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The Interaction of Human Low Density Lipoproteins with Long-chain Fatty Acid Anions

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The interaction of human low density lipoprotein with five long-chain fatty acid anions has been studied by measuring the distribution of varying quantities of each fatty acid between human serum albumin and low density lipoprotein. Two lipoprotein fractions were studied: one with density less than 1.019 and one with density 1.019 to 1.063. The fatty acids studied included lauric, palmitic, stearic, oleic and linoleic acids. The experiments were conducted in phosphate buffer, *p*H 7.45, ionic strength 0.16, at 23°. Albumin and lipoprotein were separated by ultracentrifugation, using deuterium oxide to raise the density without altering the ionic strength. The data obtained have been analyzed in terms of two classes of binding sites on each lipoprotein fraction, and the apparent association constants determined. The first class consists of a small number of sites, taken to be 4; the second consists of a much larger number of sites, taken to be 150. The five fatty acids have been compared both in terms of the absolute values of the association constants and in terms of the relative tightness with which they bind to lipoprotein compared to their binding to albumin. The two lipoprotein fractions have been shown to be qualitatively similar in their interaction with the different fatty acids; there is a slight quantitative difference, however, in that the lower density lipoprotein binds all five fatty acids somewhat more tightly. Increasing the ionic strength has been shown to alter the distribution in such a way that relatively more fatty acid becomes associated with lipoprotein. In contrast, altering the *p*H within the range to 6.8 to 7.7 has very little effect on the distribution. The implications for metabolic studies and for defining the physical state of unesterified fatty acids in plasma have been discussed.

In a recent publication[2] quantitative studies of the interaction of human serum albumin with several long-chain fatty acid anions were reported. Analysis of the data therein presented permitted the calculation of the number of sites on the albumin molecule which are able to bind fatty acid anions and of the apparent association constants for the conditions employed.

It is known, however, that serum albumin is not the only plasma protein which is capable of interacting with fatty acid ions. By means of measurements of electrophoretic mobility, Gordon[3] has demonstrated that oleate ions are bound by human β-lipoprotein, increasing its anodic mobility at *p*H 7.8. Using a system containing isolated serum albumin (fraction V) and β-lipoprotein, he found that the addition of oleate first resulted in an increase in the mobility of albumin, without affecting the mobility of the lipoprotein. As the mole ratio of oleate/albumin was increased, however, addition of more oleate resulted in a marked increase in the mobility of the lipoprotein, with a proportionately smaller increment in the mobility of albumin. These data indicated that at higher mole ratios of oleate/albumin a significant fraction of the oleate was bound to the lipoprotein, increasing its electrophoretic mobility. Furthermore, titration measurements of the unesterified fatty acids in normal human serum have demonstrated that a small fraction of these fatty acids is found associated with each of the density classes of lipoproteins.[4] It thus appeared desirable to undertake quantitative studies of the interaction of long-chain fatty acid ions with isolated human lipoprotein.

Experimental

Materials.—Both the non-radioactive and the C^{14}-carboxyl labeled fatty acids used in these studies (lauric, palmitic, stearic, oleic and linoleic acids) were identical with those described in a previous publication.[5] Solutions of the sodium salts of these fatty acids were prepared and their concentrations determined, as previously described.[5]

The human serum albumin used in these experiments was identical with the albumin preparation used in the study of the binding of fatty acid ions to human serum albumin. This albumin was a sample of fraction V, the long-chain fatty acid content of which had been reduced to 0.1 mole/mole or less by extracting the lyophilized protein with 5% glacial acetic acid in isoöctane.[6] Albumin solutions were freshly prepared for each study by dissolving some of the lyophilized protein (stored at −10°) in distilled water. The concentration of each solution was determined by comparing its optical density at 279 mμ as read in a Beckman quartz spectrophotometer to the optical density of a standard albumin solution whose concentration was known.[7] The weight concentration was converted to molar concentration by taking 69,000 as the molecular weight.[8,9] In each study the albumin solution so prepared was of concentration between 5 and 6 × 10^{-4} mole/liter.

Low density lipoprotein fractions were prepared from freshly drawn normal human serum, by a method of ultracentrifugal flotation similar to that described by Havel, Eder and Bragdon.[11] In each instance solid KBr was added to the whole serum to give a final density of 1.019, and the serum centrifuged for 16 hr. at 100,000 *g* in the 40 rotor of a Spinco Model L preparative ultracentrifuge, at 10 to 15°. The plastic centrifuge tubes were then sliced in the center of the clear zone which separated the supernatant lipoprotein of density less than 1.019 from the remainder of the serum proteins. The supernatant lipoprotein solutions were collected and the infranatant solutions adjusted to density 1.063 by the addition of more solid KBr. Centrifugation was repeated, and the supernatant lipoprotein of density less than 1.063 separately collected. This procedure resulted in the preparation of two low density lipoprotein fractions, one of density less than 1.019 and one of density between 1.019 and 1.063. Following centrifugation the lipoprotein preparations were dialyzed against 2–3 changes of large volumes of 0.16 *M* NaCl to remove the KBr and to reduce the ionic strength of the solutions. A final

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(2) D. S. Goodman, THIS JOURNAL, **80**, 3892 (1958).

(3) R. S. Gordon, Jr., *J. Clin. Inv.*, **34**, 477 (1955).

(4) E. Shafrir, *Federation Proc.*, **17**, 309 (1958); *Bull. Res. Council Israel*, **6A**, 307 (1957).

(5) D. S. Goodman, THIS JOURNAL, **80**, 3887 (1958).

(6) D. S. Goodman, *Science*, **125**, 1296 (1957).

(7) The concentration of the standard solution was determined by Kjeldahl analysis for nitrogen, using the factor 6.25 to convert weight of nitrogen to weight of protein.

(8) G. Scatchard, A. C. Batchelder and A. Brown, THIS JOURNAL, **68**, 2320 (1946).

(9) It is recognized that recent studies indicate the molecular weight of serum albumin to be close to 66,000.[10] 69,000 has been used here, however, to conform with the previous study[2] and for reasons given there.

(10) B. W. Low, *ibid.*, **74**, 4830 (1952).

(11) R. J. Havel, H. A. Eder and J. H. Bragdon, *J. Clin. Inv.*, **34**, 1345 (1955).

dialysis was conducted with a large excess of phosphate buffer, *p*H 7.45, ionic strength 0.160. The dialyses were conducted at 2° and the total time of dialysis was about 24 hr. The slightly turbid lipoprotein preparations were clarified by filtration and kept cold until used; in each case experiments with a given lipoprotein solution were carried out within 4 days of its preparation.

Three pairs of lipoprotein fractions were prepared in this fashion for the experiments herein reported. The first pair was used in the studies of palmitate binding, the second in the studies of oleate and of laurate binding, the third in the studies with stearate and linoleate. The three samples of serum from which they were prepared were all drawn 4 hr. after a low fat breakfast and were completely clear before use. The first sample of serum was initially centrifuged at 10,000 *g* for 30 minutes without addition of any salt, to remove lipoproteins of density less than 1.006. The quantity of lipoprotein material so obtained was extremely small, and this procedure was hence omitted with the other two samples. Each lipoprotein preparation was analyzed for total lipid by the method of Bragdon,[12] for protein content by the biuret method[13] and for unesterified fatty acid content by the method of Gordon.[14] The fractional protein and lipid content of each lipoprotein preparation was close to the values previously reported for normal individuals.[11,15] The concentration of each lipoprotein solution was 3 to 5 times the concentration of lipoprotein of that density in normal human serum.

Phosphate buffer was prepared as a stock solution of 10-times the desired ionic strength; dilution of this stock solution 1 in 10 resulted in a solution of *p*H 7.45, ionic strength 0.160. NaH_2PO_4 and Na_2HPO_4 were Merck and Co., analytic reagents. Deuterium oxide was supplied by Abbott Laboratories; its isotopic enrichment was given as greater than 99.5%. A solution of phosphate buffer in D_2O was prepared by diluting 10 cc. of the stock concentrated phosphate buffer (in H_2O) with 90 cc. of D_2O. The measured density of this solution at 20° was 1.0975. Toluene and acetone were Merck reagent grade solvents. Absolute ethanol (U.S.P.) was supplied by U. S. Industrial Chemical Co. Diphenyloxazole (DPO) was a product of Pilot Chemicals, Inc.

Methods.—The experimental technique employed consisted, in each study, of preparing a series of solutions containing measured amounts of a serum albumin solution and of one of the lipoprotein preparations, together with varying quantities of the fatty acid being studied. A measured volume of the corresponding C^{14}-labeled fatty acid salt solution also was added to each solution, together with enough stock concentrated phosphate buffer to make the final ionic strength 0.160, with *p*H 7.45. The total quantity of fatty acid in each series of solutions ranged over more than one order of magnitude. The volume of lipoprotein preparation used in each case was 2 to 4 times the volume of the serum albumin solution; the final ratio of lipoprotein/albumin concentrations was from 10 to 15 times the corresponding ratio of concentrations in normal human serum. The solutions were thoroughly mixed and an appropriate amount of phosphate buffer in D_2O added, to raise the final density of the solution to the density used in preparing the lipoprotein being studied. For the studies with lipoprotein preparations of density less than 1.019, 6 cc. of each experimental solution was mixed with 1 cc. of D_2O–PO_4 to make a solution of density 1.019. In the studies with lipoprotein preparations of density 1.019 to 1.063, 3 cc. of each experimental solution was mixed with 5 cc. of D_2O–PO_4, to give a final density of 1.063. The solutions were then intermittently mixed for 3 to 5 hr. to permit complete equilibration of the fatty acid between the two proteins. It is felt that this time was considerably in excess of the time actually needed to attain equilibrium in a single solution. All experiments were conducted in an air-conditioned laboratory at 23 ± 1°.

After this period of equilibration, the albumin and lipoprotein moieties were separated by centrifugation in the 40.3 rotor of the Spinco Model L preparative ultracentrifuge at 110,000 *g* for 16 hr. In every case the separation appeared complete, with a clear zone of solvent separating the concentrated lipoprotein supernate from the albumin pellet in the infranate. The plastic centrifuge tubes were sliced in the center of this clear zone, and the infranatant and supernatant solutions quantitatively and separately collected. Each supernatant solution was made to 5.00 cc., and each infranatant solution to 10.00 cc., with 0.16 *M* NaCl.

The completeness of this method of separating serum albumin from low density lipoprotein was verified by analyzing three of the albumin infranatant solutions for total lipid by the method of Bragdon.[12] The amount of lipid (including the unesterified fatty acid, bound predominantly to the albumin) found in the 3 infranatant solutions was, respectively, 2.2, 3.7 and 3.1% of the total lipid in the solution prior to centrifugation.

The concentration of fatty acid in each albumin and lipoprotein solution then was determined by measuring the concentration of radioactivity. One or two cc. of each solution was extracted with 25 cc. of a 1:1 mixture of ethanol:acetone, and a measured aliquot of each extraction solution transferred to a small vial and evaporated to dryness. To each vial then was added 15 cc. of a scintillation solution consisting of 4 g./l. DPO in toluene, and the amount of radioactivity in each vial measured with a Packard liquid scintillation spectrometer (background *ca.* 15 c.p.m.). Enough counts were recorded to bring the standard error of each measurement below 2%.

From the concentration of radioactivity (c.p.m.) and the known volume relationships the number of c.p.m. associated with the albumin and the lipoprotein in each solution was calculated. The total recovery of c.p.m. in each solution was within 5% of the calculated total c.p.m. added to the solution, with a very few exceptions where the recovery was between 90 and 95%. The total number of moles of fatty acid associated with lipoprotein and with albumin then was calculated as the per cent. of recovered c.p.m. times the known amount of fatty acid in each solution. The amount of fatty acid in each solution was taken to be the sum of the amount of pure fatty acid added to each solution plus the amount of unesterified fatty acid added with the lipoprotein preparation. The latter quantity was a very small fraction of the amount of added fatty acid in all cases except for the 2 or 3 solutions with the least amount of fatty acid in each study.[16] The average number of fatty acid ions bound per albumin molecule, $\bar{\nu}$ to H.S.A., was then determined by dividing the number of moles of fatty acid associated with albumin in each solution by the number of moles of albumin added to the solution. Finally, the corresponding average number of fatty acid ions bound per lipoprotein molecule, $\bar{\nu}$ to lipoprotein, was calculated by dividing the number of moles of fatty acid associated with lipoprotein in each solution by the number of moles of lipoprotein added to the solution. The molecular weight of lipoprotein of density less than 1.019 was arbitrarily taken to be 2×10^6 g./mole; for lipoprotein of density 1.019 to 1.063 the molecular weight selected was 1×10^6 g./mole. It is recognized that these values do not correspond to the actual molecular weights of the different lipoprotein species.[17] These values were selected, however, because of the corresponding ease of calculation which they conferred and because of the present uncertainty about the exact molecular weights of the different lipoprotein species. When these molecular weights become known with precision the results of the present studies can be converted easily to a true molar basis.

Assumptions Involved.—Before presenting the results, a brief discussion of the assumptions involved in these studies is in order. In the first place, the fatty acid associated with each of the protein moieties has been treated as if it were entirely bound to the protein. The results of previous studies of fatty acid binding to albumin[2] indicate that at all the concentrations herein employed less than 1% of the fatty acid was free in solution; the error in this assumption is hence negligibly small.

(12) J. H. Bragdon, *J. Biol. Chem.*, **190**, 513 (1951).

(13) A. G. Gornall, C. J. Bardawill and M. M. David, *ibid.*, **177**, 751 (1949).

(14) R. S. Gordon, Jr., *J. Clin. Inv.*, **36**, 810 (1957).

(15) J. H. Bragdon, R. J. Havel and E. Boyle, *J. Lab. Clin. Med.*, **48**, 36 (1956).

(16) In all cases the unesterified fatty acid added with the lipoprotein preparation was treated as if it were composed solely of the fatty acid being studied. The error in this procedure is very small because of the relatively small quantities involved (compared to the amount of pure fatty acid added).

(17) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. Colloid Chem.*, **51**, 184 (1947); J. L. Oncley and E. Toro-Goyco, *Federation Proc.*, **17**, 285 (1958).

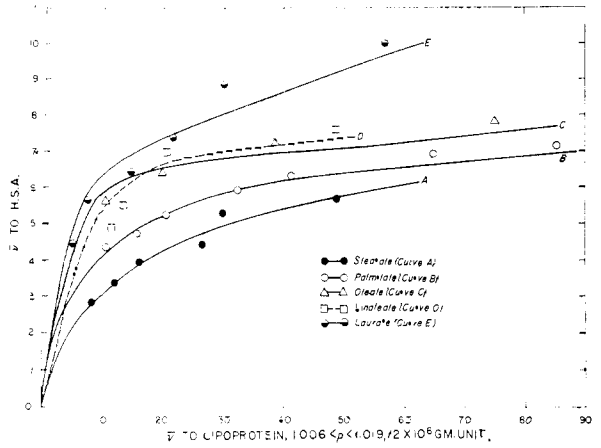

Fig. 1.—Distribution of fatty acid anions between human serum albumin and lipoprotein of density less than 1.019.

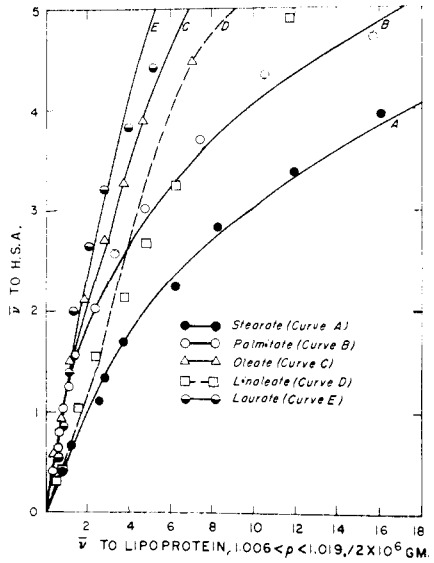

Fig. 2.—Distribution of fatty acid anions between human serum albumin and lipoprotein of density less than 1.019.

Secondly, it has been assumed that in the process of separating albumin and lipoprotein by centrifugation the equilibrium distribution of fatty acid between the two proteins was not altered. This assumption would be invalid if the substitution of D_2O for H_2O altered the binding constants, which is unlikely, or if the free fatty acid concentration changed during centrifugation. The second possibility is also unlikely, since the expected sedimentation of free fatty acids is negligible.

Thirdly, it has been assumed that the fatty acid–protein interactions herein studied are reversible equilibrium reactions. There is qualitative evidence that this is the case from the electrophoretic studies of Gordon.[3]

Finally, in the discussion which follows, the three different lipoprotein preparations of a given density, made from the three different serum samples employed, are treated as if they were all identical preparations representative of lipoproteins of that density. This assumption was tested with each study by making duplicate measurements of the binding of palmitate with each lipoprotein preparation. Four measurements, two with small amounts of palmitate and two with large amounts, were made with each of the lipoprotein preparations. The duplicate measurements for each of the two density classes of lipoproteins all agreed within 10% of each other. This is within the limits of error of the measurements. It therefore seems reasonable to assume that the 3 lipoprotein preparations of a given density would have also bound the other fatty acid ions in a quantitatively identical fashion.

Results and Discussion

The results of these studies are presented graphically in Figs. 1 through 4. In each figure, $\bar{\nu}$ to H.S.A. is plotted against $\bar{\nu}$ to lipoprotein. Figures

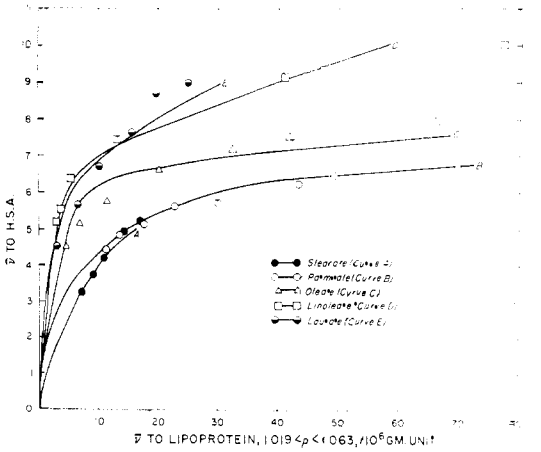

Fig. 3.—Distribution of fatty acid anions between human serum albumin and lipoprotein of density 1.019 to 1.063.

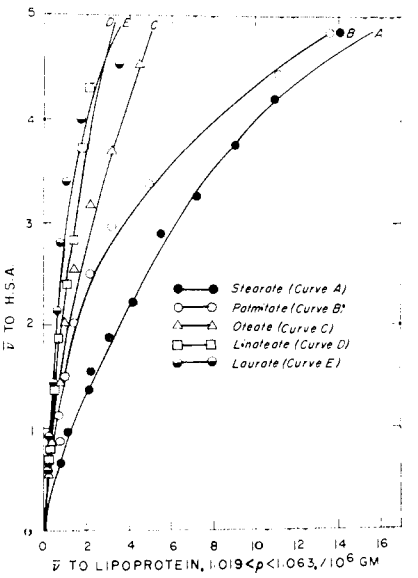

Fig. 4.—Distribution of fatty acid anions between human serum albumin and lipoprotein of density 1.019 to 1.063.

1 and 2 present the results with lipoprotein of density less than 1.019; Figs. 3 and 4 present the results with lipoprotein of density between 1.019 and 1.063. Figures 2 and 4 are enlargements of the lower left-hand corners of Figs. 1 and 3, respectively. The experimental points are omitted from the lower left-hand corners of Figs. 1 and 3 because of the crowding which would occur if they were plotted there with the scale of those figures.

Analysis in Terms of Apparent Classes and Association Constants.—As indicated by Scatchard[18–20] the binding of an anion A to several classes of sites on a protein molecule may be formulated as

(18) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

(19) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., THIS JOURNAL, **72**, 535, 540 (1950).

(20) G. Scatchard, J. B. Coleman and A. L. Shen, *ibid.*, **79**, 17 (1957).

$$\bar{\nu}_A = \sum_i \bar{\nu}_{Ai} = \sum_i \frac{n_i k'_{Ai} c_A}{1 + k'_{Ai} c_A} \qquad (1)$$

in which k'_{Ai} is the apparent association constant for each of the n_i sites in class i under any particular set of conditions, and c_A is the free (unbound) concentration of A in equilibrium with the protein.[21] The apparent association constant k'_{Ai} is related to the intrinsic association constant k^0_{Ai} by a number of factors which correct for the effects of electrostatic interactions, competition with other ions and the hydrogen ion equilibrium of the binding sites. These factors have been discussed in detail previously.[2]

In a previous publication[2] quantitative studies of the interaction of human serum albumin with six long-chain fatty acid anions (laurate, myristate, palmitate, stearate, oleate and linoleate) were reported. Analysis of the results in terms of equation 1 indicated that in every case there were 3 classes of binding sites on serum albumin, with $n_1 = 2$, $n_2 = 5$, and $n_3 = 20$. The apparent association constant for each class of binding sites was determined for each of the fatty acids studied.

Since the conditions (*p*H 7.45, phosphate buffer, ionic strength 0.160, 23°) and materials used in the previous studies were identical with those used in the present studies, the results of the previous studies were applied directly to the calculation of the values of n and k' for the lipoproteins herein studied. This was done by setting up a pair of equations of the form of equation 1 for each fatty acid. One equation of each pair applied to the binding to albumin and contains the known values of n and k' for the given fatty acid; the other equation of each pair was for the binding to lipoprotein and contained n and k' as unknowns. Since the values of c were identical for each pair of equations, it was possible to arbitrarily select values of n and k' for lipoprotein and by simultaneously solving the pair of equations to obtain a theoretical plot of $\bar{\nu}$ to H.S.A. against $\bar{\nu}$ to lipoprotein based on these values. By a method of successive approximations it was then possible to alter n and k' for lipoprotein so that a progressively closer fit was obtained between this theoretical plot and the experimental data. This process was continued until the best possible fit (by inspection) was obtained.

The initial assumption used in making these calculations was that there is a single large class of binding sites on lipoproteins, *i.e.*, that only one term need be considered in equation 1 for lipoproteins. On performing the calculations described above, however, it was found that in no case could a theoretical plot of ν to H.S.A. *vs.* $\bar{\nu}$ to lipoprotein be obtained which would satisfactorily fit the experimental data. In every case the experimental points for the lowest values of $\bar{\nu}$ fell to the right of the theoretical plot. This indicated that there must be more than one class of binding sites on the lipoprotein molecules.

The calculations were then repeated, with the assumption that there are two classes of fatty acid binding sites on lipoproteins. In every case (with one exception) it was possible to obtain theoretical plots which fitted the experimental data well, using values of n_1 of from 3 to 5 and values of n_2 of 100 or more. The final values selected were $n_1 = 4$ and $n_2 = 150$. Using these values of n, the values of k_1' and k_2' which would best fit the data were determined for each of the five fatty acids, for each lipoprotein fraction. These values are listed in Tables I and II. The curves drawn in Figs. 1 through 4 are all theoretical plots constructed as described above, using these values of n and the values of k' listed in Tables I and II (together with the values of n and k' previously reported for serum albumin). Inspection of the figures indicates that these values of n and k' fit the data very well in every case, with the single exception of linoleate binding to lipoprotein of density less than 1.019. The fact that the fit is less good in this one case has been indicated in Figs. 1 and 2 by drawing the corresponding theoretical plot in dashed lines; the reason for this one exception is not clear.

TABLE I

THE APPARENT ASSOCIATION CONSTANTS FOR THE INTERACTION OF HUMAN LIPOPROTEIN OF DENSITY <1.019 WITH FATTY ACID ANIONS

*p*H 7.45; ionic strength 0.160; 23°: lipoprotein unit of weight = 2×10^6; two classes of binding sites with $n_1 = 4$ and $n_2 = 150$.

Fatty acid anion	k_1'	k_2'
Stearate	7×10^7	1.2×10^5
Palmitate	7×10^6	2×10^5
Oleate	3×10^7	5×10^4
Linoleate	2×10^7	4.4×10^4
Laurate	7×10^5	2×10^3

TABLE II

THE APPARENT ASSOCIATION CONSTANTS FOR THE INTERACTION OF HUMAN LIPOPROTEIN OF DENSITY 1.019 TO 1.063 WITH FATTY ACID ANIONS

*p*H 7.45 ionic strength 0.160; 23°; lipoprotein unit of weight = 10^6; two classes of binding sites with $n_1 = 4$ and $n_2 = 150$.

Fatty acid anion	k_1'	k_2'
Stearate	3.8×10^7	5×10^4
Palmitate	3×10^6	1.7×10^5
Oleate	1.3×10^7	3×10^4
Linoleate	2.5×10^6	7×10^3
Laurate	1.8×10^5	1.2×10^3

The relative reliability of the values of n_1 and k_1' is fairly high, since good fits for all the curves could be obtained only within the restricted range of n_1 equals 3, 4, or 5. In several cases good fits also could be obtained using values of either 2 or 6 for n_1; in no instance, however, could equally satisfactory fits be obtained with values of n_1 equals 2 through 6 inclusive. The values of n_2 and k_2' are less precisely determined, since the data only indicated that n_2 was very large and the value $n_2 = 150$ was selected arbitrarily. The data do, however, limit the range of possible values of n_2, for they indicate that n_2 must be greater than 100, and good fits could not be obtained with values of n_2 greater than 300. For any given value of n_2 the corresponding values of k_2' are quite precisely determined.

(21) Strictly speaking, the activity of A should be used instead of the concentration. Fatty acid concentrations were used here, however, introducing a small systematic error in all of the results, of equal magnitude in all studies.

Comparison of Fatty Acids.—From the data in Figs. 1 through 4 the five fatty acids studied can be compared in terms of the relative tightness with which they bind to lipoprotein, as compared to the tightness with which they bind to albumin. This is achieved by comparing the values of the ratio of $\bar{\nu}$ to lipoprotein to $\bar{\nu}$ to H.S.A., for fixed values of $\bar{\nu}$ to H.S.A. For both lipoprotein fractions, the relative values of this ratio are: stearate > palmitate > oleate > laurate. The position of linoleate differs somewhat for the two lipoproteins and depends on the value of $\bar{\nu}$. With lipoprotein of density 1.019 to 1.063 linoleate falls between oleate and laurate, except in the region between $\bar{\nu}$ to H.S.A. equals 4.5 to 7.3, when it falls behind laurate. With lipoprotein of density less than 1.019, at low values of $\bar{\nu}$ linoleate falls between stearate and palmitate; as $\bar{\nu}$ increases, however, the curve for linoleate crosses the palmitate and oleate curves, and at value of $\bar{\nu}$ to H.S.A. greater than 7.5 linoleate falls between oleate and laurate (as with the other lipoprotein fraction). In human plasma, therefore, relatively more stearate than palmitate, more palmitate than oleate, etc., will be bound to lipoprotein than to serum albumin. This information may well prove to be of physiologic significance equal to that of the absolute values of the association constants.

The fatty acids may also be arranged according to the absolute magnitude of k_1' and k_2'. This arrangement, with respect to decreasing magnitude of k_1', is: stearate > oleate > palmitate > laurate, for both lipoprotein fractions. The position of linoleate differs for the two lipoproteins, falling between oleate and palmitate for lipoprotein of density less than 1.019 and between palmitate and laurate for lipoprotein of density 1.019 to 1.063. The corresponding arrangement according to decreasing magnitude of k_2' is identical for the two lipoprotein fractions, being palmitate > stearate > oleate > linoleate > laurate.

Comparison of Lipoproteins.—The foregoing comments indicate that the two lipoprotein fractions studied are qualitatively very similar as regards the relative tightness with which they bind the five different fatty acids. The only difference noted, in fact, was a partial difference with respect to the binding of linoleate. Comparison of the values of k_1' and k_2' listed in Table I and II also indicates that the two lipoprotein fractions are quantitatively similar as regards their binding of fatty acid ions. The differences which do exist are absolutely consistent in that in every case the values of k_1' and k_2' for lipoprotein of density less than 1.019 are greater than the corresponding values for lipoprotein of density 1.019 to 1.063. In only 3 of the 10 cases, however, does the value of one of the constants in Table I exceed the corresponding value in Table II by a factor of 3 or more, and in one of these (k_1' for laurate) the difference is less than a factor of 4. The only large difference occurs with respect to linoleate, which appears to be much more tightly bound by the lower density lipoprotein fraction.

The very great difference between the values of n_1 and n_2 permits some preliminary speculations on the mechanism of fatty acid ion interaction with lipoproteins. The fact that n_2 is so large suggests that there is relatively little specificity involved. The main factor may be that of solubility of the fatty acid hydrocarbon chain in the lipid portion of the lipoprotein, with a lining up to fatty acid ions at the lipoprotein–water interface. In a small number of instances the carboxylate end of the fatty acid ion may also interact with one or more side groups of the protein portion of the lipoprotein molecule. In such cases the interaction energy will be greater than for the majority of the bond ions, resulting in a higher association constant. This may be the mechanism involved in the small number of sites of the first class.

Effect of Ionic Strength.—The effect of increasing the ionic strength was studied for the case of palmitate binding to lipoprotein of density 1.019 to 1.063. Two series of identical solutions were prepared, one containing small amounts of palmitate and the other containing large amounts and the ionic strength progressively increased by substituting increasing amounts of concentrated NaCl in phosphate buffer–H_2O for equivalent amounts of phosphate buffer in D_2O. The results, shown in Fig. 5, demonstrate that as the ionic strength was increased relatively more and more of the palmitate was bound to the lipoprotein. The effect is quite large. In the series with small amounts of palmitate (lower curve, Fig. 5) $\bar{\nu}$ to lipoprotein was increased from 1.1 at ionic strength 0.160 to 6.5 at ionic strength 1.65. In the series with large amounts of palmitate (upper curve, Fig. 5) $\bar{\nu}$ to lipoprotein was increased from 27 at ionic strength 0.160 to 61 at ionic strength 1.65.

The effect of increasing the ionic strength was also investigated for each of the other four fatty acids, by preparing one or two duplicate solutions in each study and adjusting these to the appropriate density with KBr in H_2O. In every case the results with these solutions demonstrated a relatively larger binding of fatty acid to lipoprotein, as compared to the solutions made to density with D_2O–PO_4. The magnitude of the salt effect differed somewhat from fatty acid to fatty acid but was generally roughly similar to the results shown in Fig. 5 for palmitate. It is thus apparent that increasing the ionic strength with salt has the effect of changing the distribution of bound fatty acid between lipoprotein and albumin, so that relatively more fatty acid is bound to the lipoprotein. The mechanism of this effect (*e.g.*, whether it is due to competition between the inorganic anions and the fatty acid anions for binding sites on albumin but not for binding sites on lipoprotein) cannot be deduced from the present studies. In any event, the fact that this effect exists should be recognized when dealing with such phenomena as the physical chemistry of unesterified fatty acids in plasma.

Effect of *p*H.—The effect of changing the *p*H was also studied for the case of palmitate binding to lipoprotein of density 1.019 to 1.063, by using several different phosphate buffers of identical ionic strength. The results, shown in Fig. 6, demonstrate that with small quantities of palmitate (lower curve, Fig. 6) the distribution between al-

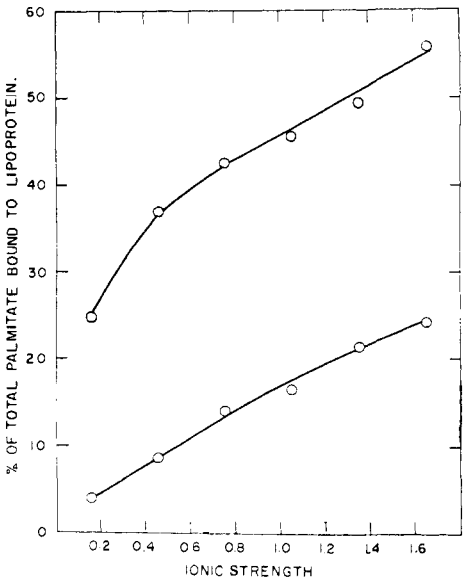

Fig. 5.—Effect of ionic strength on the distribution of palmitate between human serum albumin and lipoprotein of density 1.019 to 1.063.

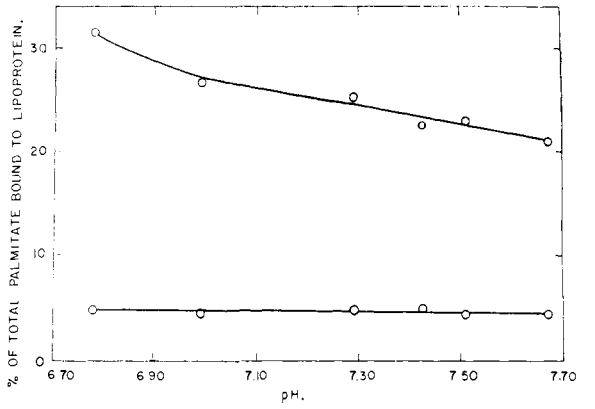

Fig. 6.—Effect of *p*H on the distribution of palmitate between human serum albumin and lipoprotein of density 1.019 to 1.063.

bumin and lipoprotein was not altered by changing the *p*H over the range studied; $\bar{\nu}$ to lipoprotein was found to be between 1.3 and 1.5 at each *p*H. When large amounts of palmitate were present, however (upper curve, Fig. 6), reducing the *p*H resulted in a progressive, small increase in the relative amount of palmitate bound to lipoprotein, with $\bar{\nu}$ to lipoprotein varying from 24 at *p*H 7.67 to 35 at *p*H 6.79. The corresponding value of $\bar{\nu}$ to albumin for the points of the lower curve of Fig. 6 was 2.1. The effect of *p*H is therefore usually negligible in physiologic studies of unesterified fatty acids in plasma, since the value of $\bar{\nu}$ to albumin of these fatty acids rarely exceeds 2.

Implications for Metabolic Studies.—The results of the present studies provide considerable information about the physical state of unesterified fatty acids present in human blood plasma. Recent studies of these fatty acids have demonstrated them to be of great metabolic significance, in representing a transport form of lipid readily available as a substrate for oxidation.[14,22-24] The normal level of unesterified fatty acid in fasting human subjects is of the order of 5×10^{-4} mole per liter of plasma.[23] This level represents a $\bar{\nu}$ of fatty acid to albumin of slightly less than one. Studies of the composition of this fatty acid fraction indicate that it consists of approximately 80% of oleic, palmitic, stearic and linoleic acids (in decreasing amounts), 5–10% palmitoleic acid and an isomer of oleic acid, 5–10% polyethenoic 20C acids and 5% of 14C to 10C acids.[25] 80 to 85% of these acids are hence represented by the five acids which have been herein studied. The extent to which these five acids are bound to low density lipoproteins in normal human plasma can be estimated from the data in Figs. 2 and 4, by taking an average value of $\bar{\nu}$ to lipoprotein corresponding to a value of $\bar{\nu}$ to H.S.A. of approximately 0.8 and multiplying by the normal concentration of lipoprotein in plasma. This calculation indicates that, for these five fatty acids, only about 0.3% of the acids will be bound to each of the two lipoprotein fractions studied. Since, at these concentrations, only about 0.01% of these fatty acids is free in solution, it is apparent that in normal plasma almost all of these five fatty acids exist bound to serum albumin.[26]

At first glance these results appear to be at variance with the results of studies based upon the titration of isoöctane extracts of the lipoprotein fractions of normal sera. These studies[4] indicated that 5 to 10% of the total acid material extractable from serum (analyzed by the method of Gordon for unesterified fatty acids),[14] was found associated with these two classes of low density lipoprotein. In addition, the effect of ionic strength on the percentage of acids associated with lipoprotein was relatively small, compared to the effects observed in the present studies. These apparent discrepancies can be resolved by the assumption that most of the titratable acids which are extracted from lipoproteins by the method employed consist of compounds other than the five fatty acids herein studied.[27] Thus, this material might consist of fatty acid(s) of chain-length longer than 18C, or of other compounds with titratable groups (*e.g.*, phosphatidylserine). It is only necessary that the substance(s) in question be predominantly associated with lipoprotein, be extractable into isooctane and have a titratable acid group. This problem is currently under investigation.[28]

Brief mention also should be made concerning previous reports[29] that a large fraction of the plasma unesterified fatty acid is normally associated with

(22) V. P. Dole, *J. Clin. Inv.*, **35**, 150 (1956).

(23) R. S. Gordon, Jr., and A. Cherkes, *ibid.*, **35**, 206 (1956).

(24) D. S. Frederickson and R. S. Gordon, Jr., *Physiol. Rev.*, **38**, 585 (1958).

(25) V. P. Dole and A. T. James, in preparation. We are indebteh to Drs. Dole and James for this information.

(26) With the additional exception of a small fraction of these fatty acids bound to high density lipoprotein. The exact magnitude of binding to high density lipoprotein is not known but unpublished observations from this Laboratory indicate it to be less than 5% for palmitate.

(27) We are indebted to Dr. Robert S. Gordon, Jr., for this suggestion.

(28) This is, of course, only one of several possible explanations. It is, however, the one which seems most likely to us.

(29) J. W. Gofman, *et al.*, *Plasma*, **2**, 413 (1954).

the high density lipoproteins (density 1.063 to 1.21). Preliminary studies in this Laboratory with this lipoprotein fraction,[30] indicate that the effect of salt upon the distribution of fatty acids between high density lipoprotein and serum albumin is similar to the effects described here for low density lipoproteins and that, in fact, only a small fraction of the plasma unesterified fatty acid is bound to high density lipoprotein. The previous observations are probably largely artifacts resulting from the very high salt concentrations used in separating the high density lipoproteins.

The data presented in Figs. 1 through 4 demonstrate that as $\bar{\nu}$ to albumin increases relatively more and more of the fatty acid becomes bound to lipoprotein. At high values of $\bar{\nu}$ to albumin a much larger percentage of the total fatty acid is therefore bound to lipoprotein. This situation exists in pathological clinical conditions, such as the nephrotic syndrome, where the serum albumin level is abnormally low, the lipoprotein concentration elevated and the unesterified fatty acid concentration normal. Under such conditions a much larger percentage of the plasma unesterified fatty acid will exist bound to lipoprotein. It is conceivable that the abnormal distribution of fatty acids might affect the metabolism of lipoproteins and/or unesterified fatty acids and hence might be involved in the metabolic abnormalities of these conditions.

(30) D. S. Goodman and E. Shafrir, unpublished observations.

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[CONTRIBUTION FROM THE LABORATORY OF THE CHILDREN'S CANCER RESEARCH FOUNDATION]

The Preparation of High Molecular Weight Polypeptides[1]

BY E. R. BLOUT[2] AND M. E. DESROCHES

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A method is described for the preparation of polypeptides of very high molecular weight, *i.e.*, with weight average molecular weights as high as 1,000,000. The method involves intermolecular condensation of lower molecular weight polypeptides using carbodiimides as the condensing agents. The method has been used with both organo-soluble polypeptides and water-soluble polypeptides and to prepare block copolymers. Examples are given of the preparation of high molecular weight poly-γ-benzyl-L-glutamate, poly-ϵ-carbobenzyloxy-L-lysine and poly-L-proline.

Polypeptides, or poly-α-amino acids, are useful models for studies of the chemical and biological properties of proteins. For these purposes it is necessary that the polypeptides have molecular weights comparable to those of proteins, *i.e.*, 50,000 to 1,000,000. Using the strong base initiated polymerization of α-amino acid-N-carboxyanhydrides (NCAs), it has been possible to prepare polypeptides in this molecular weight range from glutamic esters,[3] lysine,[4] proline[5] and and other amino acids.[6] However, with certain amino acids only lower molecular weight polypeptides (molecular weights of 10,000 to 35,000) have been synthesized. In this paper we describe a method for obtaining high molecular weight polypeptides by joining together lower molecular weight fragments.

The method makes use of the carbodiimide reagent introduced by Sheehan and Hess for the formation of peptide bonds.[7] If a polypeptide with free terminal amino and carboxyl groups is treated with a carbodiimide, then condensation may occur with formation of additional peptide bonds between polymers to yield products of increased molecular weight and degree of polymerization (DP) in the manner indicated below

$$\begin{aligned}&\mathrm{H_2N\text{-}Polypeptide\text{-}COOH} + \underset{(DP_x)}{\quad}\\ &\qquad \mathrm{H_2N\text{-}Polypeptide\text{-}COOH} + \mathrm{R{-}N{=}C{=}N{-}R'} \quad (DP_y)\\ &\longrightarrow \mathrm{R{-}N{-}\overset{\overset{O}{\|}}{C}{-}N{-}R'} + \mathrm{H_2N\text{-}Polypeptide\text{-}COOH} \longrightarrow \begin{matrix}(DP_{x+y}),\\ (DP_{2x}) \text{ and}\\ (DP_{2y})\end{matrix}\\ &\qquad\qquad \mathrm{H_2N\text{-}Polypeptide\text{-}COOH}\\ &\qquad\qquad (DP_{2(x+y)}),\\ &\qquad\qquad (DP_{2x+y}), (DP_{x+2y}), \longrightarrow \text{etc.}\\ &\qquad\qquad (DP_{3x}) \text{ and } (DP_{3y})\end{aligned}$$

It is apparent that the polypeptides indicated above may be derived from the same amino acid or different amino acids. If the latter is the case then this procedure will produce "block" polypeptides. The results obtained with polypeptides derived from four different amino acids are shown in the Table I.

It is clear from the data in the table that through the use of carbodiimides as condensing agents the molecular weights of polypeptides can be increased significantly. In several instances fourfold or greater increases have been observed.

It is assumed that this increase in molecular weight occurs because of terminal inter-molecular peptide bond formation, but definitive proof is difficult to obtain. However, indirect evidence that linear peptide bond formation is involved can be deduced from the fact that we have observed that polypeptides with one terminal amide group and one terminal amine group do not undergo molecular weight increases when treated with car-

(1) This paper is Polypeptides. XXIV. For the previous paper in this series see H. Lenormant, M. Baudras and E. R. Blout, THIS JOURNAL, **80**, 6191 (1958).

(2) Chemical Research Laboratory, Polaroid Corporation, Cambridge 39, Massachusetts.

(3) (a) E. R. Blout, R. H. Karlson, P. Doty and B. Hargitay, THIS JOURNAL, **76**, 4492 (1954); (b) E. R. Blout and R. H. Karlson, *ibid.*, **78**, 941 (1956); (c) M. Idelson and E. R. Blout, *ibid.*, **80**, 2387 (1958).

(4) (a) E. R. Blout and H. Lenormant, *Nature*, **179**, 960 (1957); (b) E. R. Blout and M. Idelson, unpublished results.

(5) E. R. Blout and G. D. Fasman, "Recent Advances in Gelatin and Glue Research," Pergamon Press, London, 1957, p. 122.

(6) E. R. Blout and co-workers, unpublished results.

(7) J. C. Sheehan and G. D. Hess, THIS JOURNAL, **77**, 1067 (1955).