quantitatively estimated by determinations of the rate of cellular respiration. The results were checked by morphological examination of the frozen incubated tissue and by *in vivo* growth. 2. Measurements of the rate of respiration were reliable as an index of survival if the cells were incubated for more than 2 hours at 37°C following freezing, and if they were suspended in a physiological medium compatible with normal survival and recovery of injured cells. 3. Krebs-Ringer-Phosphate medium was unsuitable, but equal parts of partially neutralized horse serum and Krebs-Ringer-Phosphate provided an adequate medium. 4. The respiration and survival of sarcoma-37 cells, pretreated for 2½ to 3 minutes in the ethylene glycol in Krebs-Ringer solution then frozen in liquid air was 50 to 60% of the unfrozen controls. 5. The respiration of untreated frozen sarcoma-37 was not usually significant. Morphological analyses of these tissues indicated fewer than 1% surviving cells in most experiments. 6. No significant difference in survival was observed between the rapidly and slowly frozen tissues. 7. Good correlation was observed between the morphological and respiratory characteristics of the frozen incubated tumor cells.

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Role of Heparin in *in vitro* Production of Alpha$_1$ Lipoproteins in Human Plasma. (19915)

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Recent studies on lipid transport and metabolism(1,2) have demonstrated a marked alteration of several classes of low-density lipoproteins in plasma following heparin injection. Further information concerning the nature and mechanism of action of this "lipemia clearing system" has recently been reported from this laboratory(3,4). In these latter studies it was found that the lipoprotein changes previously observed *in vivo* could also be demonstrated in an *in vitro* system employing several purified plasma proteins.

The present communication deals with the nature of the products formed during the clearing reaction. The data below demonstrate that alpha lipoproteins are formed *in vitro* in the plasma of individuals containing the essential components of the clearing system.

Methods. Eight healthy young adult males

FIG. 1. Lipoprotein boundaries before and after *in vitro* clearing of human plasma. Upper image: immediately after intrav. heparin. Lower image: after 3 hr incubation. The lipoproteins are ascending at a saline density of 1.21. Frame 1: 50000 r.p.m. Frame 2: 12 min. at max speed. Frame 3: 30 min. at max speed.

were treated in the following manner. Approximately 3½ hours following a high-fat breakfast, 25 mg of heparin (2.5 ml of Liquaemin) were injected intravenously. Within the next two minutes, 80-120 ml of blood were withdrawn and immediately transferred to a stainless-steel coil immersed in ice water. This rapid cooling delayed the enzymatic clearing reaction. Either heparin or Sequesterene was used as anticoagulant. The choice of anticoagulant had no effect on the results described below. Plasma was separated at 4°C by centrifugation for 10 minutes at 4,000 rpm. The clearing activity of 2 aliquots of plasma was irreversibly destroyed by bringing them to saline densities of 1.063 and 1.21 respectively by the addition of appropriate quantities of a concentrated NaCl-KBr solution. The remainder of the plasma was incubated at 37°C for 3 hours to allow the clearing reaction to continue. Two additional aliquots were then adjusted to densities of 1.063 and 1.21 respectively. All 4 samples were centrifuged in the Spinco preparative centrifuge at 40000 rpm (105,400 x Gravity) for 18 hours at 4°C. The plastic tubes were sliced by means of a sharp blade in a rigid holder at identical levels for each pair. The top samples at a density of 1.21, containing both low-density and alpha lipoproteins, were centrifuged simultaneously in the wedge and ordinary analytical cells of the Model E Spinco ultracentrifuge. These runs were made at 25 ± 1° at 59780 rpm. Photographs were taken during acceleration at 10000 rpm, 30000 rpm. and 50000 rpm, and at 0, 2, 6, 12, 20, and 30 minutes after attaining maximum speed.

In the plasma samples at a density of 1.063, the low-density lipoproteins accumulated at the top of the centrifuge tube. The alpha-lipoproteins, having a density greater than 1.063, sedimented toward the bottom of the tube. The 10 cm long tubes were therefore guillotined 19 mm from the top, where the solution was lipoprotein-free. The top and bottom material was quantitatively recovered and each sample analyzed in duplicate for lipid content. Conventional methods of extraction were tried, but the following proved the most satisfactory. Proteins were precipitated with sodium tungstate and the water soluble substances were removed by washing. The lipids were then extracted from the proteins with aqueous ethanol-ether. Aliquots of the extract were analyzed for free and total cholesterol by the method of Schoenheimer and Sperry(5); for lipid phosphorus by the method of Fiske and SubbaRow(6); and for total lipids by a recently described colorimetric method(7). Lipid analyses were carried out on six of the eight cases reported here.

Results. During the 3-hour incubation of all plasma samples examined, analytical ultracentrifugation revealed the qualitative shifts within the low-density lipoprotein spectrum from those of lower to those of higher density, previously described by Graham *et al.*(2) in their *in vivo* studies on the effects of intravenous heparin administration. In addition, however, there was a significant quantitative increase in the alpha$_1$ lipoprotein component, a change not previously reported. Fig. 1 illustrates these changes in case No. 4 (Table I). It may be seen in frame No. 1 that the S_f 51-300 class molecules (S_f 10-80 at density 1.063) have disappeared in the 3-hour sample and, in frame No. 3, that there is a quantitative increase in the alpha$_1$ lipoproteins. The

TABLE I. Lipoprotein and Lipid Shifts Occurring *in Vitro* in Human Plasma, 8 Cases, During a 3-Hr Incubation Period Following Intravenous Heparin. Quantities are presented as mg/100 ml of plasma. The lipoprotein changes were quantitated in the analytical ultracentrifuge. The chemical changes represent lipid shifts from the top to the bottom of preparative tubes containing plasma at a saline density of 1.063.

S_f 100-300* S_f 27-80 †	S_f 74-100 S_f 20-27	S_f 51-74 S_f 10-20	S_f 31-44 S_f 3-8	S_f 2-8 —	Phospho-lipid	Ester choles-terol	Neutral fat and fatty acid
0	0	—19	+ 9	+ 3	5	1	20
—17	— 6	—45	—15	+17	12	3	38
0	—15	—37	—14	+35	—	—	—
— 5	—13	—28	+10	+43	17	—	75
0	—15	—15	0	+44	12	2	55
0	—	—17	—	+51	19	3	62
—28	—34	—31	—43	+61	—	—	—
—54	— 5	— 2	+18	+75	28	5	100

* Flotation rate at density 1.21.
† " " " " 1.063(8).

quantitative results for 8 cases studied are presented in Table I. In several samples, the alpha$_1$ lipoproteins migrated upward as two distinct components, which we have termed alpha$_{1,2}$ and alpha$_{1,1}$ lipoproteins respectively. The more slowly rising component, alpha$_{1,1}$, appears to increase specifically during the action of the clearing system. These changes have never been observed in control samples obtained before heparin injection.

Quantitation of the very low density, high molecular weight lipoproteins of S_f 300 and above cannot be satisfactorily carried out in the analytical ultracentrifuge since these molecular classes are not sufficiently discrete to yield boundary resolution, and, in addition, scatter light intensely. Lipid analyses, however, of the samples at a density of 1.063 indicated that, during the clearing process, lipid disappeared from the top of the tube and could be quantitatively recovered from the bottom. Triglycerides and fatty acids, phospholipids, and, to a lesser extent, cholesterol esters diminished in the top fraction and increased in the bottom. The extent of these lipid shifts is presented in Table I. It should not be inferred that triglycerides were incorporated into the alpha$_1$ lipoprotein molecules. Certain unpublished chemical data indicate that, during the clearing process, triglycerides in the low-density lipoproteins are hydrolyzed. In the present experiments, therefore, the fatty acids and resulting soaps would have distributed themselves equally throughout the sample, thereby raising values in the bottom fraction. At the present state of our investigations, the figures in Table I are significant only to the extent of indicating the amounts of lipid liberated from the low-density lipoproteins.

Summary. 1. An absolute increase in high-density alpha$_1$ lipoproteins, and a concomitant decrease in certain low density lipoproteins, have been demonstrated by ultracentrifugal and chemical means. 2. These changes occurred *in vitro* in plasma obtained after heparin injections and suggest, on the basis of earlier studies on the nature of the lipemia-clearing reaction, an enzymatic conversion of one lipoprotein class to another.

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