Identification of albumin-bound fatty acids as the major factor in serum-induced lipid accumulation by cultured cells

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ABSTRACT Factors responsible for the high lipogenic activity of rabbit serum were investigated using an assay procedure based on the gravimetric determination of the 24 hr increase in cell lipid. Cellular synthesis of fatty acids was inhibited by the presence of serum in the assay medium. Approximately 90% of the increase in cell lipid produced by serum fractions was due to triglyceride accumulation.

Fractionation of rabbit serum by precipitation with ammonium sulfate or by ultracentrifugation in high density medium, both indicated that three-quarters of its lipogenic activity was associated with albumin. The lipoproteins prepared by ultracentrifugation also exhibited about one-half the activity of whole serum. The lipogenic activity of albumin was confirmed by the high potency of the albumin isolated in a nearly pure form from proteins of d>1.21 by precipitation with trichloroacetic acid and extraction with ethanol. As judged from chemical and isotopic analysis, neither the lipid content nor the lipid composition of the albumin was appreciably altered during its isolation. Of the albumin-bound lipids, only the free fatty acids, as determined by DEAE column chromatography, were present in an amount sufficient to account for the observed increase in cell triglycerides.

In control experiments with horse serum of low lipogenic activity, the proteins of d>1.21 also possessed low activity in conjunction with a low content of free fatty acid. However, the albumin isolated from the latter preparation exhibited the high lipogenic activity of rabbit serum albumin. Chemical and isotopic analysis of the recovered horse serum albumin revealed that its free fatty acid content was the same as that of rabbit serum albumin. These results indicated that the isolation of horse serum albumin was attended by a substantial increase in its free fatty acid content.

When the rabbit serum and horse serum content of media were adjusted to provide equivalent concentrations of albumin-bound fatty acids, the rabbit liver cells grown on the former media accumulated more lipid than cells grown on the latter media. This difference was shown to be due to the higher concentration of albumin per μmole of fatty acid in horse serum as compared with rabbit serum. Consequently, the albumin to fatty acid ratio also controls the lipogenic activity of a serum. A linear relationship is presented which relates the cell lipid content to the molar ratio of albumin to free fatty acids and to the absolute concentration of free fatty acids in the medium.

SUPPLEMENTARY KEY WORDS triglyceride accumulation · lipogenic activity · isolation of albumin · lipid composition of albumin · isolation of free fatty acids · ammonium sulfate fractionation · serum proteins · rabbit · horse · lysolecithin

Since cultured cells and their environment are more amenable to chemical analysis and control than their counterparts in the intact organism, this in vitro system provides an attractive model for studying the intra- and extracellular factors responsible for the accumulation of lipid by animal cells, a phenomenon associated with many of the so-called degenerative diseases. Historically, Lambert (1) in 1914 observed that the fat-staining droplets seen in chick embryo heart cells growing in chicken plasma failed to develop when the plasma was diluted 10-fold with Ringer's solution. Later Simms and coworkers, in a series of papers published between 1937 and 1956 (2–5), described a plasma factor that produced fat granules in chicken aorta and liver cells and in the intima and media of explanted arteries.

The active factor, termed lipfanogen, was inhibited by a substance present in serum called antilipfanogen. In 1961, Rose, Fuenning, and Maca (6) reported that the serum of stressed cockerels caused the appearance of lipid droplets in mouse fibroblast cells. On the basis of chemical analysis, the authors concluded that the active factor was probably hormonal in nature and that the lipid of the droplets arose from cellular synthesis. A similar point of view had been advanced earlier by Kleinzeller (7) and Grossfeld (8) although both Lambert and Simms had assumed that particle lipid was derived from serum. None of these authors, however, reported the lipid content of the cells, or provided evidence that distinguished between the alternative hypotheses concerning its origin.

Recently we found that rabbit serum, and some samples of human serum, in contrast with horse serum, produce a striking increase in lipid-staining particles in a variety of mammalian cells. These include rabbit liver cells (9), mouse fibroblast cells (10), pig and rabbit kidney cells (11), human W1-38 lung cells,[1] and, to a lesser degree, human and rat liver cells (9, 11). The lipogenic[2] activity of serum does not depend on chylomicrons and is not removed by exhaustive dialysis (10).

Chemical analysis of the cells shows that the appearance of the particles, which are approximately 1 μ in diameter and surrounded by a limiting membrane, is invariably accompanied by an increase in total cell lipid, and that this increase is due almost entirely to triglycerides (9, 10). Isolation and analysis of the particles has revealed that they contain approximately 3% protein, and 90% lipid, of which 90% is triglyceride (12). As demonstrated by fractionation experiments, the particle lipid accounts for the increase in cell triglyceride produced by rabbit serum (11). As for the source of the accumulated triglycerides, isotope experiments by Bailey (13) and ourselves (10, 11) have disclosed that low concentrations of human, horse, and rabbit sera alike suppress the synthesis of fatty acids and cholesterol by mammalian cells. Consequently, in the lipid accumulation caused by rabbit and human sera, at least the fatty acid moieties of the triglycerides are of exogenous origin.

The present paper reports experiments directed toward the isolation and identification of the serum factor(s) responsible for triglyceride accumulation in mammalian cells in the absence of *de novo* fatty acid synthesis.

[1] Mackenzie, J. B. Unpublished results.

[2] The term "lipogenic" is applied to any factor that increases the lipid content of a cell without reference to the origin of the whole lipid molecules or their constituent parts.

MATERIALS AND METHODS

Cells and Cell Culture Methods

The rabbit liver cell, clone C-15, and the rate liver cell, clone C-1, were isolated in our laboratory (9); the mouse fibroblast cell, L clone of strain NCTC-2071 (14), was kindly supplied by Dr. Virginia Evans of the National Cancer Institute; and the human liver cell isolated by Chang (15) was obtained from Microbiological Associates, Inc., Bethesda, Md. Stock cultures of the rabbit liver cell were maintained on a medium containing rabbit serum and human cord serum (9). The other cells were maintained on modified Eagle's medium containing 10% horse serum (16).

In all experiments the cells were grown at 37.5° in desiccators gassed continually with 5% CO_2 in air (16). At the start of an experiment, stock cells were trypsinized and plated in 6-cm Petri dishes containing 5 ml of the appropriate stock medium. After 18 hr, when the cells had entered the log phase of growth, cell protein was measured in three dishes, and the medium in the remaining dishes was replaced with 5 ml of one of the experimental media described below. These media were replaced daily with fresh media at pH 7.4, and the number of cells initially plated was such that the pH in an experiment never fell below 7.1.

The first experimental medium, the assay medium, had the following composition: 20 ml of horse serum, 5 ml of NCTC-109 (17), 1 ml of beef embryo extract ultrafiltrate (Microbiological Associates, Inc.), twice the amino acid, glutamine, and vitamin concentrations used in our modification of Eagle's medium (16), 80 U of insulin (U-80 Regular, Lilly USP), and Earle's salt solution (18) in a final volume of 100 ml. When serum fractions were incorporated in the assay medium, the osmolarity was kept constant by making appropriate adjustments in the volume and (or) concentration of the Earle's salt solution.

The second experimental medium, that is, the one used to compare the effects of various concentrations of horse serum and rabbit serum on cell lipid in 3-day experiments, was our modification of Eagle's medium (16). Variations in the concentration of serum were compensated for by adjustments in the volume of the Earle's salt solution.

All medium components, except serum fractions, were sterilized by filtering through a 03 porosity Selas candle (Selas Flotronics, Spring House, Pa.). Serum fractions were sterilized by filtration through a 02 porosity Selas candle.

Determination of Cell Lipid and Protein

Cells were washed free of medium, and their total lipid was quantitatively extracted and weighed to ± 5 μg

as described in previous publications (9, 10). Protein was measured in the extracted cell residue (10) by the method of Oyama and Eagle (19). The isolated cell lipid was separated into five fractions by silicic acid column chromatography (10).

Fractionation of Serum

Rabbit serum was purchased from Flow Laboratories, Inc., Rockville, Md., Microbiological Associates, Inc., and Colorado Serum Co., Denver, Colo. Horse serum was purchased from the first two of these sources. All sera were tested for lipogenic activity prior to their use in experiments.

Fractionation of rabbit serum by the procedure of Cohn et al. (20) was carried out in Pentex Biochemical,[3] Kankakee, Ill. Gel filtration was performed by applying 5 ml of serum to a 2.8 × 70.0 cm column of Sephadex G-200 and eluting with 1.0 M NaCl containing 0.1 M Tris–HCl, pH 8.0. Fractionation with $(NH_4)_2SO_4$ was done by the method of Svensson (21).

Ultracentrifugal fractionation of serum by the procedure of Havel, Eder, and Bragdon (22) was performed in a Spinco model L ultracentrifuge equipped with a No. 40 rotor. After adjusting the serum to d 1.21 with KBr, it was centrifuged for 24 hr at 100,000 g, and the lipoproteins were removed with a pipette. Residual lipoproteins were then separated from proteins of d>1.21 by slicing the centrifuge tubes at one-third of the distance from the top.

The lipoproteins were dialyzed at 4°C in a rotary dialyzer for 48 hr against several changes of Earle's salt solution minus bicarbonate. All of the other serum fractions prepared by the foregoing procedures were dialyzed at 4°C against running triple-distilled water in a rocking dialyzer for 48 hr or until the dialysate was free of Cl^-, Br^-, and $SO_4^=$. When the volume of a dialyzed fraction was too large for incorporation in the assay medium, it was concentrated by pervaporation at room temperature. Prior to assay, the serum fractions were analyzed for total protein and lipid both before and after sterilization by filtration through a 02 porosity Selas candle.

Albumin was isolated from serum proteins of d>1.21 by the trichloroacetic acid precipitation–ethanol extraction procedure of Levine (23) as modified by Korner (24). The precipitated protein was cooled in an ice bath and triturated with cold 95% ethanol containing 1% trichloroacetic acid. The blended suspension was then centrifuged at 3700 rpm in a No. 845 head of a model PR-2 International centrifuge for 15 min at 2°C, and the supernatant solution was decanted. This extraction procedure was repeated, and the combined extracts were dialyzed and pervaporated to a final concentration of 25–30 mg of protein per ml. Small amounts of insoluble material which appeared in some preparations during pervaporation were removed by centrifuging at 1500 rpm in a No. 269 head in the International centrifuge for 15 min. The clear solution of albumin was then sterilized as described above.

Analysis of Serum and Serum Fractions for Protein and Lipid

Total protein was measured by the method of Oyama and Eagle (19). Protein composition was determined by electrophoresis on cellulose acetate (Sepraphore III; Gelman Instrument Company, Ann Arbor, Mich.) in a Shandon apparatus (Colab Labs., Inc., Glenwood, Ill.) followed by dye elution as described by Kohn (25). The pH of the extracted Ponceau S solution was adjusted to about 7.0 with 2.2 N H_3PO_4, and its concentration read in a Klett photometer using a 520 nm filter. With both pooled human and rabbit sera, the standard errors for the percentage of albumin and globulins were ±0.2–±0.3.

Qualitative analysis for lipoproteins in serum and serum fractions was carried out on prestained preparations (25) by cellulose acetate electrophoresis, and on unstained preparations by filter paper electrophoresis, following the procedure of Jencks and Durrum (26).

Lipid was measured in the $(NH_4)_2SO_4$ fractions by the procedure of Bloor (27) and in all other preparations by the following modification of the method of Folch, Lees, and Sloane Stanley (28). To 0.4 ml of a serum preparation containing about 50 mg of protein, 3.2 ml of methanol was added with swirling, and the mixture was boiled for 15–30 sec. Next, 3.2 ml of chloroform was added, and the boiling was continued for 15 sec. After cooling, an additional 3.2 ml of chloroform was added, and the extract was filtered. The precipitate was washed with chloroform–methanol 2:1 (v/v), until the volume of the filtrate reached 10 ml. 2 ml of 10 mM sodium phosphate, pH 3, was then added to the filtrate to obtain the two phases. Whole serum was extracted in the same way except that 50 mM sodium phosphate, pH 3, was used to obtain the two phases.

The extracted lipid was fractionated by column chromatography on silicic acid (10) and on DEAE-cellulose charged with acetic acid. In the latter procedure, the column was eluted with the solvents of Rouser, Bauman, Kritchevsky, Heller, and O'Brien (29) except that the mixture of chloroform and glacial acetic acid was omitted. Approximately 1-mg samples of lipid were chromatographed on 0.8 × 8.0 cm columns of DEAE-cellulose charged with acetate. The eluting solvents (29) were collected in 5-g fractions (10) as follows: (*a*)

[3] Isopropanol is routinely used in Pentex Biochemical as the organic solvent in the Cohn procedure in accordance with Cohn Patent No. 2,390,074.

four fractions of chloroform–methanol 7:1 (v/v), (*b*) three fractions of chloroform–methanol 7:3 (v/v), (*c*) three fractions of methanol, and (*d*) three fractions of glacial acetic acid. The eluted fractions were evaporated to near dryness under N_2 at atmospheric pressure and dried over P_2O_5 or NaOH in vacuo (water pump). The amount of lipid in each fraction was determined gravimetrically. The average recovery of lipid applied to the columns was 95%. The composition of the lipid in the various fractions was determined by chemical analysis, silicic acid column chromatography, and silicic acid thin-layer chromatography as described under Materials and Methods.

Lecithin was eluted from the DEAE columns by the second and third chloroform–methanol 7:1 fractions, and lysolecithin was eluted by the fourth of these fractions and the first of the chloroform–methanol 7:3 fractions. The amounts of the two compounds were calculated from the phosphorus content of the corresponding fractions, taking 4.1 and 6.0% as the phosphorus content of lecithin and lysolecithin. No other phospholipids were detected by thin-layer chromatography in any of the eluate fractions or in the original lipid. The nonesterified fatty acids were eluted by the glacial acetic acid, and no other lipids could be detected in these fractions. Furthermore, when a tracer amount of palmitic acid-1-^{14}C was added to the original lipid, 99% of the isotope was recovered in the glacial acetic acid eluates. Cholesteryl esters and free cholesterol were measured by chemical analysis of the chloroform–methanol 7:1 fractions. Hydrocarbons plus cholesteryl esters were measured by silicic acid column chromatography of the original lipid samples.

Lipid fractions, as well as the total lipid, were analyzed for phosphorus by the method of King (30) and for cholesterol and cholesteryl esters by the method of Abell, Levy, Brodie, and Kendall (31). Nonesterified fatty acids were measured in serum by the double extraction procedure of Dole and Meinertz (32).

Thin-layer chromatography was carried out on silica gel prepared according to Rouser, Galli, Lieber, Blank, and Privett (33). Fatty acids and glycerides were separated in the hexane–ethyl ether–acetic acid system of Malins and Mangold (34) employing proportions of both 70:30:1 and 50:50:1 (v/v). Lysolecithin was separated from lecithin in butanol–acetic acid–water 80:20:20, and in chloroform–methanol–water 65:25:4 (v/v), according to the procedure of Rouser, Galli, Lieber, Blank, and Privett (35).

Reference compounds and their sources were as follows: palmitic acid, tripalmitin, cholesterol, and cholesteryl esters, The Hormel Institute, Austin, Minn.; egg lysolecithin, Supelco, Inc., Bellefonte, Pa.; and β,γ-dipalmito-D,L-α-lecithin, Fluka A.G., Buchs, Switzerland. The latter preparation was recrystallized from redistilled dioxane.

Palmitic acid-1-^{14}C was obtained from New England Nuclear Corp., Boston, Mass. When examined for radiochemical purity by the method of Brown and Johnston (36), over 96.5% of its radioactivity was found in the spot that migrated with the standard palmitic acid. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer (model 314EX). Each vial contained 10 ml of toluene, 80 mg of 2,5-diphenyloxazole, and 0.5 mg of 1,4-bis[3-(5-phenyloxazolyl)]benzene. Benzoic acid-1-^{14}C (Packard Instrument Co., Inc., Downers Grove, Ill.) dissolved in toluene was used as an internal standard to determine the counting efficiency.

EXPERIMENTAL PROCEDURE

Assay for Lipogenic Activity

Replicate dishes of rabbit liver cells were grown for 2 days on the assay medium. At the end of this time, three dishes of cells were continued on the assay medium alone, three dishes were transferred to assay medium supplemented with 20% rabbit serum, and three dishes were transferred to assay medium supplemented with the serum fraction to be tested. The concentration of the serum fraction was routinely adjusted to the level present in the 20% rabbit serum. After 24 hr the total cell lipid and protein present in each dish were determined, and the μg of cell lipid per μg of cell protein was calculated. The mean increase in the lipid to protein ratio produced by the serum fraction was then divided by the mean increase produced by the control rabbit serum to obtain the "lipogenic index" of the serum fraction which is defined as follows:

$$\text{Lipogenic index} = \frac{\Delta \text{ cell lipid:protein, serum fraction}}{\Delta \text{ cell lipid:protein, rabbit serum}}$$

In this assay, whole serum could be substituted for the "serum fraction," and Table 1 shows the results obtained with a typical lot of horse serum. The small standard errors for the mean lipid to protein ratios in all groups of cells are representative of those routinely obtained in the assay procedure. Consequently, in the present experiments, any increase in the cell lipid to protein ratio of 0.02 or more was highly significant as judged by the "*t*" test (37).

The mean generation time of cells grown on the assay medium alone was 24 hr and of cells grown in medium supplemented with 20% rabbit serum, 21 hr. Intermediate generation times were encountered in cells supplemented with serum fractions. Since none of the supplements reduced the mean protein content of the cells,

TABLE 1 24 hr Assay for Lipogenic Activity

Supplement	μg of Cell Lipid per μg of Cell Protein		Lipogenic Index
	Total	Increase	
None	0.236 ± 0.0015		
Horse serum	0.266 ± 0.0003	0.030*	0.24
Rabbit serum	0.362 ± 0.0010	0.126	

The test horse serum and the control rabbit serum were incorporated in the assay medium at levels of 20%. After 24 hr, cell lipid and protein were measured in triplicate dishes in each group, and the mean values and standard errors for the μg of cell lipid per μg of cell protein were calculated. The lipogenic index of horse serum is the increase in cell lipid produced by horse serum divided by the increase in cell lipid produced by rabbit serum. The lipogenic index of rabbit serum is assumed to be 1.0.

* The calculated value of t for this increase in cell lipid is 19.6 as compared with 4.6 required for P 0.01 at 4 degrees of freedom.

all increases in the lipid to protein ratio were due to true increases in cell lipid. Furthermore, within a given assay, the response to an active serum fraction was linear over a cell lipid to protein ratio of 0.24–0.55. However, two precautions should be mentioned in using this procedure. First, the cell population per dish must be restricted so that glycolysis does not lower the pH below 7.1. This is essential since acidification of the medium will in itself cause an increase in cell lipid (38). Second, the lot of rabbit serum under investigation must be included as a control in every assay. This is necessary since some fluctuation in cell response may be encountered from assay to assay even with the same batch of serum.

Lipogenic Activity of Rabbit Serum Fractions Prepared by the Cohn Procedure and by Gel Filtration

When rabbit serum was fractionated by the procedure of Cohn et al. (20) and the major fractions recombined, they exhibited less than one-sixth of the activity of the original serum (Table 2). Most of this low activity was attributable to albumin-rich fraction V which had a lipogenic index of 0.1.

Gel filtration of the same serum produced three overlapping but successively higher and broader protein peaks. The percentage of the total eluted protein under each peak, in order of emergence, was 9, 31, and 60%. The respective lipogenic index of each fraction was 0.05, 0.04, and 0.40. Repetition of the gel filtration on a different lot of rabbit serum resulted in the same distribution of protein as observed in the previous experiment. This time, however, the respective lipogenic index of each fraction was 0.30, 0.04, and 0.33. In view of these variable results and the poor recoveries of lipogenic activity, gel filtration was discontinued in favor of the classical ammonium sulfate procedure for serum fractionation.

TABLE 2 Lipogenic Activity of Cohn Fractions of Rabbit Serum

Supplement	μg of Cell Lipid per μg of Cell Protein		Lipogenic Index
	Total	Increase	
None	0.220		
Fractions II, III, IV, V, VI	0.246	0.026	0.15
Fraction V*	0.237	0.017	0.10
Rabbit serum	0.392	0.172	

Cohn fractions were incorporated in the medium at the approximate levels present in 20% serum. Concentrations (mg/ml of medium) of fractions were as follows: II, 0.6; III, 1.8; IV, 1.8; V, 8.0; VI, 0.6. Serum from which the fractions were prepared was incorporated in medium at a 20% level to provide 12.8 mg of protein per ml of medium. Results are averages of three assays with the combined fractions and five assays with fraction V alone. Conditions of the assay are given in Table 1.

* Protein in fraction V consisted of 92.8% albumin, 1.2% postalbumin, 1.6% α-globulin, and 4.4% β-globulin. The lipid content was 0.15 mg/100 mg of albumin as compared with the larger amounts present in other albumin-rich preparations (Tables 3 and 8).

Lipogenic Activity of $(NH_4)_2SO_4$ Fractions of Rabbit Serum

Maximum separation of rabbit serum albumin from globulins was obtained by adding, with constant stirring at room temperature, two volumes of serum to three volumes of a saturated solution of $(NH_4)_2SO_4$ at a rate of 1 ml/min (21). In this procedure one-half of the serum protein was precipitated, and one-half remained dissolved in the 60% saturated $(NH_4)_2SO_4$ solution. The protein composition of the soluble and insoluble fractions is given in Table 3. Albumin constituted 95% of the protein in the soluble fraction and accounted for two-thirds of the total serum albumin. Globulins constituted about half of the protein in the insoluble fraction and accounted for 95% of the total serum globulins.

The lipid content of the soluble fraction was one-third that of the insoluble fraction as measured by the method of Bloor (27). When analyzed for lipoproteins by electrophoresis both on filter paper and cellulose acetate, the soluble fraction exhibited a distinct band at the origin and a faint α-lipoprotein band. The insoluble fraction exhibited the same band at the origin as well as distinct α- and β-lipoprotein bands. The latter pattern was similar to that shown by the whole serum.

The lipogenic activity of the two fractions is shown in Table 4. On the basis of their total protein content they possessed the same activity, that is, approximately one-half the activity of serum. On the basis of lipid content, however, the soluble fraction was three times as active as the insoluble fraction.

The lipogenic activity of the soluble fraction was also compared with whole serum at equivalent albumin concentrations. Under this condition the mean lipogenic

TABLE 3 COMPOSITION OF RABBIT SERUM FRACTIONS PREPARED BY 60% SATURATION WITH AMMONIUM SULFATE

Serum Fraction	Percentage Composition of Protein						mg of Lipid per 100 mg of Protein
	Albumin	Post-albumin	α_1	α_2	β	γ	
Unfractionated	70.2	3.9	5.1	5.5	8.1	7.4	6.7
Soluble fraction	94.6	2.7			2.7		2.2
Insoluble fraction	43.6	4.5	9.4	8.8	15.2	18.7	6.5

Results for unfractionated serum are averages of six determinations. Results for each fraction are averages of duplicate determinations on three different preparations. The serum contained 64 mg of protein and 4.3 mg of lipid per ml. Average recovery of protein was 93%, divided equally between the soluble and insoluble fractions. Average recovery of lipid was 60%.

TABLE 4 LIPOGENIC ACTIVITY OF RABBIT SERUM FRACTIONS PREPARED BY 60% SATURATION WITH AMMONIUM SULFATE

Supplement	Rabbit Serum Protein	μg of Cell Lipid per μg of Cell Protein		Lipogenic Index
		Total	Increase	
	mg/ml medium			
None	0	0.232		
Soluble fraction	6.5	0.296	0.064	0.49
Insoluble fraction	6.5	0.303	0.071	0.55
Combined fractions	13.0	0.372	0.140	1.08
Rabbit serum	12.8	0.362	0.130	

Fractions were incorporated in the medium at the levels present in 20% serum. Results are averages obtained with two preparations. Conditions of the assay are given in Table 1. The compositions of the two fractions and of whole serum are given in Table 3.

index of eight such preparations was 0.77 (Table 5). Moreover, on the basis of its lipid content, the soluble fraction was three times as active as serum in increasing cell lipid.

Perhaps the simplest interpretation of the assay results summarized in Tables 4 and 5 was that three-quarters of the lipogenic activity of the rabbit serum was associated with albumin and one-quarter with one or more lipoproteins. However, all attempts to prepare a purer sample of albumin from the soluble fraction in order to test this hypothesis failed. Thus, precipitation of albumin at its isoelectric point, pH 4.7, removed neither the postalbumin nor the β-globulin contaminants (Table 3). Column chromatography on DEAE-cellulose also failed to separate the latter proteins from albumin and, in addition, resulted in the loss of one-half of the lipogenic activity. Consequently, an approach other than $(NH_4)_2SO_4$ fractionation was sought for testing the hypothesis that the lipogenic activity is associated with albumin.

Lipogenic Activity of Rabbit Serum Fractions Prepared by Ultracentrifugation in High Density Medium

As described under Materials and Methods, the centrifuged serum was separated into the following three fractions: (*a*) a top fraction of d<1.21, (*b*) a middle fraction of d about 1.21, and (*c*) a bottom fraction of d>1.21. Before assaying for lipogenic activity, the effect of dialysis and sterilizing filtration on the lipid content of the fractions was determined. These procedures caused an 11% decrease in the lipid to protein ratio in the fraction of d<1.21 and no detectable change in the other two fractions.

The serum fractions of d<1.21 and d>1.21 both possessed lipogenic activity (Table 6). On the basis of lipid content, the fraction of d>1.21 was much more active than the lipoprotein fraction. In both cases ap-

TABLE 5 LIPOGENIC ACTIVITY OF 60% AMMONIUM SULFATE-SOLUBLE FRACTION OF RABBIT SERUM COMPARED WITH WHOLE SERUM AT EQUIVALENT ALBUMIN CONCENTRATIONS

Supplement	Rabbit Serum Constituent			μg of Cell Lipid per μg of Cell Protein		Lipogenic Index
	Albumin	Total Protein	Lipid	Total	Increase	
		mg/ml of medium				
None	0	0	0	0.203		
Soluble fraction	9.0	9.5	0.21	0.299	0.096	0.77 ± 0.058*
Rabbit serum	9.0	12.8	0.86	0.328	0.125	

The soluble fraction was incorporated in the medium at the albumin concentration in 20% serum. Results are averages obtained in the assay of eight preparations of soluble fraction. Conditions of the assay are given in Table 1.

* SEM.

TABLE 6 LIPOGENIC ACTIVITY OF RABBIT SERUM FRACTIONS PREPARED BY ULTRACENTRIFUGATION AT d 1.21

Supplement	Rabbit Serum Constituent: Total Protein	Albumin	Lipid	Lipogenic Index
	mg/ml of medium			
d<1.21*	0.44	0.23	0.55	0.54
d>1.21†	13.19	8.96	0.13	0.78
Combined fractions	13.63	9.19	0.68	1.23

Results for individual fractions are averages of three separate assays. Result for combined fractions is for one assay.

* Incorporated in medium at lipid concentration present in 20% serum, minus the lipid content of proteins d>1.21.

† Incorporated in medium at albumin concentration present in 20% serum.

TABLE 7 LIPID CONTENT AND LIPOGENIC ACTIVITY OF RABBIT SERUM PROTEINS OF d>1.21 AND ISOLATED ALBUMIN

Serum Fraction	% Albumin	mg of Lipid per 100 mg of Protein	mg of Lipid per 100 mg of Albumin	Lipogenic Index*
d>1.21	74.7	0.87	1.16	0.73 ± 0.037
Albumin isolated from fraction d>1.21	98.8	1.22	1.23	0.89 ± 0.094

Lipid values are averages for three preparations each. Fractions assayed at albumin concentration present in 20% serum. Lipogenic indices and standard errors are for five preparations each.

* For difference in means, t is 1.6, and P falls between 0.2 and 0.1.

proximately 90% of the increase in cell lipid was due to the accumulation of triglycerides.

In a subsequent experiment, the lipoproteins were prepared in two fractions of d<1.063 and d 1.063–1.21 (22). The respective lipogenic indices of these preparations were 0.37 and 0.22. In the same experiment, the proteins of d>1.21 had a lipogenic index of 0.70. All together, five different preparations of proteins of d>1.21 were assayed and were found to have a mean lipogenic index of 0.73 ± 0.037. This value compared favorably with the index of 0.77 ± 0.058 obtained earlier with the albumin-rich $(NH_4)_2SO_4$-soluble fraction of serum (Table 5).

Lipogenic Activity of Rabbit Serum Albumin Isolated from Proteins of d>1.21

Dialyzed solutions of proteins of d>1.21 containing about 40 mg of protein/ml were used to prepare albumin by the trichloroacetic acid precipitation–ethanol extraction procedure (23) as described under Materials and Methods. In seven experiments employing different preparations of proteins of d>1.21, the average composition of the extracted protein, as determined by electrophoresis on cellulose acetate, was 98.8% albumin 0.9% α-globulin, and 0.3% β-globulin. The average yield of albumin from proteins of d>1.21 was 39%. In three of the experiments, the yield of lipid was also determined and found to be 42%, as compared with 40% for albumin. Furthermore, in one of these experiments the original serum had been labeled with a tracer amount of albumin containing bound palmitic acid-1-^{14}C. The lipid extracted from the proteins of d>1.21 had a specific activity of 1.53×10^5 dpm/mg, and that extracted from the albumin preparation had a specific activity of 1.63×10^5 dpm/mg.

Five of the preparations of albumin were assayed for lipogenic activity and were found to have a mean lipogenic index of 0.89 ± 0.094. This value does not differ significantly from the value of 0.73 ± 0.037 obtained with proteins of d>1.21. The results of these experiments are summarized in Table 7. One of the albumin preparations was also assayed in the mouse fibroblast (L) cell and found to have a lipogenic index of 1.01 as compared with 1.17 in the rabbit liver cell. Human and rat liver cells also responded to rabbit serum albumin with 1.4- and 1.6-fold increases in cell lipid in 24 hr. The responses to the original serum were 1.2- and 1.3-fold, respectively. Analysis of the human liver cells supplemented with albumin showed that 94% of the accumulated lipid consisted of triglycerides.

Composition of Lipid Associated with Rabbit Serum Proteins d>1.21 and Isolated Albumin

The results of the analyses of the lipid associated with proteins of the d>1.21 fraction and with the isolated albumin are summarized in Table 8. For purposes of comparison, they are expressed in both cases in terms of the albumin content of the preparation, that is, as mg of lipid per 100 mg of albumin. As can be seen, the extraction of albumin from proteins of the d>1.21 fraction was accompanied by an apparent 1.16-fold enrichment of albumin-bound fatty acids at the expense of cholesteryl esters plus hydrocarbons and free cholesterol. There was no striking change in the amounts of lecithin or lysolecithin per 100 mg of albumin, and lysolecithin was the predominant phospholipid in both preparations.

In the assay of the albumin preparations for lipogenic activity, the 45.4 mg of albumin added per dish produced a mean increase in cell lipid of 230 μg in 24 hr, of which approximately 90% was triglycerides. Even if all of the fatty acids of the albumin-bound cholesteryl esters, lecithin, and lysolecithin (Table 8) had been utilized with complete efficiency, they would have yielded only 71 μg of triglycerides with a mean fatty acid mol wt of 270.[4] The free fatty acids associated with

[4] The composite mol wt as determined by gas chromatography. (Mackenzie, C. G. Unpublished results.)

TABLE 8 Composition of Lipid Present in Rabbit and Horse Serum Proteins of d>1.21 and Isolated Albumin

Preparation	mg of Lipid per 100 mg of Albumin				
	Total Lipid	Fatty Acids	Cholesteryl Esters Plus Hydrocarbons	Lecithin	Lysolecithin
Rabbit serum					
Proteins of d>1.21	1.16	0.75	0.23*	0.03	0.13
Albumin isolated from proteins d>1.21	1.23	0.87	0.16	0.02	0.14
Horse serum					
Proteins of d>1.21	1.22	0.38	0.40†	0.38	<0.02
Albumin isolated from proteins d>1.21	1.39	0.84	0.15	0.27	0.06

Results are averages obtained in three experiments with rabbit serum proteins d>1.21 and two experiments with the other preparations.

* This fraction contained 0.14 mg of cholesteryl esters per 100 mg of albumin. The rabbit serum proteins d>1.21 also contained 0.03 mg of free cholesterol per 100 mg of albumin.

† This fraction contained 0.27 mg of cholesteryl esters per 100 mg of albumin.

the albumin, on the other hand, were present in an amount more than sufficient to account for the observed triglyceride accumulation. These same considerations also apply to rabbit serum protein of d>1.21.

Composition of Lipid and Lipogenic Activity of Horse Serum Proteins of d>1.21 and Isolated Albumin

The lot of horse serum used in these experiments contained 84 mg of protein, 34 mg of albumin, and 2.4 mg of lipid per ml as compared with 64 mg, 46 mg, and 3.6 mg, respectively, for the rabbit serum used in the preceding experiments. Its composition was representative of horse serum in general, as was its lipogenic activity. In other words, although its albumin concentration was three-quarters that of rabbit serum, it possessed only one-quarter of the lipogenic activity of the latter serum.

The composition of horse serum proteins of d>1.21 was 39% albumin, 3% postalbumin, 17% α-globulin, 20% β-globulin, and 21% γ-globulin. The lipid content, expressed in terms of albumin, was 1.22 mg/100 mg of albumin. The lipid was divided approximately equally into nonesterified fatty acids, hydrocarbons plus cholesteryl esters, and lecithin (Table 8). When assayed for lipogenic activity at the albumin concentration of 20% horse serum, the horse serum protein of d>1.21 possessed only one-fifth of the activity of rabbit serum proteins of d>1.21 assayed at an equivalent albumin concentration.

In five experiments, the yield of albumin from horse serum proteins of d>1.21 was from 3 to 11%. The average composition of the albumin preparations was 97.1% albumin, 0.9% postalbumin, and 2.0% α_1-globulin. The average lipid content determined on two of the preparations was 1.39 mg of lipid per 100 mg of albumin. However, as shown in Table 8, the mg of fatty acid per 100 mg of albumin was 2.2 times that found in proteins of d>1.21. These results were checked by preparing proteins of d>1.21 and albumin from horse serum labeled with palmitic acid-1-^{14}C. The lipid extracted from these two preparations contained 2.7 $\times$ 10^5 and 5.7 $\times$ 10^5 dpm/mg, respectively. When assayed for lipogenic activity, the isolated horse serum albumin was found to be approximately 90% as active as rabbit serum albumin.

The Lipid Content of Cells and the External Concentration of Albumin-Bound Fatty Acids

The foregoing experiments prompted us to study the lipid content of cells grown in media containing levels of horse serum and rabbit serum that provided equivalent concentrations of albumin-bound fatty acids. The horse serum and rabbit serum used in the experiments summarized in Table 8 were used for this purpose, and their concentrations of albumin-bound fatty acids were calculated as follows. First, the mg of fatty acids per 100 mg of albumin in proteins of d>1.21 were corrected for the recoveries obtained when the lipid was labeled prior to extraction with a tracer amount of palmitic acid-1-^{14}C, that is, 85% and 90%, respectively, for the rabbit serum and horse serum preparations. From these values and the 46 mg and 34 mg of albumin present per ml of rabbit serum and horse serum, the concentrations of albumin-bound fatty acids in each serum were calculated and converted to mmoles per liter using a composite fatty acid mol wt of 273.[4] On this basis, the rabbit serum contained 1.48 and the horse serum 0.52 mmoles of albumin-bound fatty acids per liter.

Replicate dishes of rabbit liver cells were grown in media containing from 10 to 48% horse serum or from 7 to 20% rabbit serum. After 3.5 days, when the cell protein per dish had increased approximately 10-fold, cell lipid and protein were determined, and the μg of

cell lipid per μg of cell protein was calculated. At equivalent concentrations of albumin-bound fatty acids, the cells grown in rabbit serum contained more lipid than those grown in horse serum in each of three experiments. Furthermore, the slopes of the lines relating cell lipid to the concentration of albumin-bound fatty acids were consistently greater for rabbit serum than for horse serum (Fig. 1). The mean slope and standard error for rabbit serum was 1.07 ± 0.003 and for horse serum 0.48 ± 0.035. The value of t for the difference in means is 11.8 as compared with 4.0 required for P 0.01.

From these results it was evident that environmental factors, in addition to the concentration of albumin-bound fatty acids, were involved in the accumulation of triglycerides by cells. Because of the high binding constants of long-chain fatty acids for bovine serum albumin (39, 40), it seemed likely that one such factor was the molar ratio of albumin (mol wt 69,000) to albumin-bound fatty acids (composite mol wt 273) in the medium. When the molar ratios of 0.45 for rabbit serum and 0.94 for horse serum are introduced in the respective slopes, they become,

for rabbit serum

$$\frac{0.48}{0.45 \text{ mole albumin/mole fatty acid}} = 1.07$$

for horse serum

$$\frac{0.45}{0.94 \text{ mole albumin/mole fatty acid}} = 0.48$$

Therefore, as a first approximation, the formula for the lipid content of the cells grown on either serum can be written as,

$$\frac{\mu\text{g of cell lipid}}{\mu\text{g of cell protein}} = \frac{0.47}{\text{moles albumin/mole fatty acid}} \times \text{mM fatty acid} + b$$

where the value of b, as determined by extrapolating the lines in Fig. 1 to the ordinate is 0.19 ± 0.036 for rabbit serum and 0.15 ± 0.02 for horse serum. For this difference in means, t is 1.12 and $P > 0.3$.

To test the formula, crystalline rabbit serum albumin (Pentex Biochemical) containing 0.035 ± 0.0036 mg of lipid per 100 mg of albumin was added to 10% rabbit serum medium in an amount that increased the concentration of albumin from 0.067 mM to 0.142 mM. From the formula it was calculated that the added albumin should reduce the lipid content of cells by 0.081 μg per μg of cell protein if none of its lipid were fatty acid, and by 0.075 μg if all of its lipid were fatty acids. In two such experiments, the observed reductions in cell lipid were 0.081 and 0.087 μg per μg of cell protein.

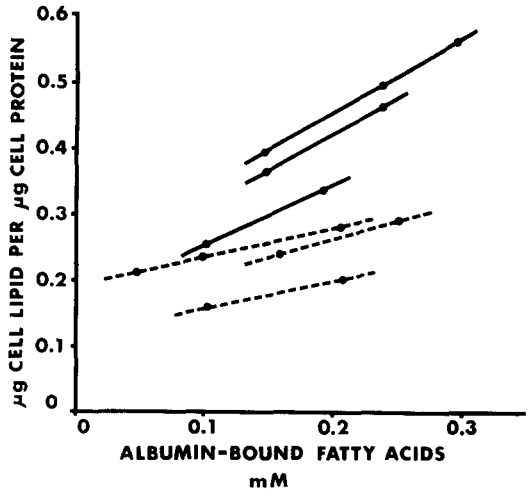

FIG. 1. Relation between the concentration in the medium of the albumin-bound free fatty acids of rabbit serum (solid lines) and horse serum (broken lines) and the lipid content of rabbit liver cells grown in culture for 3 days.

Toward the conclusion of these experiments a lot of horse serum (Microbiological Associates, Inc.) was obtained whose concentration of nonesterified fatty acids (32) was 1.22 mM and whose molar ratio of albumin to fatty acids was 0.42. The lipogenic activity of this serum was therefore compared with a more usual lot of horse serum (Flow Laboratories, Inc.) whose concentration of nonesterified fatty acids was 0.38 mM and whose molar ratio of albumin to fatty acids was 1.26. From the straight line expression given above, it was calculated that the lipid content of cells grown in media containing 20% of the two sera should differ by 0.25 μg lipid per μg of cell protein. In an experiment lasting 2.5 days, the cells grown in the high fatty acid serum contained 0.28 μg more lipid per μg of cell protein than those grown in the low fatty acid serum.

DISCUSSION

When these experiments were begun, it had been determined that the fatty acids of both the neutral and polar lipids of cells grown in media containing serum were not synthesized by the cells but rather were derived from lipids present in the serum (10, 11, 13). Geyer and Neimark (41) had shown that cultured cells could utilize the fatty acids of exogenous triglycerides as a source of energy, and Bailey, Gey, and Gey (42) had reported that cells preferentially used serum triglycerides, followed by phospholipids, as precursors of their structural lipids. It seemed possible, therefore, that the large amounts of triglycerides accumulated by cells grown in rabbit serum (9–12), and some samples of human serum, were derived from the serum lipoproteins. Consistent with such an assumption was the low lipo-

genic activity of the serum globulins and albumin prepared by the Cohn fractionation procedure in which isopropanol was used as the organic solvent (Table 2).

Subsequently, the results obtained with both $(NH_4)_2SO_4$ fractionation and ultracentrifugation suggested, however, that albumin rather than the lipoproteins of rabbit serum was responsible for most of its lipogenic activity. This hypothesis was borne out by the finding that the albumin isolated from the proteins of d>1.21 in a purity of about 99% by precipitation with trichloroacetic acid and extraction with ethanol,[5] accounted for three-quarters of the activity of whole serum. Furthermore, analysis of the isolated rabbit serum albumin indicated that its lipid content and composition had not been substantially changed by the isolation procedure (Table 8). Only the free fatty acids were present in sufficient amounts to account for the increases in cell triglycerides observed in the bioassay experiments.[6] Two additional pieces of evidence supported the conclusion that albumin-bound fatty acids were responsible for a major part of the lipogenic activity of rabbit serum. First, rabbit serum albumin that contained little or no lipid was devoid of lipogenic activity. Second, in an experiment with cells grown in serum labeled with tracer amounts of albumin-bound palmitic acid-1-^{14}C and oleic acid-1-^{14}C, the specific activity of the cell lipid was 80% of that of the free fatty acids isolated from the medium (11).

Pertinent to the foregoing conclusion is the recent isotopic evidence presented by Howard and Kritchevsky (46) that serum free fatty acids are the sources of nonsterol lipid in cultured human diploid cells WI-38. Similarly, Elsbach concluded from isotopic studies conducted several years ago that lipid utilization by polymorphonuclear leukocytes and alveolar macrophages is confined to free fatty acids (47, 48). While the foregoing workers did not report lipid droplet formation or triglyceride accumulation in their experiments, Moskowitz (49) and Geyer (50), respectively, did describe the appearance of lipid droplets in L cells when albumin-bound fatty acids or micellar fatty acids-1-^{14}C were added to serum-free media. Geyer obtained similar results using a serum-containing medium. Over 10 years ago, Fillerup, Migliore, and Mead (51) described the uptake and oxidation of albumin-bound fatty acid by Ehrlich ascite tumor cells. The experiments with Ehrlich ascites cells have been greatly extended in a series of elegant papers by Spector and Steinberg (52–55) to include effects of structure on fatty acid uptake and the rates of esterification and chemical distribution, etc., of albumin-bound fatty acids. Recently, our hypothesis (11) that increasing the H^+ concentration in the extracellular environment increases the uptake of albumin-bound fatty acids by animal cells has been confirmed by Spector who used Ehrlich ascites tumor cells, rabbit erythrocytes, and rat heart and liver slices (56).

From the results with rabbit serum, it was predicted that the proteins of d>1.21 prepared from horse serum of low lipogenic activity would have a low free fatty acid content, and this proved to be the case. However, when albumin was isolated from horse serum proteins of d>1.21, it was found, unlike rabbit serum albumin, to have undergone an apparent twofold enrichment in its fatty acids content (Table 8). Significantly, this horse serum albumin, whose free fatty acid content had been raised to that of rabbit serum albumin, was just as lipogenic as the latter preparation. Consequently, it can be concluded that the high lipogenic activity of albumin-bound fatty acids does not depend on some unique property of rabbit serum albumin or on its relatively high content of lysolecithin (Table 8).

The results of our serum fractionation and cell analysis experiments are in agreement with the tissue culture experiments of Rutstein, Castelli, and coworkers who found that the amount of lipid-staining material in MAF cells grown in medium containing serum from men maintained on various dietary regimens (57) was correlated with the free fatty acid content of the serum rather than with the levels of triglycerides, cholesterol, or phospholipids (58). Our results are also in agreement with the whole animal experiments of Carlson and Liljedahl (59) who observed in dogs that an increase in serum free fatty acids, produced either by trauma or by the administration of norepinephrine, was accompanied by the appearance of lipid-staining granules in the heart, lungs, liver, kidneys, and skeletal muscle. Moreover, the glyceride content of the liver was increased in direct proportion to the elevation of serum free fatty acids. However, as the authors pointed out, the possibility could not be ruled out that the pressor or other effects of norepinephrine were reponsible for these reactions. In view of the results obtained in the

[5] Rat serum albumin isolated by this method behaved like native albumin in free boundary electrophoresis (24) and was homogeneous as judged by immunodiffusion analysis (43). In addition, Schwert (44) found that when crystallized bovine serum albumin was subjected to this procedure, its sedimentation, electrophoretic, and solubility properties were unchanged. He did observe, however, that TCA-precipitated insulin was also dissolved by ethanol, and Korner (24) reported that rat serum albumin so prepared was indeed contaminated with insulin. However, our assay medium contained insulin.

[6] Presumably the low lipogenic activity of fraction V (Table 2) was due to its low content of nonesterified fatty acids. Chen (45) has shown that fraction V preparations from a variety of sera, including rabbit serum, often contain only 0.1 mole of fatty acid per mole of albumin. In our experiments the albumin isolated by TCA precipitation and ethanol extraction from proteins of d>1.21 prepared from various lots of rabbit serum contained from 2.2 to 3.1 moles of fatty acid per mole of albumin.

isolated cell system, it now seems certain that the hormone effect in the animals was due to the increase in the free fatty acid content of the serum.[7]

The triglyceride content of cultured cells was also increased by rabbit serum proteins of d<1.21. Since the preparation contained only 2.5% of the total serum albumin, its activity, which was about equally divided between the subfractions of d<1.063 and d 1.063–1.21, was attributable to the lipoproteins. Possible explanations for the activity of the lipoproteins include the presence of free fatty acids in these molecules, the enzymatic hydrolysis of lipoprotein–lipid during the assay procedure, and the direct utilization of complex lipids by the cells. Since evidence exists both for and against the first (60, 61) and second (62, 60) of these possibilities, the final explanation for the activity of the lipoproteins must await further experimentation.

Another important parameter in the control of cell lipid accumulation is the molar ratio of albumin to free fatty acids in the extracellular environment. Thus, increasing the albumin concentration without increasing free fatty acids decreases cell lipid, and increasing the fatty acid concentration without increasing albumin increases cell lipid. These results are in agreement with the observation of Spector, Steinberg, and Tanaka (63) that the uptake of palmitate by Ehrlich ascites tumor cells depends on the molar ratio of palmitate to albumin in the medium.

It is now of interest to examine the experiments of Simms and coworkers (2–5) on serum factors that stimulated (lipfanogen) and inhibited (antilipfanogen) the appearance of lipid granules in cultured heart cells and explanted aorta segments of the chicken. Lipfanogen, which was nondialyzable and present in the serum fraction of high density, appears to have been albumin containing bound fatty acids. Its heat stability is consistent with the fact that the albumin–fatty acid complex is the most heat resistant of the major serum proteins (64, 65). It is probable that antilipfanogen was defatted albumin since it was found only in serum, occurred almost exclusively in albumin-rich preparations, and was concentrated by procedures that would be expected to remove free fatty acids from albumin. Furthermore, it was heat-labile (4) as is albumin from which fatty acids have been removed (64).

From the experiments presented in this paper, and the results of other investigators, it appears that albumin-bound fatty acids constitute the serum factor most responsible for the accumulation of triglycerides and the appearance of lipid-rich particles in cultured cells. It is probable that in the whole animal a similar relationship exists between high concentrations of albumin-bound fatty acids and triglyceride accumulation in the tissue cells most immediately exposed to serum and its albumin containing filtrates.

[7] The lipid content of the cultured rabbit liver cells was not significantly increased by the addition of epinephrine to the medium.

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