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Cell Biology

A study of the adipose conversion of suspended 3T3 cells by using glycerophosphate dehydrogenase as differentiation marker

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ABSTRACT **The adipose conversion of 3T3 cells has been examined in stabilized suspension cultures. In 3T3-F442A cells, glycerophosphate dehydrogenase (*sn*-glycerol-3-phosphate: NAD^+ 2-oxidoreductase, EC 1.1.1.8), a key enzyme in triglyceride synthesis, increases in specific activity by more than 5000-fold and can be used as a sensitive and precise measure of the conversion. The conversion depends on an adipogenic factor present in the serum, and this factor can be assayed by the cellular enzyme response. If the cells are growing at the time they receive the adipogenic factor, the enzyme response does not become detectable until after 3 days, during which the cells first enter a resting state. If the cells are resting at the time they receive the adipogenic factor, the enzyme activity begins to increase in 24 hr or less. Only resting cells seem susceptible to the reprogramming of their differentiated state necessary for the adipose conversion. Once the conversion begins, the increase in enzyme activity is exponential over at least 2 orders of magnitude. When cells in a resting state begin the adipose conversion, their biosynthetic processes are accelerated: the rate of protein synthesis increases, they accumulate cell protein, and they may replicate their DNA and divide. The cell multiplication is not essential for adipose conversion but is a form of clonal selection that increases the proportion of adipose cells relative to nonadipose cells.**

When preadipose 3T3 cells in surface culture reach a confluent state, their growth rate decreases greatly and many of the cells undergo adipose conversion. (1). Although most studies have been carried out in surface cultures, it is known that the adipose conversion does not require the cell–cell contacts that develop under these conditions because it also takes place in separated cells stably suspended with the aid of methycellulose (2).

Among the changes that take place during the adipose conversion are increases in the activity of lipogenic enzymes (3–8) and increases in the responsiveness of the cells to hormones affecting lipogenesis and lipolysis (9–11). A key enzyme in the lipogenic process is glycerophosphate dehydrogenase (*sn*-glycerol-3-phosphate:NAD^+ 2-oxidoreductase, EC 1.1.1.8) there is a large increase in this enzymic activity during the conversion (12, 13).

The adipose conversion depends on the presence of a nondialyzable adipogenic factor in the serum (14). The chemical nature of this factor has not yet been determined, but no adipose conversion takes place in its absence. We describe here some of the changes that take place after susceptible cells interact with the adipogenic factor. In order to establish the relationship between any observed effects and the adipose conversion, we carried out most experiments of two cell lines, 3T3-F442A and 3T3-C2, known to undergo the adipose conversion with high and low frequency, respectively (15).

MATERIALS AND METHODS

Cell Culture and Enzyme Assay. Cells were inoculated into surface culture at about 5×10^4 per 100-mm petri dish and were grown in Dulbecco–Vogt medium supplemented with 10% calf serum or, in later experiments, 10% cat serum. Suspension cultures were prepared by inoculating cells of trypsinized surface cultures at $0.5–1.0 \times 10^5$ cells per ml of medium stabilized with methylcellulose (16). The final concentration of methylcellulose was 1.1%, and Ca^{2+} was decreased to 0.1 mM. Suspension cultures were supplemented with serum previously dialyzed overnight at 4°C against 10 vol of Ca^{2+}-free medium. The medium also contained insulin (5 μg/ml) and biotin (0.1 μM). The cultures were usually made in 250-ml polycarbonate erlenmeyer flasks (Corning) and were equilibrated with 10% CO_2/90% air.

At intervals, aliquots (5–10 ml) of methylcellulose-stabilized suspension containing $2.5–10 \times 10^5$ cells were diluted 1:10 in serum-free medium. The cells were centrifuged, washed once with isotonic buffer, resuspended in 0.4 ml of 25 mM Tris, pH 7.5/1 mM EDTA, and disintegrated sonically for 5–10 seconds at 30 W with the microtip of a Branson model 185 sonifer. The suspension was centrifuged at $100,000 \times g$ for 1 hr at 4°C. The final supernatants were stored at 70°C for several weeks without any change in enzyme activity.

Assay of glycerophosphate dehydrogenase was carried out by a method slightly modified (13) from that of Kozak and Jensen (17).

DNA and Protein Synthesis. Aliquots (5 ml) of methylcellulose suspension containing about 2.5×10^5 cells were diluted in 10 vol of serum-free medium. The cells were centrifuged and resuspended in 1 ml of regular or leucine-free medium for [^{3}H]thymidine or [^{3}H]leucine incorporation, respectively. In both cases the medium was supplemented with 0.5% calf serum. Incubations were carried out in bacteriological dishes at 37°C under 10% CO_2/90% air for 1 hr in the presence of 10 μCi (1 Ci = 3.7×10^{10} becquerels) of [*methyl*-^{3}H]thymidine (60 Ci/mmol; New England Nuclear) or 5 μCi of [4,5-^{3}H]leucine (60 Ci/mmol, New England Nuclear). Trichloroacetic acid-precipitable material was deposited on glass fiber filters and counted. In experiments involving the use of 1-β-D-arabinofuranosylcytosine (cytosine arabinoside; AraC) the drug was present in the washing and incubation media at the same concentration as in the suspension culture medium.

Scoring of Cells by Microscopy. Cell clusters were examined in a hemocytometer chamber under Nomarski optics. For counting of individual cells of diluted suspension cultures, the cells were first trypsinized to disaggregate any clusters. To score

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Abbreviation: AraC, 1-β-D-arabinofuranosylcytosine (cytosine arabinoside).

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individual cells by their triglyceride content, the cells were fixed for 15 min with 10% glutaraldehyde in isotonic buffer and then deposited by suction on a Teflon-coated membrane filter (FHLP 02500; Millipore). The cells were stained for 10 min with 0.3% Oil Red O in 60% isopropanol. Filters were carefully washed in distilled water and counterstained for 5–7 sec with hematoxylin to identify nuclei. After several additional washings in distilled water, filters were placed wet on glass slides and covered with glycerin jelly for microscopic examination.

RESULTS

Development of Glycerophosphate Dehydrogenase in Suspended Cells. Exponentially growing 3T3-C2 and 3T3-F442A cells were inoculated into surface cultures and fed with medium supplemented with 10% calf serum. When the cells had grown to confluence, they were trypsinized, transferred to suspension culture, and fed with medium containing 10% fetal calf serum. At intervals, aliquots of cell suspension were removed and the glycerophosphate dehydrogenase activity of the cells was determined.

The initial enzyme activity in 3T3-C2 cells was about 1 unit/mg of cell protein (Fig. 1). The value did not change for 6 days; then it increased about 6-fold. The enzyme activity in 3T3-F442A cells, initially about 6 units/mg of protein, began to increase immediately after inoculation. The increase continued to be exponential for about 5 days, the enzyme activity doubling each 15 hr. The activity then increased more slowly, and on day 11 it reached about 5000 units/mg, a level appreciably higher than that attained previously (13) and about 500 times that of 3T3-C2 under the same conditions. If the relative enzyme activity of the two lines reflects the proportion of cells undergoing adipose conversion (a reasonable supposition in the light of what follows), the conversion frequency for 3T3-C2 is less than 0.2%.

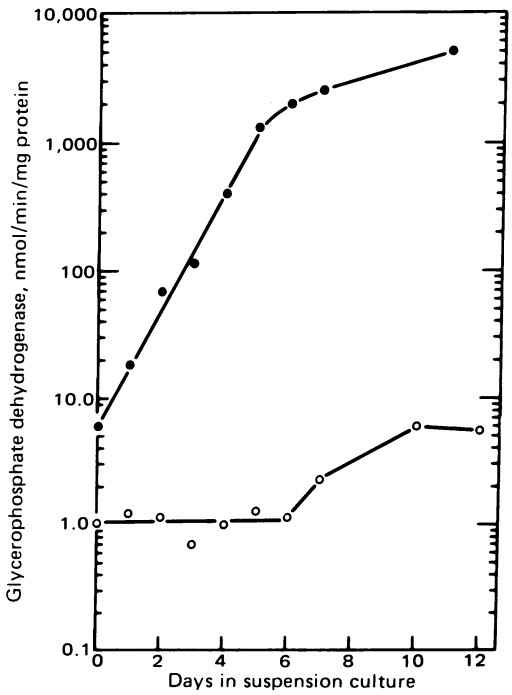

FIG. 1. Development of glycerophosphate dehydrogenase activity in cultures of suspended cells. Cells were grown to confluence in medium supplemented with 10% calf serum and then inoculated into stabilized suspension culture containing medium supplemented with 10% fetal calf serum. In 3T3-F442A cells (●), the enzyme activity increased exponentially with a doubling time of 15 hr. In 3T3-C2 cells (○), the enzyme activity did not increase for about 6 days; then increased only slightly, consistent with the low susceptibility to conversion that is characteristic of the line.

In the experiment just described, the cells were placed in suspension culture after they had become confluent in surface culture. The initial value for enzyme activity was higher in 3T3-F442A cells than in 3T3-C2 cells because the adipose conversion was already in progress at the time they were inoculated. 3T3-F442A cultures were therefore grown with special attention to eliminating all adipose cells. This was accomplished by two serial transfers, harvesting each time when the cells were sparse and in exponential growth. The initial glycerophosphate dehydrogenase activity was now 0.8–1.0 unit/mg protein, identical to that of 3T3-C2 cells harvested from similar cultures; during the period of suspension culture, it increased about 5000-fold. Because it is known that 90–100% of the enzyme of growing 3T3-F442A cells (as well as of 3T3-C2 cells) is a thermally unstable isozyme different from the stable isozyme of adipose 3T3-F442A cells (13), the increase in activity of the stable isozyme during the adipose conversion should exceed 50,000-fold.

Lag between Addition of Adipogenic Factor and Onset of Enzyme Response. To examine the kinetics of the enzyme response, it was desirable to avoid exposure of the cells to the adipogenic factor prior to their inoculation into suspension culture. For as yet unknown reasons, the serum of the cat possesses virtually no adipogenic factor (14). 3T3-F442A cells were grown in medium supplemented with 10% cat serum, harvested while still in exponential growth, and inoculated into suspension culture containing the same serum supplement. Fetal calf serum was added to a final concentration of 10%, either immediately or 3 days later. By this means it was possible to define precisely the time of interaction of the cells with the adipogenic factor and to demonstrate a lag between exposure to adipogenic factor and the enzyme response.

As expected, the enzyme activity at time zero was 0.9 unit/mg (Fig. 2). When fetal calf serum was added at time zero, the enzyme activity remained virtually constant for 3 days and then began to increase exponentially, with a doubling time of about 10 hr continuing over about 2 orders of magnitude. Thereafter, the enzyme activity increased more slowly. When the inoculated cells were allowed to incubate in suspension culture for 3 days before the addition of adipogenic serum, the lag was much less (24 hr instead of 3 days). Then the exponential increase in activity followed with about the same slope. This shows that the cells had undergone a change in suspension culture that enabled them to respond more quickly to the adipogenic factor. The remaining lag may be more apparent than real, because it could represent merely the time for the activity of the stable isozyme of adipose cells to become detectable over the background activity due to unstable isozyme.

When suspended 3T3-F442A cells were exposed only to cat serum, their enzyme activity did not change appreciably for about 6 days, and then it increased to a final level of 20 units/mg of protein. This value is only 1/200th that elicited by fetal calf serum. Adipose cells could be seen with a frequency of less than 0.5% compared with 60% for the cells exposed to fetal calf serum.

Assay of the Adipogenic Factor. Knowing the kinetics of the enzyme response to the adipogenic factor, we were able to devise a better assay for the factor. 3T3-F442A cells were grown in medium supplemented with 10% cat serum and inoculated into suspension culture with the same serum supplement. After 3 days, different amounts of calf serum or fetal calf serum were added. On days 7 and 13, the cells were assayed for glycerophosphate dehydrogenase. The enzyme activity developing in

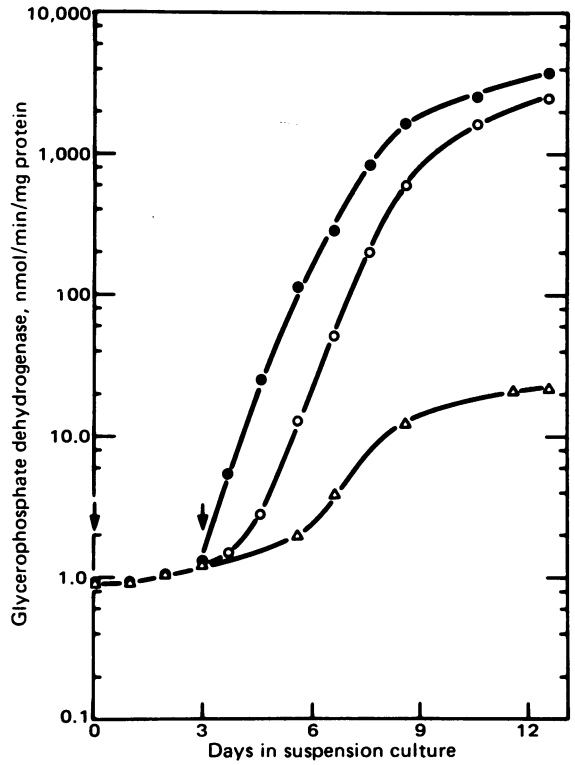

FIG. 2. Lag between first exposure to adipogenic factor and onset of enzymatic differentiation. 3T3-F442A cells were grown in surface cultures in medium containing 10% cat serum. The cells were then transferred to stabilized suspension cultures, again containing 10% cat serum. Fetal calf serum was added to a final concentration of 10%, either immediately (●) or 3 days later (○); a control received no fetal calf serum (△).

the cells depended on the amount of calf or fetal calf serum added (Fig. 3). Fetal calf serum was 5–10 times more adipogenic than calf serum. The adipogenic effect of cat serum is imperceptible on the linear ordinate.

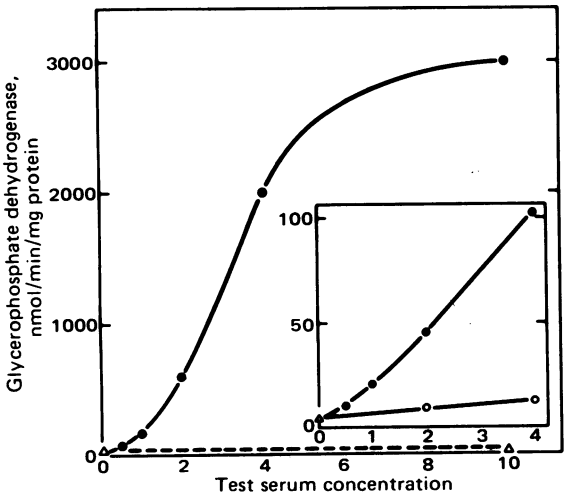

FIG. 3. Assay of adipogenic factor. 3T3-F442A cells growing exponentially in surface cultures containing 10% cat serum were harvested and inoculated as suspension cultures in tubes containing stabilized medium supplemented with 10% cat serum. Three days later, fetal calf serum (●) or calf serum (○) was added to the indicated concentrations. On days 7 (*Inset*) and 13 (*Main Curves*) after inoculation, aliquots of suspension were withdrawn and the glycerophosphate dehydrogenase activity of the cells was determined. ---, Control (cat serum only).

The number of adipose cells was also scored by their triglyceride accumulation on day 9. Although this determination is less accurate, the results were consistent with the behavior of the enzyme. The proportion of adipose cells was 60% with fetal calf serum, 5% with calf serum, and 0.5% with cat serum. It seems probable that the enzyme activity induced by the adipogenic factor depends only on the number of cells induced to undergo the conversion.

Rate of Protein Synthesis during the Adipose Conversion. It was noted earlier that in surface cultures the adipose conversion of 3T3 cells was accompanied by an increase in cell number and in the protein content per cell (3, 11, 18). To examine this aspect of the conversion, suspended cell cultures of 3T3-F442A were prepared in medium containing 10% cat serum and aliquots were withdrawn at intervals for measurement of ability to incorporate [^{3}H]leucine (Fig. 4). As shown for other 3T3 cells (19) and a related cell type, 3T6 (20), the incorporation rate began to decrease very soon after the inoculation of the cells into suspension culture and reached a level of about 10% of the initial rate in 36 hr. When fetal calf serum was then added to a final concentration of 10% on day 3, the incorporation rate began to increase and continued to do so for the remainder of the experiment, ultimately reaching about half the rate of exponentially growing cells. In contrast, the addition of fetal serum to suspended resting 3T3-C2 cells produced virtually no change in their rate of leucine incorporation.

In similar experiments, the addition of fetal calf serum to a culture of 3T3-F442A cells was found to increase their cystosolic protein content about 4-fold within 7 days. Because there was some cell multiplication (see below), the protein content per cell increased only about 3-fold; but, because this increase may be ascribed to only those cells undergoing the conversion (50%),

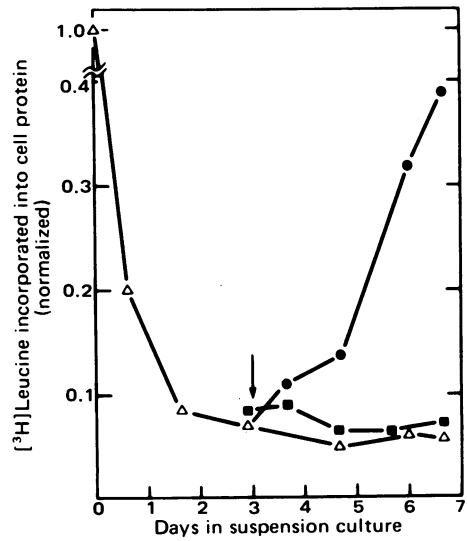

FIG. 4. Rate of amino acid incorporation during adipose conversion of 3T3 cells. Exponentially growing 3T3-F442A cells were inoculated into suspension cultures in medium supplemented with 10% cat serum. At intervals, a 5-ml aliquot was removed and the cells were exposed to [^{3}H]leucine for 1 hr. The results are expressed as total radioactivity incorporated relative to that at time zero. Within 3 days, the incorporation rate fell to less than 10% that of exponentially growing cells and remained at this low level (△). After the addition of fetal calf serum (final concentration, 10%) to one such culture (arrow), the rate of amino acid incorporation by the cells began to increase and continued to do so for at least 4 days (●). The addition of fetal calf serum to a 3T3-C2 culture resulted in no perceptible increase in its rate of amino acid incorporation (■).

the average increase in their protein content per cell must have been 5- to 6-fold. Neither the total number of 3T3-C2 cells nor their protein content was affected by the addition of adipogenic serum.

DNA Synthesis and Cell Division during the Adipose Conversion. Cells growing exponentially in medium supplemented with cat serum were inoculated into suspension culture in medium containing 10% fetal calf serum. The ability of 3T3-F442A cells to incorporate labeled thymidine decreased quickly and reached a low level (Fig. 5). Two days after inoculation and without any further addition to the culture, some cells again began to incorporate labeled thymidine; the rate of incorporation increased for several days and then remained at 10–14% of that of exponentially growing cells. The increase in cells synthesizing DNA was detectable at least 1 day earlier than the enzyme response, though the magnitude of the enzyme response was much greater.

Under the same conditions, the rate of thymidine incorporation by 3T3-C2 cells decreased similarly after inoculation but did not increase at all while 3T3-F442A cells were reaching the height of their mitogenic response. Toward the end of the experiment, the rate of thymidine incorporation did begin to increase and this was followed by a small enzyme response.

These experiments showed that the mitogenic response was linked to the adipose conversion because both susceptible cells and the presence of adipogenic serum were required. Microscopic examination of the 3T3-F442A cultures showed that, although all cells had been well dispersed at the time of inoculation, some did not remain single cells but instead multiplied to form clusters of 4–12 cells. Whereas most of the single cells were not fatty, all the cells in the clusters accumulated lipid.

The multiplication of cells undergoing adipose conversion could be prevented by suppressing DNA replication with AraC (Table 1). Under these conditions, no clusters formed and the number of fatty single cells was about the same as the sum of fatty single cells and clusters developing in the absence of the drug. Many of the fatty single cells developing in the presence of AraC were unusually large, suggesting that they would have divided in the absence of the drug.

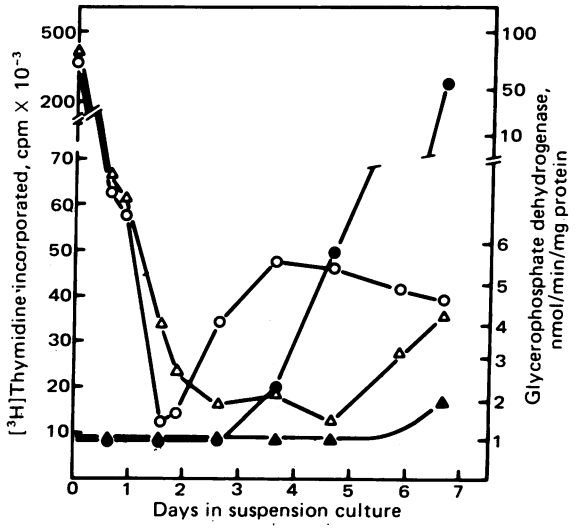

FIG. 5. Autonomous reversal of the resting state in the adipose conversion. Cells growing in medium supplemented with 10% cat serum were harvested and inoculated into stabilized suspension culture supplemented with 10% fetal calf serum. The rate at which 3T3-F442A cells incorporated [³H]thymidine decreased to less than 5% within 2 days (○), and then began to increase again, reaching a plateau several days later. The rate at which 3T3-C2 cells incorporated [³H]thymidine decreased similarly (△) but did not begin to increase again until very late. The increase in glycerophosphate dehydrogenase activity in 3T3-F442A (●) and 3T3-C2 (▲) could not be detected as early as the corresponding mitogenic response.

Table 1. Effect of AraC on production of adipose 3T3-F442A cells

	Increase in total cells, %	Cells and clusters, %		
		Single cells		Clusters
		Non-adipose	Adipose	Adipose
Control	30	89.5	4.5	6.0
3 μM AraC	0	93	7.0	0

At 3 days after inoculation of cells into suspension cultures containing 10% cat serum, AraC was added to a concentration able to prevent DNA synthesis, and fetal calf serum was then added to 5%. The cultures were scored 6 days later.

The effect of AraC on enzymatic differentiation was also examined (Table 2). The specific activity of glycerophosphate dehydrogenase increased at 3 and 6 days after adipogenic stimulation, although the magnitude of the response was clearly decreased by the drug.

Sequence of Events during Adipose Conversion. From the foregoing experiments, we may outline the development of the adipose conversion as follows.

(*i*) An adipogenic factor present in serum interacts with the cells. The magnitude of the cellular response depends on the concentration of the adipogenic factor and on the susceptibility of the cells. We assume that the interaction involves adsorption of the factor by the cells, but we have no direct evidence that this is the case.

(*ii*) If the cells are in a growing state at the time adipogenic serum is added, a period of time elapses before the cellular enzyme response begins. The enzyme begins to increase much sooner if the cells are in a resting state when the adipogenic serum is added, indicating that growing cells probably cannot respond to the adipogenic factor.

(*iii*) Cells in the process of adipose conversion undergo a mitogenic response that continues for some days. They may undergo three or four cell divisions, but eventually they become unable to divide further; fully developed adipose 3T3 cells are known to be incapable of division (1). If cells are allowed to undergo conversion in surface culture and are then transferred to suspension culture, they do not multiply or form clusters. Evidently, the cells are able to multiply only in the early stages of the adipose conversion.

One must consider the possibility that cellular DNA synthesis is required for the adipose conversion. This is particularly important because (*i*) increased DNA synthesis can be detected before increased enzyme activity and (*ii*) a replicative process must be considered as a possible explanation for the subsequent exponential increase in enzyme activity. It seems that complete

Table 2. Effect of AraC on development of glycerophosphate dehydrogenase activity

Addition of serum	Glycerophosphate dehydrogenase, nmol/min/mg protein		
		Day 6	
	Day 0	No AraC	With AraC (1 μM)
Day 0	0.9	350	50
Day 3	0.9	45	11

Cells were inoculated at 0.5 × 10^5/ml in medium containing methylcellulose and 10% cat serum; fetal calf serum was added to 10%, on either day 0 or day 3, together with AraC. Cell extracts were prepared on day 6 after inoculation.

replication of the genome is not essential for the adipose conversion because cells become fatty and develop increasing enzyme activity in the presence of AraC at a concentration able to arrest most if not all DNA synthesis. The magnitude of the enzyme response in the whole culture is decreased by AraC, but this may be a secondary consequence of prevention of cell multiplication.

Cell multiplication has an amplifying affect on the conversion because the number of adipose cells increases selectively. Although less powerful than that operating in the immune system, this form of clonal selection would produce a high density of adipose cells in a developing adipose tissue whose cell population was originally heterogeneous. This seems to be the significance of the earlier observation by Pilgrim (21) that, in the development of adipose tissue, the partly differentiated adipocytes have the highest rate of multiplication. As a result of selective multiplication, the final proportion of cells seen to be adipose in culture is not a measure of the original number of susceptible cells in the population. For example, although usually 40–60% of the total 3T3-F442A cells ultimately became adipose, the value was only 7% in the presence of AraC, the difference presumably being due to selective multiplication. In surface cultures, selective multiplication would not affect the number of colonies giving rise to fat cell clusters but is likely to be the cause of the increased number of cells commonly seen within the region of a fat cell cluster (18).

(*iv*) There remains to be elucidated a mechanism linking the action of the adipogenic factor on the cell with the increase in glycerophosphate dehydrogenase activity that follows. It seems clear that susceptible cells interacting with adipogenic factor undergo a resetting of their developmental program from one typical of fibroblasts to one devoted to triglyceride accumulation. In view of the extraordinary magnitude of the increase in glycerophosphate dehydrogenase activity that follows and the known increases in the amounts of other proteins with lipogenic enzyme activity (3, 22), it seems likely that the program requires increased formation of mRNA for the lipogenic enzymes relative to that for other cell proteins. Experiments such as those described in Figs. 2 and 5 permit the conclusion that the reprogramming takes place when the cells are in the resting state. There then follows a reversal of the down regulation that preceded the reprogramming.

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