Increase in Fatty Acid Synthetase Content of 3T3-L Cells Undergoing Spontaneous and Chemically Induced Differentiation to Adipocytes

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3T3-L fibroblasts differentiate into adipose cells when maintained in a non-growing state. The specific activity of fatty acid synthetase of differentiated cells was 25–30-fold higher than that present in 3T3-L fibroblasts or in 3T3-C2 cells that possess an extremely low incidence of differentiation to adipocytes. The results of immunochemical analysis indicate that the increased specific activity of fatty acid synthetase in the differentiated cells is due to an increase in the cellular content of this enzyme. The rate of conversion of adipose cells was accelerated by brief exposure of confluent non-growing cultures of 3T3-L cells to 3-isobutyl-1-methylxanthine. This was accompanied by an increase in the specific activity of fatty acid synthetase, which was also shown to be due to an increase in the cellular content of this enzyme. The continuous presence of 3-isobutyl-1-methylxanthine in the culture medium was not required to elicit the morphological and biochemical changes in 3T3-L cells that occurred many days after the removal of the inducer but earlier than the onset of spontaneous differentiation.

Green & Kehinde (1974, 1975) and Green & Meuth (1974) have isolated a number of sublines from the original stock of mouse embryo 3T3 cells. On prolonged maintenance in a resting state most of these sublines convert into adipose-like cells. This occurs spontaneously with various degrees of probability. This irreversible conversion occurs more rapidly when the 3T3-L cells are exposed to prostaglandin $F_{2\alpha}$ or to 3-isobutyl-1-methylxanthine, which is an inhibitor of cyclic nucleotide phosphodiesterase (EC 3.1.4.17) (Russell & Ho, 1976). Rubin *et al.* (1977) showed that, upon differentiation into adipocytes, the 3T3-L cells developed a corticotropin-responsive adenylate cyclase (EC 4.6.1.1) and that the number of insulin receptors increased several-fold (Reed *et al.*, 1977). In addition, the activities of pyruvate carboxylase (EC 6.4.1.1) (Mackall & Lane, 1977), 'malic enzyme' (EC 1.1.1.40) (Kuri-Harcuch & Green, 1977) and of a number of enzymes involved in triacylglycerol and fatty acid synthesis showed a marked increase upon conversion of these cells into adipocytes (Green & Kehinde, 1975; Grimaldi *et al.*, 1978; Mackall *et al.*, 1976).

The 3T3-L adipocytes appear to be similar to native adipocytes both morphologically and biochemically (Green & Kehinde, 1974; Mackall *et al.*, 1976), thereby providing a model system for investigating *in vitro* the short- and long-term control of lipogenesis under a variety of conditions. Fatty acid synthetase is thought to be one of the critical regu-

latory enzymes in lipogenesis, especially in developing tissues, since the capacity of developing mouse liver to synthesize fatty acids from acetate appears to correlate best with changes in the activity of fatty acid synthetase (Smith & Abraham, 1970; Vagelos, 1974). Besides the quantity of enzyme, the catalytic activity of fatty acid synthetase may be regulated by the ratio of the holo- to apo-enzyme, the concentration of effector(s) and perhaps by its phosphorylation/dephosphorylation (Qureshi *et al.*, 1975; Vagelos, 1974). Since the conversion of 3T3-L fibroblasts to adipocytes is accompanied by an increase in the specific activity of fatty acid synthetase, studies were undertaken to determine whether this reflected the increase in the amount of the enzyme per cell or changes in its catalytic activity or both. The results obtained suggest that the increased activity of fatty acid synthetase of 3T3-L cells undergoing spontaneous or 3-isobutyl-1-methylxanthine-induced differentiation is due to an increase in the amount of this enzyme. A preliminary report of these results has been presented (P. Ahmad *et al.*, 1978).

Experimental

Materials

Acetyl-CoA, malonyl-CoA and CoA were obtained from P-L Biochemicals, Milwaukee, WI, U.S.A.; 3-isobutyl-1-methylxanthine was from Ald-

rich Chemical Co., Milwaukee, WI, U.S.A.; NADPH, crystalline bovine serum albumin (fatty acid-poor), penicillin, streptomycin, dithiothreitol and EGTA were from Sigma Chemical Co., St. Louis, MO, U.S.A.; Bio-Gel A-5m was a product of Bio-Rad Laboratories, Richmond, CA, U.S.A.; [2-¹⁴C]malonyl-CoA (36.5mCi/mmol) and Omnifluor were purchased from New England Nuclear Corp., Boston, MA, U.S.A. Insulin was a gift from Eli Lilly and Co., Indianapolis, IN, U.S.A. All other chemicals were of the highest purity commercially available.

Growth of 3T3 cells

3T3-L (L1 and L2) and 3T3-C2 cells were plated at 2×10^4 cells per 20cm² dish in Dulbecco-Vogt's modified Eagle's medium (Grand Island Biological Co., Grand Island, NY, U.S.A.) supplemented with penicillin (30 µg/ml), streptomycin (50 µg/ml) and 10% (v/v) newborn calf serum (Russell & Ho, 1976). The medium was changed three times a week until the cells had reached confluence, at which time ($t = 0$) insulin (1 µg/ml) was included in the medium for all dishes.

Preparation of cell-free extracts

Dishes were rinsed twice with 5ml portions of ice-cold buffer, pH7.2, containing KCl (2.68mM), KH₂PO₄ (1.46mM), MgCl₂ (0.49mM), NaCl (137mM) and Na₂HPO₄ (8.1mM). The cells were suspended in 0.3–0.4ml of ice-cold 5mM-potassium phosphate buffer, pH7.0, containing 10mM-potassium citrate and 0.5mM-EGTA, and then homogenized manually (15 up-and-down strokes) in a Dounce homogenizer (Mackall *et al.*, 1976). The homogenate was adjusted to 250mM-potassium phosphate buffer (pH6.5)/2.5mM-dithiothreitol/7.5mM-EDTA, and centrifuged at 27000g_{av.} for 60min. The specific activity of the fatty acid synthetase in the supernatant was either determined the same day or after the supernatant had been frozen in liquid N₂ for 1–2 weeks without any noticeable change in enzyme activity.

Determination of fatty acid synthetase activity

The following methods were used to determine fatty acid synthetase activity.

(a) *Radioisotopic.* The reaction mixture (0.25ml) contained 200mM-potassium phosphate buffer, pH6.5, 6mM-EDTA, 0.04mM-dithiothreitol, 1mM-NADPH, 0.025mM-acetyl-CoA and 0.15mM-[2-¹⁴C]-malonyl-CoA (1.35 µCi/µmol). The reactions were started by adding the enzyme, incubated at 37°C for 6min and stopped by adding 0.25ml of methanolic 40% (w/v) KOH. The contents were saponified at 85°C for 1h. After adding 0.15ml of 12M-HCl the

samples were extracted twice with 5ml portions of light petroleum (b.p. 20–40°C). The combined extract was transferred to vials and evaporated to dryness under N₂ stream at 30°C. The residue was dissolved in 1ml of toluene, and the radioactivity determined by scintillation spectrometry after adding 10ml of a scintillant (2g of Omnifluor/litre of toluene). One unit of enzyme activity is the amount of protein catalysing the incorporation of 1 µmol of malonyl-CoA into fatty acids/min at 37°C.

(b) *Spectrophotometric.* The fatty acid synthetase activity was monitored spectrophotometrically by the method of Smith & Abraham (1975), except that the reaction temperature was 37°C instead of 30°C. The incorporation of 1 µmol of malonyl-CoA was found to correspond to 2–2.2 µmol of NADPH oxidized under the above assay conditions. The results of spectrophotometric assays were normalized to the radioisotopic method by dividing them by a factor of 2.

Protein determination

Protein concentrations were determined by the method of Bradford (1976), with crystalline bovine serum albumin as a standard.

Purification of lactating-rat mammary-gland fatty acid synthetase

The purification scheme for lactating-rat mammary-gland acetyl-CoA carboxylase was followed through to step (f) to achieve partial purification of fatty acid synthetase (F. Ahmad *et al.*, 1978). The synthetase was further purified by chromatography on a Bio-Gel A-5m column (F. Ahmad & P. Ahmad, unpublished results). The specific activity of the purified enzyme was about 1–1.2 µmol of NADPH oxidized/min per mg of protein at 37°C. A specific activity of about 0.8 unit/mg of protein, determined at 30°C, had earlier been reported by Smith & Abraham (1975) for the highly purified lactating-rat mammary-gland synthetase. The synthetase obtained in this laboratory appeared to be pure by the following criteria. (a) It gave a single protein band of M_r 240000–260000 on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate plus urea (F. Ahmad *et al.*, 1978). (b) The antiserum prepared against this enzyme (see below) gave a single coincident precipitin band when tested with the 100000g_{av.} supernatant of lactating-rat mammary-gland homogenate and with the purified enzyme.

Preparation of anti-(fatty acid synthetase) antibody

Preparations (0.5ml containing 0.5mg of protein) of lactating-rat mammary-gland fatty acid synthetase were used to immunize New Zealand White rabbits

at 14 day intervals. The first injection was administered as an emulsion mixed with an equal part of complete Freund's adjuvant and injected into four sites intramuscularly. Subsequent injections were given at the same sites but without the adjuvant. Blood was collected 8 days after the fourth injection. Solid (NH₄)₂SO₄ (240 g/l) was added to serum at 4°C. The γ -globulin fraction was collected by centrifugation at 27000g_{av.} for 20 min at 4°C, dissolved in 50mM-Tris/HCl buffer, pH 7.5, containing 0.15M-NaCl, and dialysed against the same buffer at 4°C for 14h. The γ -globulin fraction was stored at -20°C.

Antisera have been prepared against three different preparations of mammary-gland fatty acid synthetase. Precipitin bands similar to those shown in Fig. 2 (see below) were obtained when these antisera were subjected to Ouchterlony double-diffusion analyses against mammary-gland synthetase and the enzyme found in the cell-free supernatants of differentiated 3T3-L2 cells. However, only one of these antisera has been used during the immunochemical studies reported below.

All the data presented in this paper were obtained from duplicate determinations on at least two independent experiments. Variations between duplicate dishes were within $\pm 10\%$, whereas variations between independent experiments were within $\pm 15\%$.

Results and Discussion

Specific activity of fatty acid synthetase of differentiating 3T3-L cells

On conversion into adipocytes, the concentration of neutral lipid in 3T3-L cells increases approx. 5-fold in comparison with that in pre-adipocytes (Grimaldi *et al.*, 1978). Fig. 1 shows that, in contrast with the pre-adipocytes, the specific activity of fatty acid synthetase was markedly (25–30-fold) higher in the maximally differentiated 3T3-L2 cells. The specific

activity of fatty acid synthetase of the control cell line (3T3-C2), maintained under similar experimental conditions to those of the 3T3-L2 cells, was comparable with that of the pre-adipocytes (results not shown). Since during differentiation of 3T3-L2 cells the protein concentration increased by about 3-fold, i.e. from 0.23 mg/plate (at confluence) to 0.70 mg/plate (16–18 days after confluence), clearly the difference in enzyme activity must be correspondingly greater. These results are similar to those reported by Mackall *et al.* (1976), who used 3T3-L1 cells.

Fig. 1. Specific activity of fatty acid synthetase during maintenance of 3T3-L2 cells in a non-growing state 3T3-L2 cells were grown in 20 cm² dishes as described in the Experimental section. At and beyond confluence, cells were removed at the indicated times, homogenized and the fatty acid synthetase activity was determined in duplicate by the radioisotopic procedure. These results represent the average of four independent experiments. The mean protein content was 0.23 mg/plate at confluence and about 0.7 mg/plate at day 14 and beyond.

Table 1. Effect of 3-isobutyl-1-methylxanthine on fatty acid synthetase activity of 3T3-L cells

Cells were grown and harvested as described in the Experimental section. Confluent cultures (day 0) were treated for 24, 48 and 72 h with 0.5mM-isobutylmethylxanthine. On day 3, cultures were rinsed and fed with medium containing insulin (1 μ g/ml) for 7 days. At the end of this period (day 10 after confluence) cells were homogenized and the supernatants were assayed for fatty acid synthetase activity by the radioisotope procedure. These data are representative of three independent experiments.

Time of incubation with isobutylmethyl- xanthine (h)	Protein (mg/plate)	Fatty acid synthetase	
		(munits/plate)	(munits/mg of protein)
0	0.12	0.48	4.0
24	0.15	0.63	4.2
48	0.16	0.74	4.6
72	0.18	3.70	20.6

Effect of isobutylmethylxanthine on the specific activity of fatty acid synthetase from 3T3-L cells

Russell & Ho (1976) observed that when confluent 3T3-L fibroblasts were treated with 3-isobutyl-1-methylxanthine for 1–4 days, they differentiated more rapidly to adipocytes than did the untreated cells. The accumulation of fat-droplets in isobutylmethylxanthine-treated cultures could be observed within 3–4 days after removing the xanthine derivative (T. R. Russell, unpublished observations). Since fatty acid synthetase appears to be a suitable indicator of differentiation in adipose tissue, we measured the specific activity of this enzyme in 3T3-L2 cells that had been incubated with isobutylmethylxanthine for various times (Table 1). In contrast with spontaneously differentiating cells, the specific activity of the synthetase was approx. 5-fold higher in cells treated with isobutylmethylxanthine for 3 days and then maintained in a medium that contained insulin. Independent experiments showed that the synthetase specific activity remained unchanged during incubation of the 3T3-L cells with isobutylmethylxanthine for periods of 3 days. In addition the accelerated appearance of adipocytes and early increase in fatty acid synthetase activity in the susceptible cell lines did not require the continued presence of this xanthine derivative. Isobutylmethylxanthine failed to trigger differentiation in the 3T3-C2 cell line, which has an extremely low probability (almost zero) of differentiation (Russell, 1978).

Fig. 2. Double-diffusion patterns of fatty acid synthetase from lactating-rat mammary gland and 3T3-L adipocytes

The agar gel (1% w/v) contained 50mM-Tris/HCl buffer, pH7.5, and 0.15M-NaCl. The centre well contained anti-(lactating-rat mammary-gland fatty acid synthetase) antibody (0.98mg). Purified lactating-rat mammary-gland fatty acid synthetase (8.7 μ g) was present in wells 1 and 3. Well 2 contained 0.228mg of cellular protein derived from 3T3-L adipocytes. The double-diffusion plate was developed and washed, and the protein stained with Coomassie Brilliant Blue, and destained as described by F. Ahmad *et al.* (1978).

Immunochemical titration of fatty acid synthetase

Previous studies have shown that in liver and adipose tissue the contents of acetyl-CoA carboxylase and fatty acid synthetase, as well as of other enzymes of importance to lipogenesis, undergo co-ordinate changes when the rate of fatty acid biosynthesis fluctuates in response to metabolic alterations. These conditions include dietary, hormonal and developmental changes (for review see Vagelos, 1974; Numa & Yamashita, 1974). Therefore, using immunochemical methods, we attempted to test whether the increased specific activity of fatty acid synthetase of adipocytes arising from isobutylmethylxanthine-treated or spontaneously differentiated 3T3-L cells was due to an increase in the cellular content of this enzyme. Antibodies raised in rabbits against lactating-rat mammary-gland fatty acid synthetase showed that there was a reaction of partial identity between the purified rat mammary-gland synthetase and the enzyme present in the supernatant of maximally differentiated 3T3-L cells (Fig. 2). No precipitin lines were observed when experiments were conducted in

Fig. 3. Immunotitration of fatty acid synthetase of spontaneously differentiated and of 3-isobutyl-1-methylxanthine-triggered 3T3-L2 adipocytes

Both the spontaneously converted and the isobutylmethylxanthine-treated 3T3-L2 adipocytes (17 days after confluence) were homogenized, and constant amounts of fatty acid synthetase activity [0.56 munit (●) and 1.49 munits (○) for spontaneous and 0.93 munit (▲) for isobutylmethylxanthine-treated] of differentiated cells were separately incubated in duplicate with various amounts of anti-(lactating-rat mammary-gland fatty acid synthetase) antibody and assayed spectrophotometrically as described in the Experimental section. The amount of antibody required to inactivate the synthetase activity completely was estimated by extrapolations of the linear portions of the titration curves to zero synthetase activity. These values were used to calculate the equivalence-point [anti-(fatty acid synthetase) antibody required to inactivate 1 munit of fatty acid synthetase].

Table 2. Immunotitration of fatty acid synthetase of 3T3 cells at various times during adipose conversion

Cell-free extracts from various days after confluence were prepared and immunotitrated as given in the Experimental section. Extracts containing between 0.5 and 0.9 munit of fatty acid synthetase could be titrated alone (see Expts. 1 and 3), whereas extracts containing negligible enzyme activity had to be indirectly titrated in combination with active samples to determine any equivalence-point displacement (Expts. 2, 4 and 5). The synthetase activity was determined spectrophotometrically. Amounts of protein added (μ g): day 17 (19); day 0 (19.2); day 25 (20); 3T3-C2 (24); day 3.5 (16). Expts. 1 and 2 used 3T3-L2 cells; Expts. 3, 4 and 5 used 3T3-L1 cells. All determinations were done in duplicate.

Expt.	Extracts titrated	Fatty acid synthetase activity (munit)	Anti-(fatty acid synthetase) anti-body required to inhibit (μ g)	
			Total fatty acid synthetase activity	1 munit of fatty acid synthetase activity
1	Day 17 alone	0.88	19	21.6
2	Day 17+ day 0	0.87	19.5	22.4
3	Day 25 alone	0.56	10.6	18.9
4	Day 25+3T3-C2 (day 10)	0.65	12.8	19.7
5	Day 25+ day 3.5	0.57	11.7	20.5

the presence of cellular protein (0.23mg) obtained from the 3T3-C2 cells or pre-adipocytes. These antibodies inhibited the activity of fatty acid synthetase of rat mammary gland and 3T3-L cells.

The quantity of fatty acid synthetase was estimated by immunochemical titrations (Fig. 3). Various amounts of the antiserum were added to a constant amount of fatty acid synthetase activity. The amount of antibody required to inactivate fatty acid synthetase completely was estimated from extrapolations of the linear portions of titration curves to zero fatty acid synthetase activity. About 20 μ g of the anti-(fatty acid synthetase) antibody inactivated 1 munit of enzyme activity present in the cell-free extract derived from 3T3-L2 cells triggered to differentiate to adipocytes with isobutylmethylxanthine. Almost identical results were obtained (Table 2) when the enzyme activity present in the spontaneously differentiated 3T3-L1 and 3T3-L2 cells was titrated [20 and 21 μ g of anti-(fatty acid synthetase) antibody inactivated 1 munit of enzyme activity respectively]. Therefore isobutylmethylxanthine appears to promote an increase in the cellular content of fatty acid synthetase rather than an activation of its pre-existing molecules. However, the mechanism by which isobutylmethylxanthine promotes a somewhat rapid induction of synthetase in the 3T3-L cells is not yet clear.

There is a stoicheiometric relationship between the fatty acid synthetase of the 3T3-L cells and the anti-(rat mammary-gland fatty acid synthetase) antibody used (Fig. 3). This is illustrated by comparing the immunotitration curves constructed from 0.55, 0.93 and 1.49 munits of fatty acid synthetase activity. In all the cases the amount of enzyme activity removed was proportional to the amount of antibody added. Independent experiments showed

that control γ -globulin did not remove fatty acid synthetase activity.

Immunotitration experiments (Table 2) were also conducted on synthetase of maximally differentiated 3T3-L2 cells to which had been added cellular protein of pre-adipocytes (3T3-L cells; 0 and 3.5 days after confluence) or of 3T3-C2 cells (10 days after confluence), containing marginal enzyme activity. Addition of these proteins neither affected the fatty acid synthetase activity significantly (limit of detection about 0.1 munit) nor displaced the equivalence point (compare Expt. 1 with 2 and Expts. 4 and 5 with 3 of Table 2). This suggests the almost complete absence of immunologically reactive fatty acid synthetase in the pre-adipocytes and in the 3T3-C2 cells. These results also indicated the absence of an inhibitor or of an activator of fatty acid synthetase in the cell-free extracts of pre-adipocytes or of those of 3T3-C2 cells.

The results presented above strongly suggest that the increase in the specific activity of fatty acid synthetase from chemically induced or spontaneously differentiating 3T3-L cells is due to an increase in the cellular content of this enzyme. The content of acetyl-CoA carboxylase has also been found to increase during differentiation of 3T3-L1 cells (Mackall *et al.*, 1976). Therefore it is likely that the increased rate of lipogenesis observed in the differentiated 3T3-L cells may result from an increase in the rates of synthesis of additional enzymes of importance to lipogenesis.

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