic acid were dissolved in 100 ml saturated KOH, filtered and made up to 600 ml with water. Strips were immersed in the resultant potassium silicate for 2 minutes, excess silicate removed, and then the strips were immersed in concentrated HCl for 2 minutes. The strips were washed free of chloride ions with water, then washed twice in methanol and twice in ethyl ether, and allowed to dry in air. All strips were equilibrated for half an hour in vapor of the chromatographic chamber before being lowered into the solvent. Usually, about 2 hours were required for the solvent to advance to the top of a 35 cm strip. Reproducible results were obtained with 10, 20, and 50 μl samples of a 1% solution of various phosphatides. In most instances 20 μl samples were used. Strips were dried in air and then stained for choline(9,10), phosphate esters (11), unsaturated groups(12), primary and secondary amino groups(7), sugars(13), and long chain fatty acid groups by use of saturated solution of Oil Red O, a fat soluble dye, in 60% ethanol.

Results. Extent of ninhydrin reactions was determined quantitatively on 0.04% dispersions of soybean phophatides in water and in 5% dextrose solutions, at various pH values before and after autoclaving. Colorimetric measurements were made at 570 mμ with Beckman Spectrophotometer, Model B. These data, Table I, show that at acid pH values there was a definite increase in ninhydrin color, and therefore in the primary and secondary amino groups, with phosphatide dispersions after autoclaving. A type of browning other than the Maillard type is indicated. In that phosphatide dispersion which was alkaline prior to autoclaving, there was no significant increase in optical density. Evidently, another type of browning not resulting in increase in primary or secondary amino groups occurred.

The same ninhydrin procedure was used to measure changes in optical density after autoclaving of solutions of dextrose alone, 1% solutions of choline chloride in water and in dextrose, and 1% dispersions of pure yeast lecithin in water and in dextrose. Changes in optical density on autoclaving at different initial pH values were also observed. These changes are also given in Table I.

TABLE I. Ninhydrin Reactions.

Sample	pH changes on autoclaving	Increase in optical density on autoclaving*
Soybean phosphatides		
In water	5.98–5.68	1.375
In 5% dextrose	5.93–5.28	1.325
Idem, pH 4	4.00–4.52	1.125
" " 8	8.00–5.50	.100
Yeast lecithin (Hanahan)		
In water	6.90–6.82	.040
In 5% dextrose	6.98–5.42	.146
Idem, pH 4	4.05–4.00	.108
" " 8	8.12–5.25	.098
Choline chloride		
In water	5.93–6.10	–.017
In 5% dextrose	5.79–4.27	.028
Idem, pH 4	4.02–4.78	.081
" " 8	7.99–4.52	.031
5% dextrose sol.		
Unmodified	6.50–4.45	.019
pH 4	3.92–3.86	.040
" 8	7.99–4.32	.031

* 570 mμ.

A chromatographic study of the browning reaction products was initiated to determine whether cephalin and lecithin both entered into the observed browning reaction. The chromatographic technics of Hanahan and of Dieckert were compared. The data are summarized in Table II. Glass fiber strips impregnated with silicic acid were used to chromatograph various phosphatides, brown material isolated from emulsions containing soybean phosphatides, and choline chloride. A 1% dispersion of various phosphatides and of choline chloride in 5% dextrose solution and intimate mixtures of "dry" phosphatides or choline chloride and dextrose(1:5 by weight) were prepared. A comparison of the chromatograms of these substances before and after autoclaving was made. The R_f values are given in Table III.

TABLE II. R_f Values Obtained by Chromatography on Cellulose and Glass Fiber Strips.

	R_f values							
	Reineckate positive & phosphomolybdic acid positive spots		Ninhydrin positive spots		Positive spots for unsaturation		Positive spots for Lipids	Positive spots for Phosphate esters
Materials	Cellulose	Glass fiber	Cellulose	Glass fiber	Cellulose	Glass fiber	Cellulose	Glass fiber
Soybean phosphatides (supplied by pharmaceutical co.)	.74	.27	.06 .65	.02 .81	.72	.26 .78	.77	.04 .30
Soybean phosphatides (fractionated in this laboratory)	.74	.23	.07 .65	.05 .81	.72	.23 .81	—	.05 .31
Yeast lecithin (supplied by D. J. Hanahan)	.68	.07 .31	—	—	.80	.07 .31	—	.04 .31

Discussion. The method employing glass fiber impregnated with silicic acid required only 2 hours for solvent to travel as far as it did in 16 hours by using cellulose strips. Cephalin and lecithin travel at greatly different speeds on glass fiber whereas on paper strips, their R_f values are not too different. Sensitivity determinations for choline with both Reineckate(9) and phosphomolybdic acid(10) tests were made on both cellulose and glass fiber strips. With the phosphomolybdate test, 100 μg of phosphatides were required to give a good positive test for choline on glass fiber strips, but 200 μg were required on cellulose strips. When the Reineckate test for choline was used, 400 μg of phosphatides were required for a good positive test for choline on glass fiber strips, whereas only 300 μg were required on cellulose strips.

The R_f values obtained with ether solutions of animal phosphatides or pure yeast lecithin were identical with those obtained with water dispersions. The R_f values for soybean phosphatides in water were unlike those in ether, but like those in dextrose solution because soybean phosphatides contained naturally occurring sugars, whereas animal phosphatide and yeast lecithin did not. In the presence of dextrose solutions, yeast lecithin formed a compound which gave a positive choline spot with an R_f value of 0.55. The choline chloride, animal lecithin fraction, and soybean lecithin fraction formed compounds with dextrose which gave choline positive spots with R_f values of 0.43-0.44. In all cases except with choline chloride, positive phosphate ester spots were also obtained at these new R_f values, indicating that the reaction products with dextrose still contained the phosphate ester group. There is evidence of reaction between choline-containing phosphatide compounds and dextrose to produce a type of compound containing choline. This compound is produced whether or not the lecithin fraction is autoclaved, and in the mixture of phosphatides, regardless of whether or not water is present during autoclaving process. The resultant complex has an R_f value greater than that of the original choline-containing compound. It is evident from Table III that choline chloride and pure yeast lecithin formed other reaction products with dextrose. The ninhydrin test was strongly positive and gave R_f values for products of choline chloride and lecithin of 0.72 and 0.91, respectively. The reaction product between pure yeast lecithin and dextrose also gave a strong positive test for phosphate ester group at an R_f value of 0.90.

In those phosphatides containing cephalins, a choline positive spot with R_f value from 0.72 to 0.80 was obtained when dextrose and water were present. This choline positive spot was not obtained with choline chloride or yeast lecithin in presence of dextrose, and therefore was probably the result of interaction of primary and secondary amino groups with dextrose to produce tertiary amino complexes.

TABLE III. Summary of Chromatographic Data.

Materials	R_f values: Reineckate positive and phosphomolybdic acid positive spots (lecithin fraction)				Ninhydrin positive spots (cephalin fraction)		
Pure yeast lecithin							
Ether solution	.07	.31					
Water dispersion	.07	.30					
Dispersion in 5% dextrose solution	.05	.29	.55			.61	
Idem, after autoclaving	.09	.31	.55				.91
Water dispersion of product autoclaved dry with dextrose	.05	.28					.92
Choline chloride							
Dextrose solution		.24	.45			.71	
Autoclaved dextrose solution			.44			.72	
Water dispersion of product autoclaved dry with dextrose		.24				.73	
Soybean phosphatides*							
Ether solution		.25			.05		.81
Water dispersion			.44	.80†	.04	.74	
Autoclaved water dispersion			.43	.77	.04	.74	
Dispersion in 5% dextrose solution		.28	.44	.77	.04		.80
Autoclaved dispersion in 5% dextrose		.29†	.44	.75	.04	.75	
Water dispersion of product autoclaved dry with dextrose			.50			.72	
Water dispersion of autoclaved dry phosphatides		.28	.56		.04	.73	
Animal phosphatides‡							
Ether solution	.03	.26			.05	.74	.82
Water dispersion	.03	.26			.04	.73	.82
Autoclaved water dispersion	.03	.26			.04	.73	.82
Dispersion in 5% dextrose solution	.05			.72	.06		.79
Autoclaved dispersion in 5% solution	.04			.73	.04	.70	
Water dispersion of product autoclaved dry with dextrose		.29*	.44	.75	.04	.75	
Brown material isolated from emulsions							
Water dispersion	.03	.26	.52	.80	.03	.76	

* Contains naturally occurring sugars. † Weak spot. ‡ Small amount of lecithin.

The brown material isolated from emulsion containing soybean phosphatides appears to be the result of the reaction between dextrose and both lecithin and cephalin, as choline positive spots with R_f values of 0.52 and 0.80 were obtained on the chromatograms.

Staining of chromatograms of the brown material for unsaturated groups, long chain alkyl groups, and for organic phosphate esters resulted in spots at the same R_f values as those obtained for the ninhydrin positive and choline positive spots. The only exceptions were at those spots with R_f values <0.1. Therefore, the brown material did not contain just amino acids, amines or free choline as degradation products. Dextrose alone had an R_f value of 0.60.

Summary. This investigation gives experimental evidence that at autoclaving temperature (121°C) a type of browning occurs which involves reaction of dextrose with lecithins as well as with amino-groups of cephalins. Therefore, use of pure lecithin as an emulsifier in an oil emulsion containing dextrose in the water phase would not eliminate formation of the brown material. The complex browning reaction is responsible for formation of a colloidal material found in phosphatide-containing fat emulsions prepared for intravenous alimentation, and might be a

possible explanation of certain adverse physiological results occasionally experienced with the use of these emulsions.

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Received September 17, 1956. P.S.E.B.M., 1957, v94.

Production of Mucopolysaccharides by Synovial Cells in a Simplified Tissue Culture Medium. (22853)

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Acid mucopolysaccharides of connective tissue ground substance and synovial fluid "mucin" have assumed increasing importance in the light of mounting evidence that these substances are abnormal in various rheumatic disease states(1,2). Information regarding specific cell or cells of origin of the several acid mucopolysaccharides is contradictory (3,4,5) and biochemical data concerning their biosynthesis in normal tissue are fragmentary. Since most of the information concerning biosynthesis has been obtained from study of microbiologic systems capable of synthesizing mucopolysaccharides(6), it would be desirable to initiate parallel exploration at the level of the mammalian tissue cell. While circumstantial evidence of production of mucopolysaccharide by synovial cells has been obtained by tissue culture methods, previous studies are open to the criticism that the mucopolysaccharides demonstrated may well have been present in the initial culture medium. As early as 1933, Vaubel(7) reported the production of synovial "mucin" by rabbit synovial tissue grown *in vitro* in tissue culture. The production of a mucin clot on acidification of the culture medium, which contained splenic extract, was taken as evidence of "mucin" production. Vaubel regarded this phenomenon as specific for the synovial cell. Murray *et al.*(8) subsequently successfully cultured adult rat synovium *in vitro*. Their observations tended to confirm Vaubel's belief that the synovial cell in tissue culture was morphologically distinct from the "common fibroblast." Although they did not study the products of cellular metabolism in their culture media, they were unable to confirm Vaubel's finding of metachromatic staining of the cytoplasm with toluidine blue. More recently Grossfeld *et al.*(9) advanced further evidence of *in vitro* mucopolysaccharide production using cultures of rat subcutaneous tissue as well as normal and arthritic human synovium. In the culture medium which had supported synovial cell growth they measured mucin clot formation and turbidity following acidification with acetic acid. They noted that these phenomena could be prevented by

* Fellow of National Arthritis and Rheumatism Foundation.
† The Rackham Arthritis Research Unit is supported by grant from Horace H. Rackham School of Graduate Studies.