Effects of prolonged incubation and cell concentration on lipogenesis from glucose in isolated human omental fat cells

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ABSTRACT The behavior of human omental fat cells in vitro has been examined in order to define conditions under which glucose is converted to glyceride-glycerol and glyceride fatty acids.

Synthesis of glyceride fatty acids from glucose reached maximal rates only after several hours of incubation in Krebs–Henseleit bicarbonate buffer, with or without added bovine albumin. Conversion of glucose to glyceride fatty acids was readily demonstrable with concentrated cell suspensions and was stimulated 3- to 8-fold by insulin. With dilute cell suspensions, little fatty acid was synthesized even after prolonged incubation in the presence of insulin.

Conversion of glucose to glyceride-glycerol was linear during 6-hr incubations in buffer and unaffected by the concentration of the cell suspension. In the presence of bovine albumin, glyceride-glycerol synthesis was readily demonstrable at all cell concentrations used, although synthesis was faster in dilute suspensions.

Thus, different incubation conditions produce widely divergent patterns of glucose metabolism in human omental fat cells.

SUPPLEMENTARY KEY WORDS glucose metabolism · buffer composition · insulin

STUDIES on the metabolism of segments or shreds of human adipose tissue in vitro have yielded useful information on the tissue's over-all functions. Adipose tissues oxidize glucose, release glycerol and free fatty acids, and utilize glucose for glycerol and fatty acid synthesis (1–5). In general, metabolic activity is low, replication of metabolic parameters is poor (1, 4–7), and responsiveness to hormones is affected by the method of preparing the tissues (4, 8, 9). Furthermore, considerable difficulties have been experienced in detecting metabolic changes in the presence of insulin, as judged by the fact that pharmacological concentrations of hormone have usually been employed (3, 4, 7, 8, 10–13). Only recently have physiological concentrations of insulin been shown to facilitate glucose metabolism in human adipose tissue in vitro (5, 14, 15). On the other hand, suspensions of isolated human fat cells provide good replication of metabolic data, are highly sensitive to insulin (16), and have a theoretical advantage in metabolic studies where differences in tissue size, shape, and cellularity are anticipated. However, fatty acid synthesis from glucose and pentose cycle activity have hitherto not been readily demonstrable in human fat cells (16, 17), and this raises the possibility of cellular damage during collagenase treatment.

The present investigation was undertaken to define in vitro conditions which enable isolated human fat cells to utilize glucose for synthesis of both glyceride-glycerol and glyceride fatty acids. We have shown that fatty acid synthesis from glucose takes place in omental fat cells prepared from adults. The major determining factors were the duration of incubation and the concentration of cells.

Abbreviations: TLC, thin-layer chromatography; FFA, free fatty acids.

MATERIALS AND METHODS

Subjects

Suspensions of fat cells were prepared from omental adipose tissue obtained from one male and nine females

TABLE 1 CLINICAL DATA

Subject	Age	Sex	% Ideal Body Weight*	Operation
A	28	F	107	Total hysterectomy
B	33	F	120	Tubal ligation
C	39	F	93	Total hysterectomy
D	45	M	118	Cholecystectomy
E	33	F	95	Total hysterectomy
F	44	F	113	Total hysterectomy
G	38	F	108	Cholecystectomy
H	57	F	103	Cholecystectomy
I	29	F	101	Cholecystectomy
J	31	F	125	Cholecystectomy

* Determined from the average weights for men and women between 20 and 24 yr (18).

between the ages of 28 and 57 yr (Table 1). None showed evidence of recent weight change, diabetes, or hepatic or thyroid dysfunction. One patient was more than 20% above her ideal body weight. All subjects were undergoing elective surgery and were fasted for up to 12 hr prior to operation. Tissues were obtained as soon as possible after induction of general anesthesia, placed in warm (37°C) isotonic saline, and processed within 15 min.

Methods

Analytical techniques and methods for preparing and incubating fat cells have been detailed elsewhere (16). Additional procedures and modifications of previously described techniques (16) used in the present investigation were as follows.

Concentration of Fat Cells. Washed fat cells were concentrated by flotation into a solid mass and pooled. 0.5 ml aliquots were dispensed into incubation flasks containing 0.5–1.0 uc of glucose-U-^{14}C in 0.5 ml of buffer either without prior dilution (concentrated cells) or previously diluted 1:1 by volume ("semiconcentrated cells") or 1:5 by volume ("dilute cells") with buffer.

Incubation Conditions. Fat cells were incubated in duplicate flasks at 37°C under an atmosphere of 95% O_2–5% CO_2 for periods of 1–6 hr. Modified albumin–bicarbonate buffer containing 1 mg of glucose per ml was routinely used for the preparation of fat cells with collagenase and frequently employed in subsequent incubations with labeled glucose. The buffer was prepared by the addition of 40 mg of bovine albumin per ml of Krebs–Henseleit bicarbonate buffer which contained half the suggested concentration of calcium ion and had not been equilibrated with 95% O_2–5% CO_2. The pH was adjusted to 7.4 with 1 N sodium hydroxide. Krebs–Henseleit bicarbonate buffer containing half the suggested concentration of calcium ion and 1 mg of glucose per ml was also used for incubations with labeled glucose. This buffer was equilibrated with 95% O_2–5% CO_2 until the pH stabilized at 7.4 ± 0.02.

Separation of Lipid Classes. Adipose cell lipids were extracted by the method of Folch, Lees, and Sloane Stanley (19), and lipid classes were separated by TLC on Silica Gel G. Fractions were made visible and recovered for measurements of ^{14}C by previously described techniques (20). Preliminary separations in hexane–diethyl ether–glacial acetic acid 80:20:1.5 were employed to separate cholesteryl esters from triglycerides; monoglycerides and phospholipids were recovered as a combined fraction. The amounts of FFA, cholesterol, and diglycerides were too low to be clearly defined as individual fractions. Hence, these lipids were recovered, concentrated, and rechromatographed in the same solvent system to yield a clean separation of FFA. Cholesterol was subsequently separated from diglycerides in diethyl ether–benzene–ethanol–acetic acid 40:50:2:0.2.

Calculations. The quantities of glucose converted to CO_2 and glycerides were calculated from the initial specific activity of glucose in the incubation medium and the radioactivity in the products. The concentrations of fat cells were such that the volumes of buffer and hence the quantities of unlabeled glucose dispensed into incubation flasks varied over a 5-fold range. Therefore the specific activity of glucose was calculated after subtracting the volume of cellular glyceride from that of the total incubation mixture. Glycerides were determined as carboxyl ester (21) and the values converted to volume on the assumption that cellular lipid was triolein.

Corrections were not made for variations in the concentration of insulin in the incubation medium due to the presence of fat cells. Insulin was always added in excess (1 mU) to minimize the effects of such changes in concentration and to allow for destruction of the hormone during prolonged incubations of concentrated cell suspensions (22).

In general, we have omitted indices of the dispersion of metabolic parameters between subjects from the figures in order to keep them uncluttered. 5-fold differences in the metabolic activity of fat cells were observed between subjects. Similar variability has been recorded previously (16) and may be partly attributed to the fact that data were expressed in terms of cellular glyceride and not cell number (7). Likewise, the effect of age on the metabolic activity of fat cells (5) has been ignored. We emphasize that the present investigation was not designed to establish "normal values." Our primary objective was to determine the responses to a given experimental situation; such responses proved to be consistent from one subject to another.

TABLE 2 PERCENTAGE DISTRIBUTION OF GLUCOSE-U-^{14}C IN ADIPOSE CELL LIPIDS DURING INCUBATION

Lipid Class*	Duration of Incubation (Hr) 1	2	4	6
			%	
Triglycerides				
Control	83.9	79.6	81.9	81.3
Insulin	83.5	77.5	90.8	86.8
Free fatty acids				
Control	1.3	1.2	1.6	1.0
Insulin	1.1	1.3	1.4	1.7
Cholesterol				
Control	0.2	0.5	0.6	0.6
Insulin	0.6	0.6	0.3	1.0
Diglycerides				
Control	12.2	13.9	10.1	15.0
Insulin	11.4	13.8	4.6	7.9
Monoglycerides plus phospholipids				
Control	2.4	4.8	5.8	2.1
Insulin	3.4	6.8	2.9	2.6

0.5 ml aliquots of fat cell suspensions from subject A, equivalent to 202 mg of triolein, were incubated in 0.5 ml of modified albumin–bicarbonate buffer. Insulin, 1 mU/ml. Lipids were extracted in chloroform–methanol and the classes separated by TLC on Silica Gel G.

* Radioactivity of cholesteryl esters was negligible after these incubations.

RESULTS

Distribution of ^{14}C in Lipid Classes after Incubation with Glucose-U-^{14}C

The pattern of incorporation of glucose into adipose cell lipids was fairly constant during 6 hr of incubation of concentrated fat cells (Table 2). It was not influenced by the presence of insulin. 80–90% of the recovered ^{14}C was present in triglycerides and 5–15% in diglycerides. There was no labeling of cholesteryl ester, and virtually no radioactivity was recovered in free cholesterol. Radioactivity in the phospholipid–monoglyceride fraction did not exceed 6.8% of the total lipid-^{14}C, and less than 2% was found in FFA. The distribution of radioactivity in lipids observed in this study was similar to that previously recorded in incubations of human subcutaneous adipose tissue (4).

Time Course of Conversion of Glucose to Glyceride-Glycerol and Glyceride Fatty Acids

The conversion of glucose to glyceride-glycerol and fatty acids by concentrated suspensions of omental fat cells is shown in Fig. 1. On the basis of the data in Table 2, we assumed that most of the total lipid radioactivity was present in diglycerides and triglycerides. In the presence or absence of insulin the rate of glyceride-glycerol synthesis was maximal during the first 2 hr of incubation and diminished to a constant rate thereafter. Insulin stimulated the synthesis of glyceride-glycerol by an average of 40% during these incubations. The conversion of glucose to glyceride fatty acids was negligible for the first 2 hr without insulin, but then increased at an apparently rising rate. Even when insulin was present, the synthesis of fatty acids was negligible during the 1st hr; but during the 2nd hr fatty acid synthesis was stimulated 8-fold. The over-all effect of insulin was to produce a steadily increasing rate of fatty acid synthesis after an initial delay during the 1st hr of incubation.

Further (unpublished) studies revealed that fatty acid synthesis from glucose in whole segments of omental adipose tissue followed a similar time course, which indicated that the findings with isolated fat cells were not

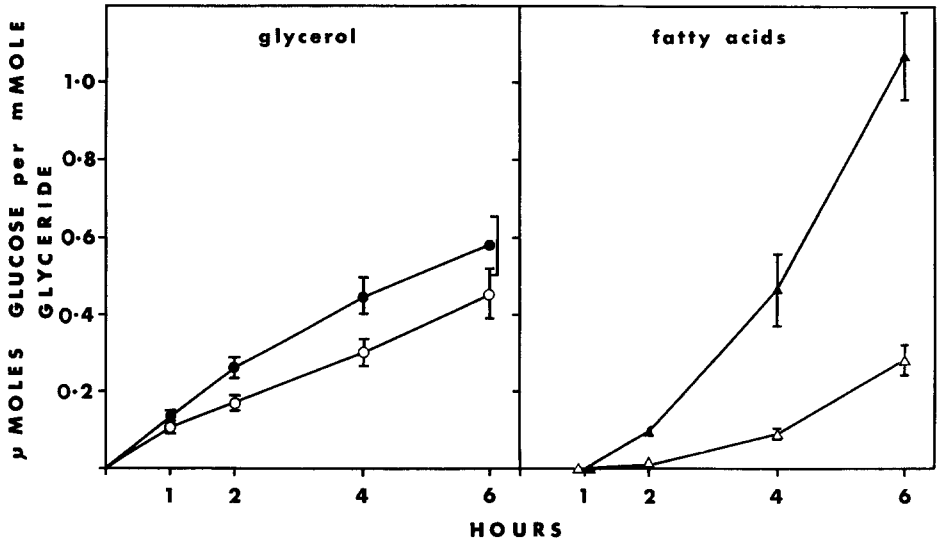

FIG. 1. Effect of insulin on the time course of conversion of glucose-U-^{14}C to glyceride-glycerol and glyceride fatty acids by omental fat cells. 0.5 ml aliquots of fat cell suspensions from subjects B, C, D, and E—equivalent to 223, 165, 281, and 314 mg of triolein—were incubated with 0.5 ml of modified albumin–bicarbonate buffer. Each point represents the mean of four experiments. Bars represent 1 SEM. Glyceride-glycerol, ○; glyceride fatty acids, △. Closed symbols represent the addition of 1 mU of insulin.

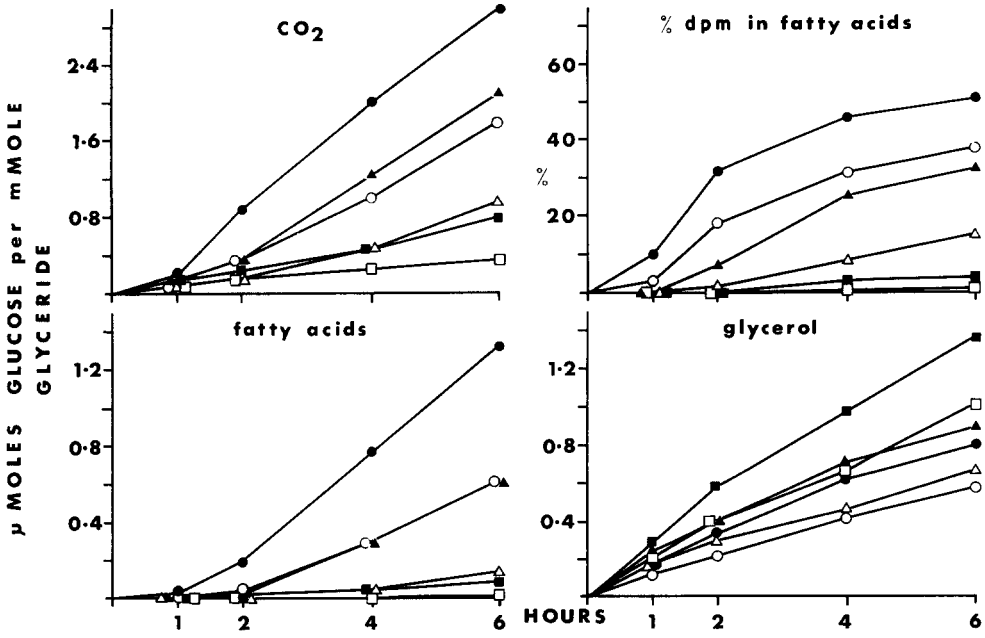

FIG. 2. Effects of cell dilution on the time course of glucose-U-^{14}C metabolism by omental fat cells in modified albumin–bicarbonate buffer. Incubation flasks contained 0.5 ml of buffer and 0.5 μC of glucose-U-^{14}C. 0.5 ml aliquots of fat cells were dispensed either without prior dilution (concentrated cells), or previously diluted by 1:1 volume (semiconcentrated cells) or 1:5 (dilute cells) with modified albumin–bicarbonate buffer. Fat cells were obtained from subjects F, G, and H and, when used without prior dilution, were equivalent to 252, 289, and 249 mg of triolein per flask. Each point represents the average of three experiments. Concentrated cells, ○; semiconcentrated cells, △; dilute cells, □. Closed symbols represent the addition of 1 mU of insulin.

dependent on the preparative procedures employed. We were also able to show (unpublished observations) that pentose cycle activity was present in concentrated suspensions of omental fat cells, as judged by the incorporation of glucose-1-^{14}C and glucose-6-^{14}C into CO_2. The passage of glucose through this pathway was increased in the presence of insulin; and in fact was maximal at the 1st hr of incubation. Thus, it appeared that the initial delay in the conversion of glucose to glyceride fatty acids was not related to diminished pentose cycle activity.

Effects of Cell Dilution on Glucose-U-^{14}C Metabolism

In the present investigation we have generally used concentrated suspensions of fat cells (approximately 250 mg of triglycerides per flask), compared with 15–44 mg per flask in earlier studies in which fatty acid synthesis from glucose was not demonstrated (16). For this reason the effects of cell dilution with modified albumin–bicarbonate buffer on glucose-U-^{14}C metabolism were examined (Fig. 2). When dilute cells, averaging 53 mg of triglyceride per flask, were incubated without insulin there was virtually no conversion of glucose to glyceride fatty acids within 6 hr. In the presence of insulin a low level of fatty acid synthesis was demonstrable by the 4th hr of incubation. With semiconcentrated cells, averaging 133 mg of triglyceride per flask, much higher rates of fatty acid synthesis were found than with dilute cells, but these rates were clearly lower than with concentrated cell preparations (265 mg of triglycerides per flask). Insulin was observed to affect fatty acid synthesis with concentrated and semiconcentrated cell suspensions by the 2nd and 4th hr of incubation, respectively. Glucose oxidation was also affected by cell dilution and followed a pattern similar to that of fatty acid synthesis. On the other hand, glucose incorporation into glyceride-glycerol occurred at all dilutions, and was higher with dilute than with semiconcentrated cell preparations; the lowest values were observed with concentrated cells. An additional finding was the effect of cell dilution on the distribution of glucose radioactivity between glyceride-glycerol and glyceride fatty acids. The percentage of the glyceride-^{14}C in fatty acids, with or without insulin, was in the order of concentrated > semiconcentrated > dilute cell suspensions. With concentrated fat cells an average of 52% (range 32–77%) of the total glyceride radioactivity was recovered in fatty acids at 6 hr in the presence of insulin.

When various concentrations of fat cells were incubated in albumin-free Krebs–Henseleit bicarbonate buffer (Fig. 3), synthesis of glyceride fatty acids from glucose was again maximal with concentrated cell suspensions, whereas glyceride-glycerol was formed at similar rates with all three concentrations of cells. It will

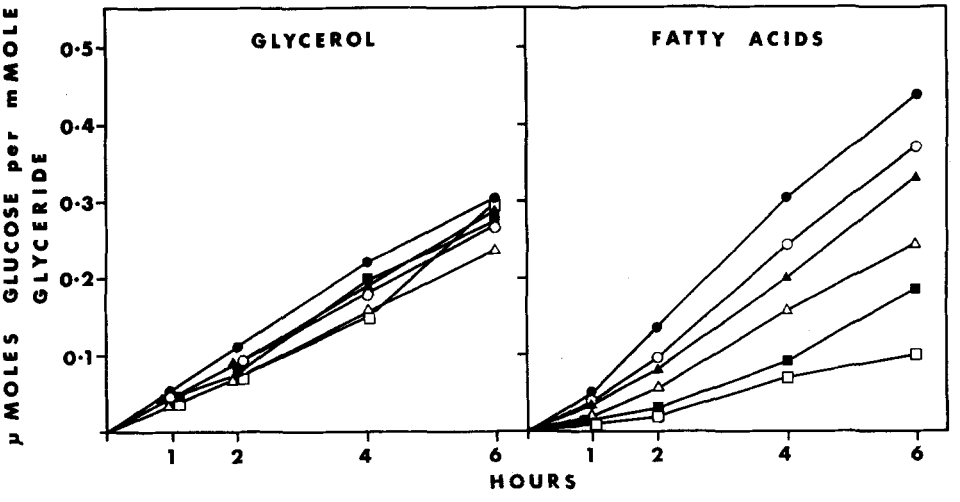

FIG. 3. Effects of cell dilution on the time course of glucose-U-^{14}C metabolism by omental fat cells in Krebs–Henseleit bicarbonate buffer. Incubation flaks contained 0.5 ml of buffer and 0.5 μC of glucose-U-^{14}C. 0.5 ml aliquots of fat cells were dispensed either without prior dilution (concentrated cells) or previously diluted 1:1 by volume (semiconcentrated cells) or 1:5 (dilute cells) with Krebs–Henseleit bicarbonate buffer. Fat cells were obtained from subjects I and J and, when used without prior dilution, were equivalent to 280 and 356 mg of triolein per flask. Each point represents the average of two experiments. Concentrated cells, ○; semiconcentrated cells, △; dilute cells, □. Closed symbols represent the addition of 1 mU of insulin.

also be noted that glyceride-glycerol synthesis was linear with time when fat cells were incubated in Krebs–Henseleit bicarbonate buffer (Fig. 3), whereas in modified albumin–bicarbonate buffer the rate of glyceride-glycerol synthesis tended to diminish slightly after the first 1 or 2 hr of incubation (Figs. 1 and 2). Furthermore, the initial delay in fatty acid synthesis, which was a striking feature of incubations in modified albumin–bicarbonate buffer (Figs. 1 and 2), was less pronounced when Krebs–Henseleit bicarbonate buffer was used (Fig. 3). In view of these differences further experiments (not shown) were carried out to determine the extent to which changes in pH, pCO_2, and FFA concentration might influence glycerol and fatty acid synthesis and thereby account for the effects of cell dilution and buffer composition on glucose metabolism. The principal findings are summarized as follows.

(*a*) Adjustment of the pH of modified albumin–bicarbonate buffer between 7.0 and 7.8 caused no change in the rate of conversion of glucose to glyceride-glycerol and glyceride fatty acids. This pH range was well outside the changes in pH observed during incubations of concentrated cell suspensions.

(*b*) Thorough equilibration of Krebs–Henseleit bicarbonate buffer (± albumin) with 95% O_2–5% CO_2 maintained the conversion of glucose to glyceride-glycerol at a linear rate over a period of 6 hr, but did not change the time course of fatty acid synthesis from that shown in Fig. 1.

(*c*) The presence of bovine serum albumin was shown to be responsible for the initial delay in fatty acid synthesis, which was more pronounced in modified albumin–bicarbonate than in Krebs–Henseleit bicarbonate buffer.

(*d*) During incubations of concentrated cell suspensions in modified albumin–bicarbonate buffer there was a net uptake of FFA from the mediun, which caused the FFA concentration to fall from 0.4 to 0.2 μmole/ml during the first 2 hr. When the molar ratio of FFA:-albumin was adjusted to produce a range of FFA concentrations corresponding to that observed during incubations of concentrated cells, synthesis of glyceride fatty acids was unaffected. However, glyceride-glycerol synthesis was stimulated as the FFA concentration was raised.

These observations indicated that the minor differences in the pattern of glucose conversion to glyceride-glycerol and glyceride fatty acids in Krebs–Henseleit and modified albumin–bicarbonate buffers were probably due to differences in gassing the media or to the presence of albumin. Differences in buffering capacity appeared to be unimportant. The effects of cell dilution on synthesis of glyceride fatty acids could not be attributed to any of the factors examined. However, the enhanced ability of dilute cell suspensions to convert glucose to glycerol when incubated in modified albumin–bicarbonate buffer may have been caused by the maintenance of higher FFA levels in the medium than in concentrated cell suspensions.

DISCUSSION

We have shown that under appropriate conditions

omental fat cells prepared from fasted adult subjects are able to convert glucose to glyceride fatty acids. This ability has been demonstrated in whole pieces of human adipose tissue by a number of workers (3–6, 12, 23) but not previously in preparations of human fat cells (16, 17). Under the incubation conditions employed here, the rates of fatty acid synthesis and glucose oxidation were nonlinear with time; they became maximal after a lag period of one or more hours. The delay in fatty acid synthesis was thought to be partially accounted for by the inclusion of albumin in the incubation medium. This delay appeared to be unrelated to the method of preparing the fat cells, because similar findings were recorded with segments of human omental adipose tissue. The temporal dissociation between the rate of fatty acid synthesis and the activity of the pentose cycle observed here, and the delayed onset of fatty acid synthesis from glucose reported in parametrial fat cells and epididymal adipose tissue from fasted rats (24–26) suggest that enzymes involved in fatty acid synthesis may be rate-limiting in tissues from humans fasted prior to surgery. Citrate cleavage enzyme is barely demonstrable in human adipose tissue obtained at surgery (27), so it is conceivable that levels of this enzyme or fatty acid synthetase build up gradually during incubations in the presence of glucose.

The concentration of fat cells was also of major importance in determining the rate of synthesis of glyceride fatty acids. With dilute suspensions of fat cells, such as those used in earlier studies (16), fatty acid synthesis from glucose was barely detectable, whereas more concentrated fat cell preparations rapidly converted glucose to glyceride fatty acids between the 2nd and 6th hr of incubation. Inhibitory effects of dilution on fatty acid synthesis in isolated fat cells have not been described before, and it is of considerable interest that the pattern of glucose metabolism and the response to insulin came to resemble the metabolic characteristics of human adipose tissue segments (5) as the cell concentration was increased. The concentration–dilution effects could not be attributed to changes in the pH or FFA concentration of the incubation medium, and the basis of these phenomena is obscure. It should be emphasized, however, that the present studies have not excluded the possibility of the concentration–dilution effects being attributable to variable contamination of the cells with collagenase. In other words, at appropriate concentrations collagenase might exhibit effects analogous to those of bacterial proteases (28). If this were so, then collagenase would have to exhibit highly specific effects, because glyceride-glycerol synthesis was unaffected by dilution in Krebs–Henseleit bicarbonate buffer. Furthermore, other workers have failed to show changes in glucose oxidation, FFA release, and oxygen consumption with different concentrations of rat (29) or human (30) fat cells.

In contrast to fatty acid synthesis in omental fat cells, the conversion of glucose to glyceride-glycerol was maximal at 1st hr of incubation and, in the absence of albumin, was unaffected by cell concentration. In the presence of albumin, dilute cell suspensions synthesized glyceride-glycerol somewhat more rapidly than concentrated cells. The latter phenomenon appeared to be caused by small changes in the FFA concentration of the buffer during incubation. Such changes in FFA concentration did not affect the rate of fatty acid synthesis from glucose; and the apparent discrepancy between the present findings and those of Turtle and Kipnis (31) with rat epididymal fat cells may be explained by the fact that we were concerned with the effects of very low concentrations of FFA. In view of these effects, the use of bovine serum albumin in studies such as these should be avoided if possible.

The different responses of glyceride-glycerol and glyceride fatty acids to variations in environmental conditions imply that widely divergent patterns of glucose metabolism will be obtained with human omental fat cells depending on the incubation conditions employed. These peculiarities of the preparations of isolated human fat cells have created a rather unusual situation wherein it is possible to carry out metabolic studies which may or may not lead to synthesis of fatty acids from glucose.

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References

1. Östman, J. 1965. *Acta Med. Scand.* **177:** 183.
2. Björntorp, P. 1967. *Acta Med. Scand.* **182:** 717.
3. Fessler, A., J. C. Beck, and D. Rubinstein. 1967. *Metabolism.* **16:** 438.
4. Hirsch, J., and R. B. Goldrick. 1964. *J. Clin. Invest.* **43:** 1776.
5. Gries, F. A., and J. Steinke. 1967. *J. Clin. Invest.* **46:** 1413.
6. Björntorp, P. 1967. *Acta Med. Scand.* **181:** 389.
7. Salans, L. B., J. L. Knittle, and J. Hirsch. 1968. *J. Clin. Invest.* **47:** 153.
8. Björntorp, P., and A. Martinsson. 1967. *Acta Med. Scand.* **181:** 359.
9. Martinsson, A. 1967. *Acta Med. Scand.* **182:** 787.
10. Björntorp, P. 1966. *Acta Med. Scand.* **179:** 229.
11. Pozza, G., A. Ghidoni, and C. Basilico. 1963. *Lancet.* **i:** 836.
12. Fessler, A., and J. C. Beck. 1965. *Biochim. Biophys. Acta.* **106:** 199.
13. Kahlenberg, A., and N. Kalant. 1964. *Can. J. Biochem.* **42:** 1623.

14. Owen, J. A., R. W. Lindsay, J. H. Gaskin, and G. Hollifield. 1967. *Metabolism.* **16:** 47.
15. Björntorp, P. 1967. *Acta Med. Scand.* **181:** 389.
16. Goldrick, R. B. 1967. *J. Lipid Res.* **8:** 581.
17. Galton, D. J. 1968. *J. Lipid Res.* **9:** 19.
18. Documenta Geigy. 1962. Scientific Tables. Ben Johnson & Co. Ltd, Great Britain. 6th edition. 623.
19. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. *J. Biol. Chem.* **226:** 497.
20. Goldrick, R. B., and J. Hirsch. 1963. *J. Lipid Res.* **4:** 482.
21. Skidmore, W. D., and C. Entenman. 1962. *J. Lipid Res.* **3:** 356.
22. Crofford, O. B. 1968. *J. Biol. Chem.* **243:** 362.
23. Kuo, P. T., L. Feng, N. N. Cohen, W. T. Fitts, Jr., and L. D. Miller. 1967. *Amer. J. Clin. Nutr.* **20:** 116.
24. Fain, J. N., V. P. Kovacev, and R. O. Scow. 1965. *J. Biol. Chem.* **240:** 3522.
25. Galton, D. J., and J. N. Fain. 1966. *Biochem. J.* **98:** 557.
26. Herrera, M. G., G. R. Philipps, and A. E. Renold. 1965. *Biochim. Biophys. Acta.* **106:** 221.
27. Shrago, E., J. A. Glennon, and E. S. Gordon. 1967. *J. Clin. Endocrinol. Metab.* **27:** 679.
28. Kuo, J. F., I. K. Dill, and C. E. Holmlund. 1967. *J. Biol. Chem.* **242:** 3659.
29. Rodbell, M. 1965. *In* Handbook of Physiology. A. E. Renold and G. F. Cahill, Jr., editors. American Physiological Society, Washington, D.C. Section 5: 471–482.
30. Galton, D. J., and G. A. Bray. 1967. *J. Clin. Invest.* **46:** 621.
31. Turtle, J. R., and D. M. Kipnis. 1967. *Biochim. Biophys. Acta.* **144:** 583.