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Effect of Lipoprotein Lability on Immunological Properties of Human Low Density Lipoproteins.* (25094)

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The effects of aging, freezing and thawing, and heating on immunological properties of human low density lipoproteins were studied to estimate the extent of changes produced by these procedures. Various reports(1-6) have indicated that lipoproteins are labile molecules readily denatured by auto-oxidation and aggregation reactions which may also alter the serological properties of the lipoproteins.

Methods. S_f 3-9 and S_f 10-400 lipoprotein fractions were prepared from human serum by ultracentrifugal flotation procedures(7), characterized by chemical composition(3), and injected into rabbits to produce antisera (7). The lipoprotein antisera did not react with human albumin, γ-globulin, high density lipoproteins, or lipoprotein free serum(7). Agar diffusion and precipitin ring tests were used in the immunological studies. The agar diffusion experiments were performed by plate method of Rheins *et al.*(8). In precipitin ring tests, doubling dilutions of an S_f 3-9 lipoprotein fraction in 0.2 ml volumes were layered over 0.2 ml of anti S_f 3-9 serum in precipitin tubes. These tubes were incubated at room temperature 30 minutes, then observed for appearance of a precipitin ring. Electrophoretic patterns were obtained with Spinco Model R paper electrophoresis system. Duplicate strips were stained with bromphenol blue for protein and sudan black B for lipid. The absorbance of lipoprotein solutions was measured with a Coleman Junior spectrophotometer at 430, 460, and 490 mμ against a distilled water blank. A standard S_f 3-9 lipoprotein fraction containing 1 mg of protein/ml was used in aging, freezing and thawing, and heating experiments. This lipoprotein solution was dialyzed against 15 volumes of 0.15 *M* NaCl containing no ethylenediaminetetraacetic acid (EDTA). In aging studies, S_f 3-9 lipoprotein fractions were stored at 4°C, then titrated at stated intervals. Lipoprotein aliquots were stored with and without aqueous merthiolate, added to a final dilution of 1:10,000, as a preservative. Two procedures were used in freezing and thawing experiments. Procedure I: Five ml of the S_f 3-9 lipoprotein fraction was frozen at –20°C in the deepfreeze and then thawed immediately at 37°C in a water bath. Aliquots were removed for analysis before the first and after first, fifth, and tenth freeze-thaw cycles. Procedure II: Aliquots of an S_f 3-9 lipoprotein fraction, 2.5 ml, were frozen at –40°C in a dry ice-acetone bath, then thawed immediately at 37°C in water bath. Samples were removed for analysis after first, fifth, and tenth freeze-thaw cycles. An S_f 3-9 lipoprotein fraction was warmed at 56°C in water

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TABLE I. Effect of Freezing and Thawing on Titer of S_f 3-9 Lipoprotein Fraction Using Precipitin Ring Test.

No. of cycles	Titer Method I	Method II
0	1:64	1:64
1	"	1:128
5	1:32	1:64
10	1:64	1:128

bath for the heating studies. Samples were withdrawn after 15, 30, and 45 minutes for analysis.

Results. Preliminary aging studies indicated that S_f 3-9 lipoproteins, stored without added merthiolate, lost their specificity toward both S_f 3-9 and S_f 10-400 antisera in precipitin ring and agar diffusion tests after 6 to 8 weeks storage at 4°C. Staining revealed that lipoprotein solutions were grossly contaminated with microorganisms. When S_f 3-9 lipoprotein fractions were isolated and stored aseptically or aqueous merthiolate to final dilution of 1:10,000 was added to the lipoprotein fractions, there was no loss of titre and no bacterial contamination during 8 months storage at 4°C. An S_f 3-9 lipoprotein fraction was frozen and stored at –20°C for 7 months with no loss of antigenicity in precipitin ring titrations.

Ten freeze-thaw cycles did not affect the titre (Table I), agar diffusion pattern, or paper electrophoretic pattern of an S_f 3-9 lipoprotein fraction. However, absorbance of the lipoprotein fraction and presumably lipoprotein denaturation increased with freezing and thawing (Table II). Heating at 56°C for 45 minutes did not alter antigenicity, agar diffusion pattern, paper electrophoretic pattern, or absorbance of S_f 3-9 lipoprotein fractions.

Discussion. Low density lipoproteins are probably destroyed by bacterial action when lipoproteins are stored at 4°C. Since lyophilization or freezing will alter the physical properties of the lipoprotein fraction, aqueous merthiolate may be added to lipoprotein solutions to improve their storage for several weeks employed in chemical and physical investigations. Although freezing and thawing increased absorbance of a lipoprotein solution (indicating lipoprotein aggregation and denaturation), the immunological properties and paper electrophoretic pattern were unchanged. Furthermore, heating at 56°C, a procedure used to inactivate complement, did not alter the immunological properties of lipoprotein fractions. Thus lipoprotein lability does not affect immunological studies with low density lipoproteins utilizing agar diffusion pattern and precipitin ring technics. The ability to fix complement and immunodiffusion constants are altered when high density lipoproteins are aged and denatured(6). Complement fixation and immunodiffusion are, therefore, sensitive procedures for measuring lipoprotein denaturation, while precipitin methods are suitable for estimating lipoprotein concentration(7,9).

TABLE II. Effect of Freezing and Thawing on Absorbance of S_f 3-9 Lipoprotein Fraction.*

No. of cycles	Absorbance 430 mμ	460 mμ	490 mμ
0	.269	.269	.211
1	.269	.270	.215
5	.289	.285	.228
10	.315	.309	.249

* Lipoproteins frozen at –40°C and then thawed immediately at 37°C.

Summary. Human low density S_f 3-9 lipoproteins can be frozen and thawed, stored at –20°C, and heated to 56°C for 45 minutes without altering their immunological characteristics in precipitin ring and agar diffusion tests. Lipoprotein fractions stored at 4°C are decomposed by bacterial action. Aqueous merthiolate added to final dilution of 1:10,000 prevents bacterial contamination in lipoprotein fractions stored at 4°C without altering immunological characteristics of the lipoproteins. Freezing and thawing increases turbidity of lipoprotein solutions but does not alter lipid and protein patterns obtained with paper electrophoresis.

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Protection Afforded Mice Against Tourniquet Trauma Induced Mortality By Ganglion Blocking Drug Chlorisondamine Dimethochloride. (25095)

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Wiggers and co-workers(1) reported that dogs were afforded significant protection against lethal effects of severe hemorrhage by preadministration of the adrenolytic drug dibenamine. They postulated that this protection was due to improved circulation in those tissues whose circulation is usually most severely curtailed as a result of sympatho-adrenal activation, which is a generalized reaction to severe hemorrhage or trauma. Many reports have since described the significant protection afforded experimental animals against lethal effects of traumatizing and/or hemorrhagic procedures, by *pre*-administration of one or more adrenolytic, sympatholytic and/or ganglion blocking drugs. References in 3 of these papers(2,3,4) include most of the literature. Some reports include data on mortalities when the test drug was administered after severe trauma or hemorrhage. The common finding has been failure of the drug to afford test animals significant protection when so administered. In tourniquet trauma, precipitation of the shock syndrome does not occur until after removal of tourniquets. Hence in this type of trauma it should be possible to assure arrival of drug at postulated vital centers before precipitation of the shock syndrome, by lengthening the period of occlusion of circulation to the hind legs, to permit injection of the drug prior to removal of hind leg ligatures. Alternatively, the innate severity of the shock syndrome might be minimized somewhat, at least in theory, by removing the ligatures earlier, and injecting the drug after such removal, when it would presumably be less capable of combating shock. Experiments were therefore initiated with the ganglion blocking drug, chlorisondamine dimethochloride (CD), with the above relationships in mind. While the original problem has not been resolved, our experiments revealed a protective capability of the drug (CD) against tourniquet trauma induced mortality, injected at time of ligature removal, sufficiently great to warrant this report.

Methods. Mice used were Carworth Farms males, about 3 months of age and 20-30 g body weight. Tourniquet trauma was inflicted by occluding circulation to both hind legs for about 2 hours using rubber bands(5). Control mice were injected with water, test mice with an aqueous solution of chlorisondamine dimethochloride. Other procedures are indicated in Table I.

Results. Table I gives data for 2 experiments, performed in the same manner, with similar results. The drug dose (5 mg/kg) was one which earlier experiments indicated would significantly increase the likelihood that mice would survive tourniquet traumatization, when the drug was injected at time of ligature removal. Experiments of Table I were designed to test whether degree of protection was measurably altered when the drug was administered 15 minutes, rather than zero minutes, after removal of hind leg ligatures. Since it was considered essential that the time between application of ligatures and injection of drug or vehicle (water) be kept constant (at 2 hours), this necessarily resulted in a ligation period of 120 minutes for mice of Groups A_1