Triacylglycerol Biosynthesis in the Adipose Tissue of the Obese–Hyperglycaemic Mouse

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Obesity in obese–hyperglycaemic mouse is associated with an increase in number and size of adipocytes. Adipocytes from the obese mouse showed increased incorporation of [^{14}C]acetate and [^{14}C]glucose into triacylglycerol. This increased capacity of triacylglycerol formation was correlated with increased activities of various triacylglycerol-forming enzymes measured in the microsomal fraction of adipose tissue from obese mice. Microsomal fractions from lean and obese mice contained *sn*-glycerol 3-phosphate acyltransferase, phosphatidate phosphohydrolase and diacylglycerol acyltransferase. Phosphatidate phosphohydrolase was also detected in the soluble fraction. In the presence of Mg^{2+} , the phosphatidate phosphohydrolase from the soluble and the microsomal fractions was active towards membrane-bound phosphatidate. Among the three enzymes studied here, the increase in Mg^{2+} -dependent phosphatidate phosphohydrolase was most prominent in adipose tissue of obese mice.

A widely studied form of experimental obesity in mice was first described at the Jackson Memorial Laboratory (Ingalls *et al.*, 1950). Several metabolic differences have been observed between these obese mice and their non-obese littermates (Bray & York, 1971). The obese mice are hyperglycaemic, and measurements of lipogenesis from [^{14}C]acetate have shown increased rates of fatty acid synthesis in the obese animals. The activity of the fatty acid-synthesizing enzymes is correspondingly increased in both liver and adipose tissue of the obese mice. Esterification of the excess of fatty acids and storage as triacylglycerol may explain the obesity in this animal. However, the biosynthesis of triacylglycerol by adipose tissue has not been directly studied in the obese mouse.

Triacylglycerol formation in adipose tissue may occur by more than one pathway (Steinberg *et al.*, 1961; Roncari & Hollenberg, 1967; Polheim *et al.*, 1973; Jamdar & Fallon, 1973a), although synthesis from *sn*-glycerol 3-phosphate predominates (Polheim *et al.*, 1973). In the present investigation, the optimal reaction conditions and several properties of enzymes involved in the *sn*-glycerol 3-phosphate pathway have been described in adipose tissues from lean and obese animals.

Materials and Methods

1,2-Diacyl-*sn*-glycerol, phosphatidate and palmitoyl-CoA were synthesized and the purity was deter-

mined as described by Jamdar & Fallon (1973a,b). The fatty acid composition of diacylglycerol by weight was 36% palmitic acid, 37% oleic acid, 15% stearic acid and 12% linoleic acid. 1,2-Diacyl-*sn*-glycerol was dispersed in 0.1% Tween 20 and phosphatidate was dispersed in water by sonification for 20s with a Branson Sonifier (model W 185, Ultrasonics, Plainview, NY, U.S.A.) equipped with microtip probe. *sn*-[1,3- ^{14}C]Glycerol 3-Phosphate (sp. radioactivity 30mCi/mmol) was purchased from ICN Chemicals and Radioisotope Division, Irvine, CA, U.S.A. [1- ^{14}C]Palmitoyl-CoA (sp. radioactivity 56mCi/mmol) and [U- ^{14}C]glucose (sp. radioactivity 240mCi/mmol) were obtained from New England Nuclear Corp., Boston, MA, U.S.A. Sodium [1,2- ^{14}C]acetate (sp. radioactivity 96.8mCi/mmol) was purchased from Schwarz/Mann, Orangeburg, NY, U.S.A. Most of the other chemicals were of AnalaR-grade quality and were purchased from the sources reported by Jamdar & Fallon (1973a).

Male lean and obese (C57 BL/6J and C57 BL/6J-*ob/ob*) mice were purchased from Jackson Memorial Laboratories, Bar Harbor, ME, U.S.A. Initial studies were conducted with adipose tissues from albino mice to determine optimum incubation conditions. Male albino mice (CF₁ strain) of the same age as the obese mice were purchased from Carworth Farms, New York, NY, U.S.A. The incubation conditions developed were found to be satisfactory for the assay of various enzymes in adipose tissues from both lean and obese mice. At the time of death, lean and obese mice were 8–10-weeks old. This period corresponds to

the active phase of lipid deposition in obese mice (Bray & York, 1971).

Preparation of subcellular fractions

Epididymal adipose tissues from twelve albino, six lean and two obese mice were separately pooled and homogenized with 3 vol. of 0.25M-sucrose/1 mM-Tris/HCl (pH 7.5)/1 mM-EDTA/1 mM-dithiothreitol (Medium A). The homogenization was performed at speed 5 for 30s at 4°C with a Tekmar Tissumizer (Tekmar Instruments, Cincinnati, OH, U.S.A.). The homogenate was centrifuged at 600g for 15min and separated into upper fat-cake, a pellet (containing nuclei, cell debris and other tissue fragments) and an intermediate layer. This intermediate layer, called the fat-free homogenate, was used directly or was fractionated into mitochondrial and microsomal fractions as described earlier for rat adipose tissue (Jamdar & Fallon, 1973a). The final mitochondrial and microsomal pellets were washed once with buffer and suspended in buffer (Medium A) by gentle homogenization. This final suspension was used in all assays.

The purity of the subcellular fractions obtained from lean and obese mice was estimated by measuring appropriate marker enzymes as described by Jamdar & Fallon (1973a). Succinate dehydrogenase (EC

1.3.99.1) was used as a marker for the mitochondrial fraction. Diacylglycerol acyltransferase (EC 2.3.1.20) was used as a marker for the microsomal fraction and α -glycerophosphate dehydrogenase (EC 1.1.99.5) was a marker for the cytosol. The distribution of diacylglycerol acyltransferase indicates that mitochondrial and soluble fractions were slightly contaminated with the microsomal fraction. The studies on α -glycerophosphate dehydrogenase suggest that mitochondria and microsomal fractions were substantially free from contaminating soluble fractions (Fig. 1).

In some experiments, subcellular fractions were prepared from both adipocytes and non-adipocytes isolated by collagenase digestion as described by Rodbell (1964), except that the incubation medium did not contain glucose and the concentration of collagenase was 1 mg/ml. Adipocytes were separated from non-adipocytes by suspending them in the incubation medium and by centrifugation (Rodbell, 1964). Adipocytes and non-adipocytes were washed twice with Medium A to remove adherent albumin and collagenase. During these washes, adipocytes from lean mice remained intact. This was apparent from the absence of any oil droplets. However, the adipocytes from obese mice showed considerable damage. Subcellular fractions were prepared from both adipocytes and non-adipocytes after homogenization in Medium A.

Fig. 1. Intracellular distribution of marker enzymes and protein

The enzymes were assayed as described by Jamdar & Fallon (1973a). The results are expressed as relative specific activity with respect to protein by taking the activity of fat-free homogenate as 1. (a), (b), Succinate dehydrogenase; (c), (d), diacylglycerol acyltransferase; (e), (f), α -glycerophosphate dehydrogenase; (g), (h), protein. Abbreviations: MT, mitochondria; MC, microsomal fraction; SF, cytosol.

Conversion of [^{14}C]acetate and [^{14}C]glucose into lipid

Small fragments of adipose tissue (60–100 mg wet wt.) were incubated in the presence of [^{14}C]acetate or [^{14}C]glucose as described by Christophe *et al.* (1961*a,b*). In the final volume of 2 ml, the reaction mixture contained 1.5 ml of Krebs–Ringer bicarbonate medium containing 3% (w/v) albumin, 0.5 mM-sodium acetate, 1 μCi of sodium [^{14}C]acetate and 20 mM-glucose. In the glucose-incorporation experiments, acetate was replaced by 2 μCi of [^{14}C]glucose (2 $\mu\text{Ci}/\mu\text{mol}$), so that the final concentration of glucose was 20.5 mM. The contents were incubated at 37°C in 20 ml plastic scintillation vials with O_2/CO_2 (95:5) as the gas phase. Under these conditions, the incorporation of radioactive substrates into lipid by adipose tissue from both lean and obese mice was linear with time up to 180 min. In the standard assay the incubation was 2 h. Tissue lipids along with the medium were extracted by chloroform/methanol (2:1, v/v), and were purified as described by Folch *et al.* (1957). A portion of the lipid sample was dissolved in 10 ml of Liquifluor (New England Nuclear Corp.) in toluene. The incorporation of radioactivity into total lipids was measured in a Beckman LS 250 liquid-scintillation counter at room temperature (24°C). The other sample (25 μl) was subjected to t.l.c. to determine the incorporation of radioactivity into triacylglycerols.

A portion of adipose tissue used in the metabolic studies was fixed in OsO_4 and the number of adipocytes was determined in a Coulter counter (Hirsch & Gallian, 1968). The rate of lipid formation is expressed as μmol of lipid formed/2 h per 10^6 adipocytes. The results of this experiment are shown in Fig. 2.

Enzyme assays

The acylation of *sn*-glycerol 3-phosphate was studied in the presence of either palmitoyl-CoA or palmitate, ATP, CoA and Mg^{2+} by using various subcellular fractions. In a final volume of 0.75 ml, the reaction mixture contained 24 mM-Tris/HCl buffer, pH 7.5, 50 mM-KCl, 0.42 mM-*sn*-glycerol 3-phosphate, 0.1 μCi of *sn*-[^{14}C]glycerol 3-phosphate, 0.7 mM-dithiothreitol, 1.05 mM-ammonium palmitate, 3 mM-ATP, 3.6 mM- MgCl_2 , 0.01 mM-CoA and 1.25 mg of fatty acid-poor albumin. The reaction was started with 0.1–0.2 ml of enzyme containing 80–120 μg of microsomal protein or 250–300 μg of homogenate protein. Incubation was under air at 37°C in a shaking water bath. The reaction was linear with time for 6 min when microsomal fractions were used and for 20 min when fat-free homogenate was used as the enzyme source.

In some experiments, the palmitate-activation mixture (palmitate, ATP, CoA and Mg^{2+}) was replaced by 0.065 mM-palmitoyl-CoA. Above this

concentration, the reaction rate decreased slightly. The rate of lipid formation was linear for 6 min in the presence and absence of 0.5 mM- Mg^{2+} with microsomal protein (80–120 μg). The major portion of palmitoyl-CoA was protein-bound under this condition. Omission of albumin resulted in a 90% decrease in the reaction velocity. After 6 min incubation at 37°C, the reaction was stopped by the addition of 3 ml of chloroform/methanol/1 M-HCl (2:1:0.025, by vol.).

The radioactive lipids formed during the reaction were extracted as described by Van den Bosch & Vagelos (1970) and dried under N_2 . The dry lipids were dissolved in 0.5 ml of benzene or chloroform/methanol (2:1, v/v). Samples were applied in a volume of 0.1 ml and separated on thin-layer plates coated with silica gel G (E. Merck, Darmstadt, Germany) slurried in 0.1 M-sodium borate. The lipid classes were identified by appropriate standards in an adjacent lane. The separation of phosphatidate was performed with the solvent system chloroform/methanol/3 M- NH_3 (65:35:8, by vol.) and chloroform/methanol/acetic acid/water (50:25:8:4, by vol.) (Renkonen, 1968; Blank & Snyder, 1970). Neutral lipids were separated with hexane/diethyl ether/acetic acid (73:25:2, by vol.) (Pieringer & Kunnes, 1965). In some studies, lipids were extracted to identify lysophosphatidate formation (Long *et al.*, 1967; Daae, 1972). The lipids were located by exposure of the plates to iodine, which, after sublimation, was removed at room temperature; appropriate areas from the plates were scraped directly into scintillation vials containing 10 ml of Liquifluor in toluene, and the radioactivity in the different lipid fractions, was then measured. The rate of glycerolipid formation was expressed as nmol of *sn*-glycerol 3-phosphate incorporated into lipids/min per 10^6 adipocytes.

In the presence of *sn*-glycerol 3-phosphate and the palmitoyl-CoA-generating system, adipose-tissue homogenates and microsomal fractions formed phosphatidate, diacylglycerol and triacylglycerol, with less than 5% of the lipid recovered as lysophosphatidate (Tables 2–4). The recovery of lysophosphatidate was not improved further with the use of a solvent system that favours the extraction of lyso compounds (Long *et al.*, 1967; Daae, 1972). Replacement of the palmitoyl-CoA-generating system by palmitoyl-CoA caused a significant decrease in the rate of microsomal lipid formation. A major portion of the lipid formed from palmitoyl-CoA was phosphatidate. It is expected that, similarly to the liver, phosphatidate formation by adipose tissue is mediated by two enzyme components (Yashimata & Numa, 1972). The addition of 0.5 mM- Mg^{2+} to the palmitoyl-CoA system resulted in the formation of neutral lipids. The increase in the formation of neutral lipid in the presence of Mg^{2+} was taken as an indicator of microsomal phosphatidate phosphohydrolase

activity (Jamdar & Fallon, 1973a). The results of these studies are reported in Table 4.

Phosphatidate phosphohydrolase was also assayed in the presence of both aqueous-dispersed and membrane-bound substrates as previously described (Jamdar & Fallon, 1973b). When aqueous phosphatidate was used, the release of P_i was a measure of phosphatidate phosphohydrolase activity. Phosphatidate phosphohydrolase activity with membrane-bound substrate was estimated by the formation of radioactive diacylglycerol from radioactive membrane-bound phosphatidate and by the ratio of neutral lipid to phosphatidate formed from *sn*-[^{14}C]glycerol 3-phosphate (Tables 4 and 5).

To measure the activity of cytosol phosphatidate phosphohydrolase, membrane-bound phosphatidate was prepared in the presence of *sn*-[^{14}C]glycerol 3-phosphate and palmitoyl-CoA as described by Jamdar & Fallon (1973b). Adipose-tissue microsomal fractions isolated from epididymal fat-pads of albino mice were used in the preparation of substrate. This preparation was heated at 100°C for 5 min to destroy intrinsic microsomal phosphatidate phosphohydrolase activity and was sonicated before use to prepare a uniform suspension in deionized water. The membrane-bound substrate contained 227 nmol of phosphatidate and 3.46 nmol of diacylglycerol/mg of protein respectively. For the phosphatidate phosphohydrolase assay the reaction mixture (total volume 0.8 ml) contained 60 μ mol of Tris/maleate buffer, pH 6.9, 23 nmol of membrane-bound phosphatidate and 0.8 μ mol of $MgCl_2$ (where present). The reaction was started by the addition of soluble protein isolated from adipose tissue from lean and obese mice and the contents were incubated at 37°C. Under these conditions, the reaction was linear with time for at least 20 min at 80–200 μ g of soluble protein. In the absence of Mg^{2+} , the utilization of membrane-bound substrate by cytosol enzyme did not occur.

1,2-Diacyl-*sn*-glycerol acyltransferase was measured in the presence of 1,2-diacyl-*sn*-glycerol dispersed in Tween 20. The standard assay was in a final volume of 1 ml containing 50 mM-Tris/HCl, pH 7.5, 1.2 mM-1,2-diacyl-*sn*-glycerol, 0.25 mM-dithiothreitol, 2 mM- $MgCl_2$, 0.08 mM-palmitoyl-CoA, containing 0.1 μ Ci of [^{14}C]palmitoyl-CoA, and 1.25 mg of fatty acid-poor albumin. The contents were incubated at 37°C for 2 min. The reaction was started by adding 0.1 ml of microsomal fraction (0.08–0.12 mg of protein) and was linear with time up to 10 min. The formation of [^{14}C]triacylglycerol was taken as a measure of the enzyme activity.

Results and Discussion

Recent studies from several laboratories suggest that the metabolic activity of adipose tissue should be expressed on a cellular basis rather than on the basis

of wet weight, triacylglycerol or protein concentration (Salans *et al.*, 1968). This consideration is probably more important in studies with adipose tissue of obese mice because obesity is accompanied by an increase in the number and the size of adipocytes and also by an increase in the number of non-adipocytes (Hellman *et al.*, 1963). Therefore the results of the metabolic studies and the rates of different enzyme reactions measured in the present studies were calculated in relation to adipocyte number.

Adipocytes from obese animals were enlarged and showed significantly higher rates of triacylglycerol formation from both [^{14}C]glucose and [^{14}C]acetate (Fig. 2). There was a slight but significant increase in the total number of adipocytes in adipose tissue from obese mice (Table 1). These results are similar to those of Batchelor *et al.* (1975) and Johnson & Hirsch (1972). Homogenates and microsomal fractions prepared from isolated adipocytes were usually less active than the corresponding fractions derived from whole adipose tissue (Table 2). Therefore the different subcellular fractions were prepared as a routine from whole adipose tissue instead of from adipocytes.

However, adipose tissue is comprised of both adipocytes and non-adipocytes (Rodbell, 1964). The results expressed on the basis of adipocytes would be

Fig. 2. Incorporation of (a) [^{14}C]acetate, and (b) [^{14}C]glucose into lipids

Adipose-tissue fragments were incubated in duplicate in the presence of radioactive glucose or radioactive acetate as described in the Materials and Methods section. Each value is mean $\pm$ S.D. of three independent experiments. □, Total lipid; ▨, triacylglycerol. L, lean; O, obese. Total lipid indicates the radioactivity in phospholipid, diacylglycerol, unesterified fatty acid and triacylglycerol fractions. Adipocytes from obese mice showed significantly ($P < 0.05$) higher rates of incorporation into the triacylglycerol fraction.

Table 1. *Determination of number and size of adipocytes from epididymal adipose tissues from lean and obese mice*

The number of adipocytes was determined by the method of Hirsch & Gallian (1968) by counting cells fixed with OsO₄ in a Coulter counter. Fat-cell size was expressed by the amount of lipid present per cell.

Group and no. of animals	Body wt. (g)	Epididymal pad wet wt. (g)	10 ⁻⁶ × no. of adipocytes		
			(per g wet wt. of tissue)	(Total)	Lipid (μg/cell)
Lean (14)	23.31 ± 3.00	0.30 ± 0.06	3.80 ± 1.19	1.13 ± 0.34	0.11 ± 0.02
Obese (10)	37.25** ± 3.33	1.96* ± 0.36	0.77** ± 0.18	1.51* ± 0.44	0.63** ± 0.18

* Results were analysed by the Students' *t* test. *P* < 0.05.

** *P* < 0.01.

Table 2. *Comparison of lipid formation by homogenates and microsomal fractions isolated from different sources*

Homogenates and microsomal fractions were prepared from whole adipose tissue, adipocytes and non-adipocytes from lean mice. Glycerolipid formation was measured in the presence of *sn*-[¹⁴C]glycerol 3-phosphate, palmitate, ATP, CoA and Mg²⁺ as described in the Materials and Methods section. Assays were done in duplicate. Each value is the mean ± S.D. from three independent experiments.

Fraction from		Glycerolipid formation (nmol/min per mg of protein)			
		Phosphatidate	Diacylglycerol	Triacylglycerol	Total
Whole tissue	Homogenates	2.3 ± 0.24	0.75 ± 0.045	2.45 ± 0.52	5.51 ± 0.52
	Microsomal fraction	15.91 ± 4.00	1.11 ± 0.40	1.82 ± 0.41	18.95 ± 4.50
Adipocyte	Homogenates	1.57 ± 0.18	0.37 ± 0.09	0.55* ± 0.06	2.51* ± 0.23
	Microsomal fraction	3.74* ± 0.39	0	0	3.74* ± 0.39
Non-adipocyte	Homogenates	0.36* ± 0.03	0	0	0.36* ± 0.03
	Microsomal fraction	0.42* ± 0.08	0	0	0.42* ± 0.08

* Significantly different from whole adipose-tissue fractions (*P* < 0.01).

valid only if the contribution by non-adipocytes to lipid formation were negligible. Lipid formation by homogenates and by microsomal fractions derived from non-adipocytes was much lower than in those from adipocytes (Table 2). Non-adipocytes contained no factors that activated lipid formation in adipocytes. A marked increase in the non-adipocyte population of adipose tissue of obese mice caused a rise in the proportion of adipose-tissue protein content derived from non-adipocytes. Therefore the calculation of enzyme-reaction rates was based on adipocyte number rather than on the total tissue protein content.

The capacity for triacylglycerol formation in tissue homogenates was significantly increased in adipose tissue of obese mice. Homogenates of tissues from lean and obese mice formed phosphatidate, diacylglycerol and triacylglycerol in the proportions 42:13:45 and 28:14:58 respectively. Thus the ratio of neutral lipids to phosphatidate formed was higher in fat-free homogenates from obese mice compared with that from lean animals (Table 3).

Since the adipocytes and adipose-tissue homogenates from obese mice showed an increased capacity for triacylglycerol formation, further studies of the

individual enzyme activities involved in triacylglycerol formation were performed. *sn*-Glycerol 3-phosphate acyltransferase activity in the microsomal fraction was measured in the presence of either palmitoyl-CoA or palmitate, ATP, CoA and Mg²⁺. In the presence of palmitoyl-CoA, adipose-tissue microsomal fractions from both obese and lean mice formed phosphatidate as the major product and lesser amounts of di- and tri-acylglycerol (Table 4). The addition of Mg²⁺ (0.1–0.5 mM) resulted in greater formation of neutral lipids from phosphatidate by activating microsomal phosphatidate phosphohydrolase. The effect of Mg²⁺ on the formation of neutral lipid was specific. Other bivalent cations, such as Ca²⁺, Mn²⁺, Fe²⁺, Ba²⁺, Ni²⁺ and Co²⁺ (at 0.5 mM), did not show this effect.

The rate of lipid formation in the presence of palmitate, ATP, CoA and Mg²⁺ was always greater than that with palmitoyl-CoA in adipose-tissue microsomal fractions from either lean or obese mice. The proportion of neutral lipids formed was also greater under these conditions. The differences between these two incubation systems have been reported in rat adipose-tissue microsomal fractions (Jamdar & Fallon, 1973a). The adipose-tissue micro-

Table 3. Comparison of glycerolipid formation by fat-free homogenates of adipose tissue from lean and obese mice

Assays were done in duplicate in the presence of *sn*-[¹⁴C]glycerol 3-phosphate, palmitate, ATP, CoA and Mg²⁺ as described in the Materials and Methods section. Each value represents mean $\pm$ s.d. from four or five independent experiments. In each experiment epididymal adipose tissues from six lean and two obese mice were pooled to prepare fat-free homogenates. A portion of this adipose tissue was used for the determination of the number of fat-cells.

Homogenates from	[¹⁴ C]Glycerol 3-phosphate converted into lipid (nmol/min per 10 ⁶ adipocytes)				(b)+(c) (a)
	Total	Phosphatidate (a)	Diacylglycerol (b)	Triacylglycerol (c)	
Lean mice	8.07 $\pm$ 0.93	3.25 $\pm$ 0.36	1.07 $\pm$ 0.06	3.71 $\pm$ 0.76	1.43 $\pm$ 0.35
Obese mice	21.33* $\pm$ 2.87	5.93 $\pm$ 0.78	2.93* $\pm$ 0.69	12.53* $\pm$ 2.29	2.60* $\pm$ 0.39

* Significantly different from lean animals ($P < 0.01$).

Table 4. Glycerolipid formation by adipose-tissue microsomal fraction from lean and obese mice

Assays were done in duplicate in the presence of (a) *sn*-[¹⁴C]glycerol 3-phosphate, palmitate, ATP, CoA and Mg²⁺ or (b) *sn*-[¹⁴C]glycerol 3-phosphate and palmitoyl-CoA with and without Mg²⁺. The final concentration of Mg²⁺ was 0.5 mM in the incubation system (b). Each value is the mean $\pm$ s.d. of four to six independent experiments. In each experiment, microsomal fractions were prepared from epididymal adipose tissues pooled from six lean and two obese mice.

Glycerolipid formation (nmol/min per 10 ⁶ adipocytes)					
Microsomal fractions from	Phosphatidate (a)	Diacylglycerol (b)	Triacylglycerol (c)	Total	(b)+(c) (a)
(a) Lean	3.18 $\pm$ 0.80	0.22 $\pm$ 0.08	0.36 $\pm$ 0.18	3.76 $\pm$ 1.05	0.17 $\pm$ 0.035
Obese	4.69* $\pm$ 0.55	0.42* $\pm$ 0.08	0.75* $\pm$ 0.23	5.86* $\pm$ 0.84	0.23* $\pm$ 0.038
(b) Lean -Mg ²⁺	1.79 $\pm$ 0.26	0.11 $\pm$ 0.01	0.085 $\pm$ 0.05	1.98 $\pm$ 0.32	0.11 $\pm$ 0.014
+Mg ²⁺	1.26 $\pm$ 0.24	0.37 $\pm$ 0.13	0.76 $\pm$ 0.10	2.41 $\pm$ 0.25	0.93 $\pm$ 0.23
Obese -Mg ²⁺	3.30* $\pm$ 0.49	0.44* $\pm$ 0.02	0.28* $\pm$ 0.12	4.0* $\pm$ 0.53	0.21** $\pm$ 0.09
+Mg ²⁺	2.17* $\pm$ 0.39	1.05* $\pm$ 0.11	3.06* $\pm$ 0.66	5.60* $\pm$ 0.72	2.17** $\pm$ 0.39

* Significantly different from lean animal ($P < 0.01$).

** Significantly different from lean animals ($P < 0.05$).

somal fractions of obese mice were significantly more active in formation of total lipids and neutral lipids than were those from lean mice (Table 4). These results indicate that *sn*-glycerol 3-phosphate acyltransferase is more active in adipocytes of obese mice.

Because of the evidence that formation of neutral lipid is proportionately greater in adipocyte microsomal fractions from obese mice, further studies of phosphatidate phosphohydrolase were undertaken. Phosphohydrolase activity was detected in both microsomal fractions and cytosol (Table 5). The dual location of phosphatidate phosphohydrolase in microsomal and cytosol fractions has previously been reported in the liver and the intestine (Hübscher *et al.*, 1967; Johnston *et al.*, 1967). Phosphatidate phosphohydrolase was distinguished as being either Mg²⁺-dependent or Mg²⁺-independent as previously described (Jamdar & Fallon, 1973b). In the absence of Mg²⁺, the utilization of membrane-bound substrate did not occur. These findings are similar to

those reported for rat adipose tissue. This suggests that the Mg²⁺-dependent phosphatidate phosphohydrolase may be essential for acylglycerol biosynthesis in mouse adipose tissue as well.

There was a significant increase in the activities of both the Mg²⁺-dependent and Mg²⁺-independent phosphatidate phosphohydrolases in the microsomal and cytosol fractions of adipose tissue from obese mice (Table 5). The activities of soluble phosphatidate phosphohydrolase measured with membrane-bound substrates in lean and obese mice were 0.70 $\pm$ 0.15 and 3.06 $\pm$ 0.78 nmol/min per 10⁶ adipocytes respectively ($P < 0.01$). The relative increase in phosphatidate phosphohydrolase activity in adipose tissue of obese mice was much greater than the approximately 2-fold increase in the activity of *sn*-glycerol 3-phosphate acyltransferase.

The activities of diacylglycerol acyltransferase in microsomal fractions of adipose tissue from lean and obese mice were 2.50 $\pm$ 0.36 and 9.18 $\pm$ 1.88 nmol/min per 10⁶ adipocytes respectively. Although these

Table 5. *Phosphatidate phosphohydrolase in microsomal fractions and cytosol from adipose tissues of lean and obese mice*
 Assays were done in duplicate in the presence of aqueous phosphatidate with and without Mg²⁺. The final concentration of Mg²⁺ was 2.5 mM. Activity is expressed as nmol of P_i released/min per 10⁶ adipocytes. Each value is the mean $\pm$ s.d. of four to six independent experiments. In each experiment adipose tissues from six lean and two obese mice were pooled separately to prepare different subcellular fractions.

		Phosphatidate phosphohydrolase		Mg ²⁺ -dependent phosphatidate phosphohydrolase	
Cell fractions		-Mg ²⁺	+Mg ²⁺		Cytosol+ microsomal
Lean mice	Microsomal	1.21 $\pm$ 0.16	4.40 $\pm$ 1.23	3.14 $\pm$ 1.13	25.0 $\pm$ 1.81
	Cytosol	0	21.83 $\pm$ 1.29	21.83 $\pm$ 1.29	
Obese mice	Microsomal	4.24* $\pm$ 0.20	21.15* $\pm$ 2.18	16.19* $\pm$ 2.11	121.0* $\pm$ 5.43
	Cytosol	11.63* $\pm$ 3.50	115.64* $\pm$ 6.75	104.00* $\pm$ 6.21	

* Significantly different from lean animal ($P < 0.01$).

Table 6. *Determination of adipocyte and non-adipocyte protein from adipose tissue from lean and obese mice*

In each experiment epididymal fat-pads from four lean and four obese mice were pooled separately to prepare the fat-free homogenates from whole fat-pads and isolated adipocytes. Right fat-pads were used for the preparation of whole adipose homogenates and left fat-pads for the preparation of adipocytes. Adipocytes were prepared as described in the Materials and Methods section in the absence of bovine serum albumin. Protein from fat-free homogenates was determined as described by Lowry *et al.* (1951). The adipocyte protein was corrected for collagenase contamination, which was 33% regardless of the cell size (Salans & Dougherty, 1971). Non-adipocyte protein is whole-homogenate protein minus corrected adipocyte protein. Each value is the mean $\pm$ s.d. of three independent experiments.

		Adipose-tissue wt. (g)		Protein recovery (mg)		Corrected adipocyte protein (mg)	Non-adipocyte protein (mg)
Mice		For adipocyte preparation	For whole fat-pad homogenate	Adipocyte homogenate	Whole fat-pad homogenate		
Lean		0.58 $\pm$ 0.35	0.57 $\pm$ 0.34	2.04 $\pm$ 0.91	5.90 $\pm$ 0.35	1.36 $\pm$ 0.60	4.33 $\pm$ 0.99
Obese		6.33* $\pm$ 0.50	6.47* $\pm$ 0.46	5.10* $\pm$ 0.94	26.79* $\pm$ 3.36	3.40* $\pm$ 0.63	23.88* $\pm$ 3.10

* Significantly different from lean animals ($P < 0.01$).

changes are significant, the measurements of diacylglycerol acyltransferase with substrate dispersed in Tween 20 may not adequately reflect the activity of this enzyme in microsomal fractions (Fallon *et al.*, 1975). However, the results shown in Table 4 indicate that the relative formation of triacylglycerol in microsomal fractions from obese mice is greater than that of diacylglycerol, an observation compatible with accelerated acylation of diacylglycerol in these preparations.

The activities of the individual enzymes provide evidence that the entire pathway of esterification via *sn*-glycerol 3-phosphate is accelerated in adipocytes of obese mice. The most notable increase in activity was observed for phosphatidate phosphohydrolase, although this reaction does not appear to be rate-limiting in adipose tissue as suggested for liver (Vavrecka *et al.*, 1969; Lamb & Fallon, 1974). The result of these changes is a marked increase in the capacity of adipocytes of obese mice to form triacylglycerol.

In addition, a remarkable increase in non-adipocyte protein concentration was noted in adipose tissue of obese mice (Table 6). A proportion of this protein may have been derived from the more fragile adipocytes during their preparation from adipose tissue of obese mice. However, it is unlikely that such contamination could account for the observation. It is concluded that obesity is accompanied by the proliferation of non-adipocytes in adipose tissue.

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