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FURTHER CHARACTERIZATION OF THE HUMAN SERUM D 1.063–1.21, α_1–LIPOPROTEIN *

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The high density lipoprotein class floating at a solvent density between 1.063 and 1.21 g per ml can be fractionated by flotation in solvents of intermediate density into two major subfractions (1, 2), which according to Shore (3) have a protein moiety with identical amino acid composition and C- and N-terminal amino acids. Homogeneity of the D 1.063–1.21 lipoprotein in terms of protein moiety has also been indicated by immunochemical studies (4). It would appear, therefore, that the human serum high density lipoprotein of D 1.063–1.21 constitutes a group of molecules identical as to protein moiety and possibly differing only in lipid complement. The experiments reported below were designed to test this hypothesis. The serum D 1.063–1.21 lipoprotein was arbitrarily fractionated into three fractions floating respectively at solvent densities of D 1.063–1.125, 1.125–1.168, and 1.168–1.21. The results dealing with some of the physicochemical and biological properties of these lipoprotein subfractions form the object of this report.

MATERIAL AND METHODS

The source of the lipoproteins was pooled sera from healthy human male subjects, fasted for at least 12 hours. All separations of lipoproteins were performed in a Spinco model L ultracentrifuge, 30.2 rotor, at 79,420 G and 16° C for 24 hours. Sera were first adjusted to D 1.063 by addition of solid NaCl. After ultracentrifugation, the top fractions, containing lipoproteins of densities between 1.006 and 1.063, were discarded and the remainder adjusted to D 1.125 by addition of solid KBr. After ultracentrifugation, the top fractions were collected (lipoproteins of D 1.063–1.125), the remainder adjusted to D 1.168 by adding solid KBr and ultracentrifuged. The top fractions thus obtained (lipoproteins of D 1.125–1.168) were removed and the remainder, brought to D 1.21 with solid KBr, ultracentrifuged to float the lipoproteins of D 1.168–1.21. Aliquots from the same normal human pooled sera were used to prepare the whole high density lipoprotein class of D 1.063–1.21 according to the method of Lewis, Green and Page (5). From each of these lipoprotein classes, possible contaminating serum proteins were removed by ultracentrifugation in a medium of density 1.21, as previously reported (6). Ultracentrifugal analysis of these purified lipoproteins was performed in a model E ultracentrifuge, at 52,620 rpm and 26° C.

All lipoprotein samples were dialyzed against large volumes of 0.15 M NaCl at 4° C for 24 hours before use. Protein (7), cholesterol (8), and phospholipid (9) content of each lipoprotein was also determined. Removal of lipids from the D 1.063-1.21 lipoprotein class was performed according to Scanu, Lewis and Bumpus (10).

I^{131}-labeling of the lipoprotein proteins was performed by a technique essentially identical to that of McFarlane (11). Free iodine was removed by passing the radioiodinated protein three times through an anion exchange resin, Ioresin (Abbott Laboratories, North Chicago, Ill.). The labeled proteins were estimated to have about 1 atom of iodine per molecule.

Moving boundary electrophoresis was determined by the Longworth modification of the Tiselius method employing barbital buffer, pH 8.6, ionic strength 0.1. Paper electrophoretic analyses were carried out in a Durrum cell (12) with barbital buffer of pH 8.6, ionic strength 0.05. Bromphenol blue was used for protein staining and Sudan black for lipid staining. Scanning of the strips was performed in a Spinco Analytrol, model RA. Starch gel electrophoretic analyses were conducted at 26° C according to Smithies (13) with the discontinuous system of buffers (Tris–boric acid, pH 8.2) proposed by Poulik (14). Amido Schwartz 10 B Bayer was used for protein staining and oil red O Spinco for lipid staining. When labeled samples were used the curve of radioactivity of the starch patterns was obtained as reported previously (6).

Studies of the ultraviolet absorption spectra of the various lipoprotein fractions were carried out in a Beckman DK-2 automatic ratio recorder, with 0.01 per cent solutions of protein in 0.15 M NaCl.

Amino acid composition of lipoprotein proteins was determined by the use of a Spinco amino acid analyzer, model 120, at 50° C. For the analysis, lipoproteins were extracted with a 3:1 ethanol:ether mixture for 2 hours

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at 26° C, and the delipidated protein was hydrolyzed in 6 N HCl at 110° C for 18 hours. The hydrolysate was dried by evaporation, and the HCl removed by the addition of distilled water and by drying, three times. The final dry residue was dissolved in a 5-ml solution composed of 1 ml of 1 per cent HCl and 4 ml sodium citrate buffer, pH 2.2. The acidic amino acids were separated with 150-cm columns of a cation exchange resin, particle size 31–41 μ (Aminex-MS, blend Q-150, Bio-Rad Laboratories, Richmond, Calif.) with a sodium citrate buffer, pH 3.25 to 4.25, as eluents. The basic amino acids were separated on a 15-cm column of an ion-exchange resin, type 15 A, particle size 19 to 25 μ (Spinco Division, Palo Alto, Calif.). The amount of material used for each column was 0.1 μmole of high density lipoprotein protein. The reproducibility of the method was within a limit of 3 to 4 per cent.

In the *in vivo* experiments lipoproteins labeled in the protein moiety with I^{131} were injected into human subjects, dogs, and mice. The radioiodinated material was sterilized before use through Swinny filter discs contained in special B-D adapters (Becton, Dickinson and Co., Rutherford, N. J.). The sterility of the material was checked by bacteriological analysis. The human subjects used in these experiments were two apparently healthy males, 40 to 45 years of age, weighing 60 to 70 kg, who were hospitalized throughout the experiment in the metabolic unit of the Medical Department of the Brookhaven National Laboratory, Upton, N. Y. A third human subject was an apparently healthy 35 year old employee of the Cleveland Clinic Foundation. All subjects received daily 20 drops of Lugol's solution in their drinking water to prevent radioiodine uptake by the thyroid.

The dogs used in the experiments were male mongrels, 1 to 2 years of age, weighing 10 to 14 kg. They were kept on a regular balanced diet, with the addition to their drinking water of 10 drops of Lugol's solution.

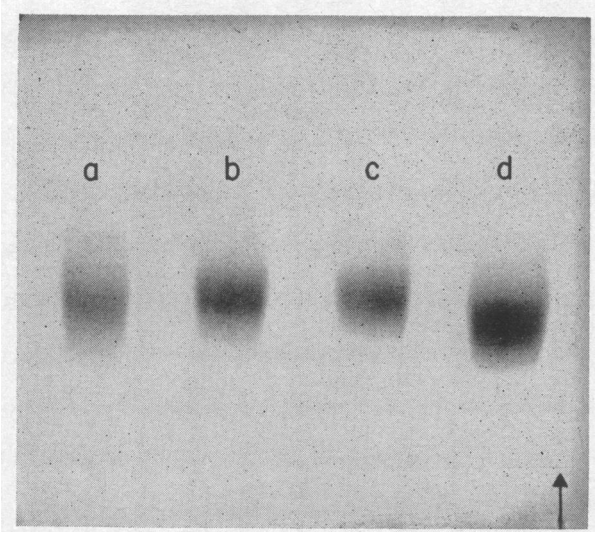

FIG. 1. STARCH GEL ELECTROPHORETIC PATTERN OF HUMAN SERUM HDL_t AND ITS SUBFRACTIONS I, II, AND III. Staining: amido Schwartz. a = HDL I, b = HDL II, c = HDL III, and d = HDL_t.

TABLE I

Percentage distribution of protein, cholesterol, and phospholipids in the D 1.063-1.21 lipoprotein and subfractions

Lipoprotein class	Flotation coefficient	Protein	Cholesterol	Phospholipids
	$-S$	%	%	%
D 1.063–1.21	5.0	50.0	18.5	33.2
D 1.063–1.125	5.0	46.0	20.0	33.0
D 1.125–1.168	4.7	53.0	18.5	29.4
D 1.168–1.21	3.9	58.0	14.5	27.5

In the human subjects and in the dogs, venous blood was withdrawn at intervals to determine the rate of disappearance from circulation of the radioiodinated lipoprotein protein. Radioactivity was also determined in the urine samples collected daily. Measurement of radioactivity of serum and urine samples from human subjects (3 ml) and from dogs (1 ml) was carried out in a sodium iodide crystal scintillation detector (Tracer-Lab, Inc.) with a counting efficiency by means of an I^{131} standard of about 33 per cent.

In the human subjects, whole-body measurements of radioactivity were also performed by use of the Brookhaven iron-room, whole-body counter, with an 8 × 4 inch KI crystal and 100-channel gamma ray spectrometer.

The male albino mice were of an average weight, 25 g. They were fed a regular Purina chow diet and were given distilled water containing 0.1 g per 100 ml of sodium iodide. Whole-body measurements of radioactivity were performed according to the technique of Terres, Hughes and Wolins (15) by placing the mouse in a nylon container which was then inserted into the well of the scintillation detector. Prior to each measurement the urinary bladder of the mouse was emptied by compression.

All counts were corrected for physical decay to the time of injection and then plotted as a percentage of the radioactivity injected against time on semilogarithmic paper. The half-time values of disappearance of the radioiodinated protein from plasma or from whole body were determined from the exponential straight portion of the curve believed to represent degradation of the injected protein. The lines were fitted to the data by the method of least squares.

In the human subjects and in the dogs, the daily urinary excretion of radioactivity was also determined. These values, corrected for physical decay, were expressed as a percentage of the total radioactivity injected per day. About 95 per cent of the radioactivity lost from the body was recovered in the urine.

RESULTS

The spectrophotometric analysis of HDL_t[1] and its subfractions I, II, and III was run in triplicate.

[1] The abbreviations used in the text are: HDL_t = high density lipoprotein of D 1.063–1.21; I = HDL 1.063–1.125; II = HDL 1.125–1.168, and III = HDL 1.168–1.21. αP is the protein from HDL_t after removal of the lipid.

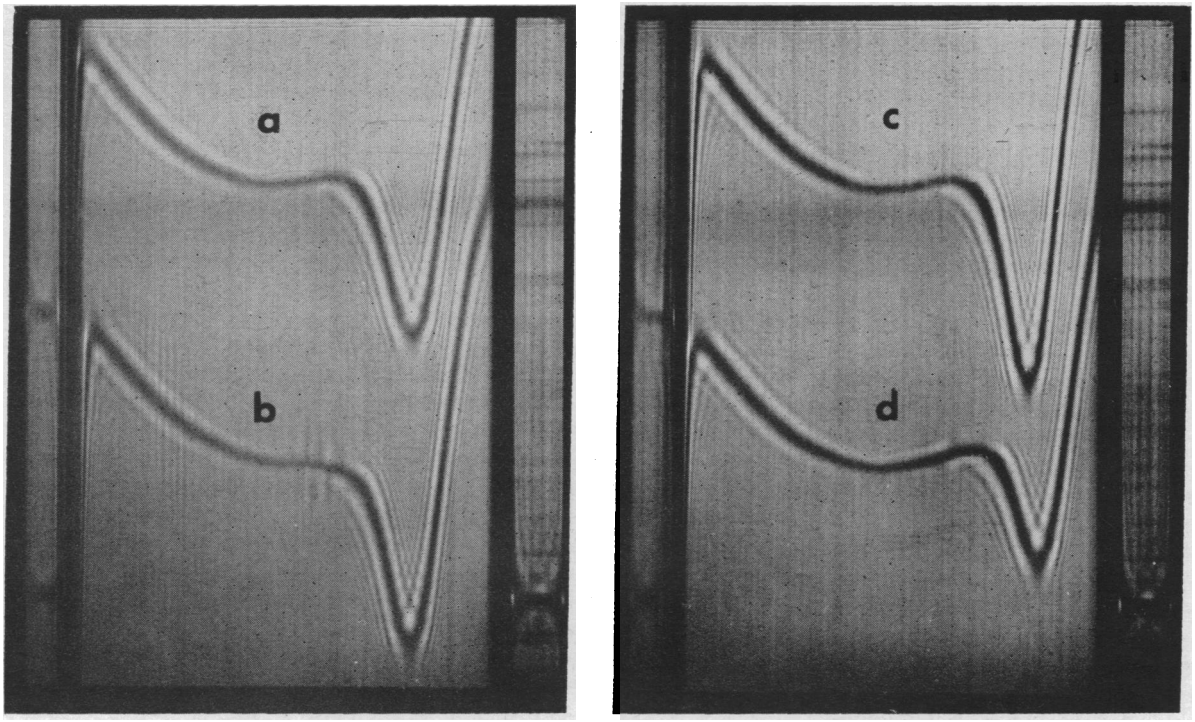

FIG. 2. ANALYTICAL ULTRACENTRIFUGAL ANALYSIS IN A MEDIUM OF DENSITY 1.21 OF HDL_t AND ITS SUBFRACTIONS I, II, AND III. Each sample contained 1 mg of lipoprotein protein. a = HDL_t, b = HDL I, c = HDL II, and d = HDL III; 52,640 rpm, 36 minutes, 26° C.

Each lipoprotein fraction had an identical ultraviolet spectrum with a peak of maximal deflection at 280 mμ. By free boundary electrophoresis, HDL_t showed a single peak with a mobility of -5.00×10^{-5} cm^2 per v per second. The three HDL subfractions exhibited a similar electrophoretic mobility.

Paper electrophoretic analysis did not show sig-

TABLE II

*Amino acid composition of the protein moiety of human serum HDL 1.063-1.21 and subfractions**

Amino acid	D 1.063–1.125	D 1.125–1.168	D 1.168–1.21	D 1.063–1.21	αP†
Aspartic	40.7	42.8	46.2	42.0	42.6
Threonine	29.8	27.3	26.4	26.1	23.7
Serine	38.6	37.9	39.2	41.7	39.2
Glutamic‡	100.0	100.0	100.0	100.0	100.0
Proline	34.7	38.2	36.2	38.4	37.5
Glycine	23.1	23.8	28.1	28.1	26.1
Alanine	41.0	38.2	37.6	38.7	39.2
Valine	29.0	27.9	28.8	25.9	24.8
Methionine§	3.59	2.53	2.62	3.10	2.62
Isoleucine	5.08	4.42	4.96	3.83	3.26
Leucine	72.0	74.0	74.8	69.7	73.1
Tyrosine	18.3	18.4	20.7	18.6	12.7
Phenylalanine	19.2	20.3	21.6	23.1	23.1
Cysteine					
Lysine	47.7	48.6	49.8	49.2	51.2
Histidine	5.70	8.20	6.4	6.70	7.90
Arginine	32.4	30.5	33.6	34.1	36.2

* Per cent glutamic acid = 100. Traces of methionine sulfoxides were found, and no cysteic acid.
† αP is the protein from HDL_t after removal of the lipids.
‡ 96 moles per mole of HDL protein, assuming mol wt of 75,000 (10).
§ Values corrected to 100 per cent recovery, assuming a 5 per cent loss of methionine in the procedure employed (15).

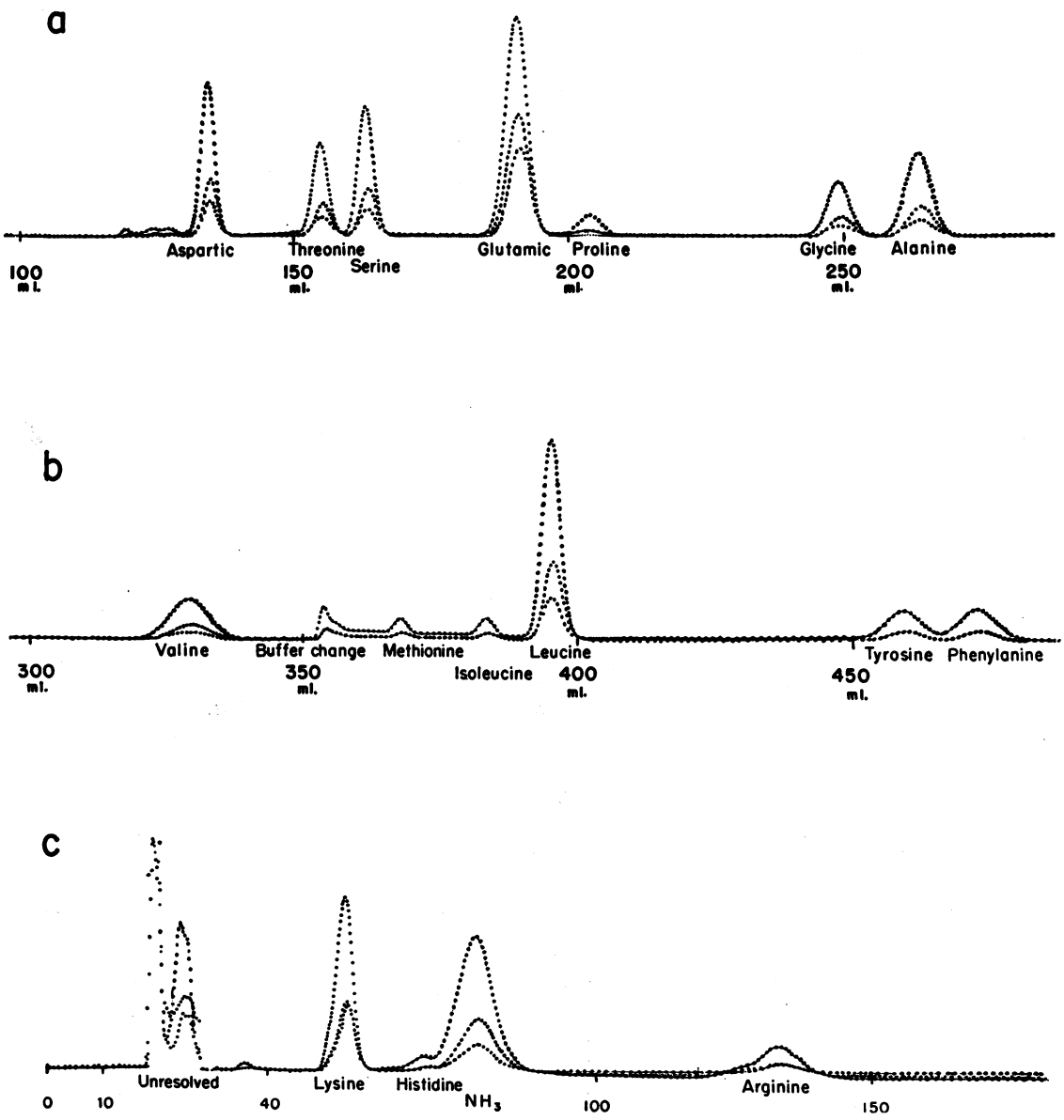

FIG. 3. TYPICAL CHROMATOGRAM OF A SAMPLE OF HDL_t PROTEIN, IN ACID HYDROLYSATE. a, b, and c indicate the sequence of the chromatogram.

nificant difference in the position of the boundaries of HDL_t and its subfractions. The boundary was in the area occupied in a reference electrophoretic pattern of normal human serum, by α_1-globulin. By starch gel electrophoresis, the HDL subfractions exhibited a slightly different velocity of migration, the electrophoretic mobility being as follows: III > II > I. A representative pattern is shown in Figure 1. HDL_t and its subfractions all exhibited a major broad dark-stained boundary in an area almost half-way between the origin and the albumin zone. In front of each boundary there was a little hazy area indicating the possible existence of a minor, poorly defined boundary. This was shown by both protein and lipid staining.

By the analytical ultracentrifuge in a medium of D 1.21, HDL_t showed a peak of maximum deflection with a coefficient of $-S$ 5. The three HDL subfractions, also analyzed in a solvent of D 1.21 (Figure 2), floated at different rates with $-S$ values of I = 5, II = 4.7, and III = 3.9. These analyses were run in triplicate and showed

a variation of less than 0.2 per cent. Within these three subfractions, the protein distribution was as follows (HDL_t protein = 100 per cent): 47 per cent in I, 37 per cent in II, and 16 per cent in III. We have reported in Table I the chemical composition of HDL_t and subfractions in terms of their relative percentage of protein, cholesterol, and phospholipids. These data show that the HDL subfractions that floated at higher density had a higher content of protein and consequently less cholesterol and phospholipids.

The amino acid composition of the protein of HDL_t and subfractions is reported in Table II. The data include those obtained on human serum αP.[2] All these proteins appeared to have a similar amino acid composition. In Figure 3 we have reported as an example a typical chromatogram obtained from an hydrolysate of HDL_t protein. Of significance is the absence of the peaks corresponding to tryptophan, cystine, and cysteic acid, and the low content of methionine. Small peaks emerging just ahead of aspartic acid suggest the presence of trace amounts of methionine sulfoxides. Two other minor peaks were not identified.[3]

[2] αP is the protein from HDL_t after removal of the lipids.

[3] For comparison, similar chromatographic analysis was performed on the human serum D 1.006–1.019 and D 1.019–1.063 low density lipoproteins. These two classes of lipoproteins showed an essentially identical amino acid composition (data referred to glutamic acid = 100): aspartic = 88.5, threonine = 55.4, serine = 81.0, glutamic = 100, proline = 36.5, glycine = 40.3, alanine = 58.5, valine = 29.6, methionine = 0.72, isoleucine = 34.3, leucine = 97, tyrosine = 24.5, phenylalanine = 47.2, cysteine = 0, lysine = 57.6, histidine = 15.1 and arginine = 28.9.

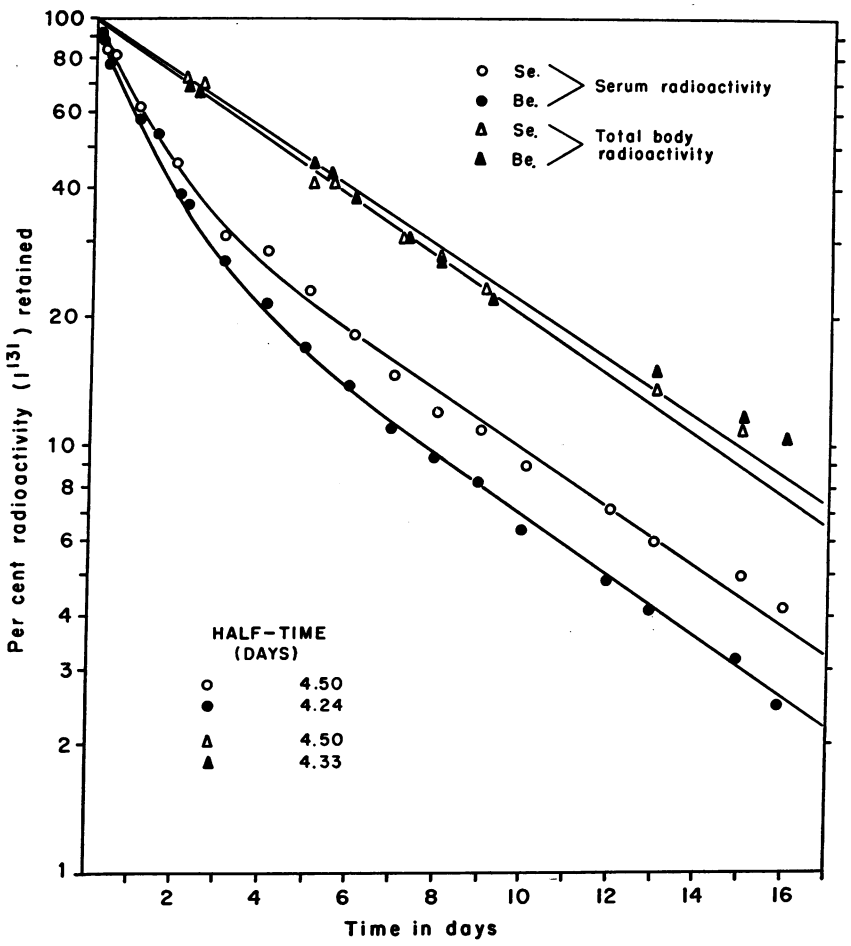

FIG. 4. CURVES OF DISAPPEARANCE OF HUMAN SERUM HDL_t, LABELED IN THE PROTEIN MOIETY WITH I^{131} FROM THE WHOLE BODY AND FROM THE PLASMA OF TWO NORMAL HUMAN SUBJECTS.

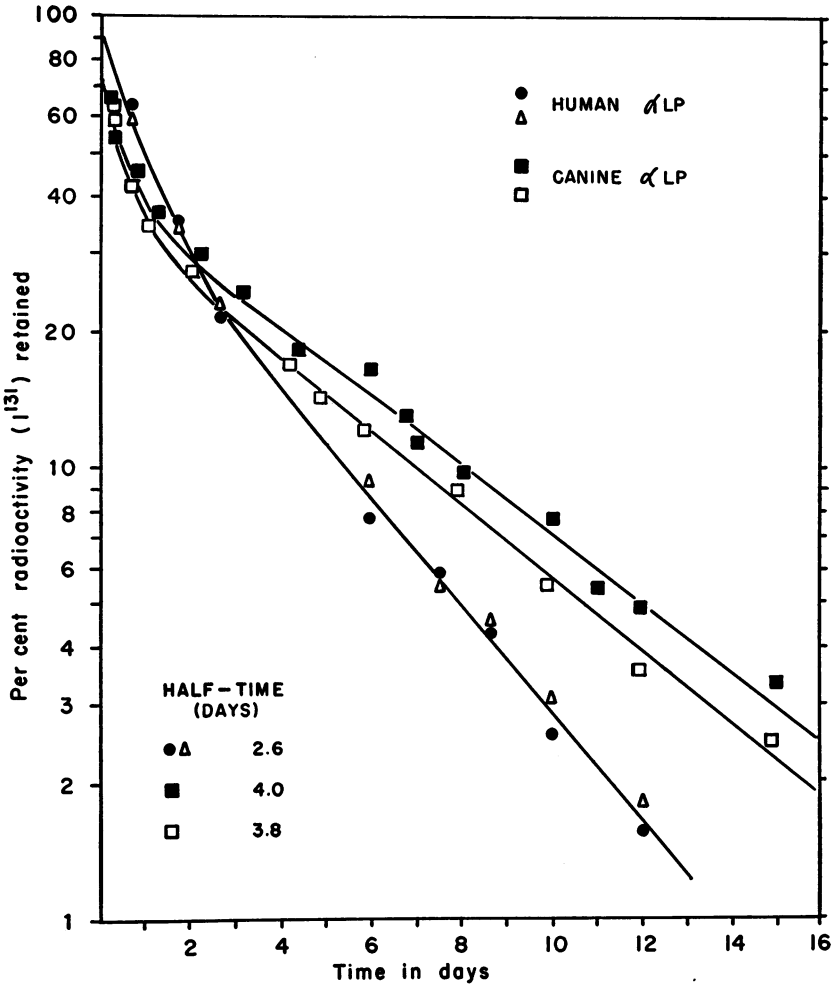

FIG. 5. CURVES OF DISAPPEARANCE OF HUMAN SERUM HDL_t, LABELED IN THE PROTEIN MOIETY WITH I^{131} FROM THE PLASMA OF TWO NORMAL DOGS.

In vivo studies

Human subjects. Two subjects were injected intravenously with approximately 1 mg of HDL_t protein of a specific radioactivity of 14 μc per mg. The curves of disappearance of radioactivity from circulation are reported in Figure 4. In both cases the phase of the curve indicating metabolic degradation of the injected radioiodinated lipoprotein protein was monomial with a half-time in days of 4.24 and 4.50. Values of half-time were very similar whether obtained from measurement of radioactivity in plasma or in whole body. The injected labeled lipoprotein protein traveled in the plasma with the D 1.063–1.21 lipoprotein class. No radioactivity was encountered in the other plasma liproprotein. A third human subject was given intravenously 1 mg of labeled human serum αP of a specific radioactivity of 25 μc per mg. This protein was removed from circulation with a half-time of 3.9 days and traveled only with the D 1.063–1.21 lipoprotein class. The distribution of radioactivity in the three HDL subfractions was also studied. Each protein had identical specific radioactivity.

Dogs. Two normal dogs were injected each with 1 mg of human serum HDL_t protein and two other dogs with 1 mg of canine serum HDL_t. In both preparations the radioiodinated protein had a specific activity of approximately 25 μc per mg. The results, shown in Figure 5, indicate that both human and canine HDL_t proteins, after the first period of equilibration, were removed

from circulating plasma according to a monomial exponential line. However, the human HDL_t protein was removed from circulation more rapidly (half-time, 2.6 days) than canine HDL_t protein (half-times, 3.8 and 4.0 days). In both experiments the injected labeled protein traveled in the plasma D 1.063–1.21 lipoprotein class.

In another series of experiments two dogs were injected intravenously with I^{131}-labeled human serum αP. Each dog was given a 1-mg sample of specific radioactivity of 25 μc per mg. The half-time values of plasma disappearance were similar to those obtained with the labeled native human HDL_t protein and likewise traveled in the D 1.063–1.21 lipoprotein class. Forty-eight hours after the injection of the labeled material, the recipients' HDL fractions I, II, and III contained protein of the same specific radioactivity.

Mice. They were divided into four groups, each including 12 animals. Human serum HDL_t and its subfractions I, II, and III were labeled in the protein moiety with I^{131} and each fraction employed for one group of mice. Each mouse was injected intravenously with 0.1 mg of labeled protein of a radioactivity of approximately 10 μc. Whole-body counting was used to follow the rate of degradation (16) of the injected labeled protein. The results summarized in Figure 6 show that the protein moiety, whether of HDL_t or its three subfractions, was degraded by the mice at identical rate. To study the distribution of radioactivity among the plasma lipoproteins, groups of 6 mice were bled by heart puncture after opening of the chest under ether anesthesia. The ultracentrifugal analysis of the pooled plasma showed that the radioactivity was contained only in the D 1.063–1.21 lipoprotein class. No attempts were made to study further the distribution of radioactivity in the three HDL subfractions.

Another group of 12 mice was injected with I^{131}-labeled human serum αP and the blood withdrawn 12 hours later. The ultracentrifugal analysis showed that all radioactivity was contained in the plasma D 1.063–1.21 lipoprotein class.

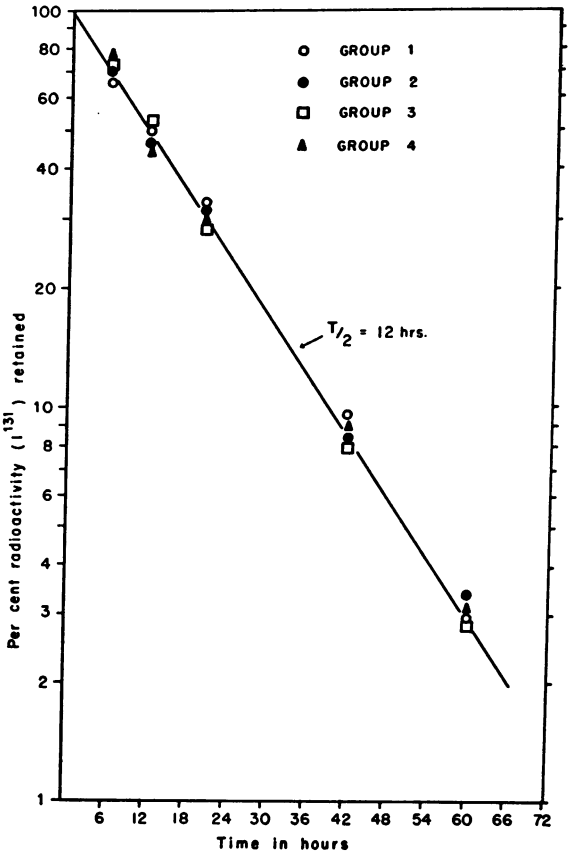

FIG. 6. DISAPPEARANCE OF RADIOACTIVITY FROM WHOLE BODY IN FOUR GROUPS OF MICE INJECTED RESPECTIVELY WITH WHOLE HDL_t AND ITS SUBFRACTIONS I, II, AND III, ALL LABELED IN THE PROTEIN MOIETY WITH I^{131}.

DISCUSSION

Our data have shown that the whole class of human serum high density lipoproteins floating between D 1.063 and 1.21 (HDL_t) can be further fractionated by ultracentrifugation in solvents of intermediate densities to yield subclasses which differ in flotation coefficient and electrophoretic mobility in starch gel. In these studies, the two intermediate solvent densities arbitrarily chosen, D 1.125 and 1.168, divided the HDL_t class into the three subfractions of D 1.063–1.125 (I), D 1.125–1.168 (II), and D 1.168–1.21 (III). It is likely, however, that more fractions could be obtained with solvents of closer intermediate densities. It is interesting to note that the difference in ultracentrifugal flotation and in starch gel electrophoresis exhibited by the HDL subfractions was not accompanied by differences in either amino acid composition or biological properties of their protein moiety, suggesting, although not proving, that the whole plasma HDL of D 1.063–1.21 has single protein constituents, a conclusion which is in

agreement with the previously reported immunochemical studies (4). This observation and the finding that HDL subfractions had a different lipid complement (see Table I) appear to support the hypothesis that a whole family of lipoproteins differing only in lipid content constitute the HDL class of lipoproteins of human plasma. A similar concept formulated on the basis of studies on density gradient ultracentrifugation has been recently presented by Oncley (17). Whether the demonstration of lipoprotein subfractions in human plasma depends on the laboratory procedures employed or reflects real existence in the living organism remains to be established. If this is the case, however, these lipoproteins may represent intermediate phases of an active lipid transfer from sites in which lipoproteins are fully saturated with lipids to sites in which lipoproteins have a smaller lipid complement. This process of lipid transfer may be favored by the labile type of association between protein and lipid in the human serum HDL (6) and by the great avidity for lipids exhibited by its protein moiety (6, 18). This avidity for lipids of the human serum HDL protein (αP), previously shown by experiments *in vitro* (6), has now been corroborated by the present results *in vivo,* also indicating that this protein recombines preferentially with the lipids of its own lipoprotein class. Of particular interest is the observation that this recombination can occur between human αP and plasma HDL of other animal species (mice, dogs).

The absence of cysteine in the HDL protein has been previously reported from this laboratory on the basis of paper chromatographic analysis (10). This finding seems supported by the present chromatographic studies showing that acid hydrolysates of HDL protein do not contain cystine or cysteic acid, oxidative products of cysteine. Our studies, however, do not rule out the possibility that small amounts of this sulphur-containing amino acid are present in the HDL protein and remained undetected by the methodology used. A final answer to this problem may come from studies involving the technique of activation analysis or enzymatic cleavage of the HDL protein.

The chromatographic method used in our experiments is known to give a recovery for methionine of 95 per cent (18). It is also known that methionine may be partially degraded, during acid hydrolysis, to methionine sulfoxides, small amounts of which were found present in our chromatogram. Even after correction for these losses, however, the methionine content of HDL protein remained low. Failure to recover tryptophan in the HDL protein hydrolysates can be explained by its loss during acid hydrolysis (10).

SUMMARY

1. The human serum high density lipoprotein (HDL) of solvent density between D 1.063 and 1.21 g per ml was fractionated by ultracentrifugation in solvents of intermediate density into three subclasses: D 1.063–1.125, D 1.125–1.168, and D 1.168–1.21. Their lipoprotein protein had identical absorption spectra and amino acid composition. When labeled with I^{131} and injected into mice, they showed the same half-time of metabolic degradation (12 hours).

2. These three HDL subfractions showed differences in flotation coefficient when analyzed at D 1.21 and had different electrophoretic mobility in starch gel. These differences appeared to depend on the lipid content of these fractions.

3. Human serum HDL, labeled in the protein moiety with I^{131} and injected into human subjects and dogs, disappeared from circulation according to a monomial exponential curve. When the delipidated protein (10) was injected into either human subjects or dogs, it recombined with serum lipids and travelled with its own HDL class.

4. It is postulated that human serum HDL represents a single family of lipoproteins with identical protein component able to carry various amounts of lipids.

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