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Effects of Fasting and Insulin Administration on Polyribosome Formation in Rat Epididymal Fat Cells*

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Polyribosomes from rat epididymal fat pads may be prepared in a synthetically active state and high yield by quick-freezing the tissue in liquid nitrogen, followed by grinding to a frozen powder. The number of initiated (active) ribosomes is limiting in a cell-free protein synthesis assay derived from the frozen preparation that measures completion of nascent polypeptide chains. When starved rats are killed 30 min after a single injection of insulin (0.8 unit, subcutaneously) there is a shift in the ribosomal distribution pattern (on sucrose density gradients) in the direction of larger polyribosomes. This is accompanied by a 2-fold increase in protein synthetic activity of fat cell ribosomes as measured *in vitro*. When isolated epididymal fat cells are treated with insulin, with or without added glucose, a 2-fold increase in the rate of incorporation of [^{3}H]leucine into trichloroacetic acid-insoluble protein is noted within 40 min.

The parallel increases in the number of initiated ribosomes, in their synthetic activity *in vitro*, and in the apparent protein synthetic activity of the whole cell indicate a rapidly evolving action of insulin at the level of peptide chain initiation.

Rates of synthesis of proteins in fat cells are responsive to a variety of endocrine and nutritional influences making these cells ideal candidates for investigating the nature of, and interrelationships between, the multiple intracellular control mechanisms that are likely to operate in normal mammalian cells *in vivo*. Yet, because of difficulties in working with adipose tissue and in obtaining subcellular components in their native state, detailed investigations into regulatory mechanisms at the subcellular level have not heretofore been possible. A major obstacle has been that fat cells have only a rim of cytoplasm so that intracellular water volume is only about 4% of the weight of fresh tissue (1).

Experiments using these methods demonstrate that a brief (30 min) insulin treatment of rats increases the number of active (initiated) ribosomes within fat pads. Parallel experiments with isolated fat cells demonstrate a similar increase in incorporation of radiolabeled amino acids into protein. These data suggest a rapid action of insulin on protein biosynthesis acting at the level of the initiation reaction.

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EXPERIMENTAL PROCEDURES

Preparation of Postnuclear Supernatant from Epididymal Fat Pads—Male Sprague-Dawley rats (120 to 140 g) were decapitated and epididymal fat pads were rapidly excised, rinsed in saline (0.9% NaCl solution), blotted, and then immediately frozen by immersion in liquid nitrogen. The total elapsed time between killing and freezing was 30 to 40 s. Frozen fat pads from individual animals were ground in liquid nitrogen using prechilled mortars and pestles and the fine powder was then transferred to 5-ml disposable (heavy wall) glass centrifuge tubes maintained at liquid nitrogen temperature. After all samples were processed in this manner, 0.5 ml of HKMED buffer (40 mM Hepes,[1] pH 7.6, 170 mM KCl, 20 mM $MgCl_2$, 0.2 mM Na_2EDTA, 4 mM dithiothreitol) was added to each of the tubes which were then immersed ≈20 s in a cold water bath (10°C) with intermittent blending on a Vortex mixer in order to rapidly thaw the mixture. All subsequent steps were at 0°C. The tubes were centrifuged for 1 min at 10,000 rpm (Sorvall SM-24 rotor), the infranatants were recovered with Pasteur pipettes, and heparin was added (500 μg/ml). Aliquots of these lysates were then used immediately for either sucrose density gradient analysis or for cell-free protein synthesis assay.

Sucrose Density Gradient Analysis of Ribosomes—Aliquots of the postnuclear supernatant from fat pads were layered on 15 to 50% convex sucrose density gradients (10 ml of 50% sucrose + 2 ml of 15% sucrose in 20 mM Tris-HCl, 100 mM KCl, 5 mM $MgCl_2$, pH 7.5) and centrifuged for 75 min at 41,000 rpm in a Beckman SW 41 rotor. All buffers were pretreated with diethyl pyrocarbonate (0.05%) to destroy RNase (2). Gradients were scanned at 260 nM with an ISCO model UA-4 absorbance monitor. Where indicated, areas under prepolysomal and polysomal regions of the scans were cut and weighed in order to estimate the percentage of polyribosomes in each preparation. Gradients presented are typical of those obtained from experiments performed many times.

Assay for Synthetic Activity of Fat Cell Polyribosomes (Run Off of Nascent Chains)—Cell-free protein synthesis assays were performed at 37°C in conical plastic Eppendorf tubes. The reaction mixture consisted of 85 mM KCl, 20 mM Hepes (pH 7.6), 10 mM $MgCl_2$, 0.25 mM each of 19 amino acids, 20 μCi of L-[4,5-^{3}H]leucine (50 to 60 Ci/mmol), 5 μg of L-leucine, 5 mM ATP, 0.5 mM GTP, 50 μl of desalted rat liver high speed supernatant, and fat pad postnuclear supernatant (0 to 40 μl) in a total volume of 200 μl. Background tubes contained an addition of puromycin to 1 mM. High speed supernatant was prepared by homogenizing rat liver in HKMED buffer containing 500 μg/ml of heparin, centrifuging at 50,000 rpm for 2 h (Beckman 65 Ti rotor) and desalting the resulting supernatant on a Sephadex G-25 column. Aliquots of this preparation were stored under liquid N_2 and were never refrozen after thawing.

The reactions were run for 30 min; aliquots of 50 μl were then removed and plated onto Whatman filter paper discs (2.3 cm diameter). These discs were immediately immersed in ice-cold 10% trichloroacetic acid containing 1% NaH_2PO_4 and 0.1% DL-leucine for 30 min. The discs were then processed through the following wash sequence to remove nonprotein bound radioactivity: 5% trichloroacetic acid, 5% trichloroacetic acid at 85°C for 30 min, 5% trichloroacetic acid, ethanol:ether (1:1, v/v), and finally anhydrous ethyl ether. Radioactivity was counted with a toluene-2,5-diphenyloxazole mixture in a liquid scintillation counter.

Materials—Trizma base, RNase-free sucrose, dithiothreitol, di-

[1] The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ethyl pyrocarbonate, heparin, and L-amino acids were purchased from Sigma; L-[4,5-^{3}H]leucine (50 to 60 Ci/mmol) was purchased from Amersham/Searle; Hepes was a product of Research Organics; crystalline, glucagon-free, porcine insulin was a generous gift from Dr. R. Chance of Lilly Research Laboratories.

RESULTS

Homogenate Preparation—In pilot studies we found that highly satisfactory polyribosomal preparations could be obtained when individual fat pads were quick-frozen, ground to powder in liquid nitrogen, and then rapidly thawed. Fig. 1*B* illustrates a typical UV scan of a sucrose gradient using the postnuclear supernatant obtained from about 200 mg of tissue processed via this quick-freeze method. A sucrose gradient using the pooled postnuclear supernatants from six fat pads (≃1.2 g of tissue) processed by traditional homogenization methods is also shown (Fig. 1*A*). This comparison shows that the relative yield using homogenization of fresh tissue is very small; there is also a substantial loss of large polyribosomes and an increase in 80 S monomers. In view of the disparity in recovery between these two methods, it is not possible to account for the differences in polysomal patterns. However, the changes created by a small delay in the processing of the frozen tissue (Fig. 2) suggest that the differences in polyribosomal profiles (in Fig. 1, *A* and *B*) may be due to ribosomal run off from the mRNA's or to degradation of polyribosomes by RNase during the homogenization procedure. In Fig. 2*A* the frozen preparation was allowed to thaw in an ice bath over 10 min; in Fig. 2*B* the thawing was accomplished within 30 s. The slower thawing results in a reduced total recovery as well as a change in pattern, with relatively fewer large polyribosomes and an increase in ribosomal dimers.

The simplicity of the freeze-grind procedure allows estimates of recovery. Typical data, presented in Table I, show the distribution of total cellular RNA between the postnuclear supernatant and the "nuclear pellet." (The latter also includes other membranous cellular debris.) Separate estimates of RNA recovery in the postnuclear supernatant are based on the orcinol assay and on cutting and weighing the UV profiles of sucrose density gradients. To minimize any time-dependent changes that might occur, our standard procedure avoids homogenization after thawing the frozen powder. Nevertheless, we routinely recover about three-fourths of the total cellular RNA in the postnuclear supernatant, as shown in columns 4 and 5. More importantly, this relative yield did not change under the three treatment conditions tested (fed, starved, and starved + insulin). Finally, the polyribosomal profiles obtained from the postnuclear supernatant and those recovered after homogenizing the nuclear pellet in the presence of detergent are qualitatively very similar, as shown in Fig. 2, *B* and *C*. Thus, the polysomes obtained routinely from

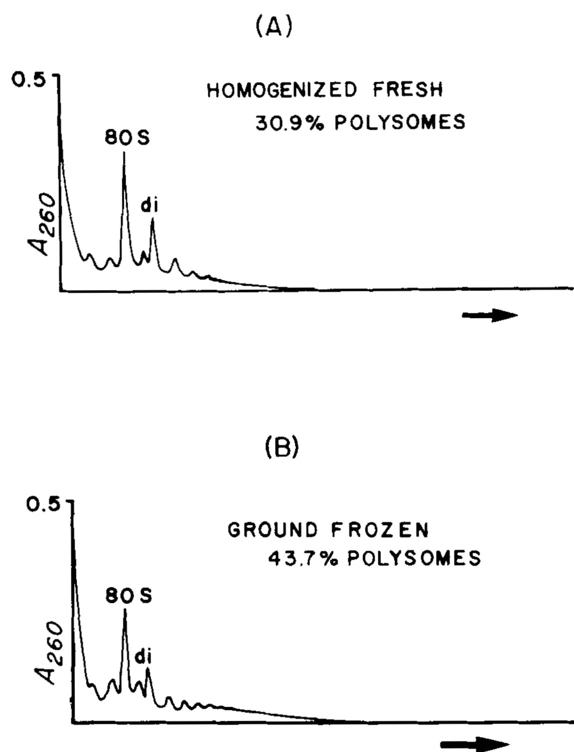

FIG. 1. **Comparison between sucrose gradient profiles of fat pad ribosomes from frozen ground and from homogenized fresh tissue.** Animals were killed by decapitation, the fat pads were rapidly excised, rinsed in saline, blotted, and then (*A*) pooled from six animals and homogenized at 2°C with a motor-driven Teflon-glass homogenizer in 0.5 ml of HKMED buffer (40 mM Hepes, pH 7.6, 170 mM KCl, 20 mM $MgCl_2$, 0.2 mM Na_2EDTA, 4 mM dithiothreitol) or (*B*) immediately frozen in liquid nitrogen. The frozen fat pads from a single animal were ground in liquid nitrogen and thawed with 0.5 ml of the same buffer. For both *A* and *B*, postnuclear supernatants were prepared and aliquots were layered on top of sucrose gradients which were centrifuged and scanned as described under "Experimental Procedures." The peak marked 80 S is that for ribosomal monomers and monosomes; the peak marked di is that for ribosomal dimers (nominally 114 S). *Arrows* represent the direction of sedimentation.

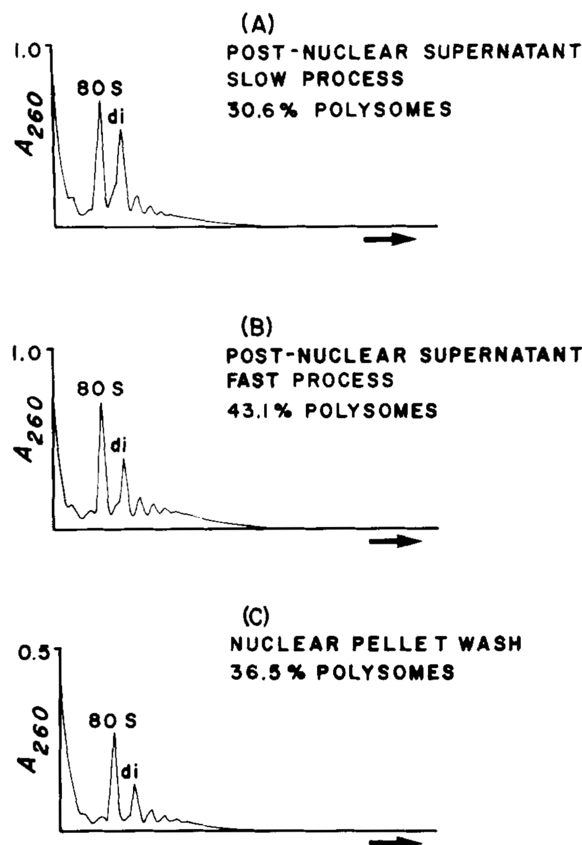

FIG. 2. **Comparisons between sucrose gradient profiles of ribosomes from slowly and rapidly thawed frozen fat pads.** Sucrose gradient profiles were produced from frozen fat pads as described under "Experimental Procedures" and Fig. 1*B*, except that the frozen powder was thawed slowly (*A*) over a 10-min period at 0–2°C before dilution with buffer; or rapidly (*B*) (within 30 s) by brief immersion in a 10°C water bath. For the gradient in *C*, the nuclear pellet from *B* was homogenized with a motor-driven Teflon-glass homogenizer in 0.5 ml of the same buffer (but with 0.1% Triton X-100 added) and recentrifuged, and the resulting (second) postnuclear supernatant was analyzed on sucrose density gradients as above. *Arrows* represent the direction of sedimentation.

TABLE I

Distribution of RNA between postnuclear supernatants and nuclear pellets derived from lysates of frozen fat pads

Fat pads from nine individual rats (three/condition) were frozen, ground, and processed as described under "Experimental Procedures." The frozen powder was thawed with 800 μl of HKMED buffer and centrifuged 1 min at 12,300 × *g* to produce a first postnuclear supernatant and a nuclear pellet. This first postnuclear supernatant was removed and aliquots were assayed for RNA content both by the orcinol determination and by weighing the UV scans from sucrose density gradients as described under "Experimental Procedures." RNA remaining in the nuclear pellet was extracted by adding 800 μl of HKMED buffer containing 0.5% Triton, homogenizing with a motor-driven Teflon-glass pestle, and recentrifuging as above to yield a second postnuclear supernatant. Aliquots of this nuclear pellet extract were assayed for RNA by the same two procedures as above. Percentages shown represent RNA recovered in the first postnuclear supernatant relative to the total (first postnuclear supernatant + Triton extract of nuclear pellet). Standard errors were less than 10% of the mean.

Condition	RNA yield (orcinol)		Relative recovery of RNA in first postnuclear supernatant[a]	
	First postnuclear supernatant	Nuclear pellet	Orcinol reaction	UV scans
	μg/fat pad		%	
Fed	149.1	51.9	74.2	76.0
Starved	118.7	33.5	78.0	82.2
Starved + insulin	121.6	44.1	73.4	77.7

[a] Postnuclear supernatant/(postnuclear supernatant + nuclear pellet) × 100.

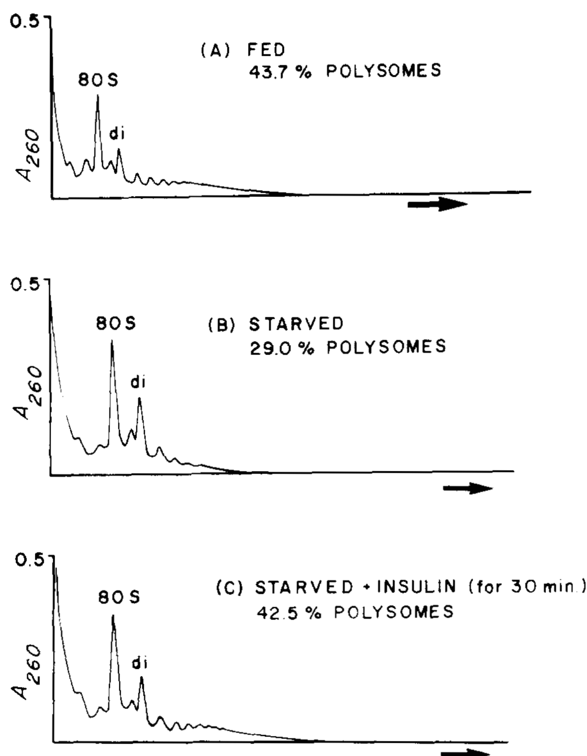

FIG. 3. **Effects of fasting and of insulin treatment on sucrose gradient profiles of fat pad polyribosomes.** Rats were starved overnight (16 h) or fed *ad libitum*; insulin (0.8 unit, subcutaneously), was administered 30 min prior to death. The percentage of polysomes in each preparation was determined by drawing the baselines as shown and then cutting and weighing the region corresponding to trisomes and larger ("polysomes") and dividing by the weight of the entire scan ("total ribosome population"). *Arrows* represent the direction of sedimentation.

the postnuclear supernatant without homogenization are representative of those in the total cellular population.

Since speed in the processing of broken cell preparations is essential in order to obtain the least degraded polyribosomes, we also searched for possible time-dependent changes that might occur between killing the animal and freezing the adipose tissue. Fed rats were killed by decapitation; one fat pad was frozen in liquid nitrogen at 20 s and the other at 90 s. The 70-s delay in freezing does result in a small loss (about 20%) in total ribosomal yield, but otherwise no dramatic changes in ribosomal distribution are evident on sucrose gradients (about 55% polysomes in each case, results not shown). These and similar experiments lead to the conclusion that profiles routinely obtained by freezing fat pads within 40 s after death are representative of those within the living animal.

Assays of Biosynthetic Activities of Ribosomes from Quick-frozen Cells—Postnuclear supernatants from quick-frozen cells are found to be active in cell-free protein synthesis. Activity is largely eliminated either by 1 mM puromycin or by 1 μg/ml of ribonuclease (results not shown). Since there is little or no reinitiation during this cell-free assay, we have used changes in radioactivity in nascent chains *in vitro* as an additional measure of changes in the number of synthetically active ribosomes *in vivo*. To circumvent the possibility that changes in endogenous pools of amino acids significantly affect incorporation, large amounts (about 200 μM) of unlabeled L-leucine were routinely added to each reaction mixture. We

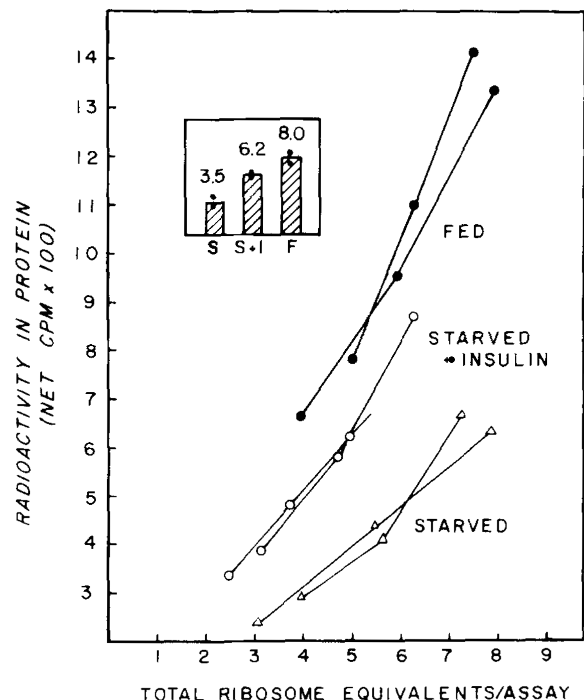

FIG. 4. **Effect of fasting and of insulin administration on the number of initiated ribosomes in fat cells.** Run off of nascent polypeptide chains was measured (as described) in cell-free protein synthesis assays on aliquots of the same lysates used to produce the profiles shown in Fig. 3. Each *curve* represents assays of a single fat pad lysate performed at three different concentrations of ribosomes. The ribosomal equivalents added to each assay were calculated from subsequent UV scans of the original material. Radioactivity in protein (*y* axis) represents net incorporation of [^{3}H]leucine after subtraction of puromycin-resistant counts. Data points on the *inset* were derived from the larger graph by comparing the protein synthetic rate of each group at a single convenient ribosome concentration (here, 5 ribosome units/assay). *Numbers above bars* depict mean values (net counts per min × 100). *S*, starved; *S & I*, starved + insulin; *F*, fed.

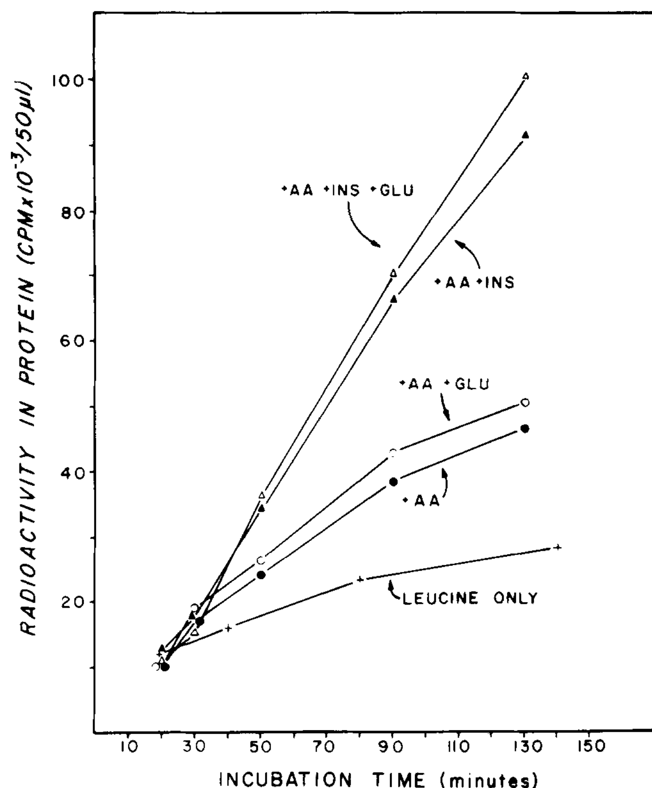

FIG. 5. **Effect of insulin on incorporation of [4,5-^{3}H]leucine into protein by isolated fat cells.** Fat cells were isolated from rat epididymal fat pads by digestion of the tissue stroma with crude bacterial collagenase as described by Rodbell (6). The isolated cells were incubated at 37°C with shaking (packed cell volume of 6 to 8%), using plastic Ehrlenmeyer flasks which was gassed with 95% O_2, 5% CO_2. The Krebs-Ringer phosphate buffer used contained (in a total volume of 1 ml) 3% bovine serum albumin, 10 µCi/ml of [4,5-^{3}H]-leucine, 1 mM L-leucine, and the following additions where indicated: 19 additional L-amino acids (*AA*) (0.10 mM each), glucose (*GLU*) (1 mg/ml), and insulin (*INS*) (100 µunits/ml) which was added at 10 min. Duplicate aliquots (50 µl) of cell suspension were removed at times indicated, precipitated by trichloroacetic acid on filter paper discs, washed, and counted as described under "Experimental Procedures." Data points, representing counts per min in protein from 4 µl of packed cells, are the means of values from three experiments which have been corrected for background (always less than 10% of total counts per min) and then normalized.

have calculated that the leucine added to each assay was at least 50 to 100 times larger than endogenous leucine from adipose cells (3). Other experiments (not shown) demonstrate that the added liver supernatant factors are more than sufficient to ensure that the number of initiated fat pad ribosomes is the determinant limiting the final level of radioactive amino acid incorporation (although the rate-limiting step during the reaction *in vitro* is the rate of elongation).

Effects of Fasting, Feeding, and Insulin Administration in the Intact Animal—In order to determine the influence of starvation or brief insulin treatment on the number of initiated (active) ribosomes *in vivo*, experiments measuring changes both in ribosomal distribution and in their synthetic activity (completion of nascent chains) *in vitro* were performed, as in Figs. 3 and 4. Within 30 min after a single injection of insulin (0.8 unit, subcutaneously) to starved animals, we observe shifts on sucrose gradients in the direction of larger polysomes (an increase in the number of initiated ribosomes, Fig. 3) and increases (approximately 2-fold) in protein synthetic activity as measured *in vitro* (Fig. 4). In these experiments the ribosomes were assayed for synthetic activity at three different concentrations. Activity per unit of ribosome is then taken from such curves (Fig. 4, *inset*). In other experiments, we consistently find that simultaneous administration of glucose (50 mg, intraperitoneally) along with insulin produces no additional response (results not shown).

Effects of Insulin on Protein Synthesis in Isolated Fat Cells—In order to compare the emergence of insulin effects on ribosomes *in vivo* with another measure of action on protein biosynthesis, we attempted to measure the onset of insulin stimulation using the incorporation of radiolabel from very large amounts (as compared to *in vivo* pools) of leucine into cellular proteins. Because such measurements are difficult to make in whole animals (4), we utilized isolated fat cells incubated in the presence or absence of 19 additional amino acids, glucose, and/or insulin. Results of typical experiments are summarized in Fig. 5. An equimolar mixture (0.1 mM) of 19 amino acids stimulates leucine incorporation by about 50%. Glucose has very little additional effect. Insulin (100 µunits/ml), with or without glucose, produces a 2-fold increase in incorporation. The relative magnitude and time course of these insulin effects in isolated cells (Fig. 5) are consistent with those effects in the animal that are measured by changes in polyribosome profiles (Fig. 3), or by changes in ribosomal synthetic activity *in vitro* under conditions that allow run off (Fig. 4). These observations also support earlier reports that insulin increases amino acid incorporation into fat cell protein in the absence of glucose (5).

DISCUSSION

Adipocytes represent easily obtained normal cells that exhibit a variety of responses to nutritional and hormonal stimuli both *in vivo* and *in vitro*. Since these responses include apparent changes in protein biosynthesis, adipocytes represent potentially ideal candidates for the study of regulatory details. Yet to our knowledge, the only previous study at the subcellular level has been a comparison of sucrose gradients obtained by combining the homogenates of about 20 fat pads from either fed or fasted rats (7). The approach reported here allows sucrose gradient analysis and simultaneous assays for ribosomal activity *in vitro* from a single fat pad. Thus, more detailed investigations of subcellular components in this tissue are possible. The use of quick-freezing and the apparent stability of components (when frozen within the first minute of killing the animals) makes it highly likely that the arrangement of ribosomes is representative of their native state within the cell. The observation that the additional ribosomes recovered from the nuclear pellets (further homogenized with detergent) display sucrose gradient patterns nearly identical with those more readily recovered from the cytoplasm reinforces this conclusion.

Several earlier studies have reported that insulin increases the incorporation of radiolabeled amino acids into fat cell proteins (3, 4, 8–12). Although insulin is known to increase uptake of amino acids in muscle, effects on transport in fat cells have generally not been seen (3, 13, 14). Minemura *et al.* (3) concluded that in the absence of an action on amino acid transport or protein degradation, the predominant effect of insulin was on the protein synthetic reaction *per se*. However, as discussed by Rannels *et al.* (4), direct evidence supporting this hormone effect requires that the specific activities of precursor amino acids be measured, either in peptidyl- or aminoacyl-tRNA. Such measurements would be extremely difficult because of the large amounts of intracellular amino acids relative to those in tRNAs, and because of the small amounts of material recoverable in fat cells.

The strategy used here, measurement of changes in relative amounts of subcellular components and in ribosomal activity,

at least provides an assurance that hormone action has occurred at steps other than those that alter the specific radioactivity of intracellular amino acids. Since the assays *in vitro* measure completion of nascent polypeptide chains under conditions where the number of initiated ribosomes is the limiting variable, it seems reasonable to interpret the result of insulin action as an increase in the rate of initiation relative to that of elongation. The coincidence of this event with a step up in incorporation of amino acids into protein in isolated cells (in our experiments, Fig. 5, and in those of others, Refs. 3, 5, 8–12) would make a hormone-induced decline in rates of elongation unlikely. Hence, an action of insulin at the level of initiation is strongly suggested.

Insulin effects on protein synthesis in muscle have been well-documented by the work of Wool *et al.* (15–17), Jefferson *et al.* (18–20), and others (reviewed in Ref. 5). Although it appears that insulin is required to maintain optimal rates of initiation of peptide chains, the mechanism of action remains obscure. Our previous studies on thymic lymphocytes strongly suggest a link between the adenylate energy charge (inhibition is associated with a small rise in AMP levels) and initiation-limited protein synthesis (21–26). Regulation at the level of initiation here could be directly mediated by changes in guanine nucleotides that in turn respond to adenine nucleotides (27, 28), or possibly by direct AMP inhibition. Studies to assess the actions of insulin on isolated fat cells via these or other mechanisms are now in progress.

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