Separation and Further Characterization of Human Adipose Tissue Neutral and Alkaline Lipolytic Activities*

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SUMMARY

Neutral lipolytic activity (hydrolysis of olive oil at 37°, pH 7.0) and alkaline lipolytic activity (hydrolysis of tributyrin at 47°, pH 8.0) have previously been demonstrated in human adipose tissue.

Neutral lipolytic activity has currently been shown to be present in a large molecular weight substance, associated with lipid, and separable from most alkaline lipolytic activity by ammonium sulfate precipitation and Sephadex G-200 gel filtration.

Alkaline lipolytic activity (ALA) has been shown to exist as three separable fractions: ALA-I, ALA-II, and ALA-III. ALA-I represents a small percentage of the total alkaline lipolytic activity, is eluted from Sephadex with neutral lipolytic activity, exists as a lipid-protein complex of ALA-II, and has no mobility during starch gel electrophoresis. ALA-II is a smaller molecule than ALA-I and exists primarily as four electrophoretically separable bands of activity. ALA-III is considerably smaller than ALA-II, is a subunit of ALA-II, and exists primarily as a fifth band of electrophoretically separable activity.

EXPERIMENTAL PROCEDURE

Methods

Source and Preparation of Tissue—During the course of these experiments, pieces of subcutaneous adipose tissue weighing 20 to 500 g were obtained shortly after induction of anesthesia from 40 individuals undergoing major surgery. Sufficient tissue was therefore available so that each experiment was performed with adipose tissue from a single individual, and, in some instances, multiple experiments were performed with one piece of tissue.

One gram of tissue per ml of 0.15 M NaCl was homogenized in a Waring Blendor at room temperature. Centrifugation at 800 to 1000 × g for 30 min at 4° produced three layers: a fat cake on top, a sediment on the bottom, and an aqueous layer in the middle of the tube. This aqueous middle layer was recovered, referred to as the adipose tissue extract, and used as the source of lipolytic activity (NLA and ALA).

Although a partial separation of NLA and ALA was previously achieved by centrifugation and ether extraction (2), a more complete separation seemed desirable to establish the separate identity of the proteins responsible for each activity and permit additional study of each.

The present paper reports separation of NLA and ALA by (NH₄)₂SO₄ precipitation and Sephadex gel filtration. Furthermore, it will be shown that ALA is eluted from Sephadex as three fractions, each of which has a different electrophoretic migration on starch gel. The largest of these molecules appears to exist as a lipid-protein complex, and the smallest as a subunit of the intermediate sized ALA molecule.
Assay of NLA and ALA—One milliliter of effluent from a column or 1 ml of an adipose tissue extract, diluted to give activity equivalent to that in 150 to 200 mg of tissue, was placed in each assay system, which had a final volume of 4 ml.

The NLA system contained 25 µmoles of olive oil, 50 mg of bovine albumin Fraction V, 20 µmoles of sodium phosphate buffer, and 20 µmoles of Tris-HCl buffer, pH 7.0. This was incubated at 37° for 30 min.

The ALA system contained 11 µmoles of tributyrin, 50 mg of bovine albumin Fraction V, 20 µmoles of sodium phosphate buffer, and 20 µmoles of Tris-HCl buffer, pH 8.0. This was incubated at 47° for 30 min.

Aliquots of each assay system were obtained before and after incubation, extracted (5), and titrated (6) for free fatty acids. Activity was expressed as microequivalents FFA per ml of sample per hour.

In several instances when activity was low, the assay systems were modified by increasing both the incubation time and the amount of the sample to be assayed. In such cases, linearity of the reaction was verified, as had previously been done for the usual conditions (2).

Hydrolysis of α-Naphthyl Acetate—Hydrolysis of α-naphthyl acetate was determined quantitatively on several occasions by a colorimetric method (7) which utilizes α-naphthyl acetate as substrate and α-naphthol as standard.

Protein Determination—The protein content of adipose tissue extracts before and after fractionation was determined by the method of Lowry et al. (8) with crystalline albumin as standard.

When various plasma proteins were filtered through Sephadex, the protein content of the effluent was monitored by determination of the optical density at 280 mp. Hemoglobin concentration was read at 440 mp.

Starch Gel Electrophoresis—Vertical starch gel electrophoresis (4, 9, 10) was performed with 0.165 M phosphate-citrate buffer, pH 7.0. Samples containing 50 to 100 µl were placed at the origin, and a voltage gradient of 8 volts per cm was applied for 17 hours at 4°.

After electrophoresis, the gel was sliced horizontally and the cut surfaces were tested for their ability to hydrolyze α-naphthyl acetate. This was accomplished by incubation at 37° for 30 min in a solution containing 5 µg of α-naphthyl acetate dissolved in 0.25 ml of acetone and 10 µg of fast blue 2B salt per 25 ml of 0.067 M phosphate buffer, pH 7.5. Black bands of precipitate appeared at the sites of esterase activity. In several instances, areas of the starch gel were assayed directly for NLA and ALA as previously described (4).

Ammonium Sulfate Precipitation—Adipose tissue extract which had been frozen at -20° for several days was thawed and filtered through glass wool. An aliquot was saved as control, and 1.98 g of additional (NH₄)₂SO₄ was added to the remaining adipose tissue extract at 4°. This was stirred and then allowed to stand for 30 min at 4°, after which it was centrifuged at 1700 X g for 30 min. The supernatant fluid was removed, and the precipitate was suspended in 2.5 ml of 0.008 M phosphate-citrate buffer, pH 7.0. The supernatant fluid was subjected to three further precipitations by successively adding 1.58, 1.76, and 1.98 g of additional (NH₄)₂SO₄. In each case, the precipitate and supernatant fluid were handled as described initially. The control adipose tissue extract, the resuspended precipitates, and the supernatant fluid derived from 100% saturation with (NH₄)₂SO₄ were dialyzed against 5 liters of 0.008 M phosphate-citrate buffer, pH 7.0, which was changed once during a 24-hour period.

Postdialysis volumes were noted, after which NLA, ALA, and protein concentration were determined on each fraction. Recoveries were calculated by reference to activity and volume of the starting material.

Sephadex Gel Filtration—Ten grams of Sephadex G-200, particle size, 40 to 120 µ, were allowed to swell for 2 to 3 weeks in 0.008 M phosphate-citrate buffer, pH 7.0, at 4°. This slurry was poured into a glass column 2.5 cm in diameter and allowed to settle gradually at 4°, and then to equilibrate for 24 to 48 hours at a flow rate of 10 to 15 ml per hour. Under these conditions, the height of the Sephadex ranged between 55 and 60 cm. In the course of these studies, 21 such columns were prepared and as many as eight separate specimens were filtered through a single column.

The void volume (V₀) of each column was determined by filtering 2 ml of 0.2% dextran blue 2000 through the column and analyzing for the fraction in which maximum concentration occurred.

Generally, 25 ml of fresh adipose tissue extract were placed on a column at 4° and eluted in 5-ml fractions at a rate of 5 to 10 ml per hour with 0.008 M phosphate-citrate buffer, pH 7.0, at a hydrostatic pressure of 50 to 75 cm. Each 5-ml fraction and the original adipose tissue extract were analyzed for NLA, ALA, protein content (8), and the electrophoretic mobility of esterase activity.

For comparison of the elution volumes of lipolytic activities with those of some proteins of known molecular weight, human γ-globulin, human crystalline albumin, and human crystalline hemoglobin were filtered through the column separately. Serum was also placed on a column, and the elution of pseudocholinesterase (11) was determined.

Materials

Olive oil was obtained as "clearing factor emulsion" from the Upjohn Company through the courtesy of Drs. Paul Schurr and Thomas Vecchio. The composition of this emulsion is olive oil, 15% (w/w); polyglycerol ester of oleic acid, 1% (w/w); oxyethylene oxypropylene polymer, 0.5% (w/w); and anhydrous dextrose, 4.17% (w/w). Tributyrin, 99%, was obtained from Mann Research Laboratories; 1100 µmoles were added to 10 ml of 1% polyvinyl alcohol, Grade 52-22, obtained from du Pont. This was emulsified in a VirTis 23 homogenizer.

Bovine Fraction V plasma albumin, human crystalline α-bumin, human Fraction II γ-globulin, and human crystalline hemoglobin were all obtained from Pentex. Rabbit plasma albumin and γ-globulin, purified and kindly supplied by Dr. Paul Binette, were also used for elution through Sephadex. α-Naphthyl acetate was obtained from Mann; fast blue 2B salt, }
Neutral and alkaline lipolytic activity in ammonium sulfate fractions of adipose tissue

Extracts of human adipose tissue were subjected to increasing concentrations of (NH₄)₂SO₄ as described in "Methods." The various precipitates were suspended in 0.008 M phosphate-citrate buffer, pH 7.0, dialyzed to remove (NH₄)₂SO₄, and compared with a control extract (0% saturation) which was subjected only to dialysis. The results depicted below are from a typical experiment. Recoveries were representative of all such experiments and were as follows: 80% (NLA), 78% (ALA), and 76% (protein).

<table>
<thead>
<tr>
<th>Saturation with ammonium sulfate</th>
<th>Lipolytic activity</th>
<th>Protein</th>
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<tbody>
<tr>
<td>%</td>
<td>NLA</td>
<td>ALA</td>
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<tr>
<td>0%</td>
<td>3.6</td>
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<td>25%</td>
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<td>50%</td>
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<td>75%</td>
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<td>100%</td>
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* Control.
* The supernatant fluid obtained after 100% saturation with (NH₄)₂SO₄ contained protein (0.67 mg per ml) and ALA (1.8 μeq of FFA per ml per hour).

The protein in the tissue extract was eluted in two peaks (Fig. 1). The first corresponded with ALA-I. The second, and larger, of the two contained hemoglobin, and corresponded roughly with ALA-III.

Combination of Ammonium Sulfate Precipitation and Sephadex Gel Filtration—On three separate occasions, 60 ml of an adipose tissue extract was brought to 50% saturation with (NH₄)₂SO₄. The precipitate and supernatant fluid were collected and dialyzed as described. Based on postdialysis volumes, 83% of the original extract was filtered through a Sephadex G-200 column.

**RESULTS**

**Separation of Neutral and Alkaline Lipolytic Activities**

*Ammonium Sulfate Precipitation*—Most NLA was separated from ALA by (NH₄)₂SO₄ precipitation. Table I depicts data from a representative experiment. In an average of four such experiments, 65% (62 to 75%) of the original NLA precipitated at or below 50% saturation with (NH₄)₂SO₄, and 14% (8 to 19%) remained in the supernatant fluid. Only 14% (8 to 18%) of the ALA precipitated at 50% saturation with (NH₄)₂SO₄, and 53% (40 to 74%) remained in the supernatant fluid. Total recovery of protein averaged 75%.

*Sephadex Gel Filtration*—Most ALA was separated from NLA by Sephadex gel filtration. Fig. 1 depicts the results of a typical column, in which 52% of the applied NLA was eluted as a major peak immediately after the void volume (V₀). An additional 13% was recovered in the remaining fractions.

ALA was eluted in three separate fractions, subsequently referred to as ALA-I, ALA-II, and ALA-III (Fig. 1). For all tissues studied, ALA-I ranged between 1.5 and 14% of the applied ALA and was eluted in the same volume as NLA. The major portion of ALA was recovered in two fractions, ALA-II and ALA-III. In the example shown (Fig. 1), 80% of the applied ALA was recovered in these fractions, collected between 125 and 215 ml.

γ-Globulin, albumin, and hemoglobin were filtered separately through the same column, and the elution volumes were compared with those of ALA-II and ALA-III. In four experiments ALA-II was eluted between the elution volumes for γ-globulin and albumin, and ALA-III was eluted close to the elution volume for hemoglobin.

The protein in the tissue extract was eluted in two peaks (Fig. 1). The first corresponded with ALA-I. The second, and larger, of the two contained hemoglobin, and corresponded roughly with ALA-III.

**Fig. 1.** Sephadex G-200 gel filtration of a human adipose tissue extract. Twenty-five milliliters of adipose tissue extract were filtered through Sephadex G-200 with 0.008 M phosphate-citrate buffer, pH 7.0, and collected in 5-ml fractions as described in the text. Each fraction was analyzed for NLA, ALA, and protein content. The elution of dextran blue (void volume, V₀), pseudocholinesterase (CH), γ-globulin, albumin, and hemoglobin was determined as described in the text. The three fractions of ALA are designated by Roman numerals I, II, and III.
as resuspended precipitate, followed by 20% of the original extract as supernatant fluid.

Fig. 2 illustrates the elution pattern for ALA and protein in a typical experiment. A small amount of ALA remained in the precipitate, but the supernatant fluid contained most of the activity as ALA-II and ALA-III. Although the relative amounts of ALA-II and III remained unchanged, the activity of ALA-II increased considerably in relation to protein content. In the example cited, the activity per mg of protein was 27 times that of the original tissue extract, and in another instance it was 36 times greater.

NALA, a much less stable enzymatic activity (2), has been more difficult to recover after this combination of procedures. However, it was demonstrated that the NLA of an (NH₄)₂SO₄ precipitate was eluted in a volume identical with the elution volumes of NLA already obtained.

Variation among Tissues from Different Individuals—When fresh tissue extracts from different individuals were filtered through the same column within a period of 1 to 2 weeks, certain variations were observed even though the void volume was shown to remain constant. Fig. 3 illustrates some of these variations.

The amount of ALA-I did not correlate with NLA and has varied from small amounts (Fig. 3B) to amounts greater than the NLA present (Fig. 3C). Even when ALA-I was greater than NLA (Fig. 3C), the recovery of applied activities in this peak was 60% for NLA and only 14% for ALA.

It was considered possible that pseudocholinesterase or a monoglyceride lipase (12) contributed to the activity of ALA-I. Although pseudocholinesterase is present in a significant number of adipose tissue extracts (4), 100 μM eserine sulfate, a known inhibitor, did not alter ALA-I in two separate experiments. Small but detectable amounts of monoglyceride lipase activity were present in the fractions coinciding with ALA-I.

Fig. 3 also illustrates the variations among individual tissues of ALA and its distribution between Fractions II and III. These variations have not correlated with the clinical or anesthetic condition of the patient.

**Fractionation of ALA**

ALA-I, II, and III—ALA was eluted in three fractions, ALA-I, ALA-II, and ALA-III (Fig. 1), each of which had the ability to hydrolyze α-naphthyl acetate (Fig. 4).

**Starch Gel Electrophoresis**—Eluates obtained after Sephadex gel filtration of adipose tissue extracts were subjected to starch gel electrophoresis, and the starch gel was analyzed for hydrolysis of α-naphthyl acetate. Fig. 5 shows results which are typical of those obtained for consecutive eluates containing ALA-I and II. To the right of this figure are noted various areas of the starch gel which were previously designated A through E (4).

11 In the preceding studies it was shown that the major portion of staining occurred as five bands of activity (Bands 1 through 5), which migrate 6 to 8 cm toward the anode, to an area designated as C. This area contained 80% of the recoverable ALA, and the origin (Area D) contained 9%. Negligible amounts of activity were distributed throughout the other areas.
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a-NA, Hydrolysis

FIG. 4. Comparison of the hydrolysis of a-Naphthyl acetate with NLA and ALA in eluates of a Sephadex G-200 column. Twenty-five milliliters of an adipose tissue extract were filtered through a Sephadex G-200 column as described in the text. The hydrolysis of a-naphthyl acetate (a-NA) was determined for each individual fraction and expressed as milligrams of a-naphthol (a-N) released per ml of eluate per hour. This was compared with the assay values for NLA and ALA in each fraction.

The eluates which contained NLA and ALA-I were the only ones that contained a dark band of staining at the origin after electrophoresis. These eluates were subjected to a second starch gel electrophoresis in which sections of the starch were assayed and found to contain NLA and ALA at the origin.

An anodal band of esterase activity (CH) which migrated to Area B was previously shown to be pseudocholinesterase activity (4).12 This band was seen occasionally in the early Sephadex fractions (Fig. 5), but not others.

Sephadex fractions which contained ALA-II and III exhibited an electrophoretic mobility of a-naphthyl acetate hydrolysis (Figs. 5 and 6) identical with that shown to be due to ALA (4). When these fractions were subjected to electrophoresis and the starch gel was subsequently assayed, ALA was found only in Area C. The electrophoretic patterns differed slightly for ALA-II and III in that ALA Band 5 was more prominent in Fraction III (Fig. 6).

The eluates obtained after Sephadex gel filtration of the (NH₄)₂SO₄ fractions were also subjected to starch gel electrophoresis. Typical electrophoretic patterns for the precipitate and supernatant fractions are seen in Fig. 7. The staining at the origin and in Area C corresponded with the known patterns of elution for NLA, ALA-I, ALA-II, and ALA-III, respectively.

It has previously been demonstrated (4) that human adipose tissue contains esterase activities which migrate to a prealbumin region designated as Area A (Fig. 5) and a cathodal region designated as Area E (Fig. 5). The staining in both of these areas, when present, was eluted from Sephadex in a volume corresponding with ALA-III.

Relationship between ALA-I and ALA-II—The Sephadex fractions containing NLA and ALA-I coincided with a milky fraction which was extracted with chloroform-methanol (2:1) or Dole’s extraction mixture (2-propanol-heptane-1 N H₂SO₄, 40:10:1) (5). These extracts were subjected to thin layer chromatography with a solvent system of hexane-diethyl ether-acetic acid (90:10:1) (13), and it was shown that the milky fractions and no others contained varying amounts of mono-, di-, and triglycerides. It seemed possible that ALA-I was associated with this lipid and that a lipid-protein complex of ALA could explain the presence of some ALA at the origin of a starch gel after electrophoresis (4) and in the lipid-containing fractions of a Sephadex column. Several experiments were performed to explore this point.

Ether extraction16 of adipose tissue yielded aqueous material

12 Between 25 and 100 ml of adipose tissue extract were treated with diethyl ether at 4° as previously described (2). The particulate interface was suspended in 0.008 M phosphate-citrate buffer, pH 7.0, and filtered through glass wool to remove large particles. The aqueous phase was re-extracted with cold ether, and the residual ether was evaporated under nitrogen. Under
Fro. 6. Esterase activity on starch gel after electrophoresis of Fractions from a Sephadex G-200 column. An adipose tissue extract was filtered through a column of Sephadex G-200 and analyzed as described in the text. The values for NLA and ALA are similar to those depicted in Fig. 1. Starch gel electrophoresis was performed on single representative fractions for ALA-I, II, and III and compared with an aliquot of the original adipose tissue extract (C) which was filtered through the column. After electrophoresis the gel was sliced horizontally and stained for esterase activity.

which contained no ALA-I and no staining at the origin after starch gel electrophoresis. No increase in ALA-II or ALA-III was detected. Similarly, no NLA has been recovered after Sephadex gel filtration of either the aqueous or particulate portion of an ether extract.

In an alternative approach, 1 ml of an ALA-I fraction from Sephadex was shaken gently for 5 min in an ice-brine bath with an equal volume of diethyl ether. The aqueous phase was then subjected to starch gel electrophoresis. Although the original ALA-I contained stainable activity only at the origin after electrophoresis, the aqueous material resulting from the ether extraction contained clearly demonstrable bands of activity such as those seen with ALA-II (Fig. 8). In this same preparation, a decrease in activity was noted at the origin. Conversely, it was possible to produce material which remained at the origin during electrophoresis by the addition of lipid to an eluate of ALA-II.

similar conditions, 67% of the original ALA was present in the aqueous phase and 10% in the interface; 25% of original NLA was present in the interface and only 9% in the aqueous phase (2).

14 Small increases in these already large values would have been undetectable.

When a similar preparation of ALA-II and lipid was passed through Sephadex, the fractions collected immediately after the void volume contained both a small peak of ALA and stainable activity which did not migrate during starch gel electrophoresis. Incubation of ALA-II with lipid did not produce detectable NLA or monoglyceride lipase activity.

Relationship between ALA-II and ALA-III—A variable quantitative relationship between ALA-II and III has already

15 ALA-II fractions from a previous Sephadex column were mixed with Lipomul (a 15% emulsion of cottonseed oil produced by Upjohn) to yield a final concentration of 1% lipid and then placed at 37°C for 4 hour. Appropriate controls containing mixtures of lipid and 0.9% NaCl or lipolytic activity and 0.9% NaCl were prepared for comparison. These controls contained no activity at the origin of a starch gel after electrophoresis.

16 ALA-II in this experiment was shown to have the typical electrophoretic pattern for ALA, and no staining at the origin.
been shown (Fig. 3). Since ALA Band 5 was more prominent in Fraction III than in II (Fig. 6), and since Band 5 occurs with storage at room temperature (4), it was thought that ALA-III might be produced by prolonged storage of ALA-II. Therefore, on three separate occasions, the fractions from a column which contained ALA-II were combined, allowed to stand at 25° for 10 days, and then refiltered through the same Sephadex column. Under these conditions, a major portion of ALA-II was converted to ALA-III (Fig. 9). In this same experiment, prolonged standing resulted in the formation of several bands of activity which migrated less rapidly than Band 4 during electrophoresis on starch gel. These bands were eluted in a volume comparable to that of ALA-III (Fig. 10).

On several occasions, a transformation from ALA-II to ALA-III was seen to occur at 4° over a period of several days. Fig. 11 illustrates such an experiment in which total ALA did not change but a precursor-product type of relationship existed between ALA-II and ALA-III.

**Fig. 8.** The appearance of ALA on starch gel electrophoresis after an ether extraction of ALA-I. An adipose tissue extract was filtered through a column of Sephadex G-200 and analyzed for ALA. The eluate that contained maximum amounts of ALA-I was subjected to extraction with diethyl ether in an ice-brine bath. In the example cited, two separate ether extractions were performed. The first extraction (I-E) was carried out for 15 min with mechanical stirring. The second extraction (I-E') was carried out with gentle hand agitation for 5 min. The aqueous phase from each extraction was compared on starch gel electrophoresis with the individual ALA Fractions I, II, and III and with the control adipose tissue extract (C) that was originally filtered through the Sephadex column.

**Fig. 9.** The conversion of ALA-II to ALA-III by storage. Twenty-five milliliters of an adipose tissue extract were filtered through Sephadex G-200, and ALA was determined (Sephadex Column 1). Selected fractions of ALA-II (shaded area) were combined, stored for 10 days at 25°, filtered through the same column, and analyzed for ALA (Sephadex Column 2). The low levels of activity in eluates of Sephadex Column 2 were due primarily to dilution. To account for this, a modified assay system was used in addition to the standard method (see "Methods").

One milligram of trypsin inhibitor per mg of extract protein partially inhibited the production of ALA-III when the extract was allowed to stand at 20° for 10 days. In contrast, 16,000 U.S.P. units of penicillin and 20 mg of streptomycin per ml of extract did not inhibit the formation of ALA-III at 20° even though the extract was proven to be sterile after 10 days.

On two occasions, ALA-III was obtained after Sephadex gel filtration and refiltered through Sephadex to eliminate any minor contamination from ALA-II. The trailing portion of this recycled ALA-III fraction was then concentrated 5 to 6 times and filtered through Sephadex for a third time. In each instance, the resulting eluates contained both ALA-II and ALA-III. Fig. 12 gives a typical elution pattern, which was substantiated by starch gel electrophoretic patterns characteristic of each fraction.

**DISCUSSION**

A number of lipolytic activities (12, 14-24) other than lipoprotein lipase (25) have been demonstrated in animal adipose tissue by a variety of assay systems. At least one of these activities is associated with the fat (12, 23, 24), and at least one other with the aqueous (14-22) portion of an adipose tissue preparation.
FIG. 10. Starch gel demonstration of the conversion of ALA-II to ALA-III. Fractions from the Sephadex gel filtration experiment depicted in Fig. 9 were subjected to starch gel electrophoresis and subsequent determination of a-naphthyl acetate hydrolysis. ALA-II after storage (II-1) and the ALA fractions from the second column (I-2, II-2, and III-2) were compared with a fresh adipose tissue extract as control (C).

FIG. 11. Precursor-product relationship of ALA-II and ALA-III. Eighty milliliters of an adipose tissue extract were prepared and stored at 4°C. Shortly after preparation (Day 0), the next day (Day 1), and 3 days later (Day 3), 25 ml of this extract were filtered through a Sephadex G-200 column. The void volume did not vary throughout the 3 days of the experiment. ALA was determined and graphed in a manner similar to the preceding figures. The total activity of ALA-II and III was calculated, expressed as microequivalents of FFA released per hour and plotted with respect to time.

FIG. 12. The conversion of ALA-III to ALA-II. Twenty-five milliliters of an adipose tissue extract were filtered through Sephadex G-200 in the usual manner (Sephadex Column 1). ALA-III was collected, concentrated to 25 ml, and resubjected to the same column (Sephadex Column 2). The trailing portion of ALA-III from this second column was concentrated 6 times and filtered through another column (Sephadex Column 3). Because the height and void volumes of this latter column were less than those of the previous column, the elution volumes of ALA-II and ALA-III were slightly different.

Recent studies from this laboratory have shown that human adipose tissue also contains at least two lipolytic activities other than lipoprotein lipase (2). One of these, neutral lipolytic activity (NLA), is assayed at pH 7.0 and 37°C, with olive oil as substrate, and the other, alkaline lipolytic activity (ALA), at pH 8.0 and 47°C, with tributyrin as substrate. Studies which related triglyceride concentration to both lipolysis and absorbance of the assay system suggested that NLA represents hydrolysis of an emulsified ester, and ALA, hydrolysis of a soluble ester. These activities differed in their requirement for albumin as a fatty acid acceptor in vitro, and in their inhibition characteristics, thermal stability, and pH dependence of an extract. NLA is primarily associated with the fat moiety, and ALA with the aqueous portion, of a tissue homogenate, a condition which made it possible to achieve partial separation by centrifugation and by extraction with diethyl ether.

Recently (4) it has been shown that human adipose tissue extracts hydrolyze a-naphthyl acetate and that the activities responsible for this hydrolysis can be identified after electrophoresis in starch gel by a staining method which employs naphthol esters as substrates and fast blue 2B as coupling agent. As
that ether extraction of human adipose tissue yielded 77% recovery of ALA-II with lipid (Lipomul) produced ALA-I. These data indicated that ALA-I existed as a lipid-protein complex of the smaller molecular size by virtue of its elution from Sephadex G-200, and each has a characteristic electrophoretic pattern which represents a portion of the previously demonstrated pattern of esterase activity in human adipose tissue (4). Some tentative properties of these three fractions of ALA are summarized in Table II.

The fractions containing NLA and ALA-I contained lipid which was identified by thin layer chromatography. Extraction of this lipid with ether converted ALA-I to ALA-II (defined by starch gel electrophoresis) (Fig. 8). Conversely, incubation of ALA-II with lipid (Lipomul) produced ALA-I. These data indicate that ALA-I exists as a lipid-protein complex of the smaller molecule, ALA-II, and are similar in some respects to gel filtration studies of pancreatic lipase (26). The fact that a similar delipidation of NLA has not been demonstrated may be due in part to its instability, since it has been shown previously that ether extraction of human adipose tissue yielded 77% recovery of ALA and only 35% recovery of NLA (2).

Most of the ALA present in an adipose tissue preparation exists as two fractions, ALA-II and ALA-III (Fig. 1), each of which is eluted after ALA-I and thus is smaller than ALA-I. The electrophoretic data (Figs. 6 and 7) indicate that ALA-II is composed mainly of Bands 1 to 4, and ALA-III mainly of Band 5. The presence of some Bands 1 to 4 in ALA-II and of some Bands 5 to ALA-II (Fig. 9) and Band 6 to ALA-III (Figs. 9 and 11), by a decreased rate of this conversion as the concentration of ALA-III increases (Fig. 11), and by the production of ALA-I\textsuperscript{II} from ALA-I\textsuperscript{III} (Fig. 12). These data, combined with the fact that ALA-III is eluted after ALA-II, suggest that ALA-III is a subunit of ALA-II.

Since ALA Band 5 is smaller than Bands 1 to 4, the presence of five electrophoretic bands of ALA cannot be explained by two subunits freely associating into five tetramers, as is the case with lactate dehydrogenase (27). In addition, the four bands of activity associated with ALA-II do not appear to be a series of polymers, as is exemplified by haptoglobin (28), because of the relatively small elution volume from Sephadex and the fact that the same bands run from the leading to the trailing edge of the fraction (Fig. 5). There are several possible explanations for the existence of Bands 1 to 4 in a single Sephadex fraction (ALA-II): (a) an artifact in vitro, (b) sequential loss (29) or addition (30) of a charged molecule, and (c) conformational change (31). It is also possible that ALA-III undergoes conformational change, thus offering an additional explanation for the several bands seen in ALA Fraction III.

Thus, NLA is a large molecule which is separable from most ALA. ALA exists in three fractions, each characterized by elution from Sephadex and starch gel electrophoresis. The ability of ALA to become bound to lipid (ALA-I) may have relevance to the mechanism of lipase action both in vitro and in vivo. The reason for the variation in the relative amounts of ALA-II and III in adipose tissues of different individuals (Fig. 3) is not certain. While this may have significance in vivo, it is quite possible that interconversion of ALA-II and ALA-III proceeds during the experiment, producing the variable relationship.

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REFERENCES