Hormonal regulation of the differentiation of rat adipocyte precursor cells in primary culture

O. Wiederer and G. Löffler[1]

Institut für Biochemie, Genetik, und Mikrobiologie, Universität Regensburg, Regensburg, FRG

Abstract A reproducible cell culture system is described that allows the study of adipose conversion in fibroblast-like cells isolated by collagenase digestion of epididymal and perirenal adipose tissue from male rats weighing 70–200 g. Adipose conversion as measured by lipid accumulation and increase in glycerophosphate dehydrogenase (GPDH) activity during differentiation strongly depends on the density at which cells are inoculated and starts only when cells are confluent and when physiological amounts of corticosterone and insulin are added. β-Estradiol, testosterone, thyroxine, triiodothyronine, and growth hormone do not affect the differentiation process. Methyl-isobutylxanthine added during the first 2 days after confluence, added with insulin and corticosterone, potentiates the effect of insulin on GPDH activity and accelerates triglyceride accumulation. The effect of methyl-isobutylxanthine seems to be mediated by increased cyclic AMP concentrations, inasmuch as it may be replaced by forskolin. — **Wiederer, O., and G. Löffler.** Hormonal regulation of the differentiation of rat adipocyte precursor cells in primary culture. *J. Lipid Res.* 1987. **28:** 649–658.

Supplementary key words adipose conversion • insulin • corticosterone • human growth hormone • triiodothyronine • β-estradiol • testosterone • methyl-isobutylxanthine • cyclic AMP • forskolin

In recent years cell culture systems have been described which allow the study of adipocyte formation from precursor cells. Clonal lines obtained from fibroblast-like cells (1–3) have been used for this purpose, as well as primary cultures from a stromal vascular fraction derived from collagenase digests of rat, sheep, pig, bovine, and human adipose tissue (4–8). In clonal cell lines the adipogenic activity of growth hormone, glucocorticoids, and thyroid hormones, in addition to insulin, is clearly established (9–11). In contrast, the hormonal control of the adipose conversion in primary culture systems derived from the stromal vascular fraction of adipose tissue from different sources has been only partially elucidated. We describe here a simple culture system for adipocyte precursors obtained from adipose tissue of rats weighing 70–200 g. These cells are able to undergo a nearly complete adipose conversion depending on the presence of insulin and glucocorticoids, each in physiological concentrations.

MATERIALS AND METHODS

Materials

Medium 199E, FCS, antibiotics, and collagenase (CLS Worthington) were obtained from Biochrom (Berlin). Bovine insulin was generously provided by Hormonchemie (Munich). MIX, bovine serum albumin, and triolein were from SERVA (Heidelberg). Forskolin was a generous gift from Dr. Metzger (Hoechst AG Frankfurt/Main). Cortisol, corticosterone, β-estradiol, testosterone, thyroxine, and triiodothyronine were from Sigma (Munich) and hGH was from Hormonchemie (Munich). Cyclo-AMP-RIA (AMPcK CEA SORIN) was from Isotopendienst West (Dreieich). All other reagents were of analytical grade and were obtained from commercial sources.

Animals

Male Wistar rats weighing between 64 and 200 g were housed for at least 1 week at a temperature of 23°C, a 12-hr light cycle, and at constant humidity before they were killed. The rats were allowed access to a standard chow (Altromin, Lage) and water ad libitum. In the morning after having had unrestricted access to food animals were killed by bleeding under diethylether anesthesia.

Preparation of preadipocytes and cell culture

Tissue preparations and cell isolation by collagenase digestion were performed according to Björntorp et al. (4). Isolated stromal vascular cells were suspended in growth medium consisting of Medium 199 with Earles salts (M199E) supplemented with 10% FCS, penicillin

Abbreviations: FCS, fetal calf serum; GPDH, glycerophosphate dehydrogenase; hGH, human growth hormone; MIX, methyl-isobutylxanthine; TCA, trichloroacetic acid.

[1]To whom reprint requests should be addressed at : Institut für Biochemie, Genetik, und Mikrobiologie, Universität Regensburg, Universitätsstrasse 31, D 8400 Regensburg, FRG.

(100 U/ml), and streptomycin (0.1 mg/ml), and counted in a hemocytometer. The cells were seeded at a density of 2–3 × 10^4 cells/cm^2 in culture dishes (35 mm diameter, Nunc, Biochrom) in 2 ml of growth medium. Cultures were kept at 37°C in an atmosphere of 5% CO_2 in air saturated with water, and the media were changed the following day. Cells reached confluence 4 days after plating at a density of about 1–1.5 × 10^5 cells/cm^2, and differentiation was induced by replacing growth medium with M199E medium supplemented with 5% FCS, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and hormones as indicated. Medium was changed every second day.

Trypsinization of cells was done after the medium had been discarded by addition of 2 ml of trypsin solution (0.25%) and incubation for 20 min at 37°C. Cells were then centrifuged for 10 min at 750 *g* and the pellet was suspended in growth medium and adjusted to the cell densities as indicated.

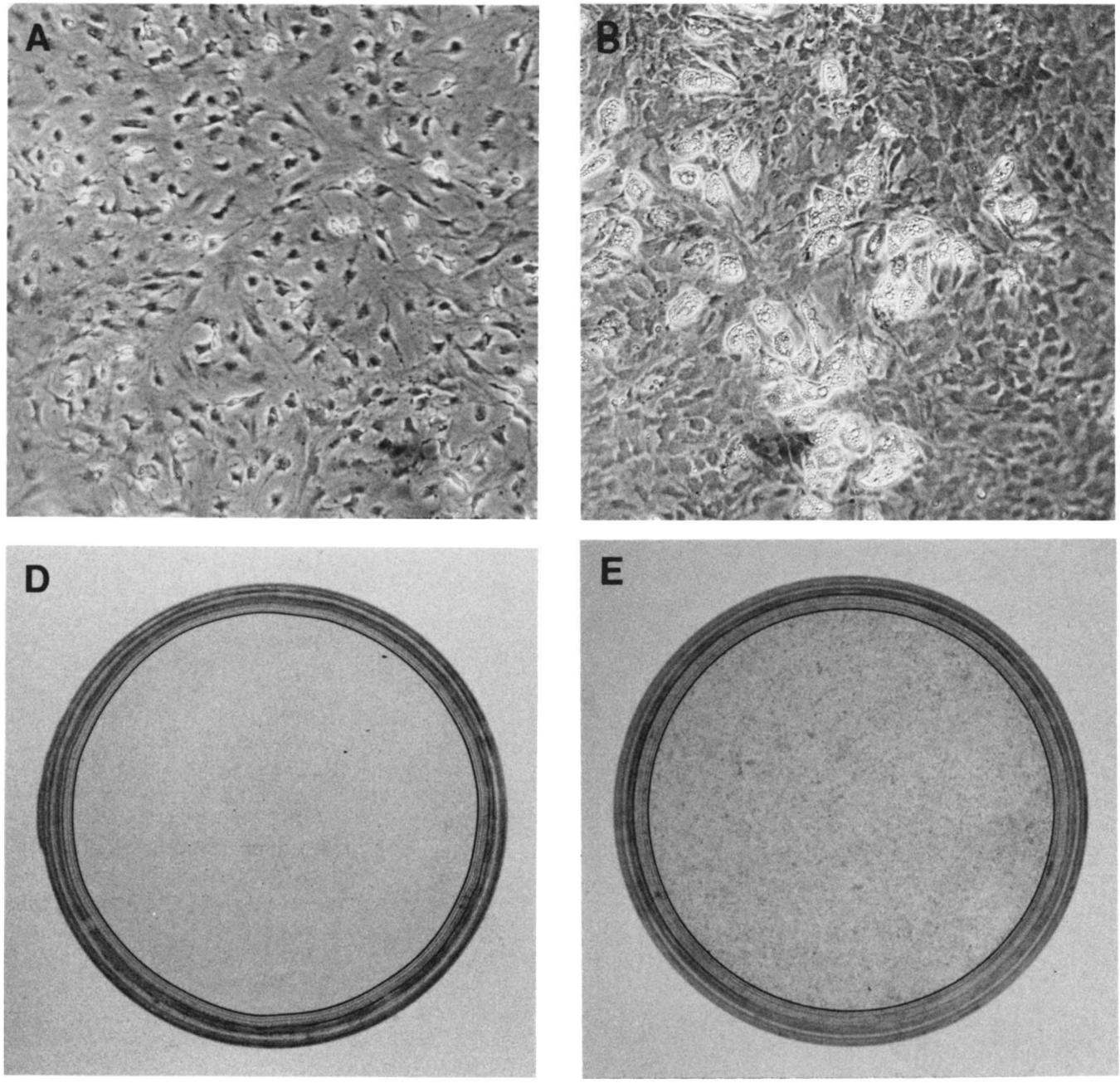
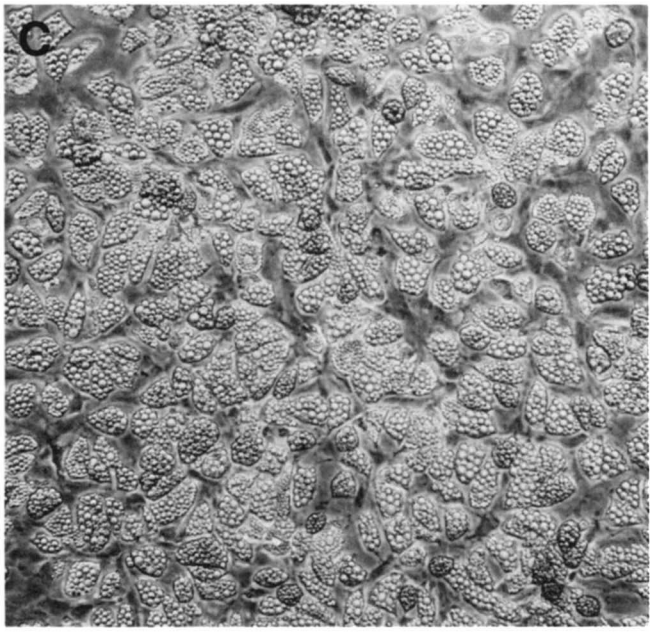


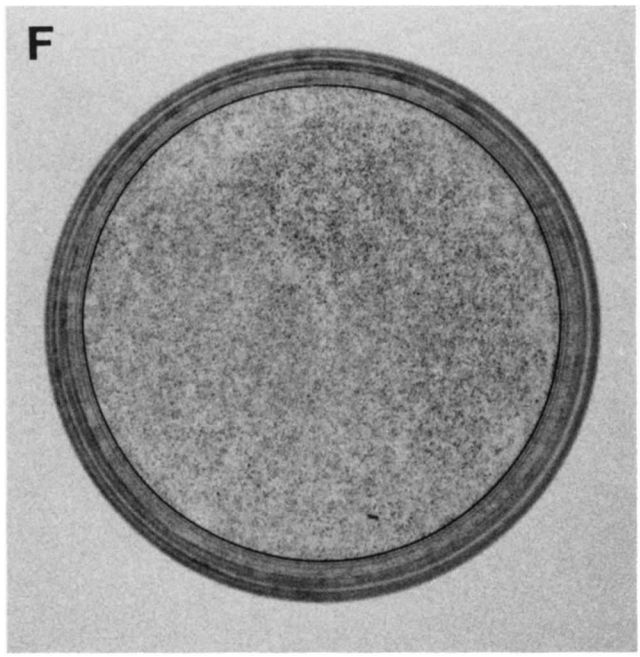
Fig. 1. Photomicrographs of rat adipose tissue precursor cells. A: Confluent preadipocytes from perirenal rat adipose tissue 4 days after inoculation at a density of 3 × 10^4 cells/cm^2 and cultured in growth medium. B: Preadipocytes from perirenal rat adipose tissue kept for 8 days after having reached confluence in the presence of insulin (10^{-8} M) and MIX (0.5 mM) for the first 2 days. C: Preadipocytes from perirenal adipose tissue kept for 8 days after having reached confluence in the presence of insulin (10^{-8} M), corticosterone (10^{-7} M) and, for the first 2 days after confluence, MIX (0.5 mM). For further details, see Methods. Magnification 125 ×. D, E, F: Oil Red O stainings of cultures treated as in A, B, and C, respectively.

At the times indicated the cultures were harvested. In sonicated cell extracts GPDH-activity was determined as a measure of adipose differentiation (12). Triglyceride concentration was determined enzymatically as triglyceride glycerol after alkaline hydrolysis of sonicated cell extracts (13). The accuracy of the assay was established with triolein as a standard. Proteins were determined by the method of Lowry et al. (14) using bovine serum albumin as a standard. DNA content of cells was determined according to Hinegardner (15) with herring sperm DNA as a standard.

For the determination of cyclic AMP, cells were broken by addition of 100 μl of ice-cold TCA (100%) into the medium. The cells then were harvested by scraping and sonicated for 5 sec. The suspension was centrifuged for 10 min at 10,000 *g*. To get rid of TCA the supernatant was extracted three times with 1 ml of diethylether acidified with 0.1 N HCl and the ether phase was discarded. The aqueous phase was evaporated to dryness under vacuum at room temperature. The residue was dissolved in 1.0 ml of buffer and cyclic AMP content was estimated by radioimmunoassay. For the visualization of cellular triglycerides, cultures were fixed with 10% formalin in phosphate-buffered saline for 4 hr at 4°C and stained with Oil Red O as previously described (16).

Charcoal-treated serum was prepared by stirring 20 ml of FCS with 1 g of charcoal (Norit A, Serva) for 30 min at room temperature. After centrifugation for 30 min at 17,000 *g*, the charcoal-treated serum was filtered through a 0.22-μm filter prior to use.





RESULTS

When stromal vascular cells obtained from either epididymal or perirenal adipose tissue and inoculated at a density of approximately 2×10^4 cells/cm^2 were kept in the growth medium described above, they reached confluence about 4 days later at a density of approximately $1\text{–}1.5 \times 10^5$ cells/cm^2. During that time DNA content increased from 2.6–3 μg/dish to 9–10 μg/dish, suggesting a doubling time of approximately 30 hr (data not shown). During this exponential growth phase only a minimal lipid incorporation was observed (**Fig. 1 A, D**). An impressive adipose conversion of these cells occurred when they reached confluence and, in addition, when the medium was enriched with insulin, corticosterone and, for the first 2 days after confluence, with MIX. Under these conditions about 80% of the cells acquired a rounded shape and became filled with numerous vacuoles, which stained intensively with Oil Red O (Fig. 1 C, F). From **Fig. 2** it follows that triglycerides accumulated only when cells were cultured in the presence of insulin together with corticosterone. In cultures in which MIX was present for the first 2 days after confluence, the rate of triglyceride accumulation increased. Maximal triglyceride concentrations were obtained under these conditions after 9–11 days. The decrease thereafter was probably the result of cell detachment. Neither MIX nor insulin alone had any significant effect on triglyceride concentration in control cultures kept in the absence of added hormones (Fig. 2B). Corticosterone as well as the combination of insulin and MIX moderately increased triglyceride accumulation, as may also be seen from the micrographs shown in Fig. 1 B, E.

In **Fig. 3** the time course of GPDH activities in preadipocyte cultures under various conditions is depicted.

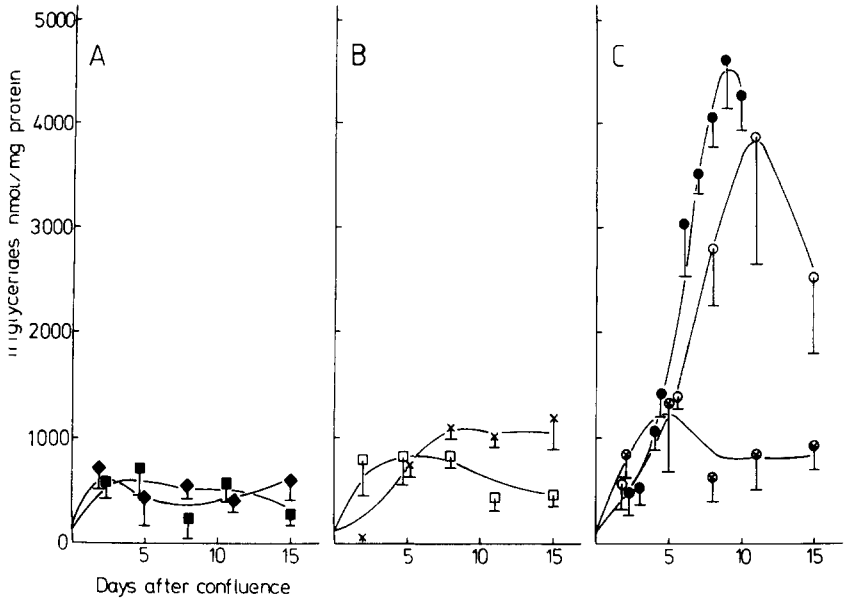


Fig. 2. Triglyceride accumulation by differentiating adipose tissue preadipocytes. Preadipocytes were prepared from rat perirenal adipose tissue and cultured as described in Methods. Differentiation was induced after having reached confluence by addition of: A, (♦) no addition, (■) MIX (0.5 mM) for the first 2 days after confluence; B, (□) insulin (10^{-8} M), (X) insulin (10^{-8} M) and, for the first 2 days after confluence, MIX (0.5 mM); C, (⊗) corticosterone (10^{-7} M), (○) corticosterone (10^{-7} M) and insulin (10^{-8} M), (●) insulin (10^{-8} M), corticosterone (10^{-7} M) and, for the first 2 days after confluence, MIX (0.5 mM). Cells were harvested as indicated for the determination of triglycerides and protein. Each point is the mean of three or four different primary cultures ± SEM.

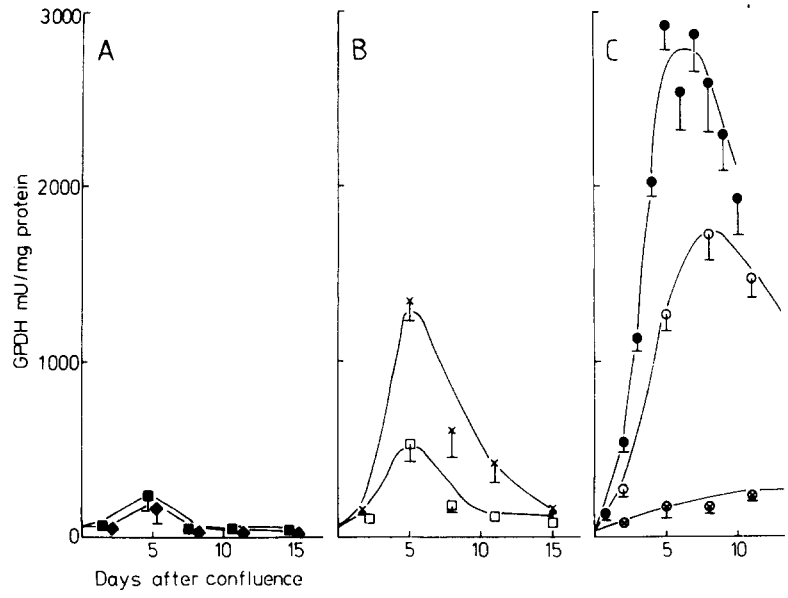


Fig. 3. GPDH activity in differentiating adipose tissue preadipocytes. Preadipocytes were prepared from rat perirenal adipose tissue and cultured as described in Methods. Differentiation was induced after having reached confluence by addition of: A, (♦) no addition, (■) MIX (0.5 mM) for the first 2 days after confluence; B, (□) insulin (10^{-8} M), (X) insulin (10^{-8} M) and, for the first 2 days after confluence, MIX (0.5 mM); C, (⊗) corticosterone (10^{-7} M), (○) corticosterone (10^{-7} M) and insulin (10^{-8} M), (●) corticosterone (10^{-7} M), insulin (10^{-8} M) and, for the first 2 days after confluence, MIX (0.5 mM). Cells were harvested as indicated for the determination of GPDH activity and protein. Each point is the mean of three or four different primary cultures ± SEM.

In control cultures to which no hormones were added, GPDH activity generally was below 40 mU/mg of protein at the start of differentiation and never exceeded 200 mU/mg protein during the next 15 days. Treatment of such cultures with MIX did not change this pattern. Addition of insulin alone in a concentration of 10^{-8} M resulted in a transitory and moderate increase of GPDH activity 5 days after confluence; however, the activity leveled off 3–4 days later. This effect of insulin was intensified by additional treatment of the cells with MIX. Corticosterone alone or in combination with MIX (data not shown) only minimally increased GPDH activity during 15 days of culture. The only conditions which gave rise to a more prolonged increase of GPDH activity during a culture time of 15 days are shown in Fig. 3C. In cells treated with the combination of insulin and corticosterone, a maximal GPDH activity of 1.7 U/mg protein was obtained 5–8 days after confluence. This value corresponds to the GPDH activity found in mature adipocytes (1.5 U ± 0.11 U/mg protein, n = 24). Additional treatment with MIX during the first 2 days after confluence led to GPDH activities of nearly 3 U/mg of protein. Beyond days 8–10, GPDH activities in differentiated cells showed a marked tendency to decrease, probably as a result of increased cell detachment (see also Fig. 2). Preadipocytes from perirenal adipose tissue consistently showed a greater tendency to differentiate and exhibited higher GPDH activities as compared to preadipocytes prepared from epididymal fat pads (see Figs. 4–6, and Table 2).

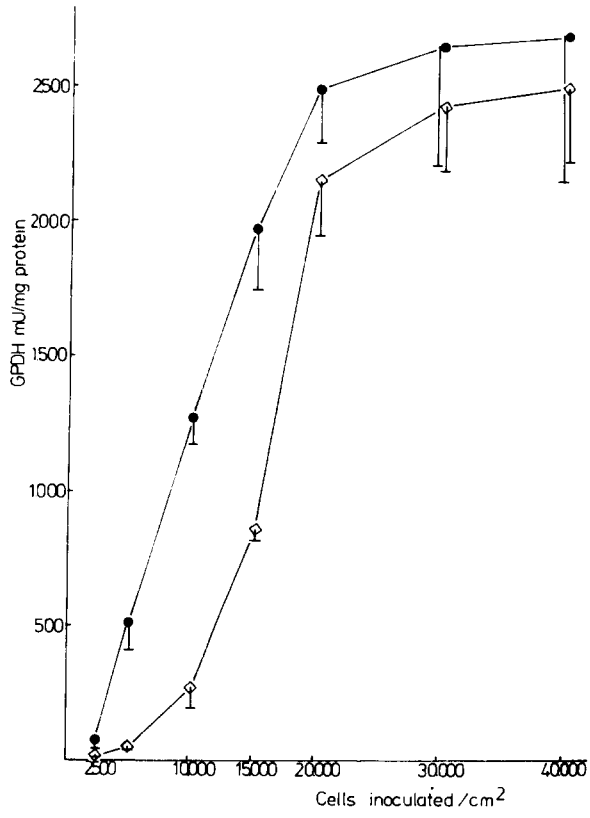


Fig. 4. Dependency of adipose conversion of rat adipose tissue preadipocytes on inoculation density. Preadipocytes prepared from perirenal (●) or epididymal (◇) rat adipose tissue were inoculated at densities as indicated. After having reached confluence differentiation was started with a medium containing corticosterone (10^{-7} M), insulin (10^{-8} M) and MIX (0.5 mM for the first 2 days). Cells were harvested 5 days later for GPDH determination as described in Methods. Each point is the mean of three different primary cultures ± SEM.

As may be seen from the data shown in **Fig. 4,** the adipose conversion of rat preadipocytes strongly depends on the density at which the cells are inoculated in the culture dishes. When cells were plated at densities below 1.5 × 10^4/cm^2, their tendency to accumulate lipid and to increase GPDH activity was markedly reduced as compared to cells plated at a density of 2 × 10^4/cm^2 or more, despite the fact that under both conditions cultures were grown to confluence before the differentiation process was started with MIX, insulin, and corticosterone. Stromal vascular cells from epididymal fat pads as compared to perirenal preadipocytes were even more sensitive to this impairment of adipose conversion by inoculation at low densities. After the first subculture of preadipocytes, a maximal adipose conversion took place only at seeding densities of at least 60,000 cells/cm^2, corresponding to a subculture ratio of 1:2 (**Table 1**). In preadipocytes subcultured twice, even at this high inoculation density, adipose conversion was greatly diminished.

The dose response relationships of corticosterone and insulin, respectively, to GPDH activities in differentiating

TABLE 1. Dependency of GPDH activity in differentiating perirenal preadipocytes on cell density at seeding and number of subcultures

	Seeding Density	Subcultures	Subculture Ratio	Divisions Needed for Confluence (Calculated)	GPDH Activity		
	cells/cm²				*mU/mg prot*	± *SEM*	
A	30,000	0		2	2733	263.6	(4)
B	120,000	1	1:1	2	2652	144.6	(4)
C	60,000	1	1:2	3	2742	125.8	(4)
D	30,000	1	1:4	4	984	291.1	(4)
E	15,000	1	1:8	5	340	67.5	(4)
F	60,000	2	1:2				
			1:2	4	866	217.1	(3)

Cells were seeded at a density of 3 × 10^4/cm^2 in growth medium and differentiation was started after cells reached confluence at a density of 1.2 × 10^5/cm^2 (A). Parallel cultures from the same batch were trypsinized and subcultured at the densities as indicated (B, C, D, E). In addition, one culture, having been treated as in C, was trypsinized again and seeded at a density of 6 × 10^4/cm^2 (F). In each case differentiation was not started before confluency was reached and was induced by addition of insulin (10^{-8} M), corticosterone (10^{-7} M), and MIX (0.5 mM, for the first 2 days). GPDH activity was determined in sonicated cell extracts 5 days after start of adipose conversion. The number of cell divisions needed to reach confluence was calculated on the basis of DNA content. In B, C, D, E, F, the first two cell divisions arose from the primary culture (A), the next ones from the corresponding subculture.

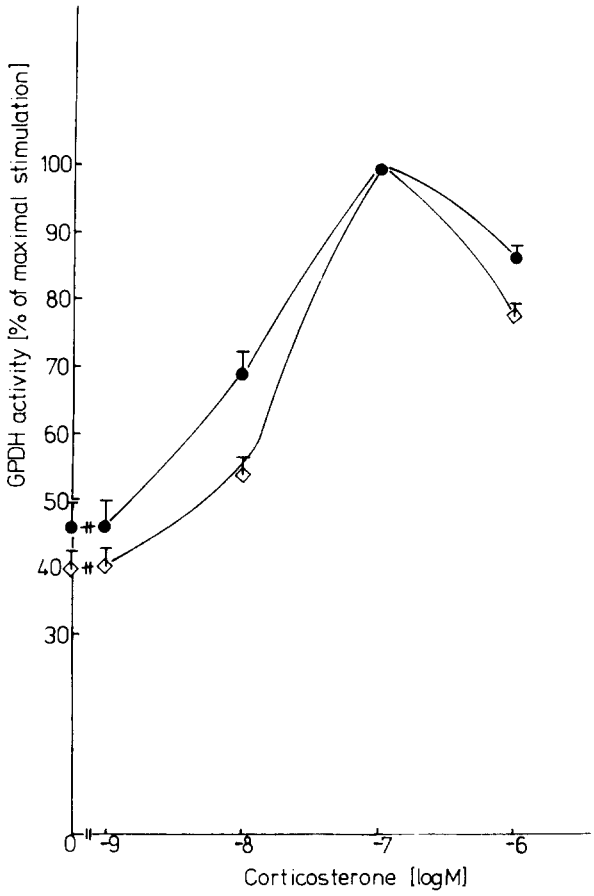


Fig. 5. Influence of corticosterone on GPDH activity in rat adipose tissue preadipocytes. Preadipocytes prepared from perirenal (●) or epididymal (◇) adipose tissue were grown to confluence. Differentiation was started by a medium containing insulin (10^{-9} M), MIX (0.5 mM for the first 2 days), and corticosterone in concentrations as indicated. GPDH activities measured 5 days later are given in % of the maximal stimulation obtained at a corticosterone concentration of 10^{-7} M. Each point is the mean of four (perirenal preadipocytes) or five (epididymal preadipocytes) different primary cultures.

adipocyte precursors from epididymal and perirenal adipose tissue are shown in **Fig. 5** and **Fig. 6.** In order to get maximal GPDH activities, in both cases MIX was added to the precursor cells for the first 2 days after start of the adipose conversion and activity was determined 5 days later. Despite the rather high GPDH activity in control cultures under these conditions, corticosterone in the presence of a physiological insulin concentration (10^{-9} M) increased GPDH activity of epididymal and perirenal precursors in a concentration range between 10^{-9} and 10^{-6} M, the maximal effective concentration being at 10^{-7} M (Fig. 5). At this glucocorticoid concentration, an effect of insulin on adipose conversion was easily demonstrated in a concentration of 10^{-10}–10^{-8} M (Fig. 6). Similar dose response curves were also obtained when the treatment of differentiating cells with MIX was omitted. Under these conditions GPDH activities, however, were considerably lower (for comparison see Fig. 3).

The data summarized in **Table 2** indicate that corticosterone may be replaced easily by cortisol but not by steroid hormones devoid of glucocorticoid activity, such as β-estradiol or testosterone. Both thyroid hormones were ineffective, although triiodothyronine has been shown to be permissive for the adipose conversion of ob17 cells (17) and stimulates GPDH activity of retroperitoneal and subcutaneous rat preadipocytes kept in a serum-free culture system (18). Growth hormone, which is an adipogenic factor for 3T3 preadipocytes and ob17 cells (9, 19–21), had no effect on GPDH activity and adipose conversion of rat preadipocytes. This was true also when cat serum instead of fetal calf serum was used as a serum substitute (**Fig. 7**).

As may be inferred from the data summarized in **Table 3,** the action of MIX on GPDH activity of preadipocytes isolated from adipose tissue is most likely due to its ability to increase the concentration of cyclic AMP. Forskolin, which has been shown to stimulate reversibly adenylate cyclase in various cells (22), increased cyclic AMP levels in preadipocytes as did MIX when added in

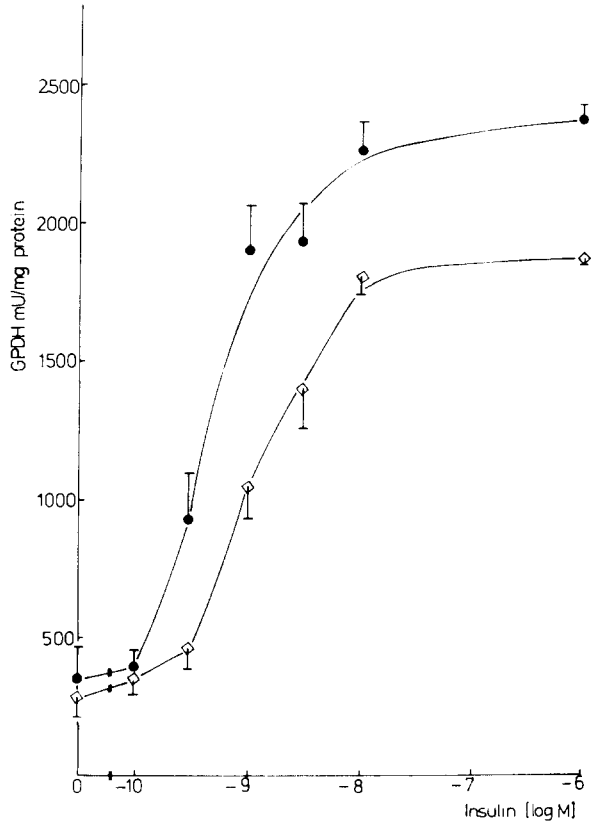


Fig. 6. Influence of insulin on GPDH activity of rat adipose tissue preadipocytes. Preadipocytes prepared from perirenal (●) or epididymal (◇) rat adipose tissue were grown to confluence. Differentiation was started by addition of medium containing corticosterone (10^{-7} M), MIX (0.5 mM for the first 2 days) and insulin in concentrations as indicated. Five days later cells were harvested for the determination of GPDH activity. Each point is the mean of four different primary cultures ± SEM.

TABLE 2. Influence of various hormones on the adipose conversion of rat adipose tissue preadipocytes

MIX	Hormone	Adipogenic Activity: Perirenal Cells Relative Units	SEM	n	Epididymal Cells Relative Units	SEM	n
	Culture in native FCS						
–		100		9	100		11
–	Corticosterone 10^{-7} M	189.9	16.2	10	133.9	14.9	12
–	Cortisol 10^{-7} M	221.7	9.9	3	125.2	15	6
–	Triiodothyronine 10^{-8} M	94.2	5.7	3	93.9	16.6	5
–	β-Estradiol 10^{-7} M	104.6	5.9	4	86.6	4.8	6
–	Testosterone 10^{-7} M	92.1	5.2	3	78	7.8	4
+		231.5	26.7	11	175.3	20.3	12
+	Corticosterone 10^{-7} M	356.2	23	11	313.3	26.6	11
+	Cortisol 10^{-7} M	302.6	13.9	3	261.9	24.8	7
+	Triiodothyronine 10^{-8} M	241.1	11.3	3	130.3	6.3	3
+	β-Estradiol 10^{-7} M	269.9	23.8	3	147.3	1	3
+	Testosterone 10^{-7} M	198.9	27.6	3	129	11.8	4
–	hGH 10^{-9} M	79.2	7.6	3	61.9	5	4
+	hGH 10^{-9} M	179.8	31.1	4	122.3	16	4
–	hGH 10^{-9} M, corticosterone 10^{-7} M	128.8	15.5	3	95.3	9.1	7
+	hGH 10^{-9} M, corticosterone 10^{-7} M	200.9	21.2	3	179	22.1	5
	Culture in charcoal-treated FCS						
–		99.1	14.7	11			
–	Corticosterone 10^{-7} M	181.2	29.6	9			
–	Triiodothyronine 10^{-8} M	93.3	8.4	6			
–	Triiodothyronine 10^{-9} M	100.9	7.8	6			
–	β-Estradiol 10^{-7} M	108.2	12.8	6			
–	Testosterone 10^{-7} M	105.8	7.2	6			
+		178.5	24.6	6			
+	Corticosterone 10^{-7} M	268.4	24.1	9			

Confluent preadipocytes, obtained from perirenal or epididymal adipose tissue as described in Methods, were induced to differentiate in a medium containing 5% FCS, 10^{-9} M insulin, and the additions as indicated. MIX was used in a concentration of 0.5 mM and was added only for the first 2 days after confluence. All values are given relative to the GPDH activity obtained in perirenal and epididymal preadipocytes, respectively, kept in the presence of 10^{-9} M insulin for 5 days after confluence. Under these conditions GPDH activities were 601 and 473.5 mU/mg of protein in perirenal and epididymal preadipocytes, respectively. By charcoal treatment the concentrations of steroid as well as of thyroid hormones were reduced to values below 10^{-13} M as measured by radioimmunoassays for β-estradiol, testosterone, thyroxine and triiodothyronine.

a concentration of 10 μM for the first 2 days after confluence. In addition, its effects on GPDH activity in differentiating preadipocytes resembled those of MIX. The data in Table 3 also clearly indicate that neither insulin nor corticosterone act by increasing cyclic AMP levels of preadipocytes and that an increase in cellular cyclic AMP levels alone failed to induce GPDH activity.

DISCUSSION

The adipose conversion of stromal vascular cells prepared from adipose tissue of various species has been described repeatedly (4–8, 23–25). Generally rather high concentrations of either FCS or human serum together with insulin concentrations several 100-fold higher than physiological have been used to induce differentiation. Under these conditions it is difficult to clearly demonstrate a hormonal regulation of the differentiation process. Gaben-Cogneville et al. (26, 27) were the first to show that the differentiation of preadipocytes from newborn rat adipose tissue depends on insulin within a physiological concentration range. In addition, evidence was presented recently by Deslex, Negrel, and Ailhaud (18) that IGF I and triiodothyronine are also involved in the adipose conversion of retroperitoneal and subcutaneous preadipocytes kept in a serum-free medium.

The conditions chosen in the present study show a clear dependency of the adipose conversion of stromal vascular cells isolated from epididymal and perirenal adipose tissue of adult rats on the presence of an adequate hormonal environment. Stromal vascular cells are not able to accumulate lipids and to acquire the characteristics of adipocytes unless they are treated with a combination of insulin and corticosterone, both in physiological concen-

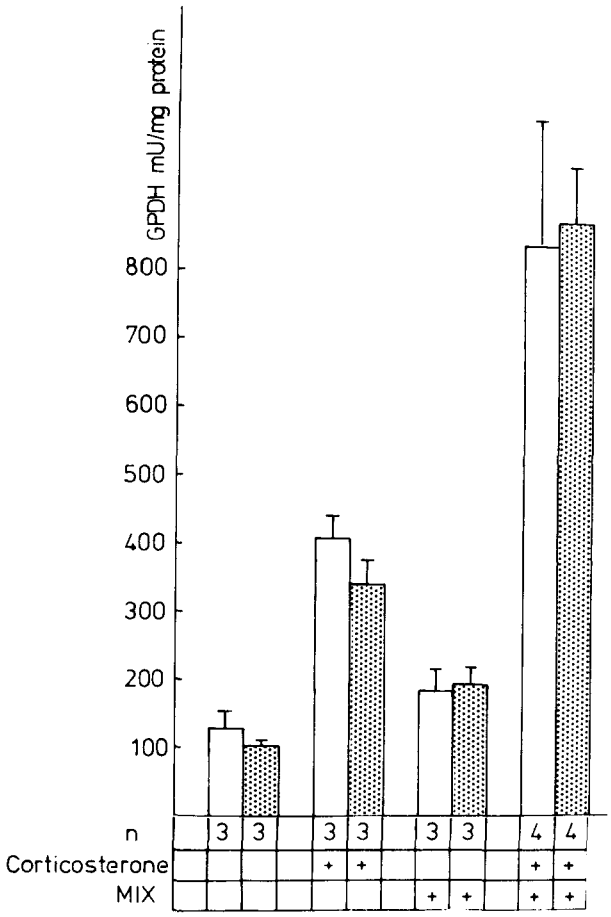


Fig. 7. Adipose conversion of rat adipose tissue preadipocytes kept in medium containing cat serum. Preadipocytes prepared from rat perirenal adipose tissue were grown to confluence as indicated in Methods. Differentiation was started by a medium containing insulin (10^{-9} M), 5% cat serum instead of FCS. Corticosterone (10^{-7} M) and MIX (0.5 mM for the first 2 days) were added as indicated. The shaded bars represent cultures containing hGH in a concentration of 10^{-9} M. Each bar represents the mean from three different primary cultures ± SEM.

trations. The rate of triglyceride accumulation is accelerated when the intracellular cyclic AMP concentration is increased during the first 2 days after confluence. Differentiated adipocytes do not appear in clusters but are distributed homogeneously in culture dishes, indicating that a rather high proportion of the inoculum is able to undergo adipose conversion.

Insulin in physiological concentrations leads to an increase of GPDH activities in differentiating stromal vascular cells. This effect is transitory and rather modest with insulin alone, and more pronounced and delayed in the presence of insulin together with a glucocorticoid hormone. An elevation of the cyclic AMP concentration during the first 2 days of differentiation potentiates the effects of insulin on GPDH activities and has no influence on glucocorticoid effects.

In 3T3 preadipocytes an accelerating effect of cyclic AMP on adipose conversion was suggested originally by Russel and Ho (28) and later, on the basis of studies with various other phosphodiesterase inhibitors, by Elks and Manganiello (29). In rat preadipocytes Björntorp et al. (30) described a significant but, on the average, a very limited effect of MIX on differentiation. From our results we conclude that cyclic AMP in rat preadipocytes accelerates the differentiation process similar to that in 3T3 preadipocytes and, additionally, potentiates the insulin effect on GPDH activity.

Growth hormone is essential for the differentiation of 3T3 preadipocytes and ob17 cells (9, 19–21). In stromal vascular cells no effect of GH is demonstrable. The assumption that added hGH is inactive, due to high GH concentrations of the FCS in the culture medium, is rather unlikely as GH also fails in the presence of cat serum to stimulate adipose conversion of rat preadipocytes. Cat serum does not promote differentiation of preadipocytes either due to a very low GH content or because cat GH does not act on murine cells (31).

From the steroid hormones studied so far only those with glucocorticoid activity stimulate adipose conversion. This has been shown for 3T3 preadipocytes (32, 33) and is true also for stromal vascular cells. Our data indicate that glucocorticoids act as permissive factors for the insulin effects on differentiation. Neither β-estradiol, which has been reported to stimulate proliferation of human fat tissue fibroblasts (34), nor testosterone has any influence on the differentiation process. Triiodothyronine, which is an essential cofactor for the adipose differentiation of ob17 cells (17), and has also been reported to be active in preadipocytes kept in a serum-free medium (18), has no effect on rat preadipocytes under our culture conditions. The reason for this discrepancy is unclear at the moment.

The ineffectiveness of thyroid or sex hormones was also preserved when charcoal-treated FCS, which is devoid of thyroid and steroid hormones, was used. This excludes the fact that already saturating concentrations of both hormones are introduced by FCS. Deslex et al. (18) have shown very recently that in a serum-free culture system the adipose conversion of retroperitoneal and subcutaneous rat preadipocytes depends on the presence of IGF I in a concentration range from 10 to 50 nM. Based on reported concentrations of IGF I in FCS (35), the IGF I concentration in our medium preparation should not have exceeded 0.5 nM and, therefore, would be ineffective.

A full differentiation with a high yield of adipose-like cells is obtained only when cell density at inoculation is kept above 2×10^4 cells/cm^2. In subcultures, this minimal cell density increases markedly. This finding extends earlier observations (6, 30). Due to the sharp decline in the capacity of preadipocytes to undergo adipose conversion at low seeding densities, it seems rather unlikely that this effect is due to an outgrowth of a cell population not

TABLE 3. Influence of MIX and forskolin on GPDH activities and cyclic AMP concentrations in preadipocytes obtained from perirenal rat adipose tissue

Insulin 10^{-9} M	Corticosterone 10^{-7} M	MIX 0.5 mM	Forskolin 10^{-5} M	GPDH mU/mg of prot	GPDH SEM	GPDH n	Cyclic AMP pmol/mg of prot	Cyclic AMP SEM	Cyclic AMP n
–	–	–	–	201.6	119.6	6	150.9	7.9	3
–	–	+	–	298.3	102.7	6	366.4	54.8	3
–	–	–	+	385.3	141.6	3	725.7		2
–	+	–	–	181.5	87.7	6	181.5	25.4	3
–	+	+	–	346	95.6	6	463.1	59.9	3
–	+	–	+	531	177.5	3	890.3		2
+	–	–	–	601.3	113.4	9	169.5	29.2	3
+	+	–	–	1141	97.1	10	122	9.5	3
+	–	+	–	1391.2	160.8	11	372.4	24.5	3
+	–	–	+	1496	193	4	435.2	80.4	3
+	+	+	–	2140.8	138.7	11	388.9	26.4	3
+	+	–	+	2333.3	262	4	550.5	112	3

Preadipocytes obtained from perirenal rat adipose tissue were grown to confluence as described in Methods. Differentiation was induced by addition of a medium containing 5% FCS and other additions as indicated. Cells were harvested 2 days later for the determination of cyclic AMP concentration and 5 days later for the measurement of GPDH activity.

determined to differentiate. Possibly tissue-specific local factors that get lost during repeated cell divisions or in subcultures are necessary to obtain a complete differentiation. Components of the extracellular matrix, whose synthesis is no longer possible under culture conditions, would be candidates for such a function.

This study was supported by a grant from the Deutsche Forschunggemeinschaft, SFB 43.

Manuscript received 15 May 1986 and in revised form 21 November 1986.

REFERENCES

1. Green, H. 1978. The adipose conversion of 3T3 cells. *In* Differentiation and Development. F. Ahmad, T. R. Russel, J. Schultz, and R. Werner, editors. Academic Press, New York. 13–36.
2. Russel, T. R. 1981. Growth and cytodifferentiation of 3T3-L1 preadipocytes into adipocytes. *Methods Enzymol.* **72:** 720–723.
3. Vannier, C., D. Gaillard, P. Grimaldi, E-Z. Amri, P. Djian, C. Cermolacce, C. Forest, J. Etienne, R. Negrel, and G. Ailhaud. 1985. Adipose conversion of ob17 cells and hormone-related events. *Int. J. Obes.* **9, Suppl. 1:** 41–53.
4. Björntorp, P., M. Karlsson, H. Pertoft, P. Pettersson, L. Sjöström, and U. Smith. 1978. Isolation and characterization of cells from rat adipose tissue developing into adipocytes. *J. Lipid Res.* **19:** 316–324.
5. Broad, T. E., and R. G. Ham. 1983. Growth and differentiation of sheep preadipocyte fibroblasts in serum-free medium. *Eur. J. Biochem.* **135:** 33–39.
6. Hausman, G. J., J. E. Novakofsky, R. J. Martin, and G. B. Thomas. 1984. The development of adipocytes in primary stromal vascular culture of fetal pig adipose tissue. *Cell Tissue Res.* **236:** 459–464.
7. Cryer, A., B. R. Gray, and J. S. Woodhead. 1984. Studies on the characterization of bovine adipocyte precursor cells and their differentiation in vitro, using an indirect-labelled-second-antibody cellular immunoassay. *J. Dev. Physiol.* **6:** 159–176.
8. Van, R. L. R., and D. A. K. Roncari. 1978. Complete differentiation of adipocyte precursors. A culture system for studying the cellular nature of adipose tissue. *Cell Tissue Res.* **195:** 317–329.
9. Morikawa, M., T. Nixon, and H. Green. 1982. Growth hormone and the adipose conversion of 3T3 cells. *Cell.* **29:** 783–789.
10. Rubin, C. S., A. Hirsch, C. Fung, and O. M. Rosen. 1978. Development of hormone receptors and hormonal responsiveness in vitro. *J. Biol. Chem.* **253:** 7570–7578.
11. Grimaldi, P., P. Djian, R. Negrel, and G. Ailhaud. 1982. Differentiation of ob17 preadipocytes to adipocytes: requirement of adipose conversion factor(s) for fat cell cluster formation. *EMBO J.* **1:** 687–692.
12. Pairault, J., and H. Green. 1979. A study of the adipose conversion of suspended 3T3 cells by using glycerophosphate dehydrogenase as differentiation marker. *Proc. Natl. Acad. Sci. USA.* **76:** 5138–5142.
13. Eggstein, M., and E. Kuhlmann. 1974. Triglyceride und Glycerin. *In* Methoden der enzymatischen Analyse. H. U. Bergmeyer, editor. Verlag Chemie, Weinheim, FRG. 1871–1878.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
15. Hinegardner, R. T. 1971. An improved fluorimetric assay for DNA. *Anal. Biochem.* **39:** 197–201.
16. Lillie, R. D. 1944. Various oil-soluble dyes as fat stains in the supersaturated isopropanol technic. *Stain Technol.* **19:** 55.
17. Gharbi-Chihi, J., O. Chabaud, and J. Torresani. 1984. The role of triiodothyronine in the regulation of synthesis rate and translatable mRNA level of fatty acid synthetase in a preadipocyte cell line. *Biochim. Biophys. Acta.* **783:** 26–35.
18. Deslex, S., R. Negrel, and G. Ailhaud. 1987. Development of a chemically defined serum-free medium for differentiation of rat adipose precursor cells. *Exp. Cell. Res.* **168:** 15–30.
19. Morikawa, M., H. Green, and U. J. Lewis. 1984. Activity

of human growth hormone and related polypeptides on the adipose conversion of 3T3 cells. *Mol. Cell Biol.* **4:** 228–231.
20. Green, H., M. Morikawa, and T. Nixon. 1985. A dual effector theory of growth hormone action. *Differentiation.* **29:** 195–198.
21. Ailhaud, G., E. Amri, C. Cermolacce, P. Djian, C. Forest, D. Gaillard, P. Grimaldi, J. Khoo, R. Negrel, G. Serrero, and C. Vannier. 1983. Hormonal requirement for growth and differentiation of ob17 preadipocyte cells in vitro. *Diabete Metab.* **9:** 125–133.
22. Daly, J. W. 1984. Forskolin, adenylate cyclase and cell physiology: an overview. *Adv. Cyclic Nucleotide Res.* **17:** 81–99.
23. Johnson, P. R., and A. L. Goldstein. 1981. Uncontrolled adipocyte differentiation is not the primary lesion in the genetically obese Zucker rat. *Int. J. Obes.* **5:** 563–570.
24. Bourgois, F., A. L. Goldstein, and P. R. Johnson. 1983. Lipogenesis in primary cultures of adipoblasts derived from genetically obese Zucker rats. *Metabolism.* **32:** 673–679.
25. Pettersson, P., R. Van, M. Karlsson, and P. Björntorp. 1985. Adipocyte precursor cells in obese and nonobese humans. *Metabolism.* **34:** 808–812.
26. Gaben-Cogneville, A. M., Y. Aron, G. Idriss, T. Jahchan, J. Y. Pello, and E. Swierczewski. 1983. Differentiation under the control of insulin of rat preadipocytes in primary culture. Isolation of homogenous cellular fractions by gradient centrifugation. *Biochim. Biophys. Acta.* **762:** 437–444.
27. Gaben-Cogneville, A. M., A. Quignard-Boulange, Y. Aron, L. Brigant, T. Jahchan, J. Y. Pello, and E. Swierczewski. 1984. Development under the control of insulin of lipogenic enzymes, lipoprotein lipase, isoproterenol and glucagon sensitivity in differentiating rat preadipocytes in primary culture. *Biochim. Biophys. Acta.* **805:** 252–260.
28. Russel, T. R., and R. J. Ho. 1976. Conversion of 3T3-fibroblasts into adipose cells: triggering of differentiation by prostaglandin F_{2a} and 1-methyl-3-isobutyl xanthine. *Proc. Natl. Acad. Sci. USA.* **73:** 4516–4520.
29. Elks, M. L., and V. C. Manganiello. 1985. Role of soluble cAMP phosphodiesterase in differentiation of 3T3-L1 adipocytes. *J. Cell. Physiol.* **124:** 191–198.
30. Björntorp, P., M. Karlsson, P. Pettersson, and G. Sypniewska. 1980. Differentiation and function of rat adipocyte precursor cells in primary culture. *J. Lipid Res.* **21:** 714–723.
31. Kuri-Harcuch, W., and H. Green. 1978. Adipose conversion of 3T3 cells depends on a serum factor. *Proc. Natl. Acad. Sci. USA.* **75:** 6107–6109.
32. Schiwek, D., and G. Löffler. 1987. Glucocorticoid hormones contribute to the adipogenic activity of human serum. *Endocrinology.* **120:** 469–474.
33. Feick, P., and G. Löffler. 1986. Adipogenic activities in rat organs. *Biol. Chem. Hoppe-Seyler.* **367:** 549–558.
34. Roncari, D. A. K., and R. L. R. Van. 1978. Promotion of human adipocyte precursor replication by 17β-estradiol in culture. *J. Clin. Invest.* **62:** 503–508.
35. Honegger, A., and R. E. Humbel. 1986. Insulin-like growth factors I and II in fetal and adult bovine serum. *J. Biol. Chem.* **261:** 569–575.