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RADIOENZYMIC ASSAY OF GLYCEROL USING ION-EXCHANGE COLUMN CHROMATOGRAPHY

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SUMMARY: Modifications of the glycerol kinase radioenzymic assay for glycerol are described. This method can be readily employed to measure glycerol kinase activity in tissue extracts as well. Ion-exchange column chromatography (QAE-Sephadex A-25) completely separates the product ^{14}C-glycerol 3-phosphate from ^{14}C-glycerol, and allows all glycerol 3-phosphate formed in an assay to be counted in a single counting vial. Increasing the Mg^{2+} concentration significantly increases activity of glycerol kinase and thus the sensitivity of the assay. These modifications provide a simple, reliable, sensitive and more rapid procedure.

INTRODUCTION: A spectrophotometric method for the determination of serum glycerol was first described by Wieland and Suyter (1) using glycerolkinase reaction complex with glycerol 3-phosphate dehydrogenase. A fluoro-spectrophotometric adaptation of this method has been reported (2) and a similar method has been described by Garland and Randle (3). The radiochemical assay for glycerol was introduced by Newsholme et.al. (4). It measures ^{14}C-glycerol 3-phosphate (G3P) formed by glycerol kinase. The radioactivity is quantitatively diluted by the presence of non-labelled glycerol in the sample, ^{14}C-G3P was then separated from glycerol by absorption onto DEAE-cellulose ion-exchange paper disks and glycerol was washed off with water. The present method uses ion-exchange column chromatography to achieve a complete separation of ^{14}C-G3P and free glycerol. It can be easily employed to measure glycerolkinase activity in crude tissue extracts.

METHODS AND MATERIALS: Standard Assay Condition for Glycerol:
The assay system contained Tris-HCl buffer, 100 mM, pH 7.6; ATP, 3.2 mM; $MgSO_4$, 8.3 mM; EDTA 1 mM; ^{14}C-u- glycerol $4X10^4$ cpm and unknown or standard glycerol (1-40 nmole) in a total volume of 120 l. The reaction was initiated by adding 0.25 ng of glycerol-kinase. After incubation at 30°C for 10 minutes, the reaction was terminated by adding 100 µl of ethanol containing 100 nmoles of G3P and mixing vigorously with

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Abbreviations: G3P, glycerol 3 phosphate; DEAE cellulose, Diethylamino-Ethyl cellulose; EDTA, Ethylenediamine Tetraacetic Acid; QAE-sephadex, Diethyl (2-hydropropyl) Aminoethy sephadex; ATP, adenosine 5-Triphosphate.

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a vortex mixer for 5 seconds. Then 800 µl of distilled water was added. The content was transferred to QAE sephadex column (0.7 x 2.5 cm) which was previously equilibrated with Tris-HCl buffer. After the loading volume was run through, 30 ml of distilled water was applied to elute the ramaining ^{14}C-glycerol. Then 4 ml of 0.1 N HCl was applied and the eluent containing G3P was collected directly into a glass counting vial and the radioactivity was estimated in a Packard Tricarb liquid scintillation spectrometer (model 3390) in the presence of 15 ml of scintillation fluid "tT 21" (5). The amount of glycerol was calculated from a standard calibration curve which was obtained from the same run. In selected cases an internal standard was added for recovery calculation. Over 95% of added standard was recovered. Glycerolkinase of tissue extracts can be measured by a method that is similar to the standard assay for glycerol.

Identification of reaction product:
The reaction mixture was applied to a DEAE-cellulose thin layer plate (0.1 mm) and air dried for 20 min. at room temperature and developed in a solvent system consisting of ammonium acetate 1M:0.2M EDTA: methanol, 70: 0.5: 20 (v/v/v) for 90 min (6). The solvent front was 10 cm. Following air drying for 20 min., the cellulose was scraped off sequentially from the origin to the solvent front, each area is 2 cm^2. The cellulose was transferred into a counting vial and extracted with 1 ml of methanol for 20 min. at room temperature before adding 10 ml of scintillation fluid for measuring of radioactivity.

Material:
^{14}-C-u-glycerol (lot number 907-2110,131c/nmole) and ^{14}-C-u-glycerol phosphate (lot number 907-227,117 mc/nmole) were obtained from New England Nuclear Corp. Boston,Mass. The initial radiochemical purity was greater than 98.3%. It was purified on QAE-sephadex column before use. Glycerolkinase, (Candida Mycoderma, Lot number 7234327) was purchased from Boehringer, 1 mg/ml suspended in 3.3 M ammonium sulphate, DEAE cellulose TLC plate, polygram cel 300, precoated plastic sheets 20X20 cm^2 0.1 mm cellulose m n 300, was obtained from Brinkman Instrument, Inc. Westbury, N.Y. 11590.

RESULTS: Recovery of GP and QAE-sephadex column chromatography: Figure 1 (top) shows the elution pattern of glycerol and G3P. Glycerol was eluted from the column in the first 5 ml of distilled water. Practically 100% of loaded glycerol was recovered in this fraction. G3P was eluted with 0.1 N HCl (Fig. 1 top) and more than 98.6± 1.3% (5 separate runs) of loaded GP was recovered in the first 5 ml of HCl.

Determination of glycerol following chloroform extraction: In certain experiments determination of the lipid content of incubation medium of fat cells was made with repeated extraction of the incubation medium with water washed chloroform. The extractant also terminates the reaction. The recovery of glycerol in the aqueous phase was 100.4± 2% (from 7 separate experiments with Krebs Ringer bicarbonate buffer) and 95.6± (from 6 separate runs of human serum).

Glycerol standard curve: We routinely used 1-40 nmole of glycerol per tube for calibration (Figure 1, bottom). Glycerolkinase (candida mycoderma) was usually 0.25 ng per tube and converted approximately 10% of the added ^{14}C-glycerol to G3P in 10 minutes at 30°C. The plot of ^{14}Co/^{14}Cs (cpm in G3P without added glycerol/cpm in G3P with

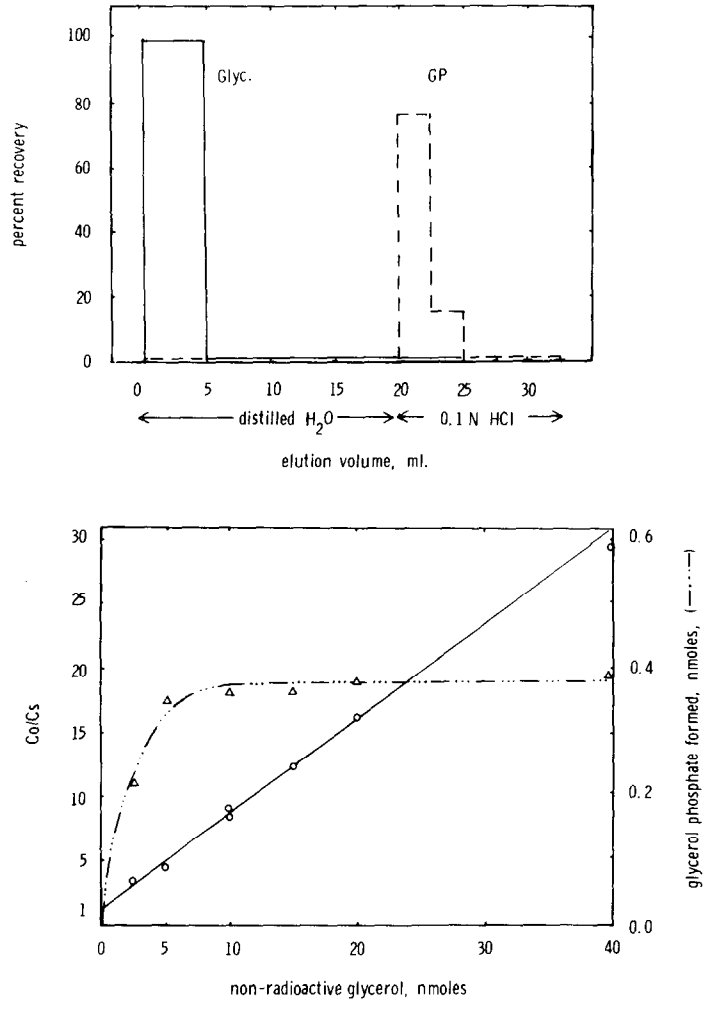

Figure 1: top. Separation of ^{14}C-u-glycerol 3-phosphate from ^{14}C-u-glycerol on QAE-Sephadex A25 column. Preparation of column and elution profile are described in Methods and Materials section. ——— glycerol; --- glycerol phosphate. bottom. Calibration curve of radioenzymic assay of glycerol. Glycerol kinase (Candida Mycoderma) 0.25 ng/tube was used.

added glycerol) versus nmoles of non-labelled glycerol added was linear.

Mg2+ and other factors effecting the slope of glycerol standard curve: Several factors have been shown to effect this radioenzymic assay. Mg^{2+} concentration and chloroform extraction should be specifically mentioned. Krebs Ringer bicarbonate buffer stimulated G3P formation (Fig. 2 top). Addition of Mg^{2+} alone can mimmick the effect of this buffer. A Mg^{2+} concentration curve on G3P formation is shown in Fig. 2, (bottom).

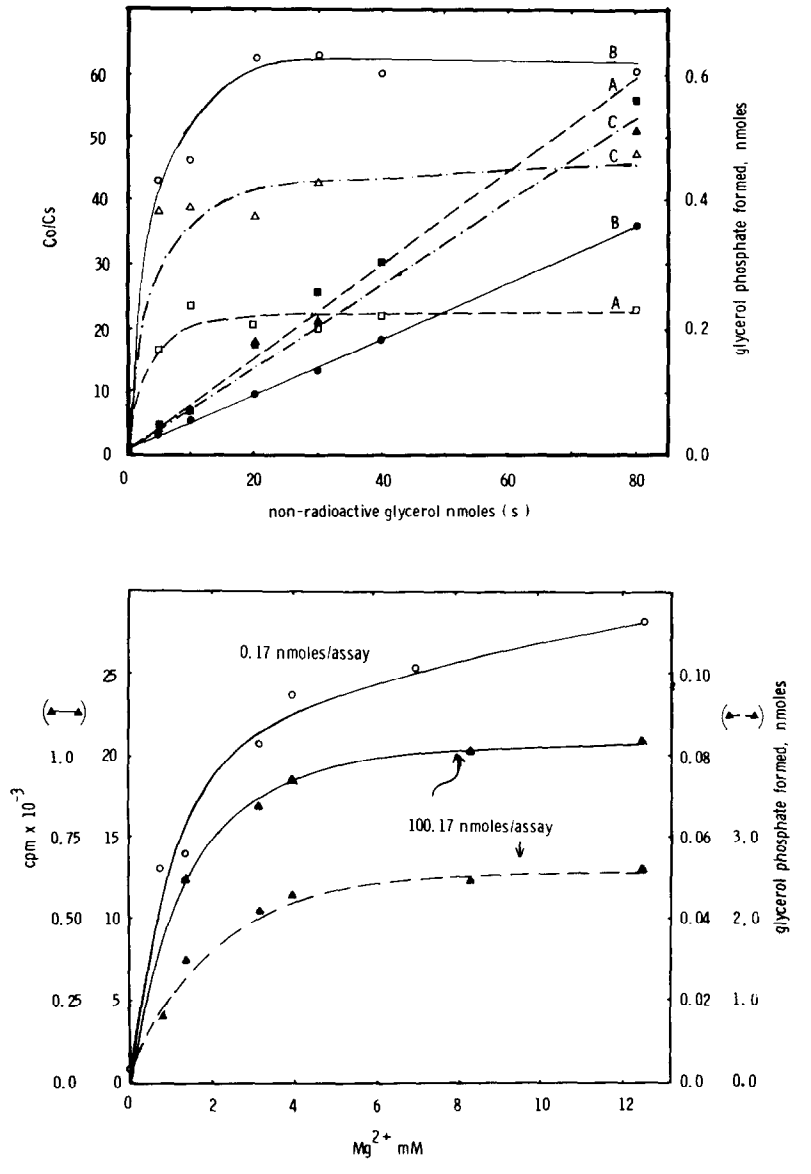

Figure 2. top. Effect of Krebs Ringer bicarconate buffer and Mg^{2+} on glycerol assay and glycerol 3-phosphate formation. There was no or very little G3P detected when the assay was carried out in the presence of no Mg^{2+}. A) Assay in the presence of 50 µl of Krebs Ringer bicarbonate buffer □--------□, GP formed; ■---------■, calibration curve. B) Assay in the presence of 50 µl Krebs Ringer bicarbonate buffer and 8.3 mM Mg^{2+}. o———o, GP formed; ●———● calibration curve. C) Assay in the presence of 8.3 mM Mg^{2+} Δ-----Δ GP formed; ▲------▲ calibration curve.

bottom. Effect of increasing concentration of Mg^{2+} on the activity of glycerol kinase (Candida mycoderma) 0———0, cpm and nmoles of GP formed, glycerol 0.17 n moles/assay; ▲———▲ cpm, ▲———▲ n mole of GP formed, glycerol 100.17 n moles/assay. Assay volume was 120 µl. Each data point is a mean of 2 experiments.

TABLE 1.Glycerolkinase activity inhibited by EDTA and stimulated by Mg^{2+}

Glyrcol, nmoles/assay	Mg^{2+}	G3P-formed, nmoles		EDTA effect %	Mg^{2+} effect,%	
		EDTA 0 mM	EDTA 1 mM		EDTA 0 mM	EDTA 1 mM
0.17	1.6	0.057	0.025	56	0	0
	4.0	0.098	0.074	24	72	196
	12.5	0.11	0.11	0	93	340
100.17	1.6	0.6	0.23	62	0	0
	4.0	0.7	0.59	16	17	157
	12.5	0.91	0.91	0	52	296

* EDTA effect is shown as % inhibition, values in the absence of EDTA are taken as 0%

** Mg^{2+} effect is shown as % stimulation, values in the presence of 1.6 mM Mg^{2+} as 0% stimulation

The optimum Mg^{2+} concentration was approximately 1.5-2.0 fold above ATP concentration (3.2mM). The inhibitory effect of EDTA can be overcome by increasing Mg^{2+} concentration in the assay. Table 1 shows results from experiments in an assay condition that ATP was 3.2 mM and Mg^{2+} was 1.6 or 12.5 mM, glycerol was 0.17 or 100.17 nmoles/tube. At both 0.17 and 100.17 nmole glycerol per tube EDTA (1.0 mM) inhibited G3P formation by increasing Mg^{2+} to 12.4mM. Based on these findings, we set up an assay condition in which a high Mg^{2+} concentration (8.3) was maintained in the reaction mixture as routine. A slightly inhibitory effect of chloroform extraction on G3P formation was observed. A calibration curve for chloroform treated standard is essential for accurate determination of glycerol in chloroform treated samples.

Application of glycerol radioenzymic assay for measuring glycerol and glycerol kinase: This modified method has been applied to measure serum glycerol from mouse and human, as well as glycerol content in rat fat cell incubation medium (Table 2). It also was adapted to measure glycerolkinase from chick, mouse and rat (Table 3). As little as 10 µg of protein from adipose tissue of rat was adequate to detect this enzyme in a single assay.

Identification of reaction product: The reaction product of a standard glycerol assay

TABLE 2 Glycerol content in serum and fat cell incubation medium

Source of glycerol	Glycerol content, mnoles/ml
Normal mouse serum	238 ± 23 (6)
Human serum	181 ± 0.01 (6)
Incubation medium	
before incubation	28.6 ± 18 (6)
no epinephrine for 30 min.	36 ± 12 (6)
epinephrine 0.1 µg/ml for 30 min.	310 ± 11.8 (6)

Results are mean ± SEM number of experiments. For fat cells, 30 mg/ml fat cells of rat epidymal fat were incubated in Krebs Ringer bicarbonate buffer in the presence of 0.1 mM caffeine for 30 min.

was shown to be G3P in two chromatographic systems (TLC and column). The results obtained with DEAE cellulose thin layer chromatography are shown. (Fig.3, left). The reaction product of glycerolkinase of crude mouse adipose tissue extracts were also identified as G3P in the same systems. The thin layer chromatographic result is shown in Fig. 3 (right) In both systems G3P formed from glycerol only after incubation with glycerolkinase. No radioactivity was detected other than glycerol and G3P.

DISCUSSION: The use of QAE-sephadex column chromatography to separate G3P from glycerol in this method allows essentially all the product formed to be radioassayed in a single counting vial. Forty complete assays require about four hours of a technicians time. It can be scale up to 100 tubes per run by reducing the amount of enzyme and increasing time of incubation for the assay. The convenience and high recovery of this method is a significant improvement over the original radioenzymic assay using a filter paper disk (4). Since in our experience it is time consuming and affected by high blanks. Another glycerol assay method using ^{32}p-ATP as tracer has been described by Thorner (7) and recently by Scheidner (6). Here too the blank values were high or processes required to separate ^{32}p-G3P from γ-^{32}p-ATP was time consuming. The volume of incubation mixture applied onto the thin layer or paper disk is also a limitation. Glycerolkinase in

TABLE 3 Application of glycerol radioenzymic assay for tissue glycerolkinase of experimental animals

Source of enzyme	Enz. protein μg/assay	glycerol in assay nmole/assay	cpm/pmole	G3P formed cpm	pmole/assay	pmole/mg/min
Chicken	46	90.7	2.10	2800	1333	965
Rat	8.8	90.7	3.95	3075	778	4423
Mouse	64	200	0.33	914	3779	1447

Glycerolkinase from adipose tissue of chicken, rat and mouse were isolated by ammonium sulfate fractionation (60, 60 and 40% by weight for chicken, rat and mourse respectively). The assay time was 30 min except 20 min for rat glycerolkinase. Results are mean of duplicates.

adipose tissue of several animals has been reported to be low or absent. It was not detectable when the photometric method was used (1,8). Using the radioenzymic assay of Newsholme glycerol kinase activity was studied in adipose tissue of rat and other animals (9-13). Activity was detectable but low. Using the present modification, the sample volume for analysis is no longer a limiting step. In view of the activity of glycerolkinase in adipose tissue of rat obtained in this paper, glycerolkinase in as little as 10 mg of isolated fat cells can be adequately measured. This will allow investigators to study regulation of glycerolkinase in isolated fat cells. Subjects regarding utilization of glycerol and regulation of glycerol kinase in mammals have been recently reviewed by Lin (14). Our convenient method should accelerate such research activities.

It should be pointed out that the possibility of unknown compounds in samples may cause interference with glycerolkinase reaction if they are assayed directly without isolation of glycerol. Therefore, an internal standard should be added in the assay for each unknown sample to assure accurate measurement. Our results indicate that glycerolkinase from Candida mycoderma is sensitive to increase in Mg^{2+} concentration. Whether adipose tissue glycerolkinase of mammalian origin also require a Mg^{2+} to ATP molar ratio greater than 1 for its optimal activity, ramains to be studied.

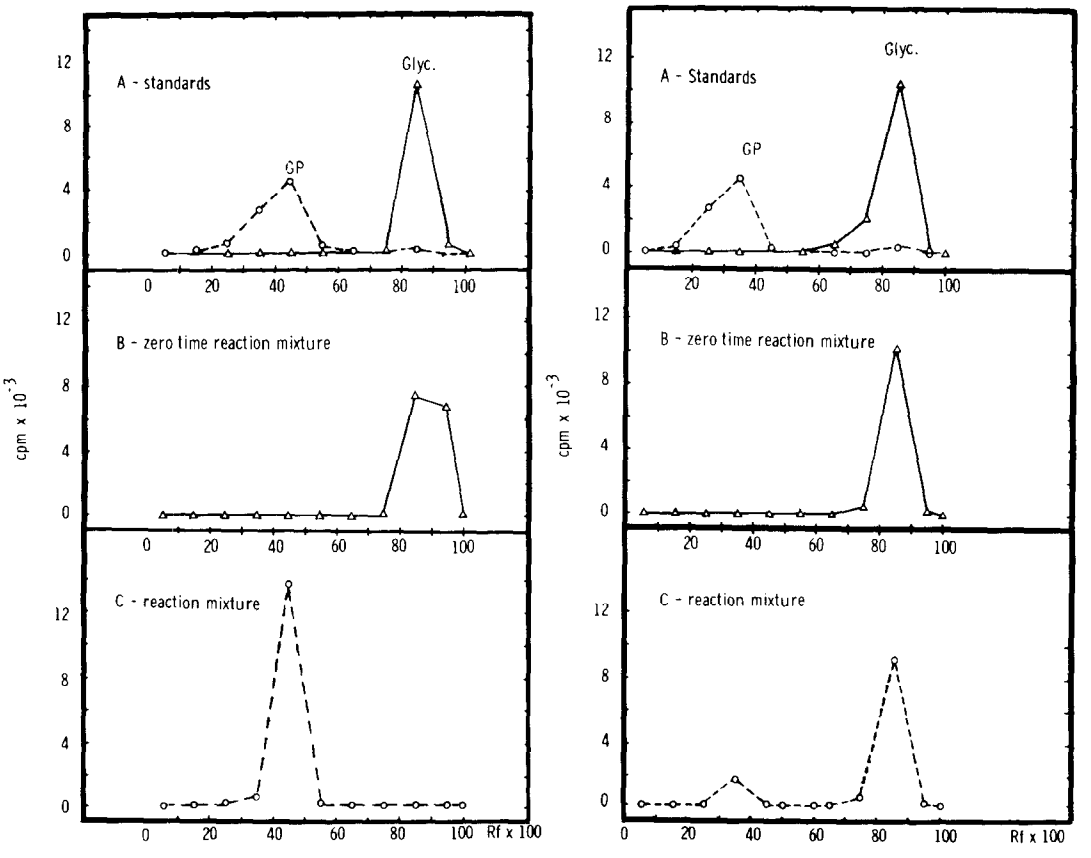

Figure 3. Identification of the reaction product of glycerol and glycerol kinase assay by thin layer chromatography. Left: glycerol kinase (40 ng/assay) and no nonlabelled glycerol ($7x10^5$ cpm/tube). Practically 100% of the applied radioactivity was recovered from the plate. A) glycerol and GP standards B) reaction mixture before incubation, 5 µl applied onto TLC plate C) reaction mixture after 20 min incubation at 30oC, 5 µl applied onto TLC plate.

Right. Glycerolkinase assay, glycerol kinase from lean normal mouse epididymal fat pad (162 µg/tube) and glycerol ($3x10^5$ cpm/tube). A) glycerol and GP standard B) reaction mixture before incubation, 5 µl applied C) reaction mixture after 20 min. incubation at 30oC, 5 µl applied. Practically 100% of the applied radioactivity was recovered from the plate.

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