METABOLISM OF ADIPOSE TISSUE IN THE FAT TAIL OF THE SHEEP

ADROUNI

UPTAKE AND RELEASE OF FREE FATTY ACIDS FROM ADIPOSE TISSUE UNDER VARIOUS EXPERIMENTAL CONDITIONS

By

Berjouhi Adrouni

Submitted in partial fulfilment of the requirements for the degree of Master of Sciences in the Department of Biological Chemistry of the American University of Beirut

Beirut, Lebanon

1965

A C K N O W L E D G M E N T
****

The author is grateful to Dr. Avedis Khachadourian for suggesting the problem and for his valuable assistance and advice throughout the course of this work and during the preparation of the manuscript.

The author also wishes to thank Miss Koharig Badjakian and Mr. Apkar Mirakian for their excellent technical assistance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

For many years adipose tissue was considered an inert site of fat storage. Investigations during the last thirty years, and especially the last few, showed that it is a metabolically active tissue where oxidative processes, as well as fat synthesis and breakdown occur.

Histology and Embryology of Adipose Tissue

Although about fifteen percent of the mammalian body by weight is adipose tissue, the large proportion of lipids to protoplasm in the cells make histological and morphological studies of this tissue rather difficult. The lack of clear distinction between white or yellow fat, characterized by having a single large lipid vacuole, and brown fat containing numerous small fat droplets in the cytoplasm, make embryological studies difficult because of morphological similarity during embryonal lipogenesis.

Isolated adipose tissue cells are spherical, but oval or polygonal when crowded. The lipid vacuole is surrounded by a thin film of cytoplasm with the flattened nucleus in a relatively thickened area on one side of the cell. Mitochondria and a Golgi complex are present in the cytoplasm (1). The cells are surrounded by a delicate network of reticular fibers. The lipid vacuole is formed by the

combination of numerous small fat droplets formed in the cytoplasm during the embryological development of the cell. The tissue is usually subdivided into lobules by thin septa of fibrous connective tissue with blood vessels inside the septa (2).

The embryological origin of adipose tissue has been a subject of controversy for a long time between investigators who believed that it is derived from primitive connective tissue cells which deposit fats (1,3), and those who claimed it is a distinct tissue arising from special mesenchymal cells (1). Clark and Clark (4) observed adipose tissue formation in vivo from cells histologically identical to ordinary connective tissue, supporting the first hypothesis. Hausberger, on the other hand, showed development of fat cells by transplanting the anlagen of perirenal fat body of newborn rats. Upon similar transplantation of embryonic connective tissue no adipose tissue was formed, implying its distinct origin from connective tissue (5). Interconversion of the two tissues has been reported by Sheldon (6).

Composition of Adipose Tissue

In a well fed animal eighty five percent of adipose tissue by weight is made up of fat, primarily triglycerides (7). Small amounts of phospholipids are found on the protoplasmic membranes. Brown fat on the other hand, contains much less neutral fat, but large amounts

of phospholipids in the mitochondria, glucolipids, cholesterol, and probably fatty acids (1,2,8) and is richer in proteins (10,11). A small pool of free fatty acids in the adipose tissue acts as a precursor for triglycerides (9).

Fat soluble vitamins are stored in adipose tissue (8).

Polysaccharides are found in the cytoplasm of fat cells. The presence of glycogen depends on the nutritional state of the animal, and is also controlled by the nervous system and insulin. Normally the rat adipose tissue is devoid of glycogen during daylight hours, variable small amounts being formed at night (1,2,8,12).

Metabolic Activities of Adipose Tissue

Many of the enzymes involved in the major metabolic pathways have been found in adipose tissue, implying the probability of different metabolic activities occuring in this tissue (1,2,8,10,11,13,14). In fact, many of the intermediary metabolic pathways have been shown in fat cells, such as glycolysis, the tricarboxylic acid cycle, the hexose monophosphate shunt, glycogenesis and glycogenolysis, fatty acid and triglyceride synthesis. Most of these reactions are under hormonal control.

Triglyceride synthesis and storage in adipose tissue is controlled by insulin. Addition of glucose or insulin to tissues from fed animals incubated in the presence of heparin and triglyceride causes

release of a lipase which hydrolyzes the triglycerides enhancing uptake by the tissue (15-17). Glucose also favors triglyceride synthesis in the tissue by providing glycerol phosphate during glycolysis (18-20, 26). Hormones controlling fat mobilization, adrenaline, noradrenaline, thyroid stimulating hormone, adrenocorticotropic hormone, growth hormone and glucagon seem to do so by stimulating a lipolytic process, many of them accompanied by an increase in oxygen and glucose uptake and lactate production indicating enhanced glycolysis (21-27).

Fatty Acid and Triglyceride Turnover in Adipose Tissue

Rittenberg and Schoenheimer were among the first people who, by use of deuterium, showed fatty acid turnover in adipose tissue with an estimated half life of 5-9 days (28,29). More recent investigators reported longer half lives for fatty acids and triglycerides. Stein et. al. (30) incubated rat epididymal adipose tissue in vivo and in vitro in equimolar mixtures of 9,10 H^3 labelled palmitic acid and 1 C^{14} linoleic acid. By comparing the radioactivity in one pad right after the incubation and the second after one, two, or more months they found mean half lives of 163 days for palmitic and 187 days for linoleic acids. Even longer half lives of linoleic acid, 350-750 days in human adipose tissue was found by Hirsch and his collaborators (31).

Gorin and Shafrir studied triglyceride turnover in adipose tissue by the release and reesterification of glycerol and estimated

an average half life of 41 days (32).

The degree of transesterification between triglyceride molecules was studied by the use of C^{14} palmitate. Only a slow intermolecular rearrangement of triglyceride fatty acids was seen as a result of hydrolysis and reesterification rather than transesterification (33).

The data from these various experiments suggest the presence of two lipid metabolizing compartments in adipose tissue; a small one in the cytoplasmic particles having a rapid turnover rate with the plasma fatty acid pool, and the main lipid vacuole with a very slow turnover (30).

Mobilization of Adipose Tissue - Free Fatty Acids in Blood

Interest in the role of free fatty acids in blood increased simultaneously with the study of adipose tissue metabolism. Experimental evidence for the presence of free fatty acids in plasma came first from Szent-Gyorgi and Tomianga in 1924 (34). Kelsey and Longenecker (1941) reported the presence of free fatty acids in the lipid fraction of bovine plasma (35). In 1947 Davis found free fatty acids in human plasma (36), and Cohn in the albumin fraction of human plasma (37). The importance of plasma free fatty acids was not considered till 1956 when Dole (38), and Gordon and Cherkes (39) reported the dependence of their levels on the nutritional state of the animal. They concluded that free fatty acid is the form in which fat is transported from depots during fasting.

The relative compostion of individual free fatty acids of plasma, on the other hand, was found to be relatively stable during fasting and ingestion of different fats. This was explained on the basis of their rapid turnover between blood and tissue pools (40).

Further investigations showed that the plasma free fatty acid level is also dependent on hormonal influences on the adipose tissue. Insulin causes a fall in blood free fatty acid levels by favoring triglyceride synthesis in adipose tissue, whereas lipolytic hormones especially adrenaline and noradrenaline favor release of free fatty acids by adipose tissue. The effect of adrenaline infusion in vivo in increasing free fatty acid release from adipose tissue is shortlived because of the simultaneous increase of glucose in blood and its utilization (18,19,41-47). Noradrenaline which has little effect on glycogenolysis causes a more sustained elevation of blood free fatty acids (42,44).

As interest in blood adipose tissue free fatty acid increased methods for the determination of their concentrations were developed. Earlier methods by Dole (38) and Gordon (48) were based on their titration with dilute alkali after extraction with organic solvents. More recently colorimetric methods were developed (49,50).

Isotopes were also used to trace fatty acids and their metabolic pathways. Schoenheimer and Rittenberg (28) used deuterium labelled fatty acids to study their turnover. Later when C^{14} labelled fatty acids were available these were used extensively to study fatty

acid and triglyceride metabolism *in vivo* and *in vitro*.

A considerable amount of literature exists about the *in vitro* study of adipose tissue metabolism, carried mainly on rat epididymal adipose tissue. Small amounts of the tissue are incubated in various media, mainly serum and Krebs phosphate buffer, for various lengths of time, and the effects of various factors studied. Uptake of free fatty acids from serum by adipose tissue *in vitro* was reported by Cahill *et. al.* (21), Stern and Shapiro (51); release into the medium by Vaughan (9), and in the presence of albumin by Gordon and Cherkes (18). There was no release of free fatty acids *in vitro* in the absence of albumin (52-54).

Various techniques have been used to study adipose tissue metabolism *in vivo*. Fatty acids labelled with C^{14} were injected intravenously to animals and their rates of removal from blood measured (55-57). Arteriovenous (A-V) differences of free fatty acid levels across various organs were also studied under different conditions to measure release and uptake by these organs. Gordon and Cherkes (39), Gordon (48), and Miller *et. al.* (58) measured A-V differences in blood perfusing the normal and fasting human myocardium *in vivo*. A-V differences in liver (59) and in muscle (60) were also studied, all showing free fatty acid uptake by these tissues. Adipose tissue, on the other hand, was found to release free fatty acids (58,61).

Although measurements of A-V differences across organs is one of the best methods to study fatty acid metabolism, it is li-

mited to tissues where anatomy and vascularization is satisfactory. In the experiments on adipose tissue mentioned above the venous blood often drained several tissues apart from adipose tissue. To overcome this difficulty we used the Syrian sheep Ories aries var. craemicandus which has a large tail composed entirely of white adipose tissue, drained by two main lateral veins which can be catheterized to obtain blood samples draining adipose tissue exclusively. This made collection of large venous samples possible to measure A-V differences across the tail. Biopsies from the tail were used for in vitro observations, making comparison of in vivo and in vitro studies possible.

So far, in the majority of reported studies analyses were done for total free fatty acid concentration, very few on the individual fatty acid composition. Kelsey and Longenecker listed the bovine plasma free fatty acids by fractionation (35). Dole studied the free fatty acid composition of human blood by silicic acid column chromatography and gas liquid chromatography (62). Miller et. al. (58) used Barker-Coleman gas liquid chromatography to determine A-V differences for individual free fatty acids across various tissues in dogs. During the last few years Argon gas liquid chromatography was introduced for individual free fatty acid study. Reed and Awdeh (63) used it to study sheep triglyceride fatty acids from different sites in adipose tissue. Garton and Duncan (64)

also used it to determine the free fatty acid composition of sheep plasma. We adopted Argon gas liquid chromatography for the quantitation of individual free fatty acids and triglyceride fatty acids in sheep serum and tail biopsies, and to study the effect of adrenaline, noradrenaline and glucose administration on fatty acid mobilization in normal and fasted sheep.

CHAPTER II

MATERIALS AND METHODS

Preparation of Sheep and Procedure of Experiment

Male, non-castrated Syrian sheep *Ories aries* var. crassicandus weighing 20-40 kg. were bought from the local market and kept on a diet of barley and water *ad libitum* for at least ten days before the experiment.

The operation was carried in the animal operating room, under partially sterile conditions. Usually all animals were fasted overnight before the experiment, but some were kept without food for 40-145 hours for the fasting experiments. Intravenous pentobarbital was used for anesthesia, with an initial dose of 25 mg/kg body weight, additional injections being given when necessary during the experiment. Blood and tissue samples were taken at least thirty minutes after anesthetizing the animal to minimize the effects of endogenous adrenaline secretion during the induction of anesthesia.

A small incision was made on the hind leg of the sheep to expose the femoral artery and vein. An indwelling Cournand needle was placed into the artery and a thin polyethylene catheter into the vein. Repeated blood samples could thus be collected and injections given into the femoral vein.

A second incision was made on the ventral side of the tail

and either the main vein or one of its tributaries was catheterized with a second polyethylene catheter to collect venous blood from the tail. A small volume of 0.3 percent ethylene diamine tetraacetic acid (EDTA) was pushed into the catheter with a syringe to prevent clotting. In several experiments clotting in the vein prevented further collection of blood. Direct venipuncture was used in these cases. Occasionally experiments had to be discontinued because of failure to obtain adequate amounts of venous blood.

Injections and infusions were administered through the femoral vein catheter either with a syringe or a constant flow infusion pump at the rate of 3-5 ml./minute. Adrenaline, noradrenaline and glucose were infused during different experiments. Sealed ampoules containing one milligram adrenaline in the form of its hydrochloride dissolved in isotonic saline were obtained from Parke, Davis and Co., Detroit, Michigan. This stock was diluted in saline and injected at the rate of 1 mgm./kgm./minute for 25-30 minutes. Two milligrams of noradrenaline-d-bitartrate monohydrate* in one milliliter acid saline pH 3, was also used. Five percent dextrose solution was used for glucose experiments.

Adipose tissue biopsies were obtained by making a new incision on the tail distal to the first incision. At the end of the experiment all incisions were neatly sutured and the animals were allowed to recover for several weeks before being used for a second time.

---

*L-Norepinephrine-d-Bitartrate Monohydrate was obtained from Sterling-Winthorp Research Institution New York.

In Vivo Experiment

The in vivo experiments consisted of collection of arterial blood from the leg and venous blood from the tail to study arterio-venous differences (A-V) of free fatty acids, glucose and lactic acid. In some experiments A-V for cholesterol, phospholipids, and total esterified fatty acids were also determined. Measurements were made on control and fasted animals before and after injection of adrenaline, noradrenaline and glucose.

Arterial and venous blood samples (20-30ml.) were collected simultaneously at various intervals before and after the injection of hormones into 50 ml. plastic centrifuge tubes containing 20 mgm. EDTA. These were kept in an ice bath until centrifugation which was carried within twenty minutes after collecting the samples. The blood samples were centrifuged in a refrigerated centrifuge at 4°C and 1000g for 15 minutes. Ten milliliter aliquots of the sera were added to suitable amounts of standard margaric acid (n-17:0) dissolved in heptane. The test tubes were covered with corks wrapped with aluminum foil, and frozen until further extraction for gas chromatography, carried within the same day. For the other determinations the sera were processed immediately or kept frozen at -4°C until analysis.

Biopsies of adipose tissue from the tail were removed after taking the first blood samples, and occasionally after the infusion

to compare the free fatty acid and triglyceride fatty acid patterns with the controls. The tissues were cut with scissors into small pieces, then passed through a syringe twice giving a pastelike mince. Frozen sections from this material revealed that most of the cells had microscopically intact cell membranes. These tissues were then weighed and added directly into the extraction mixture for gas chromatography and kept at 4°C until extraction.

In Vitro Experiment

The in vitro experiments were carried with adipose tissue obtained during the in vivo experiments thus allowing comparison of results. The tissue was prepared as described above for the in vivo studies. Two different media were used for incubation:

a) Serum from the first arterial blood sample collected from the sheep.

b) Krebs-Ringer phosphate buffer pH 7.4 containing 5% bovine serum albumin.

One gram of the homogenized tissue was added to 50 ml. Erlenmeyer flasks containing 7 ml. of the incubation medium with or without 0.1 ml. of adrenaline or noradrenaline solutions equivalent to 1 ugm. or 33 ugm. For the in vitro effect of glucose on free fatty acid mobilization from the adipose tissue 0.1 ml. of glucose solution was added to raise the medium glucose concentration to about 200 mgm./100 ml. All the flasks were covered with aluminum foil to

minimize evaporation and were incubated at 37°C in a Dubnoff shaking incubator at about 60 cycles per minute.

At the end of one or two hours incubation four or five milliliter aliquots of the media were pipetted into the extraction mixture simultaneously with the standard for gas chromatography. One or two milliliter aliquots were also pipetted for total free fatty acid dtermination by titration, or glucose and lactic acid measurements. In several experiments the tissues were washed with saline after incubation, blotted on filter paper and extracted for free fatty acid and triglyceride fatty acid analysis by gas chromatography.

Rat Experiments

Three in vitro experiments were carried on rat epididymal fat to confirm results reported in the literature and check on our experimental procedures. Male albino rats weighing 200-250 gms. were anesthetized with ether. A single medial incision was made ventrally on the abdomen, and the testes were pushed upwards, out of the incision. Small pieces of the epididymal pad fat were removed and 0.5 gm. portions were weighed and incubated in 3.5 ml. of Krebs-Ringer phosphate buffer with 5% albumin and with or without adrenaline for one hour at 37°C in the Dubnoff shaker. Three milliliter aliquots were pipetted from the medium after incubation and extracted for gas chromatographic analysis.

Chemical Analyses

1. Free Fatty Acid Determination.- Dole's double extraction method was adopted for total free fatty acid determination (65) in serum samples, incubation media and tissues. The latter were homogenized in the extraction mixture in a VirTis homogenizer for 10 minutes.

Serum was extracted with five volumes of a mixture of isopropanol: Heptane: N H_2SO_4 (40:10:1 by volume). Five minutes later two volumes of distilled water and three volumes of heptane were added. The heptane layer was then extracted with the aqueous layer of the blank and titrated against 1:1000 saturated NaOH from a microburette. Aqueous thymol blue 0.1%, diluted tenfold in absolute ethanol was used as the indicator. A standard of 500 uEq/l palmitic acid in heptane and a blank with distilled water were run simultaneously.

Almost all the interfering substances are removed by the double extraction resulting in fairly reproducible values for free fatty acids.

2. Glucose Determination.- In the earlier experiments the Somogyi-Nelson method (66) was used for the determination of glucose concentration in blood and incubation media. A $ZnSO_4$-$Ba(OH)_2$ protein free filtrate of the serum was used for this assay which is based on the reducing properties of glucose on Cu^{++} to Cu^{+}. The latter produces a blue green complex with arsenomolybdate and is measured photometrically by comparison with a standard glucose solution.

Later the glucose oxidase method was adopted to assay for glucose, to reduce the effect of interfering substances. Glucose oxidase - Glucostat - was obtained from the Worthington Biochemical Corporation, N. J. The assay is based on a coupled enzyme system where glucose, in the presence of oxygen and water, is oxidized to H_2O_2 and gluconic acid by glucose oxidase. The peroxide then reacts with a reduced chromogen in the presence of peroxidase to produce the oxidized chromogen. The absorbancy of the color developed is read at 400 mu and compared with a standard.

Comparative concentration studies of glucose solutions ranging from 0.05-0.5% by Glucostat showed that the reaction follows Beer's Law only in the lower concentrations, and aliquots were diluted accordingly.

Temperature studies showed that the method was adequate at room temperatures, 15-30°C for appropriate concentrations.

3. Lactic Acid Determination.- Lactic acid was determined colorimetrically by the Barker-Summerson method (67). The $ZnSO_4$-$Ba(OH)_2$ protein free filtrate of serum is treated with $CuSO_4$ and $Ca(OH)_2$ to remove dextrose and other interfering substances. H_2SO_4 then converts lactic acid to acetaldehyde which, when treated with p-hydroxydiphenyl, produces a purple condensation product. All the test tubes used for this assay were washed very carefully. To avoid contamination batches of $Ca(OH)_2$ and H_2SO_4 had to be selected to obtain satisfactory results.

4. Determination of Cholesterol, Phospholipids and Total Lipid Esters.- Cholesterol was determined according to the method of Abell et. al. (68). Cholesterol in the serum is liberated from lipoprotein complexes by treatment with alcoholic KOH. It is then extracted with petroleum ether and measured by color development with a modified Liebermann-Burchard reagent consisting of 20 volumes of acetic anhydride, one volume of concentrated H_2SO_4 and ten volumes of glacial acetic acid.

Stewart and Hendry's method was used for phospholipid phosphorous determinations (69). The serum is extracted with 3:1 alcohol ether mixture. An aliquot is then evaporated and the residue digested with 10 N H_2SO_4. Perhydrol is added to remove the carbon, the residue is diluted with water. Color is developed by adding 2.5% Ammonium Molybdate and a reducer, amino-naphthol-sulfonic acid solution. Optical densities are compared with that of a standard phosphate solution.

Assays for the total esterified fatty acids were carried according to the method of Stern and Shapiro (70). The esterified fatty acids are treated with hydroxylamine in alkaline solution to yield hydroxamic acids which produce a red to violet color with ferric chloride. The color is compared to a standard triacetin solution in ethanol-ether mixture.

Gas Chromatography

1. Principle.- Gas chromatography is a method of separating components of mixtures. As in all chromatograms one phase of an immiscible pair of compounds, (a liquid in this case) is held stationary in a column, absorbed on a porous non-reactive material, while the second phase (a gas) is moved continuously through it. Compounds which are volatile at the temperature of the column are distributed between these two phases. The concentration of the eluate in the effluent gas is detected as the gas passes through a chamber with a source of ionizing radiation, and plotted against time by an amplifier and recorder unit (71,72).

2. Apparatus and Conditions Used.- The Pye Argon Gas Liquid Chromatograph used in these studies consists of two cabinets, the Analyzing unit and the Amplifier-Recorder unit. The first includes the detector, the column heater and heater control circuits. An argon tank with a constant flow regulator is connected to the top of the column by rubber tubing. A soap bubble flow indicator shows the rate of gas flow as it passes through the column and the detector. The Analyzing unit is connected to the Amplifier-Recorder cabinet by means of a coaxial cable.

Pyrex columns 4 ft. long, with a 4 mm. bore diameter, obtained from Pye and Co. Cambridge, England, were used. The sup-

porting material was celite, and the stationary phase Apiezon L grease. The columns were conditioned by heating for two days at 220°C with a slow flow of argon. Chromatography of fatty acid methyl esters was carried at 198°C and 1250 volts, with a gas flow of 50-75 ml. per minute. The sensitivity of the amplifier was adjusted according to the sample used, usually avoiding the highest sensitivity to minimize any effects of impurities. The chart speed was kept at 1 in/6 min all through. Under these conditions separation of a mixture ranging from lauric to stearic acid lasted about 60 minutes.

To determine the dependability of the chromatograph we carried separations of the National Institute of Health's standard fatty acid mixture F ranging from 14:0 to 24:0 carbons, obtained from the Applied Science Laboratories, Inc. State College, Pennsylvania (73). The results are presented in table I with the true values and the average for the same mixture from three other laboratories using the Pye Argon Chromatograph.

3. Preparation of Samples.- The free fatty acids in the sera or incubation media are extracted in a manner similar to that described above for total free fatty acid extraction. Fifteen milliliters of 90% ethyl alcohol is added on the heptane layer and 1 ml. of 0.1% aqueous thymol blue indicator. The free fatty acids are then con-

Table 1

Gas Chromatographic Analysis of N.I.H. Standard Fatty Acid Mixture F.

Fatty Acid	Actual weight %	Average for 3 laboratories	Average for our laboratory
24:0	47.0	45.6	48.72
22:0	25.4	25.4	24.74
20:0	13.6	14.03	12.47
18:0	7.3	7.4	7.30
16:0	4.2	4.6	4.41
14:0	2.5	2.7	2.37

verted to their sodium salts and dissolved in the alcohol-water layer by the addition of 0.02 N NaOH. The solution is washed twice with petroleum ether to remove non-saponifiable fractions. The fatty acids are then liberated by the addition of 1 N H_2SO_4, and extracted in petroleum ether and dried over anhydrous Na_2SO_4. The free fatty acids are methylated by refluxing in 0.5 N anhydrous methanolic HCl for 2 hours on a water bath. This is then evaporated under nitrogen, dissolved in petroleum ether and washed with 5% $NaHCO_3$ to neutralize any acidity left, and dried over anhydrous Na_2SO_4. The samples are kept in the freezer until application on the gas chromatogram (38,40,74).

Appropriate amounts of a standard margaric acid solution in heptane is added to the extraction mixture simultaneously with the samples. Margaric acid (n 17:0) was obtained from L. Light and Co. Ltd. Colnbrook, England. Normally very little (3-4%) of this acid occurs in sheep serum or tissue and the arteriovenous defference is negligible, making this fatty acid a suitable internal standard.

For triglyceride fatty acid determination the tissue is extracted with a mixture of chloroform: absolute methanol (2:1 v/v) (75); water is then added and the lower chloroform layer containing the triglycerides collected. Hydrolysis and methylation of the triglyceride fatty acids is achieved by refluxing with methanolic HCl as described in the free fatty acid procedure.

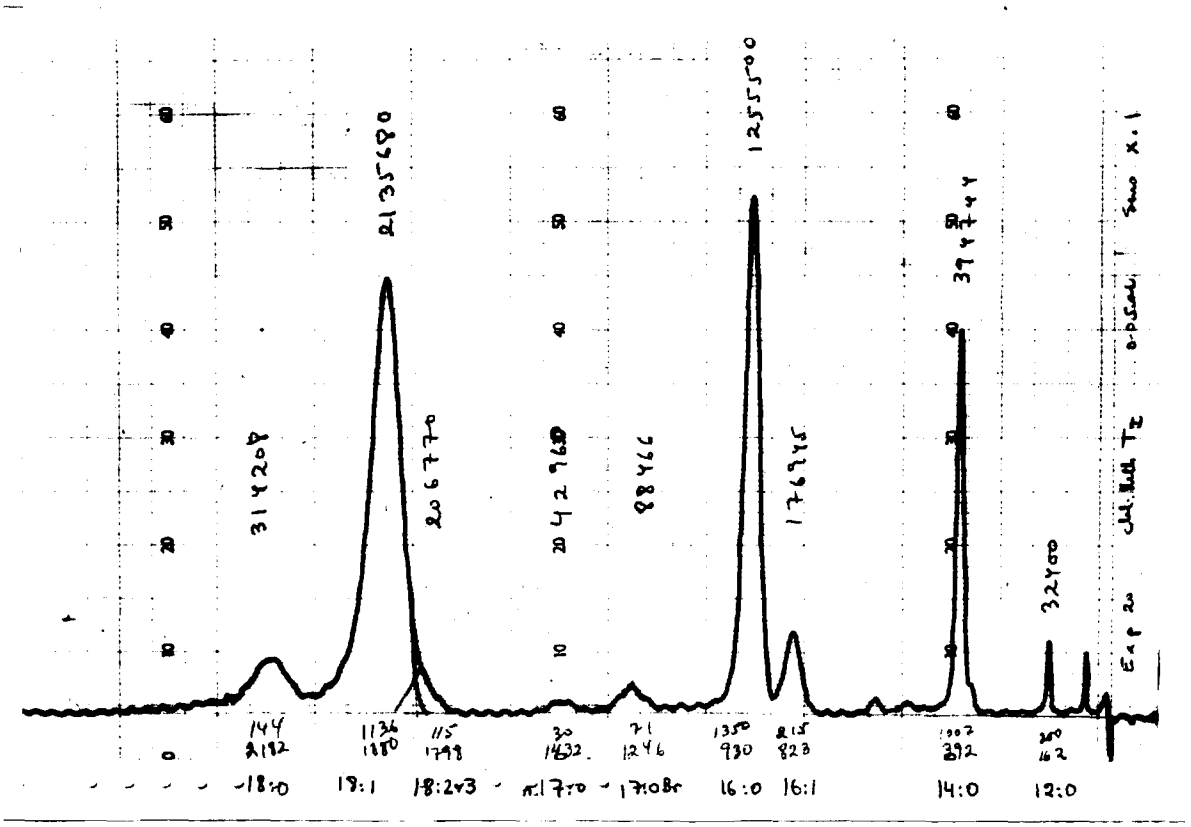
The petroleum ether solution of methylated fatty acids is evaporated under nitrogen in a centrifuge tube and an aliquot of the remaining fat applied on the column. Usually the samples are very small and must be centrifuged to make pipetting possible. The pipettes used consist of short capillary tubes with long stems and a rubber tubing at the end with capacities of 0.025, 0.050 and 0.100ul. The samples are drawn by capillary action. To load the column the gas flow is stopped with a clamp for a short time to allow the pressure in the column to fall, the glass connection on top of the column is opened and the sample applied on a small piece of glass wool resting on the support. The gas flow is started as soon as possible.

Anhydrous methanol was prepared by refluxing methanol with iodine and powdered magnesium for 90 minutes and then distilling.

Anhydrous methanolic HCl was prepared by generating HCl by the action of concentrated H_2SO_4 on NaCl in an Erlenmeyer flask. HCl gas produced was passed through $CaCl_2$ to remove moisture and trapped in a flask containing anhydrous methanol.

4. Interpretation of the Chromatogram.- Figure 1 shows a typical chromatogram. The small negative peak marks the beginning of the chromatogram, caused by oxygen entering the column during the application of the sample. With the Apiezon L column fatty acids ranging from 12-24 carbons are measured, the unsaturated fatty acid methyl esters preceding the saturated ones on the chromatogram.

Figure 1



A Typical Chromatogram of Sheep Tail Triglyceride Fatty Acids

Since only negligible amounts of longer chain fatty acids are found in sheep adipose tissue and serum the chromatograms were stopped after the peak for stearic acid.

The peaks on the chromatogram were identified by their retention times, found by comparing the distance of each peak from the beginning to that of palmitic acid.

The quantities of individual fatty acids were determined by the method of Carroll (76). The height of each peak was multiplied by the distance from the start of the chromatogram to the mid-point of the base of the peak. The relative values found in this way were converted to the absolute concentrations in milligrams by comparing with that of the internal standard.

Duplicate extractions and chromatographic runs on different blood samples were carried to test the accuracy and reproducibility of the technique, and gave satisfactory results. Table 2 shows comparisons of three separate extractions of the same blood sample.

Lipase Studies

The lipolytic activity of adipose tissue on specific triglycerides was studied by extracting the lipase(s) as described by Rizack (77). The tissue was homogenized in three volumes of 0.25M sucrose in a Potter-Elvehjem homogenizer in the cold room. The homogenate was centrifuged at 11,000 r.p.m. for 10 minutes. The surface

Table 2

Comparison of Triplicate Gas Chromatographic Analyses On A Serum Sample

Fatty Acid mg/l.	Extraction A	Extraction B	Extraction C
18:0	35.39	34.99	33.53
18:1	33.39	33.83	33.74
18:2 & 3	5.29	6.60	5.39
17:0 Br	1.58	1.81	1.67
16:0	19.98	21.27	19.04
16:1	1.30	1.22	1.24

In this and all subsequent tables the first number in the first column represents the number of carbon atoms in the fatty acids, and the second number the number of double bonds.

fat cake was discarded and the supernatent collected. The sediment contained no activity when resuspended in sucrose as described by Rizack, so we used the first supernatent.

The enzyme assay medium consisted of the following:-

0.5M phosphate buffer pH 6.8	0.6 ml
10% bovine serum albumin extracted overnight with diethyl ether	2.5 ml
Substrate	0.5 ml
Enzyme extract	1.0 ml
Distilled water	0.4 ml

One or two milliliter aliquots were pipetted before, during and after incubation to determine the free fatty acid concentration by Dole's double extraction method.

Grade A triolein, tripalmitin and tristearin obtained from the Sigma Chemical Co. were used as substrates for the enzyme extract. In preliminary experiments the triglycerides were emulsified with 1.2% lecithin in water to a final concentration of 0.2M. In later experiments lecithin was omitted, and emulsification of the triglycerides was carried in 1% albumin in the VirTis homogenizer. Olive oil emulsion obtained from the Sigma Chemical Co. was found to be unsatisfactory as a substrate because of unreproducible results.

To determine differences in lipolytic activity due to adrenaline we incubated the tissues with or without adrenaline, then extracted the lipase and assayed with the triglycerides.

CHAPTER III

RESULTS

Triglyceride and Free Fatty Acid Composition of Sheep Tail Adipose Tissue and Serum

The major constituents of adipose tissue and serum fatty acids were found to be oleic, palmitic and stearic acids, together forming more than 80% of the total fatty acids (Tables 3 and 7).

Tissues removed 30-60 minutes after injection of adrenaline or noradrenaline to the sheep did not show significant differences from non-treated sheep in their triglyceride fatty acid composition (Table 3). Fasting for two days increased the percentage of stearic acid slightly, whereas after a fast of four to six days this acid decreased by 39.14% when compared to the normal; oleic acid increased by 15% and myristic acid almost doubled (Table 3).

The free fatty acid composition in normal tissue was similar to that of triglyceride fatty acids except for stearic acid which was found in higher concentrations in the free fatty acids. Following a fast of 2-6 days there was no change in the composition of tissue free fatty acids (Table 4-A). Adrenaline and noradrenaline injections to the animal caused an increase in stearic acid, and a decrease in oleic acid (Table 4-B). Incubation in adrenaline did not alter the free fatty acid composition (Table 5), whereas incu-

Table 3

Tissue Triglyceride Fatty Acids

Fatty Acid	Normal (7 exp) % *	45 minutes after adrenaline (2 exp) % *	45 minutes after noradrenaline (1 exp) % *	40 hrs fast (2 exp) % *	90-145 hrs fast (4 exp) % *
18:0	13.03	13.86	12.96	17.27	7.93
18:1	40.16	44.17	43.77	40.10	46.28
18:2 & 3	4.14	3.97	4.85	4.88	5.39
17:0	3.51	3.87	2.91	3.83	2.14
17:0 Br	4.33	4.23	3.69	3.47	3.66
16:0	25.25	24.48	25.16	23.05	24.14
16:1	2.49	2.09	2.03	1.80	3.57
15:0	2.20	1.88	1.65	2.04	1.60
14:0	2.62	2.29	2.96	2.91	5.53

* % by weight

Table 4 A

Tissue Free Fatty Acid Composition

Fatty Acid	Normal (11 exp) %	40 hrs fast (2 exp) %	90-145 hrs fast (4 exp) %
18:0	18.11	21.73	18.27
18:1	43.85	40.41	40.99
18:2 & 3	5.50	6.61	7.17
17:0 Br	4.42	3.71	3.09
16:0	23.04	24.65	26.12
16:1	3.11	2.14	2.38

Table 4 B

Tissue Free Fatty Acid Composition

Fatty Acid	1 Experiment		2 Experiments	
	Control %	25 minutes after adrenaline* %	Control %	45 minutes after noradrenaline** %
18:0	14.11	20.21	15.97	19.78
18:1	47.79	38.20	46.84	40.43
18:2 & 3	4.33	5.94	6.04	6.06
17:0 Br	4.35	3.88	4.27	4.51
16:0	26.35	28.53	24.73	26.63
16:1	2.66	3.23	2.17	3.11

* 0.25 mg injected.

** 0.25 mg and 1 mg in two experiments.

Table 5

Tissue Free Fatty Acids After Incubation With Adrinaline (Exp. 6, 12)

1 gm tissue was incubated for 1 hour in 7 ml serum & 0.1 ml saline for the controls, and 0.1 ml adrenaline (1 ug) for the experimental.

Fatty Acid	FFA - mg/100gm tissue		
	Preincubation	Control incubation	Incubation with adrenaline
18:0	9.42	12.56	10.96
18:1	16.49	16.76	15.06
18:2 & 3	2.76	2.79	2.70
17:0 Br	1.72	2.01	1.70
16:0	8.83	10.23	10.76
16:1	0.87	0.88	0.92
Total	40.09	45.23	42.10

bation with noradrenaline caused a decrease in oleic and palmitic acid concentrations (Table 6).

Relative concentration of serum free stearic acid in normal animals was 60% higher than in the tissue, and that of palmitic acid 20% lower (Table 7). The effect of fasting on the serum free fatty acid composition was comparable to changes in tissue triglyceride fatty acids, stearic acid increasing during a two day fast but decreasing after a longer fast (Table 7).

Blood samples taken after adrenaline, noradrenaline and glucose injections showed a decrease in stearic acid. Palmitic acid increased after glucose injection (Table 7).

Arteriovenous Differences (A-V) of Free Fatty Acids and Related Metabolites

Arteriovenous differences across the tail were measured for various metabolites before and after noradrenaline and glucose infusion to the fasted sheep (4-6 days). Tables 8 and 9 sum up the results of these experiments. In spite of the increase in free fatty acid levels in blood after administration of noradrenaline, the arteriovenous differences remained unchanged. Glucose and lactic acid levels also went up in the blood, accompanied with 33% increase in glucose uptake and 44% decrease in lactic acid release. Cholesterol, phospholipid and esterified fatty acid levels did not change (Table 8).

Table 6

Tissue Free Fatty Acids After Incubation With Noradrenaline (Exp. 7, 9, 10)

1 gm tissue was incubated for 1 hour in 7 ml serum and 0.1 ml saline for the controls, and 0.1 ml moradrenaline (1 ug) for the experimental.

Fatty Acid mg/100g tissue	Preincubation	Control incubation	Incubation with noradrenaline
18:0	11.15	9.71	8.03
18:1	32.11	34.89	21.93
18:2 & 3	4.58	4.46	3.24
17:0 Br	2.79	3.06	2.20
16:0	16.61	23.67	12.66
16:1	1.77	2.09	1.28
Total	69.01	77.88	49.31

Table 7

Blood Free Fatty Acid Composition

Fatty Acid	Normal (6 exp) %	40 hrs fast (2 exp) %	90-45 hrs fast (4 exp) %	After adrenaline (3 exp) %	After noradrenaline (2 exp) %	After glucose (2 exp) %
18:0	29.43	33.73	24.91	23.58	25.69	22.03
18:1	42.17	39.90	46.60	43.61	45.10	44.48
18:2 & 3	5.79	7.34	5.82	7.49	6.61	6.46
17:0 Br	2.37	2.08	2.20	3.52	2.22	2.29
16:0	18.44	15.87	17.30	19.56	19.02	22.07
16:1	1.83	1.03	1.44	2.25	1.37	2.66

Table 8

Arteriovenous Differences Accross the Tail In Fasted Sheep After Adrenaline (Exp. 19)

Metabolite	A_I	$A_I - V_I$	A_{II} *	$A_{II} - V_{II}$
Total FFA (uEq/l.)	395	- 447	1000	- 420
Glucose (mg/100ml)	81.9	+ 9.0	103.1	+ 12.2
Lactic acid (mg/100ml)	25.7	- 9.5	44.6	- 5.2
Esterified FA (mEq/100ml)	0.445	0	0.450	- 0.02
Phospholipids (mmoles/100ml)	0.116	- 0.003	0.112	- 0.003
Cholesterol (mg/100ml)	76.86	0	68.17	- 3.57

* Second arteriol (A_{II}) and venous (V_{II}) samples were taken 30-45 minutes after the beginning of adrenaline infusion.

Table 9

Arteriovenous Differences Accross The Tail in Fasted Sheep After Glucose Infusion (Exp. 20).

Metabolite	A_I	$A_I - V_I$	A_{II}*	$A_{II} - V_{II}$
Total FFA (uEq/l.)	719	- 281	375	- 31
Glucose (mg/100ml)	69.9	+ 1.7	368.7	+ 4.7
Lactic acid (mg/100ml)	12.9	- 3.9	37.0	- 4.0
Esterified FA (mEq/100ml)	0.37	0	0.47	- 0.05
Phospholipids (mmoles/100ml)	0.119	- 0.007	0.109	- 0.004
Cholesterol (mg/100ml)	63.2	- 2.6	55.4	+ 0.84

* Second arterial (A_{II}) and venous (V_{II}) samples were taken 90 minutes after the start of glucose infusion.

Glucose infusion to a fasted animal lowered the blood free fatty acid concentration and fatty acid release markedly, while glucose uptake increased slightly. The concentration of lactic acid increased in blood without any change in its A-V difference. The change in esterified fatty acid, phospholipid and cholesterol levels and their arteriovenous differences was negligible (Table 9).

Control experiments carried on three sheep kept under anesthesia for thirty minutes showed only slight variations in the serum free fatty acid levels and the arteriovenous differences, with some increase in glucose uptake (Table 10).

As in the case of fasted animals mentioned above, normal animals responded to adrenaline injections by an increase in the blood free fatty acid and glucose levels and glucose uptake. The slight uptake of free fatty acids observed before the treatment in these experiments decreased by the hormone, but the change in A-V was insignificant (Table 11). Noradrenaline injection increased stearic acid uptake slightly, decreased release of oleic acid, and increased release of palmitic acid, but there was no net effect on the arteriovenous differences of the total free fatty acids. Glucose uptake doubled and lactic acid release decreased (Table 12). In the fasted animal treated with noradrenaline there was no change in the arteriovenous differences for total free fatty acids, but release of acids studied by gas chromatography decreased. Stearate

Table 10

Control Arteriovenous Differences (Exp. 1, 2, 4)

Free fatty acid mg/l.	A_I	$A_I - V_I$	A_{II}*	$A_{II} - V_{II}$
18:0	10.70	- 0.32	10.51	- 3.24
18:1	10.52	- 3.59	9.78	- 4.77
18:2 & 3	1.75	- 0.79	1.13	- 0.76
17:0 Br	1.72	- 1.25	1.13	- 0.69
16:0	9.00	- 1.23	6.84	- 1.76
16:1	1.03	- 0.32	1.21	- 0.19
15:0	0.53	- 0.21	0.56	- 1.42
14:0	1.09	- 1.29	0.90	- 0.73
Total	36.34	- 9.00	32.06	- 13.56
Total FFA (uEq/l.)	173	- 54	162	- 46
Glucose (mg/100ml)	66	+ 1.25	66	+ 6.60
Lactic acid (mg/100ml)	8.5	- 0.2	8.5	- 2.9

* A_{II} & V_{II} were taken 15-30 minutes after A_I & V_I.

Table 11

Arteriovenous Differences After Adrenaline Injection (Exp. 5 & 6)

Free fatty acid mg/l.	Control		After adrenaline	
	A_I	$A_I - V_I$	A_{II}*	$A_{II} - V_{II}$
18:0	13.18	+ 0.99	16.32	+ 0.62
18:1	14.15	+ 2.10	30.17	- 0.64
18:2 & 3	2.48	+ 0.73	5.18	+ 0.37
17:0 Br	1.21	+ 0.18	2.44	+ 0.22
16:0	7.07	+ 0.51	13.54	- 0.29
16:1	1.12	-	1.56	+ 0.09
Total	39.21	+ 5.02	69.21	+ 0.37
Glucose mg/100ml	90.93	+ 22.77	133.7	+ 33.7

* A_{II} & V_{II} were taken 15 minutes after the injection of 0.25 mg adrenaline intravenously.

Table 12

Arteriovenous Differences After Noradrenaline Injection (Exp. 7 & 9)

Free fatty acid mg/l.	Control		30 min. after noradrenaline*	
	A_I	$A_I - V_I$	A_{II}	$A_{II} - V_{II}$
18:0	10.75	+ 0.36	16.81	+ 1.70
18:1	21.56	- 5.52	39.69	- 2.59
18:2 & 3	3.13	- 1.58	6.52	- 1.10
17:0 Br	2.01	- 0.42	3.30	- 1.54
16:0	8.29	- 0.82	16.04	- 3.63
16:1	0.93	- 0.04	1.40	- 0.23
Total	46.67	- 8.02	83.76	- 7.39
Glucose (mg/100ml)	65.4	+ 5.9	123.1	+ 10.9
Lactic acid (mg/100ml)	6.79	- 4.20	12.17	- 1.46

* 2 mg. noradrenaline in 300 ml saline was infused over 30 minutes.

release came down almost to zero, whereas oleic and palmitic acid release continued at half the original rate (Table 13).

Release of all the free fatty acids from the tail to blood increased extensively after a long fast as shown in table 14.

Glucose infusion to a fasted animal depressed the blood free fatty acid level by 50%, with relatively small increase in glucose uptake. The lactic acid level in blood increased without any change in its arteriovenous difference across the tail. Free fatty acid release into venous blood came down considerably, while uptake of oleic, palmitic and some other acids started, stearic acid continued to be released (Table 15).

In Vitro Studies of Tail Adipose Tissue in Serum and Krebs-Ringer Phosphate Buffer

Release of all fatty acids studied by gas chromatography was observed after incubation of adipose tissue in sheep serum for one or two hours. Addition of glucose to tissues from normal sheep suppressed the release of stearic and palmitic acids (Table 16). In two other experiments with tissue from animals fasted for 90-145 hours glucose caused 30% increase in free fatty acid release over the control, mainly for oleic, palmitic and palmitoleic acid, but suppressed release of stearic acid (Table 17).

Table 18 shows average values from four experiments where

Table 13

Arteriovenous Differences In A Fasted Sheep After Noradrenaline Injection (Exp. 19)

Free fatty acids mg/l.	Control (fasted)		After noradrenaline	
	A_I	$A_I - V_I$	A_{II}*	$A_{II} - V_{II}$
18:0	26.14	- 15.20	46.50	- 0.17
18:1	49.30	- 60.26	80.58	- 35.75
18:2 & 3	8.22	- 6.35	12.10	- 4.32
17:0 Br	1.94	- 1.71	2.78	- 1.91
16:0	17.75	- 39.21	36.98	- 21.19
16:1	1.47	- 3.05	2.78	- 3.76
14:0	0.70	- 3.38	1.37	- 4.17
Total	106	-130	182.4	- 71
Total FFA (uEq/l.)	395	-447	1000	-420
Glucose (mg/100ml)	82	+ 9.0	103	+ 12
Lactic acid (mg/100ml)	26	- 9.5	45	- 5.2

* A_{II} & V_{II} were taken 30-45 minutes after the start of 900 ug noradrenaline infusion over 25 minutes.

Table 14

Arteriovenous Differences After A Long Fast

(Exp. 1, 2, 3 - control) (Exp. 19, 20 - fast)

Free fatty acids mg/l.	Control		Fasted (92 & 145 hrs)	
	A_I	$A_I - V_I$	A_I	$A_I - V_I$
18:0	10.70	- 0.32	30.34	- 15.65
18:1	10.52	- 3.59	65.98	- 62.69
18:2 & 3	1.75	- 0.79	9.80	- 6.76
17:0 Br	1.72	- 1.25	2.24	- 2.04
16:0	9.00	- 1.23	22.33	- 35.94
16:1	1.03	- 0.32	1.92	- 3.56
14:0	1.09	- 1.29	0.96	- 2.86
Total	6.34	- 9.0	134.12	- 129.50
Total FFA (uEq/l.)	173	- 54	557	- 364
Glucose (mg/100ml)	66	+ 1.25	75.9	+ 5.1
Lactic acid (mg/100ml)	8.5	- 0.2	19.3	- 6.7

Table 15

Arteriovenous Difference In A Fasted Sheep After Glucose Infusion (Exp. 20)

Free fatty acid mg/l.	Control (14½ hrs fast)		After glucose (30g/60 min.)	
	A_I	$A_I - V_I$	A_{II}*	$A_{II} - V_{II}$
18:0	34.53	- 16.10	14.86	- 4.82
18.1	82.65	- 65.12	36.17	+ 0.48
18:2 & 3	11.39	- 7.16	5.00	- 0.96
17:0 Br	2.53	- 2.36	1.72	- 0.61
16:0	26.90	- 32.62	17.75	+ 0.86
16:1	2.37	- 4.0	2.08	+ 0.16
14:0	1.27	- 2.35	3.23	+ 1.69
Total	161.58	-229.71	80.81	- 3.20
Total FFA (uEq/l.)	719	-281	375	- 31
Glucose (mg/100ml)	70	+ 2	369	+ 5
Lactic acid (mg/100ml)	12.9	- 3.9	37.0	- 4.0

* A_{II} & V_{II} were taken 90 minutes after the start of glucose infusion.

Table 16

The Effect Of Glucose Added In Vitro On The Release Of Free Fatty Acids From Adipose Tissue Suspension

1 gm. of sheep tail biopsy was incubated for 60 minutes in 7 ml. of serum with 0.14 ml. of saline for the control, and 0.1 ml. of 5% glucose for the experimental.

Free fatty acids mg/l.	Preincubation serum	Release during control incubation	Release during incubation with glucose
18:0	41.58	10.30	4.86
18:1	58.71	8.34	9.88
18:2 & 3	6.70	3.88	3.66
17:0 Br	2.63	2.99	2.16
16:0	23.07	17.33	10.11
16:1	2.16	6.49	5.49
Total	134.75	49.33	36.16

Table 17

Release Of FFA In Vitro By Tissue From Fasted Animal Incubated With Glucose (Exp. 19, 20)

FFA mg/l.	Preincubation serum A_I	Release during control incubation	Release during incubation with glucose *
18:0	30.34	26.14	20.81
18:1	66.03	77.31	129.01
18:2 & 3	9.66	12.14	11.60
17:0 Br	2.91	5.02	4.17
16:0	22.33	69.68	79.97
16:1	1.92	9.03	13.40
14:0	0.96	6.54	7.85
Total	134.15	205.86	266.81
Total FFA (uEq/l.)	557	887	1125

* 0.1 ml. of 7% glucose added.

Table 18

Release Of Free Fatty Acids In Vitro With Noradrenaline (Exp. 9-12)

Free fatty acid mg/l.	Preincubation serum	Release during control incubation	Release during incubation with 30 ug noradrenaline
18:0	39.73	46.63	- 0.24
18:1	47.40	61.72	65.29
18:2 & 3	8.79	9.83	10.06
17:0 Br	2.81	6.34	8.31
16:0	19.60	33.66	42.02
16:1	1.46	2.81	4.14
Total	119.79	160.99	129.58
Glucose (mg/100ml.)	111.64	- 15.94	- 20.84
Lactic acid (mg/100ml.)	14.72	+ 6.38	+ 8.08

tissue from normal animals was incubated with or without noradrenaline. These show considerable release in the control flasks of free fatty acids and lactic acid, accompanied with glucose uptake. In the flasks with the hormone glucose uptake has increased but the total free fatty acid release is less. Stearic acid release has almost stopped although release of oleic and palmitic acids has increased.

Response to incubation with adrenaline was varied. In two experiments there was marked increase in the release of all the free fatty acids during incubation with adrenaline (Table 19), whereas in three other experiments incubation with this hormone for one or two hours did not result in any significant difference from the controls (Table 20). Release of free fatty acids by tail biopsies removed from a fasted sheep was almost trebled when adrenaline was added to the medium (Table 21).

In three experiments release of free fatty acids into serum was compared with release into Krebs-Ringer phosphate buffer containing 5% bovine serum albumin. Although there was a marked release of the fatty acids into both media by the control tissues, release into serum was considerably more than that into buffer. Adrenaline caused further release of free fatty acids into the buffer, especially for oleic acid, whereas it suppressed free fatty acid release by tissues incubated in serum (Table 22).

Table 19

Release Of FFA In Vitro With Adrenaline - Increased Release (Exp. 5 & 6)

FFA mg/l.	Preincubation serum	Release during control incubation	Release during incubation with 1 ug adrenaline
18:0	9.88	11.95	74.82
18:1	14.15	23.59	194.00
18:2 & 3	2.48	2.19	14.87
17:0 Br	1.21	2.48	15.14
16:0	7.07	14.23	96.98
16:1	1.12	1.24	7.53
Total	35.91	55.68	402.34
Glucose (mg/100ml)	90.7		

Table 20

Release Of FFA In Vitro With Adrenaline - (Exp. 11, 12, 13)

Free fatty acid mg/l.	1 hour		2 hours	
	Control incubation	Incubation with 33 ug adrenaline	Control incubation	Incubation with 33 ug adrenaline
18:0	16.58	16.52	53.48	52.39
18:1	36.47	36.96	87.65	87.77
18:2 & 3	6.96	7.05	15.15	16.28
17:0 Br	4.12	3.65	8.27	9.13
16:0	22.68	22.16	43.51	50.70
16:1	3.05	2.59	3.69	4.35
Total	89.86	88.93	211.75	220.62
Glucose (mg/100ml)	113.42		87.10	86.72
Lactic acid (mg/100ml)	10.68		15.17	14.92

Table 21

Release Of Free Fatty Acids In Vitro By Tissue From A Fasted Sheep, Incubated With Adrenaline (Exp. 19)

Free fatty acids mg/l.	Preincubation serum	Release during control incubation	Release during incubation with 33 ug adrenaline
18:0	26.14	13.21	47.05
18:1	49.30	52.73	169.36
18:2 & 3	8.22	4.50	16.31
17:0 Br	1.94	2.24	6.92
16:0	17.75	38.41	108.91
16:1	1.47	4.02	13.50
14:0	0.70	3.57	10.94
Total	105.42	118.68	372. 9
Total FFA uEq/l.	395	894	1315
Glucose mg/100ml.	82		

Table 22

Comparison Of In Vitro Free Fatty Acid Release With Adrenaline In Serum And Krebs-Ringer Phosphate Buffer With 5% Albumin (Exp. 14, 15, 18)

Free fatty acid	FFA release into buffer - mg/l.			FFA release into serum - mg/l.		
	Preincubation	Control	+ Adrenaline*	Preincubation	Control	+ Adrenaline*
18:0	22.50	5.54	9.90	27.58	16.90	6.13
18:1	37.56	16.73	44.25	37.52	76.99	30.59
18:2 & 3	8.79	0.81	4.68	7.52	10.47	4.60
17:0 Br	5.15	- 0.54	1.91	3.12	8.13	3.13
16:0	19.10	7.65	18.14	16.16	37.29	16.52
16:1	2.37	0.29	1.60	1.58	3.72	1.33
Total	95.47	30.48	80.48	103.48	143.50	52.30

* 33 ug adrenaline added.

Rat Adipose Tissue Studies

Epididymal fat tissue was incubated in Krebs-Ringer phosphate buffer containing 5% albumin in three experiments. As reported in the literature, addition of adrenaline to the medium caused considerable increase in release of free fatty acids into the medium. Stearic acid release increased by only about 25%, whereas release of all the other fatty acids studied were more than double (Table 23, Figure 2,3).

Lipase Studies

We obtained little lipolytic activity from sheep tail biopsies when extracted directly with 0.25M sucrose. Incubation of the tissue in Krebs-Ringer phosphate buffer containing 5% albumin in the presence of adrenaline caused little increase in the lipolytic activity. Following incubation in the absence of this hormone more lipase was extracted from the tissue. The lipolytic activity extracted was too small to show significant differences in the rate of hydrolysis of triolein, tristearin and tripalmitin used in this experiment. (Table 24).

Table 23

Release Of Free Fatty Acids By Rat Epididymal Fat

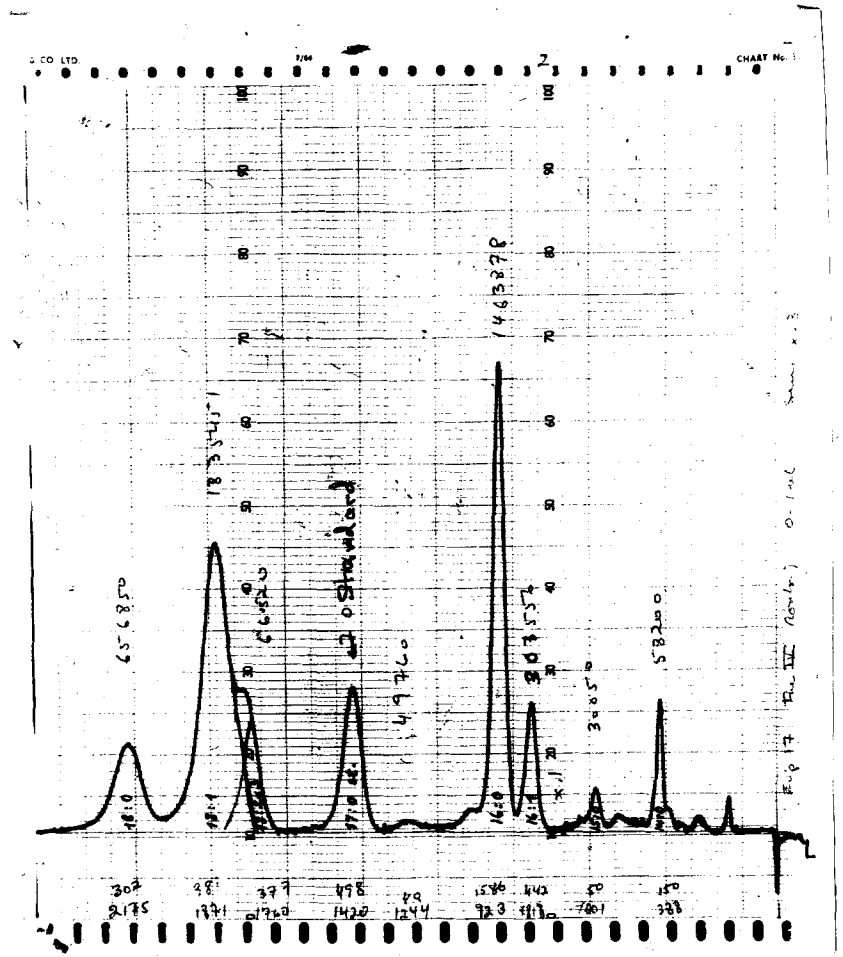
Into Buffer With Adrenaline

(Exp. 16-18)

0.5 gm. portions of rat epididymal fat were incubated for 1 hour in 3.5 ml. Krebs-Ringer phosphate buffer with 5% albumin. 0.1 ml. saline was added to the control flask, and 0.1 ml. adrenaline (33ug) to the experimental flask.

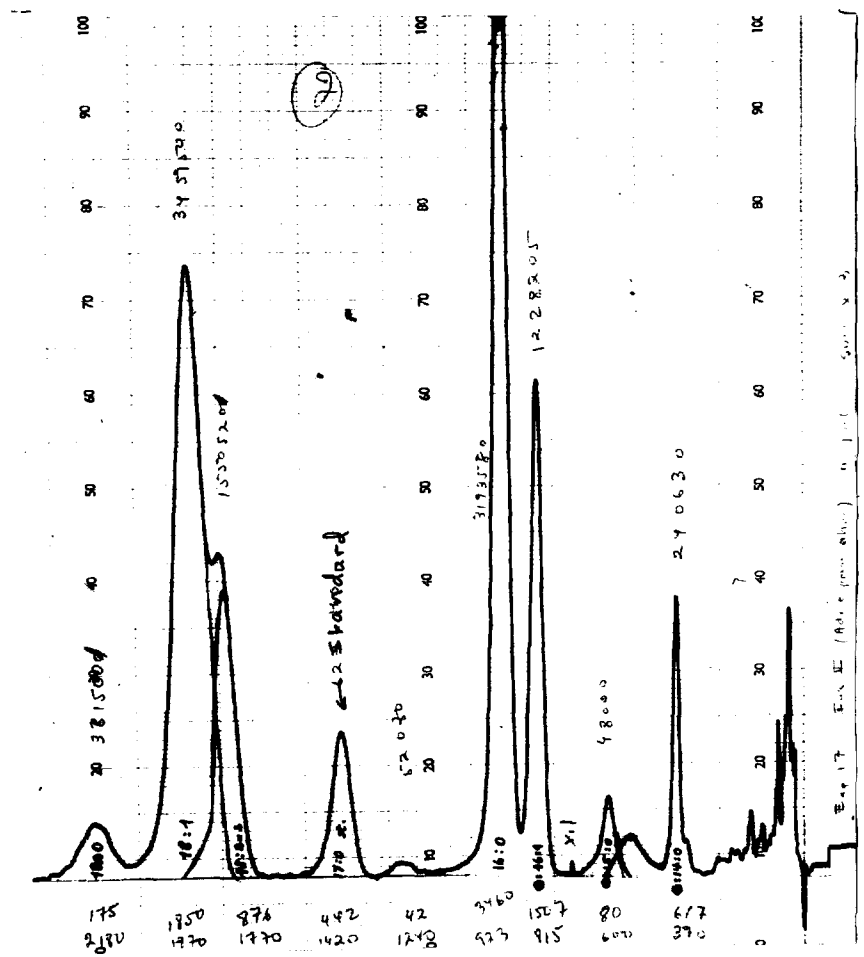
Free fatty acids mg/l.	Control incubation	Incubation with adrenaline	Release during incubation with adrenaline
18:0	32.84	40.93	8.09
18:1	72.40	196.89	124.44
18:2 & 3	19.79	78.76	58.97
17:0 Br	4.74	7.01	2.27
16:0	51.70	124.30	72.60
16:1	7.64	40.51	32.87
Total	189.11	388.40	199.29

Figure 2



Free Fatty Acids Released into Krebs-Ringer Phosphate Buffer by Rat Epididymal Fat in the Absence of Adrenaline

Figure 3



Free Fatty Acids Released into Krebs-Ringer Phosphate Buffer by Rat Epididymal Fat with Added Adrenaline

Table 24

Effect Of Adrenaline On Lipolytic Activity In Sheep Tail In Vitro

Tail adipose tissue biopsies (5 gm) were incubated at 37°C in 15 ml. Krebs-Ringer phosphate buffer with 5% albumin for 2 hours. 25 ug adrenaline was added to one flask. The lipolytic activity was extracted from the tissue and assayed as described in the text

	FFA - uEq/l/hour	
Triglyceride	Control tissue	Tissue with adrenaline
Tristearin	219	125
Triolein	313	94
Tripalmitin	219	62

CHAPTER IV

DISCUSSION

The tail of the Syrian sheep serves as a depot of fat and its lipids consist primarily of triglycerides, with a very small pool of free fatty acids. The literature contains only few reports on the composition of this tissue. Reed and Awdeh (63) studied the triglyceride composition of adipose tissue from various sites in the sheep and noted a high concentration of odd-numbered and branched chain fatty acids in the tail. The major components of the fatty acids were oleic, stearic and palmitic acids. Our results confirmed these findings.

The free fatty acid profile in the sheep tail adipose tissue is similar to the triglyceride fatty acids (Tables 3,4). These findings are consistant with those reported by Miller et. al. for the subcutaneous fat in dogs (58). Comparison of the free fatty acids in serum and those of the tissue shows similarities in all fatty acids except stearic acid. Comparable results were reported for sheep plasma free fatty acid composition by Garton and Duncan (78). The similarity in the composition of the three pools might indicate that the tissue free fatty acid pool serves as an intermediate between tissue triglycerides and serum free fatty acids.

Several investigators have demonstrated release of free fatty

acids from adipose tissue *in vivo* (58,61) and *in vitro* (9). Our results demonstrate release of all free fatty acids *in vitro*. In most animals under basal conditions there is a slight release of free fatty acids from the tail. Oleic and palmitic acids are the main ones thus mobilized, whereas stearic acid is released at a much lower rate or is taken up by the tissue (Table 10).

Many tissues have been found to take up free fatty acids *in vivo* (48,58-60), but in adipose tissue free fatty acid uptake has been reported only *in vitro* (21,51). In several sheep we could demonstrate positive arteriovenous differences for free fatty acids across the tail which decreased after 30 minutes of anesthesia.

The arteriovenous difference for glucose was positive in all the animals. Glucose uptake increased slightly after thirty minutes of anesthesia probably due to endogenous adrenaline secretion.

Mobilization of lipids from adipose tissue in the form of phospholipids, triglycerides or cholesterol was not observed in normal or fasted animals (79). Infusion of noradrenaline or glucose also did not change their arteriovenous differences across the tail significantly confirming reports by Shafrir *et. al.* (43). These results support the view that lipids are mobilized primarily in the form of free fatty acids (80).

The Effect of Catecholamines.- There was no change in the relative

concentrations of triglyceride fatty acids in the sheep tail after injection of catecholamines or their addition *in vitro*. This result is expected since the differential mobilization would be too small to be detected by our methods.

Several investigators have reported accumulation of free fatty acids in rat epididymal adipose tissue after incubation with adrenaline or noradrenaline (17,27,81). This was shown to be due to increased lipolysis in the tissue, resulting in an increased release of glycerol and free fatty acid to the medium (21,26,27,82). We could show increased release of free fatty acid by rat epididymal tissue by addition of adrenaline *in vitro*. Steinberg *et. al.* (81) reported greater accumulation of palmitic and palmitoleic acids. Hollenberg *et. al.* (83), and Meinetz *et. al.* (84) showed a relatively greater release of oleic acid into the medium *in vitro* from rat adipose tissue, in agreement with the *in vivo* findings of Spitzer in the dog (85). The lipolytic effect of the catecholamines is influenced by their effect on carbohydrate metabolism and on the concentration of glucose in the medium (18,19,24,26,27,46,47). Adrenaline, being a glycogenolytic hormone causes a rise in serum glucose level, hence counteracts its own lipolytic effect. Noradrenaline on the other hand, does not have a glycogenolytic effect, and causes a more persistent elevation of blood free fatty acid (42).

Our studies show a relative increase in the stearic acid

concentration in the tissue free fatty acids after administration of catecholamines. Uptake of stearic acid by the tail increased slightly and its relative concentration in serum fell. Palmitic acid release increased, accompanied by its decrease in the tissue free fatty acid pool. Free oleic acid in the tissue also decreased. Similar effects were noted in a sheep fasted for six days (Table 13). Following noradrenaline infusion to this animal stearic acid release stopped, while oleic and palmitic acid release decreased only by 50%. The arteriovenous differences for total free fatty acids remained unchanged while release of the free fatty acids measured by gas chromatography decreased. This might suggest that under the influence of noradrenaline there is greater release of fatty acids shorter than myristic which are not measured by the gas chromatograph. In experiments on dogs, Spitzer also observed decrease in stearic acid and increase in oleic acid in the arterial blood free fatty acid (85).

Despite the fact that the arteriovenous differences across the tail decreased following administration of catecholamines the arterial levels of free fatty acids were substantially elevated. This might indicate that the hormones caused increased mobilization of fat from other sites.

Adrenaline had varied effects in vitro. In the first two experiments there was marked increase in the release of the free fatty acids by the tissue in the presence of this hormone (Table 20).

In all the following experiments adrenaline did not have any effect *in vitro* (Table 19). In the two experiments with positive adrenaline effect the medium glucose level was lower than in the subsequent experiments when adrenaline had no effect.

Noradrenaline suppressed total free fatty acid release by the tissue *in vitro*, while glucose uptake increased. Similar to the *in vivo* experiments, stearic acid release stopped completely during incubation with noradrenaline, while that of oleic and palmitic acids increased (Table 18).

Since most reported experiments were carried on adipose tissue incubated in Krebs-Ringer phosphate buffer we repeated these experiments and found increased release of free fatty acids with adrenaline. Further experiments should be carried to find out whether the varied effect of adrenaline in the two media is due to glucose or some other factors. In one experiment we could extract less lipolytic activity from tissue incubated in buffer with adrenaline than without suggesting a suppression of lipases by the hormone independently from glucose.

The Effect of Glucose.- Glucose added *in vitro* or injected to the animal is known to inhibit the release of free fatty acids from adipose tissue (18,19,44,48,83), and to suppress the lipolytic effect of hormones (18,19,43,85,86). Contradictory results have been re-

ported on the effect of glucose on the tissue free fatty acid pool, Raben and Hollenberg (87) reporting decreased concentration, and Engel et. al. (88) reporting their accumulation in the tissue.

In normal and fasted sheep infused with glucose we found a decrease of all the free fatty acids in serum. Their relative composition was also changed, resulting in a lower percentage of stearic acid and a higher percentage of palmitic acid. Release of stearic acid from the tail continued at a lower rate, while uptake of oleic and palmitic acids started. In this experiment done on an animal that had fasted for 6 days there was no increase in glucose uptake by the tail in spite of the considerable increase in the blood glucose level resulting from its intravenous administration (Table 15). This suggests that the effect of glucose on free fatty acid transfer is independent of elevation in glucose uptake. The failure of adipose tissue to increase its uptake of glucose following hyperglycemia may be explained by the prolonged fasting which is known to suppress uptake of glucose by tissues. Since this experiment was carried only in one sheep, further work must be done to verify and explain these points.

Contrary to reports in literature (18,19,44) tail biopsies from fasted sheep released more free fatty acids when incubated with added glucose. Of the major fatty acids, release of oleic and palmitic acids increased, while that for stearic acid was suppressed.

This observation further confirms the finding *in vivo* and throws doubt on the assumption that increasing levels of glucose favor esterification of fatty acids. Release of free fatty acids *in vitro* from adipose tissue incubated in serum compared to release into Krebs-Ringer phosphate buffer with 5% albumin in the absence of glucose also supports our findings.

Effect of Fasting.- Adipose tissue releases large amounts of free fatty acids during a fast because of increased lipolysis and decreased lipogenesis (89,90).

Although we could not detect any changes on the triglyceride composition of adipose tissue in acute experiments with hormones, appreciable changes were noted by prolonged fasting. The relative concentration of stearic acid in the tissue triglycerides increased after a two day fast, but decreased considerably after a longer fast, with simultaneous increase of oleic, linoleic and myristic acids. Since stearate is released at a relatively slower rate under basal conditions its relative increase after a short fast is expected. The drastic decline in its concentration after a six day fast may be explained by the greater utilization of this acid after a long fast.

As expected the arterial blood level and the arteriovenous differences for all the fatty acids studied increased considerably after the animals were fasted.

The studies reported above show selectivity in the behavior of the various free fatty acids, particularly evident for stearic acid which is affected differently from other free fatty acids in most experimental procedures. In normal sheep it is released at a lower rate than the other major fatty acids. Adrenaline and noradrenaline infusions decrease its release while release of the other free fatty acids increases. Glucose infusions also cause a relatively smaller decrease in release of stearic acid. The few in vivo studies reported on the arteriovenous differences of individual fatty acids accross adipose tissue do not point out a similar behavior of stearic acid. Rizack (77) has reported a faster rate of lipolysis for short chain fatty acids in vitro by adipose tissue extracts, and faster hydrolysis for trilinolein, followed by tristearin and triolein. In one experiment we could not show any difference in the rate of hydrolysis for pure tristearin, triolein and tripalmitin. The activity in this extract was very low and minor differences could have been missed. If the result of this experiment can be confirmed, the selectivity for free fatty acids in vivo is then probably due to selectivity of the cell membrane as suggested by Miller et. al. (50).

Our failure to demonstrate a rise in arteriovenous differences for free fatty acids as well as a rise in free fatty acid release in vitro under the influence of catecholamines suggests a difference between adipose tissue of the tail and the epididymal pad fat that has been used extensively for studies of adipose tissue. However the rise

in free fatty acid concentration in arterial blood indicates that fatty acid mobilization is occuring from other sites of the animal. This difference of behavior of the tail fat may be instrumental in the large accumulation of triglycerides at this site.

SUMMARY

Metabolism of sheep tail adipose tissue was studied by measuring arteriovenous differences for various metabolites. Mobilization of individual free fatty acids from the tail was studied *in vivo* under different conditions and compared with *in vitro* observations on the same tissue. Analysis of the individual free fatty acids was carried by argon gas liquid chromatography.

Release and uptake of free fatty acids from the tail was seen in normal sheep. This is the only animal in which free fatty acid uptake by adipose tissue *in vivo* has been reported.

Adrenaline and noradrenaline infusions caused increased release of palmitic and oleic acids, but stearic acid release stopped. There was little change in the arteriovenous difference for total free fatty acids. *In vitro*, adrenaline caused release of free fatty acids from the tissue in two experiments, but had no effect in all subsequent experiments. Noradrenaline suppressed free fatty acid release, specially for stearic acid. This results suggest relative insensitivity of tail adipose tissue to adrenaline and noradrenaline compared to rat epididymal fat, or other tissues of the sheep, since arterial free fatty acid levels rise after injections of these hormones to the sheep.

Glucose suppressed free fatty acid release *in vivo*

without an appreciable increase in glucose uptake by the tail.. High concentration of glucose added *in vitro* caused a rise in free fatty acid release. Again stearic acid was the one least effected.

Fasting caused an increased release of all the major free fatty acids.

All the experiments showed differences in release and uptake of individual free fatty acids, primarily stearic acid. Whether this is due to selectivity of the lipase, compartmentalization of the tissue or cell membrane selectivity has still to be proved.

REFERENCES CITED

1. Kinsell, Laurence W. (ed.) Adipose Tissue as an Organ. Springfield: Charles C. Thomas, 1962.
2. Menschik, S. Anat. Rec. 116: 439, 1953
3. Bell, T. Am. J. Anat. 9: 412, 1909.
4. Clark, E.R., and E.L. Clark. Am. J. Anat. 67: 255, 1940.
5. Hausberger, F.X. Arch. Path. Anat. and Physiol. 302: 640, 1938.
6. Sheldon, E.F. Anat. Rec. 28: 331, 1924.
7. Crofford, O.B., and A.E. Renold. J. Biol. Chem. 240: 14, 1965.
8. Fawcett, D.W. J.Morph. 90: 363, 1952.
9. Vaughan, M. J. Lipid Research 2: 293, 1961.
10. Barnett, R.J., and A.M. Seligman. Science 116: 323, 1952.
11. Weiss, L.P., Tsou, K.C., and A.M. Seligman. J. Histochem. and Cytochem. 2: 29, 1954.
12. Fawcett, D.W. Endocrinology 42: 454, 1948.
13. Gomori, G. Arch. Path. 41: 121, 1946.
14. Dempsey, E.W., Bunting, H., and G.B. Wislocki. Am. J. Anat. 81: 309, 1947.
15. Hollenberg, C.H. Am. J. Physiol. 197: 667, 1959.
16. Cherkes, A., and Gordon, R.S. J. Lipid Research 1: 97, 1959.
17. Robison, D.S. Biochem. J. 74: 25, 1960.
18. Gordon, R.S., and A. Cherkes. Proc. Soc. Exp. Biol. Med. 97: 150, 1958.

19. Lopez, E., White, J.E., and F.L. Engel. J. Biol. Chem. 234: 2254, 1959.

20. Cahill, G.F. Jr., Bally, P.R., Leboeuf, B., and A.E. Renold. Fed. Proc. 18: 22, 1959.

21. Cahill, G.F., Leboeuf, B., and R.B. Flinn. J. Biol. Chem. 235: 1246, 1960.

22. Ashmore, J. et. al. Recent Progr. in Hormone Research 16: 547, 1960.

23. Leboeuf, B., and Cahill, G.F. J. Biol. Chem. 236: 41, 1961.

24. Hagen, J.H., and E.G. Ball. J. Biol. Chem. 235: 1545, 1960.

25. Jungas, R.L., and E.G. Ball. J. Biol. Chem. 235: 1894, 1960.

26. Lynn, W.S., Macleod, R.M., and R.H. Brown. J. Biol. Chem. 235: 1904, 1960.

27. Leboeuf, B., Flinn, R.B., and G.F. Cahill. Proc. Soc. Exp Biol. Med. 102: 527, 1959.

28. Schoenheimer, R., and D. Rittenberg. J. Biol. Chem. 114: 381, 1936.

29. Rittenberg, D., and Schoenheimer, R. J. Biol. Chem. 121: 235, 1937.

30. Stein, Y., and O. Stein. Biochim. Biophys, Acta 60: 58, 1962.

31. Hirsch, R.L., Farquhar, J.W., Ahrens, E.H. Jr., Peterson, M.L., and W. Stoffel. J. Clin. Nutr. 8: 499, 1960.

32. Gorin, E., and E. Shafrir. Biochim. Biophys. Acta 70: 109, 1963.

33. Hollenberg, C.H. J. Lipid Research 6: 84, 1965.

34. Szent-Gyorgi, A., and T. Tomianga. Biochem. Z. 146: 226, 1924.

35. Kelsey, F.E., and H.E. Longenecker. J. Biol. Chem. 139: 727, 1941.

36. Davis, B.D. Arch. Bioch. 15: 351, 1947.

37. Cohn, E.J., Hughes, W.I., and J.H. Weare. J. Am. Chem. Soc. 69: 1753, 1947.

38. Dole, V.P. J. Clin. Invest. 35: 150, 1956.

39. Gordon, R.S. Jr., and A. Cherkes. J. Clin. Invest. 35: 206, 1956.

40. Dole, V.P. J. Clin. Invest. 38: 1544, 1959.

41. Goodman, H.M., and E. Khobil. Proc. Soc. Exp. Biol. Med. 100: 195, 1959.

42. Havel, R.J., and A. Goldfien. J. Lipid Research 1:102, 1959.

43. Shafrir, E., Sussman, K., and D.S. Steinberg. J. Lipid Research 1: 109, 1959.

44. White, E.J., and F.L. Engel. Proc. Soc. Exp. Biol. Med. 99: 375, 1958.

45. Leboeuf, B., Cahill, G.F., and R.B. Flinn. J. Biol. Chem. 235: 1246, 1960.

46. Raben, M.S. Recent Progr. in Hormone Research 15: 71, 1959.

47. Raben, M.S., and C.H. Hollenberg. J. Clin. Invest. 38: 484, 1959.

48. Gordon, R.S. Jr., Cherkes, A., and H Gastes. J. Clin. Invest. 36: 810, 1957.

49. Duncomb, W.G. Biochem. J. 88: 7, 1963.

50. Itaya, K., and M. Ui. J. Lipid Research 6: 16, 1965.

51. Stern, J.R., and B. Shapiro. Metabolism 3: 539, 1954.

52. White, E.J., and F.L. Engel. J. Clin. Invest. 37: 1556, 1958.

53. Reshef, L., Shafrir, E., and B. Shapiro. Metabolism 7: 723, 1958.

54. Campbell, J., Martucci, A.D. and G.R. Green. Biochem. J. 93: 183, 1964.

55. Bierman, E.I., Schwartz, I., and V.P. Dole. Am. J. Physiol. 191: 359, 1957.

56. Fredrickson, O.S., and R.S. Gordon, Jr. J. Clin. Invest. 36: 890, 1957.

57. Laurell, S. Acta Physiol. Scand. 41: 158, 1957.

58. Miller, H.I., Gold, M., and J.J. Spitzer. Am. J. Physiol. 202: 370, 1960.

59. Spitzer, J.J., and P.S. Roheim. Fed. Proc. 17: 155, 1958.

60. Issekutz, B., and J.J. Spitzer. Proc. Exp. Biol. Med. 105: 21, 1960.

61. Spitzer, J.J., and F.J. Hohenleitner. J. Lipid Research 2: 396, 1961.

62. Dole, V.P. Proc. Exp. Biol. Med. 93: 532, 1956.

63. Read, W.W.C., and Z. Awdeh. J. Science of Food and Agric. 14: 770, 1963.

64. Garton, G.A., and W.R. Duncan. Biochem. J. 92: 472, 1964.

65. Dole, V.P., and H. Meinetz. J. Biol. Chem. 235: 2595, 1960.

66. Nelson, N. J. Biol. Chem. 153: 375, 1944.

67. Barker, S.B., and W.H. Summerson. J. Biol. Chem; 138: 535, 1941.

68. Abell, L.L., Levie, B.B., Brodie, B.B., and F.E. Kendall. J. Biol. Chem. 195: 357, 1958.

69. Steward, C.P., and E.B. Hendey. Biochem. J. 29: 1683, 1935.

70. Stern, I., and B. Shapiro. J. Clin. Path. 6: 158, 1953.

71. Nutrition Reviews 17: 1959, Supplement.

72. Littlewood, A.B. Gas Chromatography. New York, London: Academic Press, 1962.

73. Horning, E.C., Ahrens, E.H. Jr. Lipsky, S.R., Mattson, F.H., Mead, J.F., Turner, D.A., and W.H. Goldwater. J. Lipid Research 5:21, 1964.

74. Glick, D. Methods of Biochemical Analysis vol. VIII. New York, London: Interscience publishers, 1960.

75. Folch, J., Ascoli, I., Lees, M., Meath, J.A., and F.N. Le Baron. J. Biol. Chem. 191: 833, 1961.

76. Carroll, K.K. Nature 191: 377, 1961.

77. Rizack, M.A. J. Biol. Chem. 236: 657, 1961.

78. Garton, G.A. and W R. Duncan. Biochem. J. 92: 472, 1964.

79. Khachadourian, A.K. J. Lipid Research in print.

80. Olson, R.E., and J.H. Vester. Physiol. Revs. 40: 677, 1960.

81. Steinberg, D., Vaughan, M., and S. Margolis. J. Biol. Chem. 235: PC 38, 1960.

82. Schotz, M.G., and I.H. Page. J. Lipid Research 1: 466, 1960.

83. Hollenberg, C.H., and D.E. Douglas. Nature 193: 1962.

84. Meinetz, H. Fed. Proc. 21: 284, 1962.

85. Spitzer, J.J., and M. Gold. Proc. Soc. Exp. Biol. Med. 110: 645, 1962.

86. Wadstrom, L.B. Nature 179: 259, 1957.

87. Raben, M.S., and C.H. Hollenberg. J. Clin. Invest. 39: 435, 1960.

88. Engel, F.L., and J.E. White. Am. J. Clin. Nutr. 8: 691, 1960.

89. Laurell, S. Scand. J. Clin. and Lab. Invest. 8: 8, 1956.

90. Fritz, I.B. Physiol. Revs. 41: 52, 1961.