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Lipogenin

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LIPOGENIN

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VITA

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CHAPTER I

INTRODUCTION

In a wide variety of biological systems, fatty acid synthesis is catalyzed by two enzyme systems that function in sequence, acetyl-CoA carboxylase and fatty acid synthetase (Volpe and Vagelos, 1976). Acetyl-CoA carboxylase catalyzes the first committed step in the synthesis of fatty acid from acetyl-CoA. It is the rate limiting enzyme in short term control of fatty acid synthesis. Malonyl-CoA is formed by means of a biotin-dependent carboxylation:

$$ATP + HCO_3^- + \text{biotin-E} \xrightarrow{Me^{2+}} CO_2\text{-biotin-E} + ADP + P_i$$

$$CO_2\text{-biotin-E} + CH_3CO\text{-SCoA} \longrightarrow {}^-OOCCH_2CO\text{-SCoA} + \text{biotin-E}$$

$$\text{Sum: } ATP + HCO_3^- + CH_3CO\text{-SCoA} \xrightarrow{Me^{2+}/\ \text{biotin-E}} {}^-OOCCH_2CO\text{-SCoA} + ADP + P_i$$

Acetyl-CoA carboxylase is located in the cytoplasm, and exists in at least two major forms, an active polymer and an inactive protomer. Dissociation to the protomeric form is favored by low protein concentration, Cl^-, pH 7.5, palmitoyl-CoA and carboxylation of the enzyme. The polymeric form is favored by citrate, acetyl-CoA, high protein concentration, or pH 6.5 - 7.0 (Lane, et al, 1974). Various subunits have also been found to be important in the regulation of acetyl-CoA carboxylase. Rat acetyl-CoA carboxylase appears to contain at least two types of unlike subunits with molecular weights of 118,000 and 125,000 daltons (Numa,

et al, 1966). The subunits of animal acetyl-CoA carboxylase are tightly associated and experimental methods employed to dissociate the enzyme also result in inactivation of the subunits (Volpe and Vagelos, 1976).

Citrate and other Krebs-cycle intermediates have been shown to play important roles in the allosteric control of acetyl-CoA carboxylase. These intermediates have been shown to cause polymerization and activation resulting in increased maximal velocity of enzymatic action (Vagelos, et al, 1963). In contrast, long-chain fatty acylCoA thioesters, especially of C_{16} - C_{18} fatty acids in micromolar concentrations, cause a reversible inhibition of acetyl-CoA carboxylase. Further observations indicate that this enzyme may be regulated by phosphorylation-dephosphorylation, e.g., inactivated by phosphorylation and activated by dephosphorylation (Carlson and Kim, 1974). Certain hormones are also important in the short term regulation of acetyl-CoA carboxylase. Insulin, glucagon, and epinephrine are involved in fatty acid synthesis through their effects on acetyl-CoA carboxylase (Nepokroeff, et al, 1974). Whereas there is evidence that insulin increases the activity of the enzyme, glucagon and epinephrine inactivate acetyl-CoA carboxylase, possibly doing so by acting through cyclic AMP via a protein kinase which phosphorylates the enzyme (Robison, et al, 1971).

Fatty acid synthetase (FAS) is a multienzyme complex that catalyzes synthesis of saturated fatty acids from malonyl-CoA, as indicated by the following equation for palmitic

acid:

$$CH_3CO\text{-}SCoA + 7\ HOOCCH_2CO\text{-}S\text{-}CoA + 14\ NADPH + 14\ H^+ \longrightarrow$$
$$CH_3CH_2(CH_2CH_2)_6CH_2COOH + 7\ CO_2 + 14\ NADP^+ + 8\ CoASH + 6\ H_2O$$

(Volpe and Vagelos, 1976). The FAS of rat liver is composed of two polypeptide chains having a molecular weight of 250,000 daltons. One chain contains the prosthetic group, acyl carrier protein (ACP). The ACP appears to be a distinct polypeptide region (Stoops, et al, 1975). FAS has been purified from rat liver as a tightly associated multienzyme complex with a molecular weight of 540,000 daltons. All FAS complexes appear to contain either single or multiple copies of multifunctional polypeptide chains of similar weight (Bloch and Vance, 1977). It is thought that FAS enzymes are coded by no more than two genetically unlike gene loci giving rise to no more than two polypeptides each of which is multifunctional (Knobling, et al, 1975).

Acetyl-CoA carboxylase and FAS each undergo relatively long-term regulatory changes secondary to hormonal, nutritional, developmental, genetic, neoplastic and pharmacologic factors (Volpe and Vagelos, 1976). There is, however, very little evidence to support a role for regulation of FAS in the short term control of fatty acid synthesis. For example, insulin and glucagon have roles in the long-term regulation of both acetyl-CoA carboxylase and FAS. Insulin increases acetyl-CoA carboxylase and FAS synthesis. Clucagon decreases acetyl-CoA carboxylase and FAS synthesis. Thyroid hormone also appears to have a role in the long-term regulation of

fatty acid synthesis. This hormone increases the activity of the two enzymes (Volpe and Vagelos, 1976).

The most profound nutritional alterations in the activities of acetyl-CoA carboxylase and FAS occur in animals re-fed a high-carbohydrate, fat-free diet after a 48-hour fast. FAS may be the rate limiting enzyme during long-term high-carbohydrate feeding. In contrast, high fat feeding leads to decreased activities of acetyl-CoA carboxylase and FAS, with polyunsaturated fatty acids being most effective and acetyl-CoA carboxylase being most sensitive to this inhibition (Volpe and Vagelos, 1976).

The liver seems to be more susceptible to alterations in nutrition than are gut, skin, adipose tissue or other body tissues. Early information on this subject was provided by Boxer and Stetten (1944) who found that undernourished rats displayed a very low rate of incorporation of deuterium into the body fatty acids. After the demonstration by Rittenberg and Bloch (1945) that acetate is an intermediate precursor of the carbon chain of fatty acids, a number of studies demonstrated the impairment of fatty acid synthesis during fasting. In 1952, Medes, et al reported that fatty acid synthesis from ^{14}C-methyl labeled acetate in liver slices was greatly impaired by fasting or undernutrition. Tompkins and Chaikoff (1952) showed that fasting of rats for as little as 24 hours resulted in a pronounced reduction in the capacity of the liver to incorporate acetate carbon into

unsaponifiable lipid such as cholesterol. Hutchens, et al (1954) studied the participation of liver, gut, carcass and skin in the lipogenic responses of the adult male rat fasted up to 240 hours. Liver lipogenic mechanisms appeared to be the most susceptible to fasting, although all tissues studied showed significant decreases in the incorporation of labeled acetate with extended fasting.

In 1955, Catravas studied the in vitro synthesis of fatty acids in liver homogenates of fed and starved rats. He found that about one-fourth as much labeled acetate was incorporated into fatty acids in homogenates from starved rats than in homogenates of fed animals. Later, Catravas and Anker (1958a) reported that a factor was found to be present in the livers of normal rats and pigs and in yeast which, when added to a liver homogenate from fasted rats, increased the rate of incorporation of acetate carbon into fatty acids. The concentration of this material in the liver seemed to depend upon the nutritional state of the animal. It was isolatable from the livers of rats maintained on a high carbohydrate, fat-free diet that had been fasted for a minimum of 36 hours. It increased the rate of incorporation of labeled acetate into fatty acids in the livers of fasted rats both in vitro and in vivo. It had no effect on fatty acid synthesis in fed animals. The rate of fat synthesis in liver preparations depended upon the availability of this material and it was, therefore, suggested that its synthesis by the liver served the function of a biochemical control

mechanism. The name, lipogenin, was proposed for this stimulator of fatty acid biosynthesis by Catravas and Anker (1958b). It was shown that the injection of partially purified lipogenin preparation obtained from yeast into fasted rats resulted in a drop in blood glucose concentration and an increase in lipogenesis as measured by the level of *in vitro* incorporation of labeled acetate into fatty acids. The administration of lipogenin to fed animals did not have these effects (Catravas and Anker, 1958a).

In 1963, Catravas reported the details of his method for the purification of lipogenin from yeast. Heated (95°C) aqueous extract of yeast was extracted three times with absolute ethyl alcohol. The active material was then passed through a Dowex 1-X10 formate anion exchange column. This was then followed by electrophoresis of the active eluate on a Geon bed. Analysis of the lipogenin obtained revealed 59 per cent mannose, 3.5 per cent glucose and 36 per cent amino acids. This suggested that the active component of yeast lipogenin might be combined with the polysaccharide, mannan. Subsequent treatment of the lipogenin with mannanase yielded a lipogenin fraction which was some 1,000 times more active than the original yeast extract. The final preparation contained 30 per cent carbohydrate, mainly mannose, and 70 per cent peptide. Since the major portion of the lipogenin can be split off by mannanase without destroying the biological activity, it appears likely that the residual

carbohydrate is not required for activity, and that the peptide represents the active moiety. The partially purified lipogenin from yeast was described as being heat stable, stable to treatment with amylase and pepsin at 37°C for two hours and not precipitable by ammonium sulfate. Due to the uncertain purity of the most active material then obtained, neither molecular weight determination nor qualitative amino acid analysis was attempted.

Little attention has been directed toward studies on lipogenin since the 1960's. In 1972, Catravas and McHale reported on the effect of lipogenin on fatty acid synthesis in the livers of irradiated rats. Hepatic fatty acid synthesis, which was increased by X-irradiation of the rats, showed a marked decrease when lipogenin was added to the cell-free liver preparations from the irradiated animals. Addition of the same amount of lipogenin to liver homogenates from rats exposed to mixed neutron-gamma radiation caused a slight increase of lipogenesis, an action which is usually decreased by this type of radiation. Endogenous liver lipogenin extracted from livers of X-irradiated rats maintained its activity, whereas material extracted from animals exposed to mixed neutron-gamma irradiation was inactive.

In 1972, a group of Japanese workers (Ohkido, et al) described an investigation of lipid biosynthesis from acetate in normal rat epidermis. They noted that the incorporation of ^{14}C-acetate into fatty acids, cholesterol and phospho-

lipids in the epidermis was stimulated by adding boiled extract from rat livers.

Wieser (1973) studied the effects of lipogenin on the activation reaction and catalytic reaction of acetyl-CoA carboxylase, the enzyme essential to the first step of *de novo* biosynthesis of fatty acids. Lipogenin was obtained from the livers of rats which had been maintained on a high carbohydrate, fat-free diet, and partially purified by Dowex column chromatography. This material was found to replace the citrate requirement for activation of acetyl-CoA carboxylase. It was found to offer some measure of protection to the activation of acetyl-CoA carboxylase in the presence of ATP. The effect of lipogenin on the catalytic reaction of acetyl-CoA carboxylase was found not to be significant.

Statement of the Problem:

It is the purpose of this thesis to obtain further information relevant to the characterization of lipogenin, proposed by some investigators as an *in vivo* regulator of fatty acid biosynthesis. The asay for lipogenin activity has been based on the ability of the factor to increase the incorporation of labeled acetate into fatty acids (Catravas, 1955). Modification of the assay is to be attempted so that the effect of lipogenin on the synthesis of non-saponifiable lipids may be appraised. This approach was predicated by the observation of Ohkido, *et al* (1972) that the incorporation of labeled acetate into non-saponifiable lipids in normal rat epidermis is stimulated by the addition of a

boiled liver extract, the starting material in lipogenin isolation. Most of the previous attempts to purify lipogenin have been done with extracts of yeast. In the present work, selective ultrafiltration and column chromatographic techniques will be applied in an attempt to purify rat liver extracts.

CHAPTER II

MATERIALS AND METHODS

Preparation of Crude Lipogenin

Crude lipogenin was extracted from rat liver tissue according to the method of Catravas and Anker (1958a). Holtzman albino rats used in the experiments were obtained from the Holtzman Company, Madison Wisconsin. They were acclimated in the animal quarters for at least three or four days before being used. Female rats weighing between 120 and 140 grams or male rats weighing between 225 and 525 grams were maintained on a high carbohydrate, fat-free diet (ICN Nutritional Biochemicals, Cleveland, Ohio) for three to five days. Water was given ad libitum. The rats were then stunned, decapitated and the livers removed. After teasing away excess fat with tweezers, the livers were weighed. The livers obtained from the female rats weighed between 5.9 and 8.2 grams and those obtained from the male rats weighed between 8.1 and 18.5 grams.

Ten grams of liver were immersed for two minutes in a beaker containing 25 ml of boiling water. The beaker was cooled to room temperature in an ice bath and the liver tissue was removed from the water and ground with a mortar and pestle. After bringing the volume of the water in the beaker back to 25 ml, the ground liver was reimmersed and the

mixture boiled for an additional three minutes. The contents of the beaker were cooled and centrifuged at 700 x g at 4°C in an International Centrifuge, Model PR-2 for 15 minutes. The supernatant was lyophilized and stored at 4°C until used. The lyophilized powder provided the source of crude lipogenin.

Boiled liver extracts were also prepared by the method of Ohkido, et al (1972). Rat liver was homogenized with 0.001 M phosphate buffer (pH 7.4) in the ratio of 2 ml of buffer to 1 gram of liver. The homogenate was centrifuged at 700 x g for 15 minutes. After removal of the sediment, the supernatant was boiled in a 50 ml beaker for 15 minutes, taking care to keep evaporation to a minimum by covering the beaker with a watch glass. After boiling, the mixture was recentrifuged as before. The resultant supernatant containing the boiled liver extract was removed and lyophilized.

Preparation of Liver Mitochondrial Extract

Mitochondrial extract was prepared by a method based on a fractionation scheme described by Mahler and Cordes (1966). Five grams of minced liver tissue from fed rats were homogenized in 15 ml of homogenizing medium (0.32 M sucrose, containing 0.02 M Tris, pH 7.6 and 3 X 10^{-3}M $MgCl_2$) in a Duall glass homogenizer. This was followed by homogenization in a Kontes Teflon homogenizer. The homogenate was filtered through two layers of cheesecloth to remove the

remnants of fibrous tissue. Each 12.5 ml of homogenate was diluted to a final volume of 20 ml by adding 7.5 ml of diluting medium (0.25 M sucrose, containing 0.02 M Tris, pH 7.6, and 3 X 10^{-3}M $MgCl_2$). An equal volume of homogenizing medium was layered underneath the homogenate preparation. The entire volume was centrifuged for 10 minutes at 700 X g at 4°C in an International Centrifuge, Model PR-2. The supernatant fraction containing mitochondrial and microsomal material was thoroughly mixed and re-centrifuged for 10 minutes at 7,000 X g at 4°C in the International Centrifuge. The residue containing mitochondria was re-suspended in 10 ml of diluting medium and centrifuged for 10 minutes at 24,000 X g at 4°C in the International Centrifuge. The residue from this centrifugation, termed mitochondrial extract, was removed and lyophilized.

Purification of Lipogenin Extract by Ultrafiltration

Purification and some characterization of lipogenin was effected by filtration of the crude lipogenin extract through Amicon Stirred Standard Ultrafiltration Cells (Model 52, Amicon Corporation, Scientific Systems Division, Lexington, Massachusetts). PM-10, DM-5 and UM-2 Amicon Diaflo Membranes which have molecular weight cutoffs of 10,000, 5,000 and 1,000 daltons, respectively, were used either singly or in combination. Ultrafiltration was carried out in a cold room at 4°C. Thirty five ml of crude boiled liver extract were placed in the filtration chamber and the extract

was forced through the membrane with nitrogen at a pressure of 45 psi. Filtration was allowed to proceed until 50 to 60 per cent of the fluid passed through the ultrafilter. The ultrafiltrate and the retentate were assayed for protein content and lipogenic activity.

Purification of Lipogenin Extract by Ion Exchange Chromatography

Purification of the crude lipogenin extract was attempted by means of ion exchange columns. Dowex 1-X10 formate ion exchange columns similar to those prepared by Catravas (1963) and by Wieser (1973) were prepared as follows: The Dowex 1-X10 anion exchange resin, 200-400 mesh, chloride form was obtained from Bio-Rad Laboratories, Richman, California. The resin was suspended in water overnight. Following decantation, the resin was placed on a piece of filter paper in a Buchner funnel and over it was poured a 3 M aqueous solution of sodium formate until no more chloride ion could be detected by silver nitrate solution. The resin was then washed with water, after which it was poured as a thin slurry into a column (Kimax 17800, 11 X 300 mm) and washed with ten bed volumes of a 1:1 mixture of 6 N formic acid and 1 M sodium formate. The resin was then washed with several bed volumes of water until the effluent was neutral to litmus paper.

Lyophilized boiled liver extract from ten grams of liver tissue was dissolved in two ml of distilled water.

This solution was applied to the column and eluted with water. Ten serial fractions of 15 ml in volume were collected with the use of a Unifrac Fraction Collector (Savant Instruments Inc., Hicksville, New York). The transmission of the effluent at 280 nm was monitored with a LKB Uvicord Absorptiometer and Recorder (LKB Instruments, Inc., Rockville, Maryland). However, because of the turbidity of these fractions, maximum absorption peaks could not be detected nor selective fractions collected. Therefore, the eluate (150 ml) was combined into two 45 ml and one 60 ml fractions. Tubes 1 to 6 corresponded to the first two fractions and tubes 7 to 10 corresponded to the third fraction. These were lyophilized and reconstituted in 5 ml of water and assayed for protein content and lipogenin activity.

Further purification of the lipogenic material obtained by ultrafiltration was attempted by means of the Dowex ion exchange columns. Lyophilized ultrafiltrate and retentate from the DM-5 filters were dissolved in 2 ml of distilled water. These solutions were applied to Dowex columns and eluted, first with water then with 1 per cent formic acid. Fifteen serial fractions of 10 ml in volume were collected with water by use of the Unifrac Collector, followed by 12 fractions of 10 ml with the formic acid. The transmission of the effluent at 280 nm was monitored with the LKB Uvicord Absorptiometer. Three peaks were recorded for the water eluate and two more for the formic acid eluate.

These eluates were lyophilized separately; tubes 1 to 16 corresponding to the three water peaks, and tubes 17 to 27 corresponding to the formic acid peaks. Each of these two fractions were reconstituted in 5 ml of water.

Protein Analysis

The protein concentration of the crude lipogenin extract and the purified fractions obtained by ultafiltration and column chromatography were determined by the method of Lowry (1951) as modified by Oyama and Eagle (1956).

Assay of Lipogenin Activity

The assay of the lipogenic activity is based on the fact that this material stimulates the incorporation of labeled acetate carbon into fatty acids (Catravas, 1955).

Female Holtzman albino rats weighing 90 - 110 grams were maintained on a high carbohydrate, fat-free diet (ICN-Nutritional Biochemicals, Cleveland, Ohio) for three days and were then fasted for a minimum of forty eight hours with water being given ad libitum. The rats were then stunned and decapitated. The livers were removed and the excess fat teased away with tweezers. The livers were weighed and cut into small pieces. One gram of liver (wet weight) was homogenized with a teflon-tipped hand homogenizer in 2.5 ml of freshly prepared modified Bucher (1953) solution containing sucrose, niacinamide, magnesium chloride and potassium phosphate buffer, pH 7.5. The homogenate was centrifuged at 700

X g for fifteen minutes st 4°C. The inactive sediment was discarded. Homogenates were also prepared as described here with liver tissue from rats which had not been fasted.

Incorporation of labeled acetate into lipids synthesized by the liver homogenate was found by time study to reach a maximum after incubation for 4 hours at 37°C. Into a 25 ml Erlenmeyer flask were added 1.5 ml of liver homogenate and 0.5 ml of incubation medium (Bucher medium containing labeled acetate). When crude liver extract was assayed, the lyophilized extract obtained from 10 grams of liver was reconstituted in 2.5 ml of distilled water, of which 0.5 ml was used in the assay. Distilled water was used in place of the test material for the blank runs. When lipogenin obtained by ultrafiltration of crude liver extract was assayed, 200 ul of either the filtrate or retentate was used in the assay. Distilled water was added to give a final volume of 2.5 ml and the pH adjusted to 7.5. Controls, representing basal synthesis, were run using 0.5 ml of crude liver extract. When lipogenin prepared by ion exchange chromatography was assayed, the lyophilized eluates were reconstituted in 5 ml of distilled water, of which 100 ul aliquots were taken for assay. Distilled water was added to bring the final volume to 2.5 ml. Controls were run using 0.5 ml of crude liver extract when this material had been run through the column, or 200 ul of ultrafiltrate or retentate when either of these had been run through the column.

The final concentration of the components in the

assay mixture was as follows: sucrose, 096 M; magnesium chloride, 0.012 M; niacinamide, 0.22 M; potassium phosphate, 0.035 M; NAD, 0.008 M; potassium citrate, 0.020 M; 1-^{14}C-acetate, 0.004 M.

The mixtures were incubated in air in a shaker water bath for 4 hours. After incubation, the lipids were saponified by adding 2 ml of diethylene glycol and 2 ml of 4 M potassium hydroxide to each flask and heating the contents in an oven to 110°C for two hours. The flasks were corked and wired closed.

Initially the lipids were extracted according to the method of Folch, et al (1957). 15 ml of a 2:1 chloroform-methanol solution was added to the incubation mixture and shaken vigorously for1 hour. The lipid extract was filtered into a 30 ml separatory funnel through filter paper washed with the chloroform-methanol. This was done to remove the denatured protein. The protein was washed with 5 ml of chloroform-methanol to recover trapped lipid, and this was added to the separatory funnel. The material in the separatory funnel was washed by vigorous shaking and swirling 4 times with 8 ml of a solution containing chloroform, methanol and an aqueous salt solution in a ratio of 3:48:47. After each washing the system was allowed to equilibrate for at least two hours and the upper phase was removed by aspiration. After the fourth washing the bottom phase was drained into a pre-weighed scintillation vial and the solvent removed by evaporation. The extracted lipids were fractionated using

thin layer chromatography.

Thin Layer Chromatographic Separation of Lipids

Separation and quantitation of synthesized lipids was attempted by thin layer chromatography using a modification of Pocock, et al (1972). The lipids in the scintillation vials after extraction with chloroform-methanol were redissolved in 1 ml of chloroform. Twenty microliter aliquots of this solution and 20 ul of a standard lipid mixture (nonradioactive) containing free fatty acid, triglyceride, cholesterol, cholesterol ester and phospholipid (5×10^{-5}g) were placed on plates of glass-fiber paper impregnated with silica gel. The lipid fractions were allowed to separate by placing the plates in fresh developing solution containing 300 ml iso-octane, 90 ml benzene, 0.3 ml glacial acetic acid and 3 ml acetone. This represents a slightly greater proportion of acetone than the developing solution described by Pocock, et al (1972) and results in constant R_f values and improved separation of the lipid fractions. The plates were allowed to stand in the solution until the solvent front was approximately one inch from the upper edge of the plate (15 - 20 minutes).

Separation of Saponifiable and Non-saponifiable Lipids

When it became apparent that lipid fractionation using thin layer chromatography was not a useful technique for quantitating lipid synthesis, an alternative

method for separating and quantitating synthesized lipids was used. After incubation, the contents of each flask were transferred to 40 ml heavy duty, glass stoppered centrifuge tubes and the non-saponifiable lipids were removed by extracting twice with vigorous shaking with 10 ml of petroleum ether. The ether phase was removed by aspiration after centrifugation at 700 X g and reserved. The aqueous phase was acidified to pH 2 with 25 per cent (v/v) sulfuric acid and the free fatty acids were reextracted twice with petroleum ether. The aqueous phase was discarded. The two ether extracts were washed free from unincorporated labeled acetate, twice with 5 ml of 5 per cent (v/v) acetic acid and four times with 5 ml of water so that the washings were neutral to Hydrion pH paper. Control experiments demonstrated the absence of labeled acetate after these washings. The washed ether extracts were then transferred into preweighed 22 ml low potassium-40 content scintillation vials. The petroleum ether was removed by evaporation at 50°C in a shaker water bath at time-speedup by a stream of nitrogen. The vials were reweighed to determine the amount of fatty acid and non-saponifiable lipids present. Ten ml of a scintillation fluid (prepared fresh each day by dissolving 10 grams of PPO (2,5-diphenyloxazole) fluor in 1 liter of scintillation grade toluene) was then added to each vial and the radioactivity of the labeled fatty acids and non-saponifiable lipids was determined with a Beckman LS-250 Liquid Scintillation Spectrometer.

Quenching was not a problem in the measurement of the radioactivity of the samples. This conclusion was based upon an evaluation of a quench correction curve of a plot relating per cent efficiency versus external standard ratio (ESR) for a series of counting samples prepared by adding various amounts of unlabeled palmitate (2 to 10 mg) to 10 ml of the toluene-based fluor containing 0.025 microcuries of ^{14}C-acetate. No quenching effect was found.

The efficiency of recovery of fatty acid by this technique was evaluated by the use of radioactively labeled palmitic acid standard. Fifty microcuries of 1-^{14}C-palmitic acid (New England Nuclear, Boston, Massachusetts) was dissolved in 500 ul of benzene. The benzene was evaporated and the palmitic acid was redissolved in 1 ml of chloroform. An aliquot of this solution was diluted so that 0.5 ml contained 0.005 microcuries. This was added to 1.5 ml of liver homogenate in place of the labeled acetate in the Bucher incubation medium. The incubation, saponification, extraction and washing procedures were performed as described above, and the recovery of labeled palmitic acid was determined. By calculation, 0.005 microcuries of radioactivity represents a count of 11.1×10^3 disintegrations per minute (dpm); of this, 9.3×10^3 dpm were recovered in each of three separate experiments.

Calculations

The following equations were used to determine the statistical significance of the experimental values (Freund, 1970):

S.D. = Standard Deviation

$$= \sqrt{\frac{\Sigma (M_1 - M_2)^2}{N - 1}}$$

θ = Standard error of the mean

$$= \frac{S.D.}{\sqrt{N}}$$

Z = Standard units

$$= \frac{\bar{X} - X}{\theta}$$

where $\bar{X}$ = mean of per cent increase in synthesis

and X = basal synthesis expressed as 100 per cent

Because only increased lipogenic activity is being tested for significance, those columns in the probability table (Table II, Freund, 1970) dealing with one-tail testing were used.

If $Z > t$ (from probability table), then the difference between the control and the experimental result is statistically significant.

CHAPTER III

EXPERIMENTAL RESULTS

The Effect of Crude Lipogenin on Fatty Acid Synthesis in Liver Homogenates of Fasted Female and Male Rats

The relative amount of isotopic incorporation of labeled acetate into free fatty acids synthesized by lipogenin from crude liver extracts obtained from female and male rats is shown in Table I. The radioactivity of incorporated labeled acetate into fatty acids was expressed in terms of disintegrations per minute (dpm) per gram of wet weight of tissue per hour of incubation. The relative isotopic incorporation is expressed as a per cent of the basal control synthesis expressed as 100 per cent. The basal level reflects fatty acids synthesized in homogenates prepared from livers of fasted rats in the absence of a boiled liver extract, or other test preparation. Three experiments, with 3 assays each, were performed using crude liver extract from female rats. The same number of assays (9) were performed with male rat liver extract.

The rate of synthesis resulting from lipogenin in the crude liver extracts from female rats averaged 204 per cent (S.D. $\pm$ 45) of basal synthesis; from male rats, it averaged 126 per cent (S.D. $\pm$ 17) of basal synthesis.

EFFECT OF CRUDE LIVER EXTRACT OF LIPOGENIN ON FATTY ACID SYNTHESIS IN LIVER HOMOGENATES OF FASTED FEMALE AND MALE RATS

A. FEMALE RATS

Experiment	Control (dpm)	Experimental (dpm)	Incorporation of ^{14}C-Acetate Per Cent of Basal
1	225	545	242
		581	258
		609	271
2	7,146	12,053	169
		10,739	150
		10,430	146
3	215	444	207
		429	200
		422	196
			Mean 204
			S.D. ±45
			$p < .005$

B. MALE RATS

Experiment	Control (dpm)	Experimental (dpm)	Incorporation of ^{14}C-Acetate Per Cent of Basal
1	1,084	1,320	122
		1,309	121
		1,403	129
2	224	317	142
		336	150
		329	147
3	672	766	114
		715	106
		674	100
			Mean 126
			S.D. ±17
			$p < .005$

TABLE I. 0.5 ml of crude liver extract of lipogenin was incubated for 4 hours with 2 ml of fasted rat liver homogenate which contained ^{14}C-Acetate. After incubation and saponification, the non-saponifiable lipids were extracted with petroleum ether. The FFA's were acidified with sulfuric acid, extracted with petroleum ether and washed free of un-incorporated labeled acetate. The radioactivity of the synthesized lipids was recorded by a scintillation counter.

The Effect of Liver Extract Prepared According to the Method of Ohkido on Fatty Acid Synthesis

Three assays were performed using liver extract prepared from female rats according to the method of Ohkido, et al (1972). No increase was observed in the incorporation of labeled acetate into fatty acids over that measured for the basal controls (per cent of basal = 97%). Therefore, investigation with this extract was discontinued.

The Effect of Crude Lipogenin on Non-Saponifiable Lipid Synthesis

The increase in the rate of synthesis of non-saponifiable lipids; cholesterol, cholesterol esters and phospholipids resulting from lipogenin in the crude liver extracts of female rats is shown in Table II. The three experiments relating fatty acid synthesis with female rat liver extract described above included assays (in triplicate) of the extracted non-saponifiable lipids synthesized with incorporated labeled acetate. The synthesis averaged 114 per cent (S.D. $\pm$ 11) of basal synthesis.

The Effect of Mitochondrial Extract on Fatty Acid Synthesis

The relative amount of isotopic incorporation of labeled acetate into free fatty acids synthesized by one, two and three mg portions of a lyophilized mitochondrial extract is shown in Table III. The mitochondrial extract, obtained by differential centrifugation of minced liver

EFFECT OF CRUDE LIVER EXTRACT OF LIPOGENIN
ON NON-SAPONIFIABLE LIPID SYNTHESIS

Experiment	Control (dpm)	Experimental (dpm)	Incorporation of ^{14}C-Acetate Per Cent of Basal
1	8.0	9.3	116
		9.7	121
		10.0	125
2	5.5	5.2	95
		5.6	102
		6.3	115
3	39.0	42	108
		50	128
		46	118
			Mean 114
			S.D. ±11
			$p < .005$

TABLE II. 0.5 ml of crude liver extract was incubated for 4 hours with 2 ml of fasted rat liver homogenate which contained ^{14}C-Acetate. Lipids were extracted with petroleum ether which was then washed free of unincorporated labeled acetate. The amount of lipid synthesis was then determined using a scintillation counter.

EFFECT OF MITOCHONDRIAL EXTRACT ON FATTY ACID SYNTHESIS IN LIVER HOMOGENATES

Control (dpm)	Experimental mg Extract	(dpm)	Incorporation of ^{14}C-Acetate Per Cent of Basal
15,743	1	18,384	117
		16,581	105
		18,929	120
		Mean	114
		S.D.	±8
		p	< .05
	2	20,965	133
		12,416	79
		16,691	106
		Mean	106
		S.D.	±27
		p	n.s.
	3	15,703	100
		17,081	108
		18,458	117
		Mean	108
		S.D.	±9
		p	n.s.

TABLE III. One, two and three mg portions of mitochondrial extract prepared according to the method of Mahler and Cordes (1966) were incubated for 4 hours with 2 ml of fasted rat liver homogenate, containing ^{14}C-Acetate. FFA's were extracted as described in this paper and FFA synthesis was monitored using the scintillation counter.

tissue, was incubated with a liver homogenate obtained from fasted rats and the incorporation of radioactively labeled acetate into fatty acids was measured. The mitochondrial extract resulted in only a limited synthesis, ranging from 106 to 114 per cent of basal synthesis. No increase in fatty acid synthesis with increasing amount of mitochondrial extract occurred (108 per cent of basal synthesis for 3 mg).

Thin Layer Chromatographic Separation of Lipid Fractions

The lipid fractions which are synthesized in liver homogenates by lipogenin are shown in Figure 1. Thin layer chromatographic separation followed by staining with 2',7'-Dichlorofluorocein demonstrated the presence of non-saponifiable lipids; phopholipids, cholesterol and cholesterol esters; and saponifiable lipids; free fatty acid, triglyceride and an additional fraction which may be presumed to be diglyceride.

Table IV shows the extent of incorporation of labeled acetate into each of the fractions cut from the chromatographic plate. The disintegrations per minute found for the labeled lipid fractions are barely above background count (non-radioactive lipid factions) suggesting that thin layer chromatography may not be a sensitive enough method for assaying lipogenic activity. However, when the radioactively labeled palmitic acid standard described in the Methods section was added to a homogeniza-

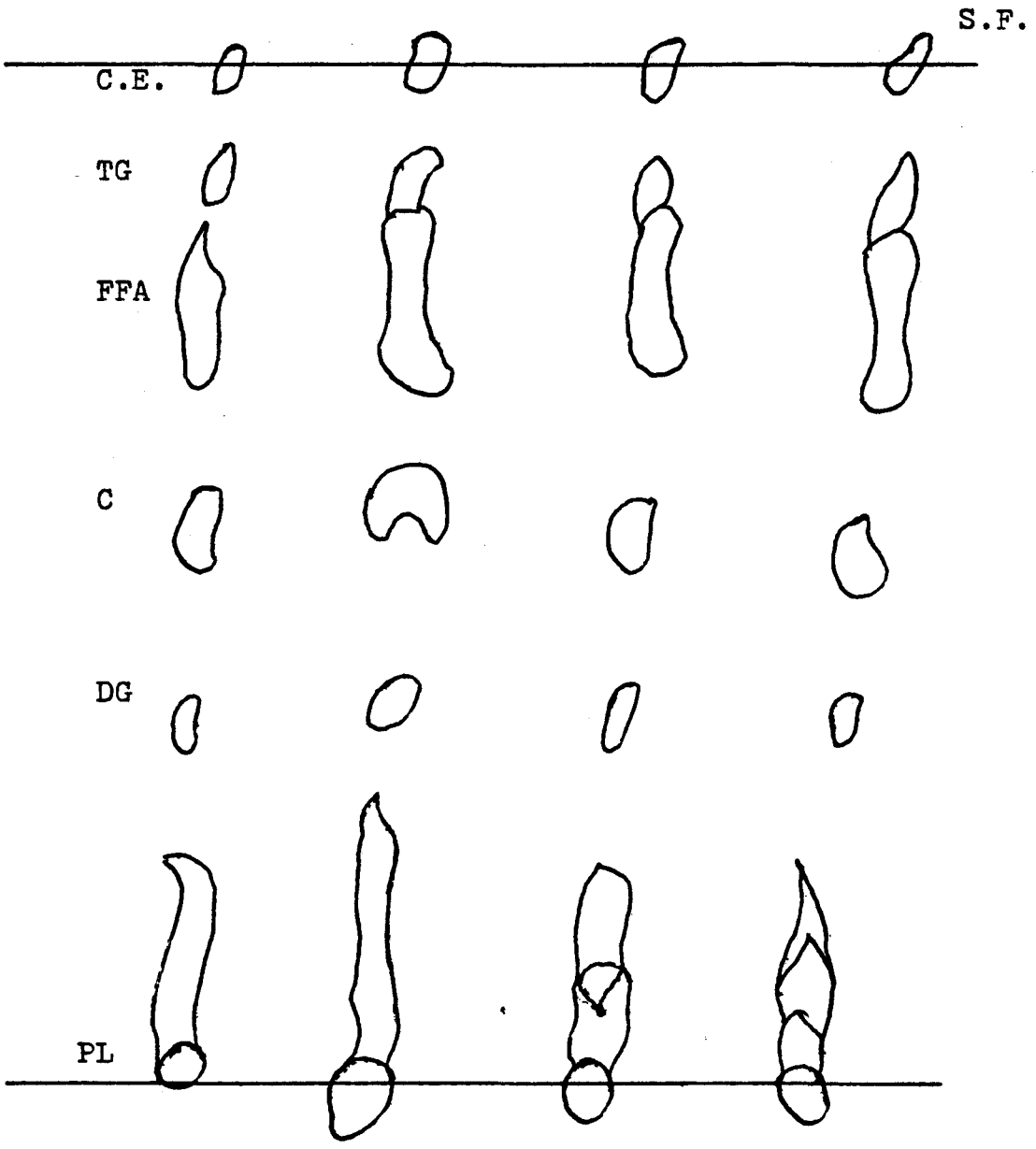

Figure 1. Migration Pattern of Standard Lipids (on left) and of Sample Aliquots.

(S.F.= Solvent Front; C.E.= Cholesterol esters; TG = Triglycerides; FFA = Free Fatty Acids; C = Cholesterol; D.G.= Diglycerides; P.L.= Phospholipids)

TLC SEPARATION OF LIPIDS SYNTHESIZED WITH LABELED ACETATE

Lipid Fraction	Standard Lipids (Non-radioactive) (dpm)	Labeled Lipids (dpm)
Cholesterol Esters	80	82
Triglycerides	62	62
Free Fatty Acids	67	76
Cholesterol	70	71
Diglycerides	59	69
Phospholipids	67	70

TABLE IV. Lipids synthesized in the presence of 0.5 ml of boiled liver extract were chromatographed on TLC paper together with six standard, unlabeled lipids. After chromatographic separation the lipid fractions were cut from the chromatography paper and the radioactivity was recorded by a scintillation counter.

tion mixture, incubated, extracted and subjected to thin layer chromatographic separation, sufficient radioactivity was detected. It is apparent from Table V that the labeled palmitic acid can be recovered and measured by this system. The count of 726 dpm obtained for the triglyceride fraction indicates that radioactively labeled palmitic acid is incorporated into triglyceride.

Purification of Female Rat Lipogenin by Ultrafiltration

Table VI shows the results obtained when 200 ul aliquots of fractions obtained after ultrafiltration through Diaflo Membrane Ultrafilters of female rat liver extract were assayed. Two experiments were performed with the PM 10 membranes. The filtrates and retentates obtained were each assayed in triplicate. These assays indicate that the active lipogenin factor is present in the filtrate. The rate of synthesis by this fraction averaged 166 (S.D. $\pm$ 16) per cent of the control level. The PM 10 retentate showed a rate of synthesis, as compared to the crude extract control, averaging 65 (S.D. $\pm$ 16) per cent. Because the active lipogenic factor passed through the PM 10 membrane, its molecular weight is estimated to be less than 10,000 daltons.

Three experiments were performed using the DM 5 membranes, and the filtrates and retentates were each assayed three times. These assays indicate that the active factor is again contained in the filtrate. The rate of

TLC SEPARATION OF LIPIDS RESULTING FROM INCUBATION WITH RADIOACTIVE PALMITIC ACID

Lipid Fraction	Labeled Lipids (dpm)
Background	83
Cholesterol Esters	83
Triglycerides	726
Free Fatty Acids	4,763
Cholesterol	89
Diglycerides	79
Phospholipids	86

TABLE V. 0.005 muCi of radioactive palmitic acid was incubated with liver homogenate in place of crude boiled liver extract. The synthesized lipids were chromatographed on TLC and the radioactivity was recorded using a scintillation counter.

RESULTS OF PURIFICATION OF LIPOGENIN FROM FEMALE RATS BY ULTRAFILTRATION

Membrane	Exp.	Controls (dpm)	Experimental (dpm)		Incorporation of ^{14}C-Acetate Per Cent of Basal	
			Filtrate	Retentate	Filtrate	Retentate
PM 10	1	37,120	70,970	18,574	191	50
			60,080	18,902	162	51
			65,551	17,437	177	47
	2	731	1,073	592	147	81
			1,200	494	164	68
			1,126	656	154	89
				Mean	166	65
				S.D.	±16	±18
				p <	.001	*
DM 5	1	226	969	167	429	74
			749	179	331	79
			724	212	320	94
	2	205	892	75	435	37
			749	80	365	39
			724	77	353	38
	3	892	1,802	580	202	65
			1,780	508	200	57
			1,872	589	210	66
				Mean	316	61
				S.D.	±93	±18
				p <	.001	*
UM 2	1	163	83	542	51	333
			57	766	35	470
			57	537	35	329
				Mean	41	377
				S.D.	±7	±60
				p	*	< .05

* no increase

TABLE VI. Crude boiled liver extract obtained from female rats was passed through a PM 10 ultrafiltration membrane. The filtrate and retentate were assayed for their ability to increase lipid synthesis when added to liver homogenates obtained from fasted female rats. The active fraction (filtrate) was then passed through a DM 5 ultrafiltration membrane or alternatively crude boiled liver extract was passed through a DM 5 membrane. Again, the filtrate and retentate were assayed for lipogenic activity and the more active fraction (filtrate) was passed through a UM 2 ultrafiltration membrane and the separated fractions were assayed for lipogenic activity.

synthesis by this fraction averaged 316 (S.D. ± 93) per cent of that obtained with the crude extract control. The rate of synthesis by the retentate was found to be 61 (S.D. ± 18) per cent of that by the crude lipogenin. This indicates that the molecular weight of the active lipogenic factor is less that 5,000 daltons.

Only one experiment was performed with the UM 2 membrane because it became apparent that the use of this membrane resulted in no further purification of the lipogenin. When the DM 5 filtrate fraction was subjected to filtration through this membrane, assay (in triplicate) of the retentate indicated that the active lipogenic fraction remained in this fraction. Thus, the molecular weight of lipogenin obtained from female rat livers must be greater than 1,000, placing its value between one and five thousand.

The Effect of Using Increasing Amounts of Lipogenin Purified by DM-5 Ultrafiltration on Fatty Acid Synthesis

Fatty acid synthesis in response to increased amounts of lipogenin purified by ultrafiltration through DM 5 membranes is shown in Table VII A. Two experiments using 100, 400 and 1,000 ul of DM 5 filtrate, rather than the 200 ul portions used for the routine assay procedure, were performed. Two assays were run at each level. When 100 ul of the DM 5 filtrate was incubated with homogenates prepared from livers of fasted rats, the incorpor-

RESULTS OF ASSAYS USING INCREASING AMOUNTS OF DM 5 FILTRATE ON FREE FATTY ACID AND NON-SAPONIFIABLE LIPID SYNTHESIS

A. FREE FATTY ACID

Experiment	Control (dpm)	Experimental (dpm)			Incorporation of ^{14}C-Acetate Per Cent of Basal		
		100ul	400ul	1000ul	100ul	400ul	1000ul
1	1,632	1,997	2,241	5,306	122	137	325
		1,751	2,924	5,619	107	179	344
2	134	171	289	680	128	219	507
		151	266	738	113	198	551
				689			514
				Mean	118	183	448
				S.D.	± 9	±34	±105
				p	$< .05$	$< .025$	$< .005$

TABLE VII A. 100, 400 and 1,000 ul aliquots of DM 5 filtrate were added to 1.5 ml of fasted rat liver homogenate and assayed to determine increased FFA synthesis.

B. NON-SAPONIFIABLE LIPIDS

Experiment	Control (dpm)	Experimental (dpm)			Incorporation of ^{14}C-Acetate Per Cent of Basal		
		100ul	400ul	1000ul	100ul	400ul	1000ul
1	127	121	173	240	95	136	189
		129	157	204	102	124	161
2	36	39	33	132	108	92	367
		36	30	81	100	84	225
				100			278
				Mean	101	109	244
				S.D.	± 5	±25	±81
				p	n.s.	n.s.	$< .025$

TABLE VII B. 100, 400 and 1,000 ul aliquots of DM 5 filtrate were added to 1.5 ml of fasted rat liver homogenate and assayed to determine increased non-saponifiable lipid synthesis.

ation of labeled acetate into fatty acids was 118 (S.D. ± 9) per cent of the control (0.5 ml of crude extract). When 400 ul of the filtrate was used, the incorporation amounted to 183 (S.D. ± 34) per cent of the crude extract control; and when 1,000 ul was used, the increased incorporation amounted to 448 (S.D. ± 105) per cent.

The Effect of Using Increasing Amounts of DM 5 Filtrate on Non-Saponifiable Lipid Synthesis

The increase in the rate of synthesis of radioactively labeled non-saponifiable lipids resulting from using increasing amounts of DM 5 filtrate is shown in Table VII B. The two experiments described in the above paragraph were done with preliminary extraction of non-saponifiable lipids. Two assays were run for each amount of DM 5 filtrate used (100, 400 and 1,000 ul). As was found with the use of crude liver extracts, the incorporation of labeled acetate into non-saponifiable lipids was minimal. When 100 ul of DM 5 filtrate was used, the incorporation averaged 101 (S.D. ± 5) per cent of the basal control, and 109 (S.D. ± 25) per cent when 400 ul was used. The use of 1,000 ul of DM 5 filtrate resulted in the only significant increase in non-saponifiable lipid synthesis: 244 (S.D. ± 81) per cent of the control synthesis.

Purification of Male Rat Lipogenin by Ultrafiltration

Table VIII shows the results obtained when 200 ul aliquots of fractions obtained after filtration through Diaflo Membrane Ultrafilters of male rat liver extract were assayed. Only one experiment was performed in which the retentate from a PM 10 membrane was assayed. The rate of synthesis of free fatty acids was found to be 61 per cent that of the crude extract control. This is in agreement with the results obtained with the female rat livers, indicating that the active fraction of lipogenin passes through the PM 10 membrane. Three experiments were performed in which male rat liver extract was forced through DM 5 membranes. In contrast to the results found with the female rat liver extracts, the active lipogenic factor was found to be present in the retentate. The rate of free fatty acid synthesis by this fraction averaged 236 (S.D. $\pm$ 55) per cent of that by the crude extract control. Thus, purified lipogenin obtained from large male rat livers must have a molecular weight between 5,000 and 10,000 daltons. Because the active fraction is retained by the DM 5 membrane, filtration through UM 2 membranes was not deemed necessary for male rat liver extract.

The Effect of Trypsin on Purified Lipogenin From Male Rat Livers

Because purified lipogenin obtained by ultrafiltration of male rat liver extract apparently has a larger

RESULTS OF PURIFICATION OF LIPOGENIN FROM MALE RATS BY ULTRAFILTRATION

Membrane	Exp.	Controls (dpm)	Experimental (dpm)		Incorporation of ^{14}C-Acetate Per Cent of Basal	
			Filtrate	Retentate	Filtrate	Retentate
PM 10	1	1,081		679		63
				685		63
				613		57
				Mean		61
				S.D.		± 3
				p		*
DM 5	1	1,081	597	2,230	55	206
			606	1,998	56	185
			971	2,264	89	209
	2	671	600	2,276	89	339
			502	1,790	75	266
			527	2,045	73	305
	3	762	490	1,722	64	226
			469	1,540	62	202
			316	1,424	41	187
				Mean	68	236
				S.D.	±16	±55
				p	*	< .005

* no increase

TABLE VIII. Crude boiled liver extract obtained from male rats was passed through a PM 10 membrane. Aliquots of retentate were assayed for ability to increase FFA synthesis. The filtrate was passed through a DM 5 ultrafiltration membrane. Aliquots of filtrate and retentate were assayed for ability to increase FFA synthesis in fasted rat liver homogenate.

molecular weight (5,000 to 10,000) than that obtained from female rat livers (1,000 to 5,000), the effect of trypsin digestion on the larger molecule was tested. Approximately 0.5 mg of trypsin was added to 450 ul of a suspension of DM 5 retentate in Tris buffer at pH 8.1. After incubation at 37°C for 4 hours, the reaction was stopped with 0.5 mg of Lima Bean Trypsin Inhibitor (LPL). A trypsin control was prepared with 450 ul of a suspension of the DM 5 filtrate fraction (an inactive fraction) in place of the active retentate fraction. 200 ul aliquots of each incubation mixture were then assayed for lipogenic activity. When trypsin was added to the DM 5 filtrate fraction, there was no increase in the incorporation of labeled acetate into fatty acids over that found for the control, in which no trypsin was added. This rules out any lipogenic action due to the trypsin per se. However, incubation of the DM 5 retentate fraction with trypsin resulted in an increased incorporation of labeled acetate amounting to thirteen times the activity found with the untreated DM 5 retentate control (Table IX).

Purification of Lipogenin by Column Chromatography

Table X shows the results obtained by attempts to purify crude lipogenin by column chromatography. Two ml of reconstituted boiled extract from female or male rat livers was passed through an 11 X 300 mm Dowex

EFFECT OF TRYPSIN ON PURIFIED LIPOGENIN FROM MALE RATS

Experiment	Controls (dpm)	Experimental (dpm)	Incorporation of ^{14}C-Acetate Per cent of Basal
1	DM 5 Filtrate	DM 5 Filtrate plus Trypsin	
	808	850	105
		770	95
2	DM 5 Retentate	DM 5 Retentate plus Trypsin	
	7,585	105,514	1,388
		95,703	1,261

TABLE IX. 200 ul aliquots of DM 5 filtrate, DM 5 retentate, DM 5 filtrate plus trypsin, and DM 5 retentate plus trypsin were added to fasted rat liver homogenates and assayed for ability to increase FFA synthesis.

EFFECT OF COLUMN CHROMATOGRAPHY ON CRUDE EXTRACT

Experiment	Controls (dpm)	Experimental (dpm)		Incorporation of ^{14}C-Acetate Per Cent of Basal
1	470	Female Rats	469	99
			449	96
			441	94
			Mean	96
2	562	Male Rats	581	103
			559	99
			531	95
			Mean	99

TABLE X. Two ml of reconstituted boiled extract from female or male rat livers was passed through an 11 X 300 mm Dowex anion exchange resin column. The fraction of water eluate having the highest protein content was assayed for lipogenic activity.

anion exchange column. The fraction of water eluate having the highest protein content was assayed for lipogenic activity. In each experiment the activity was determined three times. The eluate obtained after passing the crude lipogenin extract from female rat livers through Dowex 1-X10 formate columns showed no increase in the incorporation of labeled acetate into fatty acids over that found for the control (96 per cent). In agreement with this, the eluate obtained by passing the crude lipogenic extract from male rat livers through these columns showed no increase in the incorporation of labeled acetate into fatty acids (99 per cent of basal).

Figure 2 shows the points of maximum absorbance found for the water and formic acid eluates of lipogenic material obtained by DM 5 ultrafiltration. This material had been applied to Dowex 1-X10 formate columns. Three peaks were recorded for the water eluate and two more for the formic acid eluate. The water eluates were combined, as were the formic acid eluates. These were then lyophilized separately and each was reconstituted in 5 ml of water and analyzed for protein content. Because the protein content of the formic acid eluate was higher, the material collected in tubes 17 to 29 was used for assay. The results of the assay are shown in Table XI. Two experiments were performed using the DM 5 filtrate from female rats and two using DM 5 retentate from male

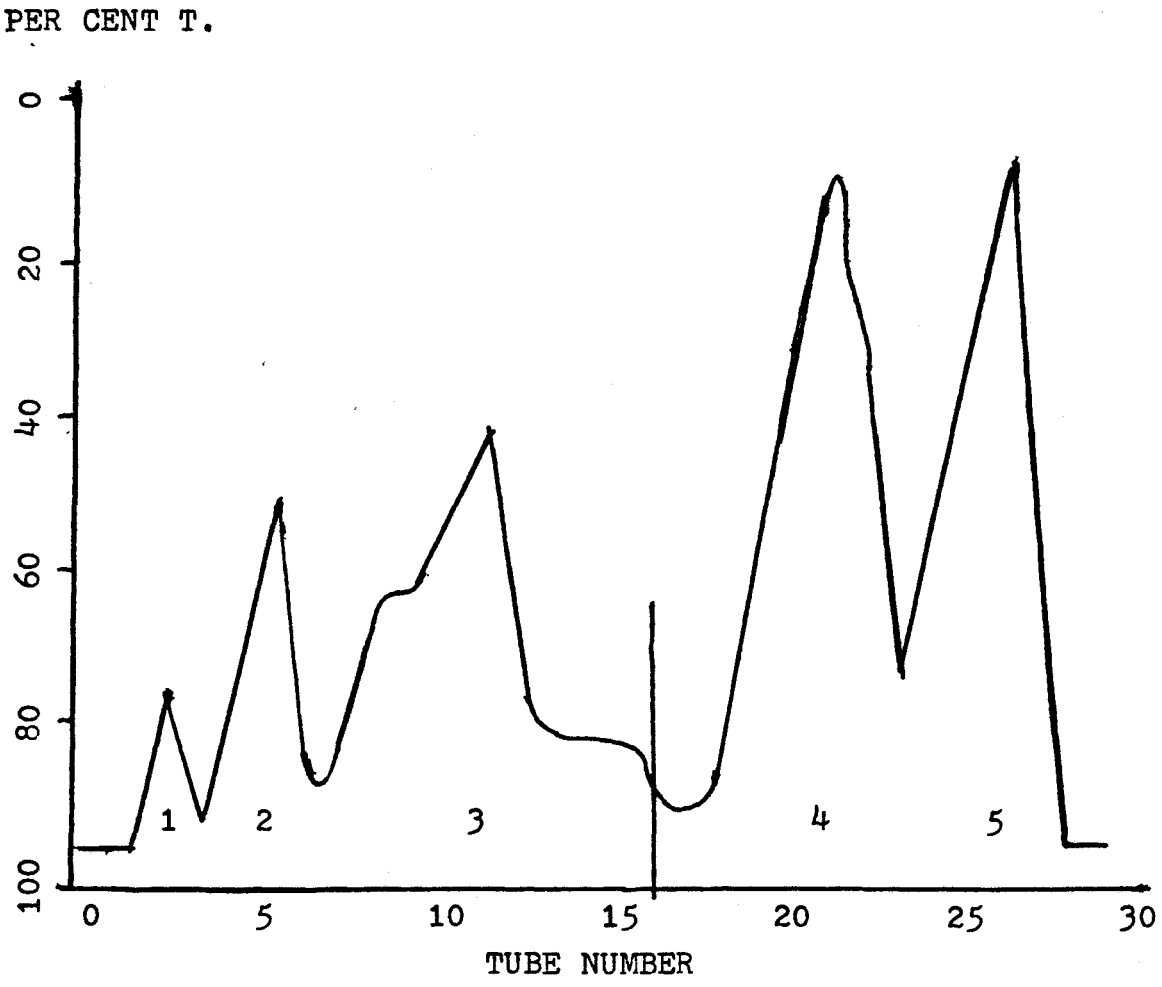

Figure 2. Ultraviolet Absorption Pattern of Protein Peaks Obtained When Lipogenin Material Was Eluted From Dowex 1-X10 Formate Columns with Water (Peaks 1-3) and with Formic Acid (peaks 4-5).

EFFECT OF COLUMN CHROMATOGRAPHY ON PURIFIED LIPOGENIN

Experiment	Controls (dpm)	Experimental	(dpm)	Incorporation of ^{14}C-Acetate Per Cent of Basal
1	3,853	DM 5 Filtrate Female Rats	4,260	111
			4,179	108
			4,133	107
2			4,679	121
			4,939	128
			4,790	124
				Mean 116
				S.D. ± 9
				$p < .01$
3	1,166	DM 5 Retentate Male Rats	1,411	121
			1,100	94
			1,423	122
			1,879	161
			1,584	136
			1,389	119
				Mean 126
				S.D. ±22
				$p < .05$

TABLE XI. Two ml of reconstituted ultrafiltrate or retentate from DM 5 filters was applied to Dowex columns. The formic acid eluate which showed maximum absorption at 280 nm was assayed for lipogenic activity.

rats. In each experiment lipogenic activity was assayed three times. The eluate from the former material showed limited incorporation of labeled acetate into fatty acids, averaging 116 (S.D. ± 9) per cent of that of the control. The eluate from the latter material (DM 5 retentate of male rat liver extract) also demonstrated limited incorporation of labeled acetate into fatty acids, averaging 126 (S.D. ± 22) per cent of that of the control.

CHAPTER IV

DISCUSSION

The method described by Catravas and Anker (1958a) for extracting lipogenin from livers of fed rats has been employed in this investigation. Grinding of livers from fed female or male rats and extraction with boiling water, followed by high speed centrifugation has resulted in material capable of synthesizing lipids in homogenates of fasted rat livers. This material, crude liver extract, is composed of low molecular weight particles in solution and also in suspension. The crude liver extract prepared from female rat liver was found to have more lipogenic activity, 204 per cent of basal control synthesis, than did the crude liver extract prepared from male rat liver, 126 per cent of basal synthesis (Table I).

Another method of extracting lipogenin, that described by Ohkido, et al (1972), was attempted. This method differs from that of Catravas and Anker in that the rat liver is homogenized in 0.001 M phosphate buffer (pH 7.4), followed by centrifugation and boiling of the supernantant for 15 minutes. Extracts prepared by the Ohkido method were found to have limited lipid biosynthesis when assayed with liver homogenates by the method described in this paper. The reported method for preparation of this extract was inadequate and some

details had to be assumed. After boiling for 15 minutes, the extract may have been denatured. This might explain why the method was not successful in our hands.

In 1958, Catravas and Anker (1958 a) stated that lipogenin might be localized in the liver mitochondria. As part of the present investigation an attempt was made to test this concept. The method of Mahler and Cordes (1966) was employed for extracting mitochondria from fed rat livers. Incubating this extract, in increasing increments, with homogenate of fasted rat livers did not result in the increase which might be expected if mitochondrial extract had lipogenic activity. Therefore, the question of which fraction of liver contains the lipogenin cannot be answered at this time.

The lipogenic activity of boiled rat liver extract and its purified fractions has been assayed by the method described by Catravas and Anker (1958 a), with several modifications. Initially, lipids synthesized with ^{14}C-labeled acetate by a homogenate of fasted rat liver containing boiled liver extract were extracted with a chloroform-methanol solution according to Folch (1957). Because little is known of the relative synthesis of non-saponifiable lipids by lipogenin, as compared to that of free fatty acid synthesis, the method of extracting the lipids was altered so that non-saponifiable lipids syn-

thesis could be determined. The lipids were saponified prior to extracting the non-sponifiable lipids. Petroleum ether was used as the solvent rather than the chloroform-methanol. After re-acidification, the free fatty acids were extracted with petroleum ether, so that the non-saponifiable lipids and the free fatty acids could be measured separately. Lipogenin - synthesized lipids were then measured in the presence of pre-formed lipids by measuring the incorporation of ^{14}C.

An attempt was also made to separate the synthesized lipids into fractions by thin layer chromatography, and to measure the amount of each fraction by radioactive determination. A procedure for adequately separating standard non-radioactive lipids was developed before attempting to separate and measure the synthesized labeled lipids. Although the chromatographed synthesized lipids could be identified by the staining procedure, attempts to measure the radioactivity of the ^{14}C incorporated in each fraction were unsuccessful. Experimental radioactivity was no greater than background activity (Table IV). Thus, for the conditions employed here, thin layer chromatography is not a sensitive enough method to detect incorporation of ^{14}C-labeled acetate into the lipid fractions synthesized in the presence of lipogenin. However, radioactive palmitate was added to a homogenization mixture, incubated, extracted and subjected to thin layer chromatography. As a result, two lipid fractions, triglyceride in addition

to fatty acid (the added palmitic acid), were found to have radioactivity greater than background activity (Table V). This establishes the lipid synthesizing capability of the homogenization medium, as well as establishing thin layer chromatography as a useful assay procedure when adequate conditions prevail.

Forcing crude liver extract through Diaflo Membrane Ultrafilters resulted in fractions of lipogenin capable of increased rate of lipid biosynthesis. The data presented in Tables VI and VIII suggest that the membranes act in separating more active material from less active ingredients present in the crude live extract. Inert suspended material and solubilized material of molecular weight greater than 10,000 must be retained by the PM 10 membranes. This "dilution" of active crude extract would explain the diminished activity of the retentate, and the resulting increased activity of the filtrate (Table VI). Another explanation for diminished activity of the retentate and increased activity of the filtrate would be the presence of an inhibitor. This inhibitor would have a molecular weight greater than 10,000 and could not be one of the known inhibitors of fatty acid synthesis, eg. glucagon or fatty acid.

The increase in lipid biosynthesis of the DM 5 filtrate and diminished rate of synthesis by retentate,

together with an active retentate and an inactive filtrate obtained when UM 2 membranes were used, places the molecular weight of female rat lipogenin between 1,000 and 5,000. The same reasoning is applied to the results found with male rat lipogenin (Table VIII). Because of the increased activity of the PM 10 filtrate over retentate and the increased activity of the DM 5 retentate over that of the filtrate, the molecular weight of male rat lipogenin, as prepared here, must lie between 5,000 and 10,000 daltons. This larger molecule is apparently less active, 236 per cent of basal (Table VIII), than the female rat lipogenin, 316 per cent of basal (Table VI).

It is conceivable that lipogenin may actually be insulin. The lowest molecular weight for insulin is approximately 6,000, and, as described in the introduction, insulin does have lipogenic properties. Catravas (1958b) found that 12 units of insulin injected into fed or fasted rats could lower blood glucose concentration. They also found that lipogenin injected into fasted rats decreased blood glucose concentration, but injection of lipogenin into fed rats caused no change in blood glucose concentration.

A column constructed with bound insulin antibodies might be used to determine whether or not lipogenin is insulin. An extract of lipogenin could be poured through

the column and comparison made of the lipogenic activity of the eluate with that of the original extract.

Catravas and Anker (1958a) reported that the rate of lipid synthesis in liver preparations depended on the concentration of the lipogenic material. To test this, assay preparations with increasing amounts of lipogenin purified by ultrafiltration through a DM 5 membrane were evaluated. In the two assays which were run, data were found supporting the proposal that increased fatty acid synthesis does occur with increasing amounts of lipogenin (Table VIII A). In addition, a five-fold increase in DM 5 filtrate (1,000 ul, compared to the 200 ul used in the routine assays) resulted in a significant incorporation of acetate (244 per cent, $p < .025$) in the synthesis of non-saponifiable lipids (Table VII B).

According to Catravas (1963), yeast lipogenin is stable in the presence of amylase or pepsin. Because the active lipogenin obtained by ultrafiltration of male rat liver extract (DM 5 retentate) has a larger molecular weight and is less active than that prepared from female rat liver, it was incubated with trypsin. When the resulting product was assayed for lipogenic activity, not only was it found to be stable, but its activity was found to be increased 13-fold (Table IX). Apparently inert polypeptide is removed from the DM 5 retentate by the action of trypsin. Because the amount

of DM 5 retentate available for tryptic digestion was limited, replicate assays were not possible. Had sufficient DM 5 retentate been available for tryptic digestion, ultrafiltration of the resultant active product through a DM 5 membrane could have been revealing. The active fraction might now be expected to be found in the filtrate. This would establish a common molecular weight for male and female rat lipogenin, i.e., between 1,000 and 5,000.

When lipogenin, prepared either as crude boiled extract or as a purified ultrafiltration fraction, was passed through a column containing Dowex anion exchange resin in the expectation of effecting additional purification, lipid biosynthesis by the resulting eluates did not fulfill this expectation (Tables X and XI). For the crude boiled liver extract, the turbidity of the eluate made it impossible to determine by direct absorption at 280 nm how best to select fractions which would result in isolation of a more active product. When lipogenin purified by passage through Diaflo membranes was applied to the chromatographic columns, elution with water resulted in three fractions containing protein but which were inactive. The remaining material which subsequently came off the column with formic acid elution might have been expected to have a two to three-fold increase in lipid biosynthesis. This was not realized. The increase in activity amounted to

only 116 or 126 per cent of basal (Table XI). It is, therefore, assumed that lipogenin was distributed among all of the fractions, and that only limited purification is realized by column chromatography.

When crude boiled liver extract from female rats was purified by ultrafiltration through DM 5 membranes, the protein content was increased from 4.0 to 6.5 mg per ml. (Table XII). This could have been due to removal of non-protein material of molecular weight greater than 5,000, e.g. polysaccharide. Since purification of boiled liver extract from male rats did not include passage through DM 5 membranes (the active fraction being the DM 5 retentate), removal of this non-protein material could not occur. This is consistant with the finding of an unaltered protein content (4.5 mg versus 4.7 mg). The protein content of 2.3 mg per ml found for the eluate from columns of crude liver extract suggests the possibility of dilution of this fraction with water.

Catravas and Anker (1958a) reported that partially purified lipogenin did not result in increased lipogenesis if liver from fed rats, rather than fasted rats, was used in their assay procedure. In the present investigation one experiment was performed in triplicate in which liver from fed rats was used in the incubation medium. In agreement with the findings of Catravas and Anker, no increased biosynthesis of lipids was realized, and, therefore, only livers from fasted rats were used in the assay procedure.

SUMMARY

In 1958 Catravas and Anker (1958 a) reported the existence of a factor found in yeast and in rat livers capable of increasing synthesis of lipids by liver homogenates prepared from fasted rats. In order to better characterize this factor, termed lipogenin, further investigations were initiated.

An assay procedure was developed during this investigation which permits evaluation of free fatty acid synthesis and non-saponifiable lipid synthesis by lipogenin.

Initial studies during this investigation included a determination of the effect of crude boiled liver extract on free fatty acid synthesis. The FFA synthesized in a liver homogenate obtained from fasted rats after incubation for four hours with the crude extract was extracted with petroleum ether. Inclusion of the crude liver extract from female rat livers resulted in fatty acid synthesis of 204 per cent of the basal control synthesis, expressed as 100 per cent, i.e., homogenate without the added crude liver extract. The increased synthesis amounted to only 126 per cent when crude liver extract from male rats was included in the homogenate.

An attempt was made to determine the effect of crude liver extract on the synthesis of non-saponifiable lipids, cholesterol, cholesterol esters and phospholipids, by in-

cubating fasted rat liver homogenates. Thin layer chromatography or petroleum ether extracts yielded qualitative support for the production of these lipids by lipogenin. However, the increase in synthesis of non-saponifiable lipids was not as great as that realized for FFA synthesis; 114 per cent above basal synthesis compared to 206 per cent, as determined by the modification of the assay developed by Catravas and Anker.

Dowex 1-X10 formate anion exchange columns were used in an attempt to duplicate the findings of previous investigators in purifying and characterizing lipogenin. Only limited increase in the synthesis of FFA was realized when eluates prepared from either female or male rat liver extracts were included in the liver homogenates. However, the use of Amicon Diaflo ultrafiltration membranes of various pore size for lipogenin purification and characterization proved to be more successful. The use of these membranes resulted in a purification of the crude boiled liver extract obtained from fed female and male rats. The rate of synthesis resulting from lipogenin obtained from female rat livers, with a molecular weight between 1 and 5 thousand daltons, averaged 316 per cent of basal synthesis. For a product obtained from male rat livers and having a molecular weight between 5 and 10 thousand daltons the rate of synthesis averaged 236 per cent of basal FFA synthesis.

Catravas and Anker (1963) reported that the incubation of yeast lipogenin, which is thought to be a polypeptide, with pepsin did not affect the lpogenic activity. When the larger lipogenin molecule (5 to 10 thousand molecular weight), obtained by Amicon ultrafiltration of male rat liver extract, is incubated with trypsin, a fraction 13 times more active and possibly having a molecular weight equal to that of female rat lipogenin (1 to 5 thousand) is obtained.

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The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

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