Effect of Diet and Insulin on the Morphology and TPNH Generating Enzyme Activities of Rat Adipose Tissue* (34521)

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Since insulin stimulates protein synthesis in adipose tissue (1), it could be involved in the selective synthesis of specific proteins in that tissue. Altered patterns of enzyme activities related to adaptive hyperlipogenesis (2) have been found parallel to changes in nucleic acid and protein synthesis in adipose tissue (3, 4, 5). Since fat feeding inhibits the adaptive hyperlipogenesis response (6, 7) and also results in a decrease of insulin content of the pancreas (8), it was pertinent to inquire whether or not adaptive increase in the TPNH generating enzymes of adipose tissue—the aggregate dehydrogenases of the direct oxidative pathway of glucose metabolism and the TPNH malic enzyme—could be affected by supplying exogenous insulin to animals refed a high-fat, carbohydrate-free diet. Accordingly, rats were refed, after a 72-hr fast, diets of widely divergent fat and carbohydrate content, and part of each group received insulin. Concomitantly, correlated microscopic observations were made of the RNA staining of adipose tissue.

Materials and Methods. Animals and diets. Young adult male CFE rats (Sprague-Dawley descent), weighing 220–250 g at the start of the experiment, were obtained from Carworth, Inc., New City, N. Y. The animals were housed in individual cages (Acme Metal Products, Inc., Chicago Ill.), and weighed daily throughout the experiment.

Prior to fasting, all animals were fed a standard laboratory diet (5-RF provided by Agway, Inc.). The sucrose and fat diet used for refeeding consisted of 350 g of a basal mixture and 800 g of sucrose, or a roughly isocaloric amount (355 g) of lard. The basal mixture consisted of the following (in grams): vitamin free casein, 200; salt mixture USP XIV, 50; vitamin supplement (Nutritional Biochemicals, Chicago, Ill.), 40; alphacel, 50. Except for the control group, which was fed the standard laboratory diet *ad libitum* throughout the experiment, all animals were fasted for 3 days and subsequently refed for another 3 days the sucrose or high-fat diet. Half of the animals on each diet were treated with 1 U/kg of crystalline insulin (Squibb), administered in two intraperitoneal injections on each day of the refeeding period up until 12 hr before sacrifice. Previous studies have shown that at this dose insulin produces a measurable drop in blood glucose (9). Individual subgroups comprised four animals each.

Enzyme assays. Immediately after the rats were decapitated, approximately 1 g of adipose tissue was removed from the upper segment of the epididymal fat pad and homogenized in 9 ml of 0.25 *M* ice-cold sucrose in a glass Teflon homogenizer. The homogenate was centrifuged for 30 min in a refrigerated centrifuge (4°) at 21,500*g*. The supernatant (*i.e.*, the clear intermediate layer below the fat cake) was used for assaying the enzymes.

The combined activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase was measured by the method of Glock and McLean (10), malic enzyme by the procedures of Ochoa (11) and Fitch and Chaikoff (12). The

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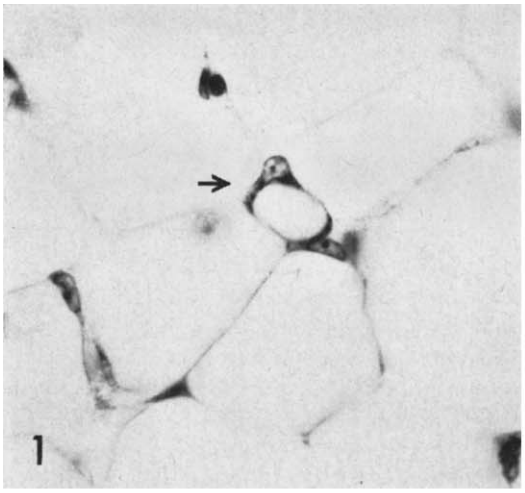
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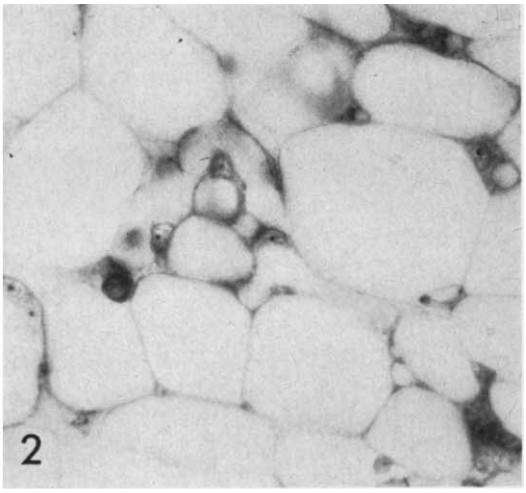
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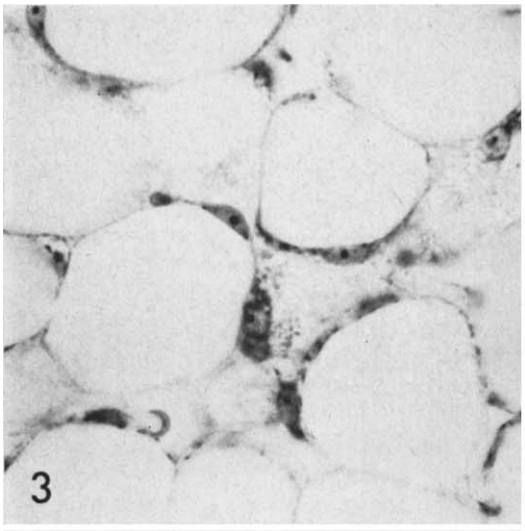
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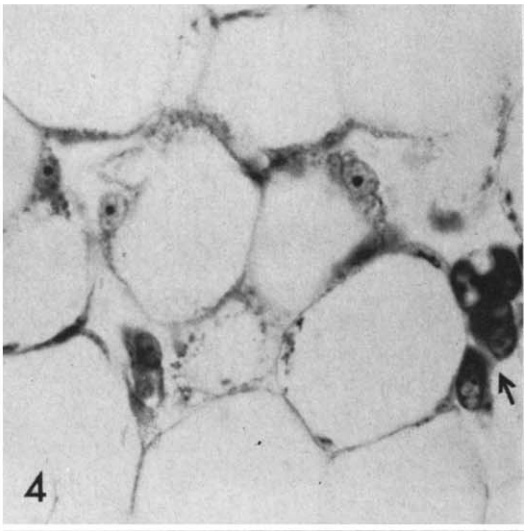
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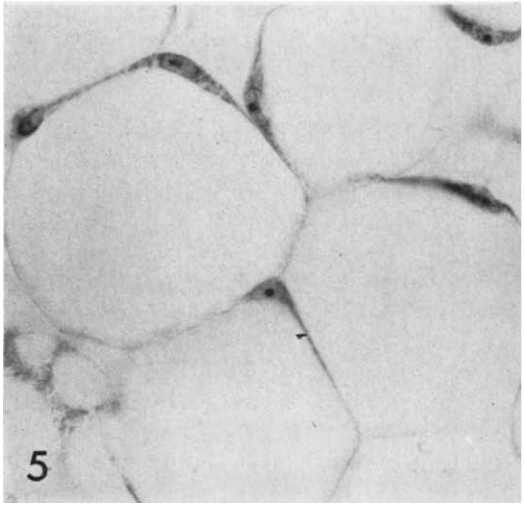
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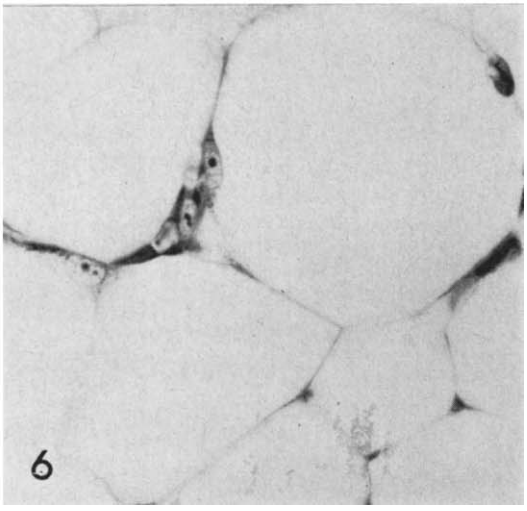
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TABLE I. Influence of Diet and of Exogenous Insulin on Activities of TPNH Generating Enzymes in Rat Adipose Tissue (Averages ± SE of Groups Comprising Four Animals Each).

Group	Body wt (g)	Wt of fat pad (g/100 g body wt)	Malic enzyme activity (U/mg protein × 100)	Pentose shunt enzymes activity (U/mg protein × 100)
Controls	291 ± 6.6	0.77 ± 0.07	2.09 ± 0.17	5.51 ± 0.14
Fasted 72 hr	214 ± 2.8[a]	0.69 ± 0.05	1.47 ± 0.15[b]	3.32 ± 0.56[b]
Refed sucrose diet	232 ± 3.0[a]	0.63 ± 0.03	11.59 ± 1.03[a]	9.16 ± 1.14[b]
Refed sucrose diet + insulin[c]	241 ± 4.5[a]	0.62 ± 0.04	13.26 ± 0.39[a]	10.02 ± 1.66[b]
Refed high-fat diet	246 ± 2.9[a]	0.84 ± 0.07	2.69 ± 0.40	6.10 ± 0.97
Refed high-fat diet + insulin[c]	249 ± 3.5[a]	0.86 ± 0.06	3.18 ± 0.47	5.84 ± 0.63

[a] Difference as compared with the control group is significant at $p < 0.01$.

[b] at $p < 0.05$.

[c] 1 U/kg crystalline zinc insulin (Squibb) per day divided into two ip injections on each of the 3 days of refeeding.

formation of TPNH was recorded in a Gilford 2000 spectrophotometer at 340 mμ. One unit of enzyme was defined, in both cases, as that amount of enzyme preparation which reduced 1.0 μmole of TPN/min at 25°.

The protein content of adipose tissue homogenates was estimated by a modification of Lowry's procedure (13).

Histology. Small pieces of epididymal adipose tissue (thin upper portion) were fixed in acetic alcohol (1 part of glacial acetic acid:3 parts of ethanol), processed, and embedded in paraffin. Relative concentrations of nucleolar and cytoplasmic RNA were studied in 10-μ sections stained with azure B at pH 4.0 (14). Azure B stains DNA orthochromatically (blue-green) and RNA metachromatically (purple). All nucleolar and cytoplasmic basophilia was removed in sections pretreated with ribonuclease (0.2 mg/ml, pH 6.5, 1 hr at 30°).

Results. Enzyme studies. In confirmation of many prior studies, fasting for 72 hr resulted in a significant decrease both in malic enzyme and pentose shunt enzyme activities (Table I). Refeeding a high-sucrose, fat-free diet caused the usual large increase in both enzymes described by Young, Shrago, and Lardy (15) and others. The concurrent administration of 1 U/kg crystalline insulin did not significantly change the refeeding response.

Fat refeeding restored the enzyme activities but produced no increase above the pre-

Micrographs of epididymal adipose tissue of the rat stained with azure B; × 650.

FIG. 1. Chow-fed control animal. A mature signet-ring cell, with a flattened nucleus containing a prominent nucleolus, is shown next to a highly basophilic, newly differentiated adipocyte (arrow).

FIG. 2. Fasted animal. Mean cell size is reduced and nuclear and nucleolar volumes are decreased. Note small cells with depleted fat stores.

FIG. 3. Sucrose-refed animal. Nucleoli are enlarged and cytoplasmic mass and basophilia are increased.

FIG. 4. Sucrose-refed, insulin-treated animal. Increased basophilia similar to sucrose-refed-alone group. Note group of intensely stained basophilic cells containing small liquid droplets (arrow). These may be either newly formed adipocytes or formerly depleted cells in the process of growth and adaptive hyperlipogenesis. Both small and large cells show enlarged nucleoli and increased cytoplasmic basophilia.

FIG. 5. High-fat-refed animal. Mean cell size is increased, and nuclear and nucleolar volumes correspond to that of the control group.

FIG. 6. High-fat-refed, insulin-treated animal. Similar to Fig. 5. Cells are enlarged, and overall basophilia approximates that of the control group.

fasting baseline levels. The addition of exogenous insulin to the regimen had no effect on the 72-hr enzyme levels detectable in the adipose tissue (Table I). In an additional experiment, malic enzyme activity was measured in adipose tissue of rats refed the high-fat diet for 72 hrs and injected with 12.5 U/kg of insulin instead of 1 U/kg. Despite the increased dose of the hormone, the enzyme activity was the same as in rats refed the same diet without insulin.

Cytological studies. When stained for nucleic acids, the mature adipocyte, or "Signet-ring" cell shows a flattened pale nucleus with one or two prominent nucleoli surrounded by a cap of weakly basophilic cytoplasm (Fig. 1). The size of the cell varied depending on how large a fat globule it contained.

After a 72-hr fast, the mean cell size was reduced as fat was mobilized. The nuclear and nucleolar volumes decreased and cytoplasmic RNA staining was reduced.

Refeeding a high-sucrose, fat-free diet for 72 hr did not result in an increase in mean cell size, but the nucleus was enlarged and a striking increase in nucleolar volume and RNA staining was evident. Concomitantly, the cytoplasmic RNA was increased, and a thickened rim of basophilic cytoplasm extending to the periphery of the fat globule could be demonstrated (Fig. 3). The morphologic picture indicated that both an increase in cytoplasmic mass and RNA concentration occurred during the refeeding period.

The morphological picture of adipocytes from rats refed the sucrose diet and treated with insulin was about the same as that seen in the sucrose-fed group (Fig. 4).

Refeeding the high-fat diet for 72 hr resulted in a marked increase in mean cell size. Nuclear and nucleolar volumes increased to approximate control values (Fig. 5). The cytoplasmic basophilia was increased over the fasting level, but was not as marked as that seen in the sucrose refed rats, in agreement with the biochemical data for total RNA previously reported (6). Coincident administration of insulin at both doses also produced large cells. The overall basophilia was not different from the chow fed control group (Fig. 6).

Discussion. The results of this study indicate that insulin is not the limiting factor for the failure of "adaptive hyperlipogenesis" to occur in adipose tissue of rats refed a high-fat, carbohydrate-free diet. It is rather the availability of carbohydrates or some derivative metabolite which is essential for the adaptive response. This does not mean that insulin does not play a significant role in diet-induced changes of adipose tissue. As noted by Hausberger (16), insulin is essential for fat formation and deposition, but only small amounts are required if a strongly stimulating dietary factor is provided. Under the experimental conditions in this study, it appears likely that the release of endogenous insulin was sufficient for effecting the characteristic enzymatic changes in the two refed groups. For the high-fat-diet group, this resulted in restoration of enzymatic activities to the prefast level. For the sucrose-refed animals, enzyme activities were increased severalfold above the prefasting level.

From the changes in the enzyme patterns on refeeding the two diets with and without insulin, and from the cytological appearance of the cells, it is possible to construct a plausible hypothesis of the interrelations of RNA metabolism and the metabolic function of the adipocyte under different circumstances. Refeeding the sucrose diet with or without exogenous insulin results in the elaborate rearrangement of the cell's biochemical machinery to perform the function which has been designated as "adaptive hyperlipogensis" (2). This is a coordinated synthesis of new enzyme proteins which are related to the synthesis of triglycerides from carbohydrate precursors—both biosynthetic pathway enzymes and coenzyme support pathway enzymes. This adaptive response is superimposed on any other stimulation of protein synthesis that may occur as a result of refeeding after a single fast, *i.e*, increase in cytoplasmic mass. Thus, the adipocyte is presented with two sets of protein synthetic problems: (1) those involved in "adaptive hyperlipogenesis" and (2) those involved in cellular hypertrophy associated with the storage of increased amount of triglycerides, *i.e.*, "repletion protein synthesis".

From the enzyme data presented in Table I, is is apparent that the protein synthetic activity of the adipocyte in the high-fat-diet refed animal revolves importantly around triglyceride storage and cell hypertrophy. Repletion synthesis occurs and lipogenic and coenzyme support pathway enzyme activities return to prefast values. In contrast, the sucrose and sucrose-insulin groups exhibit severalfold increases in enzyme activities. The accompanying increase in nucleolar and cytoplasmic RNA amounts, indicative of increased ribosomal RNA synthesis, is presumably related to a developing capacity for lipogenesis. Thus, it appears that "repletion" protein synthesis may be quantitatively less demanding on the cell's resources than is the combination of "adaptive hyperlipogenesis" and "repletion".

It is relevant to note the *in vitro* study of RNA metabolism of adipocyte by Benjamin and Gellhorn (4). Starvation for 48–72 hr resulted in a decreased rate of synthesis of "heavy," or ribosomal RNA. Refeeding glucose restored the rate to control levels within 7 hr. It would be particularly interesting to know the comparative rates of change in RNA and protein synthesis that occur during the 72-hr refeeding period between the high-fat- and sucrose-refed animals. Such data might further elucidate the pattern of regulation involved in repletion vs adaptive hyperlipogenesis.

Summary. The activities of TPN malic enzyme and aggregate hexomonophosphate shunt dehydrogenases were determined in epididymal fat pads of rats subjected to the following treatment: (1) *ad libitum* chow fed; (2) fasted 72 hr; (3) refed sucrose diet 72 hr; (4) refed sucrose diet plus insulin; (5) refed high-fat diet; and (6) refed high-fat diet plus insulin. Refeeding the sucrose diet for 72 hr resulted in more than a six-fold increase in malic enzyme activity, and approximately a doubling of the activity of the shunt enzymes over the prefast levels. Refeeding the high-fat, carbohydrate-free diet resulted in restoration of enzyme activities to prefast levels. Administration of exogenous insulin resulted in no change beyond that produced by the respective diets alone. Fasting resulted in a decrease in nucleolar and cytoplasmic RNA staining and a reduction of the mean cell size. Refeeding sucrose induced a marked enlargement of nucleoli and increased cytoplasmic basophilia with essentially no changes in mean cell size. Refeeding the high-fat diet produced a striking hypertrophy of fat cells, whereas the nucleolar and cytoplasmic RNA staining was comparable to the prefast state. Exogenous insulin did not produce marked differences in either of the two diet groups.

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