# Glycerokinase in Human Adipose Tissue

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### ABSTRACT

The presence of glycerokinase has been demonstrated in human omental and subcutaneous adipose tissue. The enzyme reaction showed a linear time course for 5 min at 30 C and pH optima at pH 7.6 and 9.0. Saturation of the enzyme was observed at 1.8 mM adenosine triphosphate (ATP) and the double reciprocal plot of activity vs. ATP concentration was nonlinear giving two apparent Km values of 0.094 and 0.518 mM. The apparent Km for glycerol, 0.112 mM, was obtained from a linear double reciprocal plot, and the enzyme was saturated at about 0.4 mM glycerol. The activity of glycerokinase in human adipose tissue excised under general anaesthesia was low and was unrelated to adipose cell size or the degree of obesity of the subject from whom the fat was obtained.

### INTRODUCTION

The development of a radiochemical assay (1) established the presence of glycerokinase (adenosine triphosphate:glycerol phosphotransferase, EC 2.7.1.30) in rat adipose tissue (2) and raised the possibility that the enzyme might be involved in the development of obesity (3). Further studies have confirmed the presence of glycerokinase in rats (4,5) and have also shown that this enzyme occurs in adipose tissue from mice (6,7), domestic chickens (8), and pigs (9), and in human subcutaneous adipose tissue (10). The objectives of the present investigation were to establish optimal conditions for assaying glycerokinase in human adipose tissue and to determine whether any relationship exists between the activity of the enzyme and adipose cell size. Since subcutaneous fat cells are almost invariably larger than omental fat cells (11), paried comparisons were performed on omental and subcutaneous adipose tissue obtained from each of a series of subjects undergoing elective abdominal surgery.

### MATERIALS AND METHODS

#### Source of Adipose Tissue

Adipose tissue was obtained from subjects undergoing elective cholecystectomy or hysterectomy. Operations were performed in the mornings following an overnight fast. Morphine and atropine or their derivatives were used for premedication. Anaesthesia was induced with thiopentone sodium and maintained with nitrous oxide and halothane. Tissues were transported to the laboratory in 0.15 M sodium chloride at 37 C and processed within 15 min. Preliminary studies to define optimal conditions for assaying glycerokinase were performed on adipose tissue from the greater omentum. Comparative studies were performed on fat obtained from the subcutaneous tissues of the anterior abdominal wall and the greater omentum of 18 subjects. Relevant clinical data on these 18 subjects as well as individual values for adipose cell size are shown in Table I. Most of the subjects were females. Their body weights standardized for age, sex, and height (12) averaged 18% in excess of ideal (range-6% to + 37%). Paired comparisons of adipose cell size (expressed as μg triolein per cell) showed that subcutaneous fat cells were 73% larger ($P<0.001$) than omental fat cells.

#### Assay of Glycerokinase

Adipose tissue was homogenized for 2 min at 0 C in 2 volumes of 2% KCl in 1 mM EDTA. The homogenate was centrifuged at 17,000 x g for 10 min at 4 C. The aqueous layer was centrifuged at 100,000 x g for 30 min at 4 C. The supernate was used directly in the assay which was performed using a modification of the procedure of Newsholme et al. (1). The reaction mixture contained the following components in a final volume of 130 μl: 0.04 μmoles glycerol -$^{14}$C (specific activity 16.2 μCi/μmole); 0.44 μmoles adenosine triphosphate (ATP); 1.3 μmoles ethylene diamine tetracetic acid (EDTA); 3.25 μmoles NaF; 13 μmoles tris-HCl, pH 7.6. The reaction was started by the addition of 50 μl of homogenate and allowed to proceed for 5 min at 30 C. The reaction was stopped by the addition of 100 μl absolute ethanol. Controls consisted of reaction mixtures to which ethanol was added before the enzyme extract. The protein was removed by centrifugation, and 20 μl of the deproteinized reaction mixture were applied transversely at one end of a 1.5 x 6 cm strip of Whatman DE 81 chromatography paper. Glycerophosphate was separated from glycerol by descending

TABLE I

Clinical Data

| Subject | Age (yr) | Sex | % IBW[a] | μg triolein per cell Adipose tissue Omental | Subcutaneous |
|---|---|---|---|---|---|
| 1 | 19 | F | 125 | 0.235 | 0.546 |
| 2 | 25 | F | 104 | 0.208 | 0.590 |
| 3 | 55 | M | 132 | 0.880 | 1.285 |
| 4 | 35 | F | 137 | 0.439 | 0.766 |
| 5 | 63 | F | 135 | 0.238 | 0.565 |
| 6 | 23 | F | 115 | 0.264 | 0.485 |
| 7 | 57 | M | 117 | 0.746 | 0.720 |
| 8 | 49 | F | 104 | 0.333 | 0.890 |
| 9 | 35 | F | 133 | 0.259 | 0.710 |
| 10 | 77 | M | 94 | 0.712 | 0.590 |
| 11 | 36 | M | 117 | 0.292 | 0.482 |
| 12 | 23 | F | 130 | 0.140 | 0.361 |
| 13 | 61 | F | 121 | 0.438 | 0.918 |
| 14 | 45 | F | 106 | 0.364 | 0.524 |
| 15 | 27 | F | 113 | 0.334 | 0.593 |
| 16 | 44 | F | 100 | 0.173 | 0.413 |
| 17 | 65 | F | 133 | 0.271 | 0.312 |
| 18 | 32 | F | 106 | 0.275 | 0.656 |
| Means ± SEM | 43.8 ± 4.0 | | 118 ± 3 | 0.367 ± 0.049 | 0.634 ± 0.054 |

[a]Percentage of ideal body weight

chromatography in distilled water. Glycerophosphate was recovered in the area closest to the origin; glycerol ran with the solvent front. The papers were dried in air and their radioactivity was measured in a liquid scintillation system using a toluene scintillator (0.05 g dimethyl POPOP and 4 g PPO per liter toluene). The product of the assay had the same Rf values as glycerophosphate when tested in the present separation system and when subjected to descending chromatography on Whatman No. I paper in the following solvent systems (13): (a) ethyl acetate:acetic acid:water = 3:3:1, (b) methyl cellosolve:methyl ethyl ketone:3M $NH_4OH$ = 7:2:3. The amount of product formed was calculated from the specific activity of glycerol in the assay system and the radioactivity measured in glycerophosphate. The data have been expressed as n moles glycerol converted per minute either per mg protein or per $10^6$ adipose cells. The coefficient of variation of replicate analyses of a single homogenate was 4.9%.

#### Analytical Methods

Protein was estimated by the method of Lowry et al. (14). Glycerol was measured enzymatically (15) in homogenates which had been deproteinized with perchloric acid and neutralized with potassium bicarbonate. The conversion of reduced trinicotinamide adenine dinucleotide (NADH) to nicotinamide adenine dinucleotide (NAD) was measured in an Aminco-Bowman spectrofluorimeter using excitation and emission wavelengths of 340 and 460 nm, respectively. The assay system was adapted for fluorimetry by reducing the concentration of NADH to 100 nmoles in a final volume of 1 ml. The concentration of glycerol was estimated from internal standards spanning the range 5-60 nmoles. The diameter of adipose cells was measured (16) on at least 500 isolated fat cells prepared (17) from a subsample of the specimen of adipose tissue and is expressed as μg triolein per cell. The latter was calculated from the mean cell volume (18) and the specific gravity of triolein. The number of fat cells in the sample of adipose tissue used in the glycerokinase assay was derived from the wet weight of the tissue as follows. A subsample of adipose tissue was weighed and then extracted for 48 hr at 4 C in isopropyl alcohol:heptane: 0.1 M sulphuric acid 4:1:0.1 v/v. The extract was converted to a two phase system (19) and the triglyceride content was measured (20) on an aliquot of the upper phase. The number of adipose cells in a given wet weight of tissue was calculated by dividing the triglyceride content by the weight of triolein per cell. Paired comparisons of the data were performed by the analysis of vairance and correlation coefficients were computed by covariance analysis (21).

#### Materials

All enzymes and cofactors for the glycerol and glycerokinase assays were obtained from

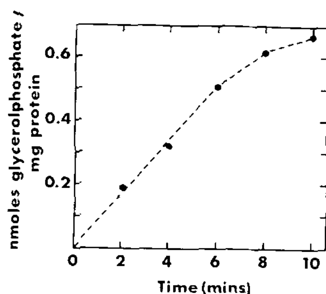


FIG. 1. Time course of glycerophosphate production at 30 C. The data represent the means of six experiments.

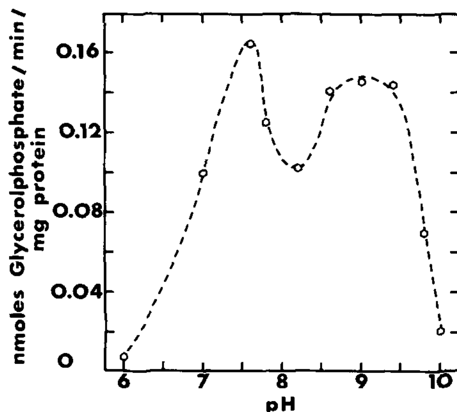


FIG. 2. Effects of pH on the production of glycerophosphate. Tris-HCl was used for all values of pH except at Ph 6.0 when tris-maleate buffer was used. The data represent the means of three experimentsl.

the Boehringer Corp. (London) Ltd. Other materials and their suppliers were as follows: collagenase (Worthington Biochemical Corp., Freehold, NJ); DL-α-(di) sodium glycerophosphate, 6 $H_2O$ (Mann Research Laboratories, Orangeburg, NY); Whatman DE81 and Whatman No. I chromatography papers (W & R Balston Ltd., England); and glycerol-$^{14}$C (Radiochemical Centre, Amersham, Bucks, England). The glycerol-$^{14}$C was purified (>99.6%) before use by descending chromatography in distilled water on Whatman DE81 paper.

## RESULTS

Preliminary studies showed that conditions for assaying glycerokinase in rat adipose tissue failed to permit a linear production of glycerophosphate withtime. This problem was not corrected by using an ATP regenerating system (2) but was overcome by carrying out the assay at 30 C. Mercaptoethanol had unpredictable effects and was therefore omitted. With the present system, the production of glycerophosphate was linear for 6 min (Fig. 1), it was 2.5-fold greater than that observed when the assay was performed at 37 C for 30 min and was proportional to the concentration of protein over the range 20-160 μg. Enzyme activity was undetectable in the absence of tissue extract and was destroyed by boiling for 5 min. Activity was also destroyed by preincubating the tissue homogenate with 10 mM iodoacetamide in 0.03 mM tris buffer, pH 7.4 for 75 min. In the latter respect, the glycerokinase in human adipose tissue exhibited similar characteristics to that in rat liver (22). As shown in Figure 2, the human enzyme exhibited pH optima at 7.6 and 9.0. For most glycerokinases, the optimum lies in the range pH 9.0-9.8 (23) although some have an optimum between pH 7.0-7.5 (24). The reason for the double peak in human adipose tissue is uncertain, but because the lower optimum is closer to physiological, a pH of 7.6 was used for all assays.

The effect of varying the concentration of ATP is shown in Figure 3. In the absence of exogenous ATP, ca. one-third of maximal activity was found and the data have been corrected for endogenous ATP. The human enzyme exhibited a downward curvature of the double reciprocal plot giving two apparent Km values of 0.094 and 0.518 mM. This unusual property appears to be characteristic of glycerokinases insofar as it has been demonstrated in rat, beef, and human liver and in *Candida mycoderma* (25) as well as in crystalline glycerokinase from *Escherichia coli* (26). The data in Figure 3 show that the enzyme was saturated at 1.8 mM ATP.

The effect of varying the concentration of glycerol is shown in Figure 4. In contrast to the findings with ATP, the double reciprocal plots of enzyme activity vs. glycerol concentration were linear with saturation occurring at about 0.4 mM. The mean ± S.E.M. apparent Km for glycerol was 0.112 ± 0.009 mM. Corresponding values for rat adipose tissue vary from 0.01 mM (2) to 1.0 mM (5). The apparent Km for glycerol with the glycerokinase in chicken adipose tissue is 0.153 mM (8) and is of the same order of magnitude as that observed in the present investigation.

Table II lists the average values for glycerokinase activity as well as concentrations of glycerol and protein measured in homogenates prepared from the tissues of subjects in Table I. When the data in Table II were expressed on a per cell basis, glycerokinase activity was similar in subcutaneous and omental adipose tissue from the same subject. The protein concentrations of the two tissues were also similar. How-

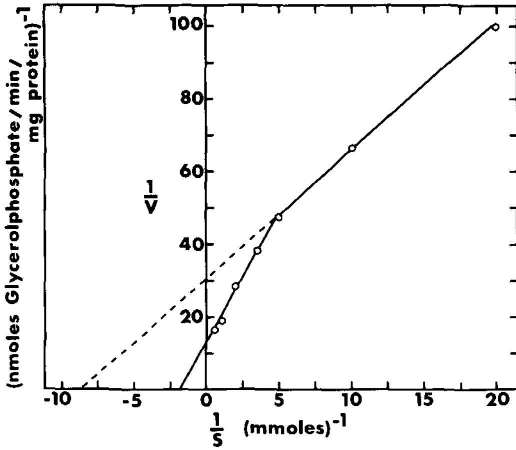


FIG. 3. Double reciprocal plot of glycerophosphate production versus the concentration of adenosine triphosphate (ATP). The data represent the means of four experiments.

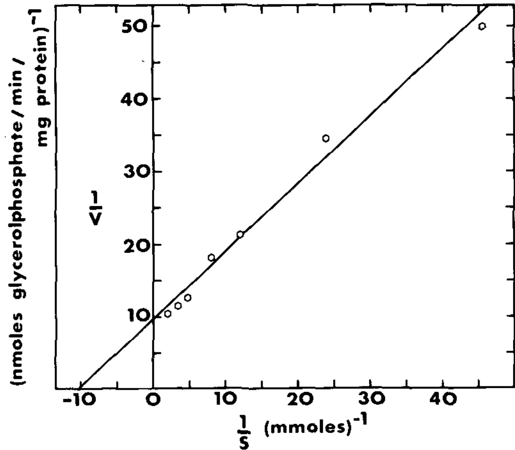


FIG. 4. Double reciprocal plot of glycerophosphate production versus the concentration of glycerol. The data represent the means of seven experiments.

ever, glycerokinase activity expressed per mg protein was significantly greater in omental than subcutaneous adipose tissue. Since glycerokinase is confined to adipocytes (4,5,27,28) and the latter contribute only about 50% of the total protein in adipose tissue (29), it appears that tissue protein is a poor reference for comparing the activity of this enzyme in fat from different sites. In effect, paired analyses have failed to shown any difference in glycerokinase activity between large and small fat cells from the same subject. Furthermore, there was no relationship between glycerokinase activity and adipose cell size in subcutaneous or omental adipose tissue. There was also no relationship between glycerokinase activity and the degree of obesity. However, it should be emphasized that the range of relative body weights of these subjects was fairly small.

The data for tissue glycerol concentrations listed in Table II can be used to estimate the error involved if dilution of the labelled glycerol by tissue glycerol is not corrected for when computing the production of glycerophosphate. Using the average values for tissue glycerol/g adipose tissue, it can be calculated that glycerokinase activity will be underestimated by 10% in subcutaneous adipose tissue and by 5% in omental adipose tissue if the glycerol content of the homogenate is not taken into account. It should be emphasized, however, that these estimates apply only to the conditions of the present experiments because it is very likely that the tissue glycerol concentrations were artificially high due to lipolysis that occurred between removal of tissues at surgery and processing in the laboratory. This can be deduced from the fact that glycerol production is increased in enlarged fat cells (11) and, as shown in Table II, tissue glycerol concentrations were higher in subcutaneous than in omental adipose tissue. Furthermore, not only were subcutaneous fat cells larger than omental fat cells, but also the concentration of tissue glycerol/$10^6$ cells was directly proportional to adipose cell size in omental (0.701; $P<0.001$)

TABLE II

Metabolic Data on Omental and Subcutaneous Adipose Tissue[a]

| | Omental | Subcutaneous | |
|---|---|---|---|
| Glycerokinase activity | | | |
| (nmoles glycerol/$10^6$ cells/min) | 0.24 ± 0.04 | 0.30 ± 0.07 | N.S. |
| (nmoles glycerol/mg protein/min) | 0.15 ± 0.003 | 0.08 ± 0.02 | $P<0.05$ |
| Glycerol ($\mu$moles/g tissue) | 0.082 ± 0.009 | 0.161 ± 0.028 | $P<0.01$ |
| Glycerol ($\mu$moles/$10^6$ cells) | 0.042 ± 0.009 | 0.139 ± 0.023 | $P<0.001$ |
| Protein (mg/g tissue) | 4.56 ± 0.50 | 5.20 ± 0.54 | N.S. |

[a]Values are given as means ± SEM

and subcutaneous ($r=0.592$; $P<0.01$) fat.

## DISCUSSION

The present investigation has confirmed the findings of Koschinsky and Gries (10) that glycerokinase is present in human subcutaneous adipose tissue. We have also shown that this enzyme occurs in adipose tissue from the greater omentum and that its activity is similar in omental and subcutaneous fat. Modifications to established assay conditions (1,2) were necessary for measuring optimal enzyme activity in human tissues. These consisted principally of lowering the incubation temperature to 30 C, reducing the incubation time to 5 min, and omitting mercaptoethanol from the reaction system. Under these conditions, the requirements of the human enzyme for ATP were similar to those of other species, and the Km for glycerol was similar to that of the glycerokinase in chicken adipose tissue. The human enzyme, however, was distinguished from other glycerokinases by exhibiting two pH optima, one at 7.6 and the other at 9.0.

The activity of the glycerokinase in human adipose tissue was very low by comparison with that in laboratory animals. The potential ability of human adipose tissue to phosphorylate glycerol was ca. 1% of that in porcine adipose tissue (9), 2% of values in hens and mice (7,8), and ca. 20% of values in rat adipose cells (4). Differences of this order are not surprising because other parameters of metabolic activity in rodent, porcine, and avian adipose tissue are generally greater than in human adipose tissue. However, they may well have been exaggerated by the effects of general anaesthesia and starvation prior to obtaining samples of tissue at surgery (8). The activities observed in this investigation were ca. 20 times greater than those reported in subcutaneous adipose tissue from lean subjects and 1.5-fold greater than those reported in subcutaneous fat from grossly obese subjects (10). The higher enzyme activities recorded here cannot be attributed to differences in nutritional state and are at least partly accounted for by differences in assay conditions. We were unable to show any relationship between adipose cell size and glycerokinase activity nor could we confirm previous reports that glycerokinase activity is elevated in adipose tissue of obese humans (10). However, in the latter connection, it should be emphasized that the subjects studied here encompassed a fairly narrow range of relative body weights.

The physiological importance of glycerokinase in human adipose tissue remains to be defined. If the enzyme is saturated with substrate, the present findings indicate that omental adipose tissue can phosphorylate 0.24 nmoles glycerol/$10^6$ cells/min. Basal glycerol production in human omental fat cells averages 0.933 nmoles/$10^6$ cells/min (11). On this basis, the true rate of glycerol production is 1.173 nmoles/$10^6$ cells/min and reutilization of glycerol represents 20.5% of the total. Similar calculations show that 14.7% of glycerol produced under basal conditions by subcutaneous fat cells (11) is reutilized. These calculations assume inter alia that the intracellular concentration of free glycerol is of the order of 0.4 mM which represented a saturating concentration of glycerol in the glycerokinase assay. On the other hand, glycerol is freely diffusible and its concentration in plasma in the post absorptive state is ca. 0.05 mM (30). If the intracellular concentration of glycerol corresponds to that in plasma, it can be calculated from the Km for glycerol that glycerokinase will phosphorylate 0.08 nmoles glycerol/$10^6$ cells/min in omental adipose tissue or reutilize 7.9% of the glycerol produced. In the case of subcutaneous adipose tissue, glycerokinase may only phosphorylate 0.1 nmoles glycerol/$10^6$ cells/min which represents a 5.2% reutilization of the glycerol produced. Thus, in the final analysis, accurate data on the effects of dietary changes and obesity on the intracellular concentration of glycerol and on the activity of glycerokinase will be required before a regulatory role for glycerokinase in human adipost tissue can be defined.

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## REFERENCES

1. Newsholme, E.A., J. Robinson, and K. Taylor, Biochim. Biophys. Acta 132:338 (1967).
2. Robinson, J., and E.A. Newsholme, Biochem. J. 104:2C (1967).
3. Mayer, J., "Overwiehgt: Causes, Cost and Control," Prentice-Hall Inc., Englewood, Cliffs, NJ, 1968.
4. Reardon, M.F., R.B. Goldrick, and N.H. Fidge, J. Lipid Res. 14:319 (1973).
5. Persico, P.A., G.M. Cerchio, and H. Jeffay, Am. J. Physiol. 228:1868 (1975).
6. Koschinsky, T., F.A. Gries, and L. Herberg, Horm. Metab. Res. 2:185 (1970).
7. Welton, R.F., R.J. Martin, and B.R. Baumgardt, J. Nutr. 103:1212 (1973).

8. Welton, R.F., R.J. Martin, R.W. Scholz, and B.R. Baumgardt, Ibid. 103:890 (1973).
9. Martin, R.J., and J.H. Herbein, Proc. Soc. Exp. Biol. Med. 151:231 (1976).
10. Koschinsky, T., and F.A. Gries, Hoppe-Seyler's Z. Physiol. Chem. 352:430 (1971).
11. Goldrick, R.B., and G.M. McLoughlin, J. Clin. Invest. 49:1213 (1970).
12. Society of Actuaries, Chicago, Build and Blood Pressure Study 1:16, 1959.
13. Mortimer, D.C., Can. J. Biochem. 30:653 (1952).
14. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall, J. Biol. Chem. 193:265 (1951).
15. Garland, P.B., and P.J. Randle, Nature (London) 196:987 (1962).
16. McCleod, K.I., R.B. Goldrick, and H.M. Whyte, Aust. J. Exp. Biol. Med.Sci. 50:435 (1972).
17. Goldrick, R.B., J. Lipid Res. 8:581 (1967).
18. Goldrick, R.B., Am. J. Physiol. 212:777 (1967).
19. Dole, V.P., and H. Meinertz, J. Biol. Chem. 235:2595 (1960).
20. Skidmore, C., and W.D. Entenman, J. Lipid Res. 3:356 (1962).
21. Bailey, N.T.J., "Statistical Methods in Biology," English Universities Press Ltd., London, 1959.
22. Bublitz, C., and E.P. Kennedy, J. Biol. Chem. 211:951 (1956).
23. Thorner, J.W., and H. Paulus, "The Enzymes," Edited by Paul D. Boyer, Academic Press, NY, 1973, p. 487.
24. Pande, S.V., R. Parvin, and T.A. Venkitasubramanian, Can. J. Biochem. 45:797 (1967).
25. Grunnet, H., and F. Lundquist, Eur. J. Biochem. 3:78 (1967).
26. Thorner, J.W., and H. Paulus, J. Biol. Chem. 248:3922 (1973).
27. Koschinsky, T., F.A. Gries, and L. Herberg, Diabetologia 7:316 (1971).
28. Thenen, S.W., and J. Mayer, Proc. Soc. Exp. Biol. Med. 148:953 (1975).
29. Rodbell, M., J. Biol. Chem. 239:753 (1964).
30. Goldrick, R.B., N. Havenstein, K.F. Carroll, and M. Reardon, Metabolism 21:761 (1972).


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