Presence of glycerokinase in guinea pig mammary gland and the incorporation of glycerol into glycerides

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SUMMARY The presence of glycerokinase in homogenates of mammary tissue from lactating guinea pigs is demonstrated. It has been shown that α-glycerophosphate-C^{14} (chromatographically identified) is formed from glycerol-1,3-C^{14} in the presence of homogenate. This reaction requires ATP and Mg^{++}. Glycerol-C^{14} is incorporated into the glycerol moiety of glycerides via α-glycerophosphate. Glucose-C^{14} can also serve as a source of glyceride glycerol.

L-α-GLYCEROPHOSPHATE IS the source of the glycerol moiety of glycerides in the pathway of glyceride biosynthesis that proceeds through L-α-phosphatidic acids and D-α,β-diglycerides (1). L-α-Glycerophosphate can be formed by the reduction of dihydroxyacetone phosphate or can arise by the direct phosphorylation of glycerol by ATP catalyzed by glycerokinase. It is presently thought that the former pathway is predominant in most mammalian tissues. Evidence is presented in this paper for the existence of a glycerokinase in mammary tissue from lactating guinea pigs as well as for the direct formation of α-glycerophosphate from glucose and for the incorporation of both glycerol-C^{14} and glucose-C^{14} into glyceride glycerol. A preliminary report of these results was presented at the April 1963 meeting of the Federation of American Societies for Experimental Biology (2).

MATERIALS AND METHODS

Glycerol-1,3-C^{14} was obtained from the Nuclear-Chicago Corporation, Chicago, Ill. Its purity was checked by cochromatography with unlabeled glycerol on Whatman No. 1 paper in three solvent systems (*n*-butanol–ethanol–water, 52:32:16; *n*-butanol–acetic acid–water, 5:1:2; upper phase of system benzene–*n*-butanol–pyridine–water, 1:5:3:3). Radioactive peaks were detected by a paper strip scanner and the strips were then stained for reducing groups with $AgNO_3$. In all three systems there was a single radioactive peak that corresponded exactly to the location of the glycerol.

Uniformly labeled glucose-C^{14} was obtained from the Nuclear-Chicago Corporation, Chicago, Ill., and was shown to be pure by paper chromatography.

"DL-α-Glycerophosphate" was a product of Sigma Chemical Company, St. Louis, Mo. It was a racemic mixture which, by our analysis, contained 76% α-glycerophosphate, 1% inorganic phosphate and, by difference, 23% β-glycerophosphate. Pure L-α-glycerophosphate was obtained from the California Biochemical Corporation, Los Angeles, Calif., and was used as a chromatographic standard. β-Glycerophosphate (<0.1% α-glycerophosphate) was a product of Eastman Kodak Company, Rochester, N.Y. Glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, phosphoglyceric acid, and nicotinamide were commercial products. They were converted to ammonium salts on columns of Amberlite 4B prior to use as chromatographic standards. ATP and CoA were purchased from Pabst Laboratories, Milwaukee, Wis. L-α-Glycerophosphate dehydrogenase, glycerokinase, and DPN were obtained from the California Biochemical Corporation, Los Angeles, Calif.

Enzymatic Assay of Glycerol and α-Glycerophosphate

Glycerol was determined by a modification of the one-stage procedure of Bublitz and Wieland (3). An excess of glycerokinase, L-α-glycerophosphate dehydrogenase,

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TABLE 1 INCORPORATION OF GLYCEROL-C^{14} AND GLUCOSE-C^{14} INTO GLYCERIDE GLYCEROL

Radioactive Precursor	Nonradioactive Additions	Incorporation
		cpm
Glycerol-1,3-C^{14}	None	13,641
"	DL-α-Glycerophosphate	552
"	Glucose, DPNH, nicotinamide	3,426
Uniformly labeled glucose-C^{14}	None	10,075
"	DL-α-Glycerophosphate	1,238
"	Glycerol	9,952

All vessels contained ATP, 15 μmoles; $MgCl_2$, 3 μmoles; CoA, 0.1 μmole; L-cysteine, 25 μmoles; NaF, 125 μmoles; phosphocreatine, 7.5 μmoles; phosphate buffer, pH 7.4, 125 μmoles, and 2 ml of a 1:5 homogenate in a total volume of 3 ml. Incubations were for 30 min at 37°. As radioactive precursors, 0.1 μmole of glycerol (150,000 cpm) and 0.3 μmole of glucose (166,000 cpm) were used. Where indicated, 20 μmoles of DL-α-glycerophosphate, glucose, and glycerol, 2.5 μmoles of DPNH, and 1 μmole of nicotinamide were added.

ATP, $MgCl_2$, and DPN were added to the samples in 1.0 M hydrazine–0.2 M glycine, pH 9.8. The reaction was allowed to go to completion (45 min at 37°) and the absorbancy at 340 mμ was determined against a "blank" identical with the first solution except for the omission of L-α-glycerophosphate dehydrogenase. The concentration of glycerol was calculated from the difference in absorbancy and the difference in the extinction coefficients of DPN and DPNH. L-α-Glycerophosphate was assayed by the same method except for the omission of glycerokinase.

Preparation of Tissue for Assay

Mammary tissue was obtained from lactating, N.I.H. strain guinea pigs which were 1 week post partum. Food and the suckling litter were removed less than 2 hr prior to the experiments. The animals were given light ether anesthesia and the mammary glands were removed, chilled on ice, weighed, squeezed through a tissue press, and homogenized for 1–2 min in a Potter-Elvehjem glass homogenizer in 4 volumes of cold 0.25 M sucrose in 0.05 M Tris buffer, pH 7.4. The homogenates were centrifuged at 800 × *g* for 10 min to remove the large particulate material and most of the floating fat. This is the preparation referred to as "homogenate."

In some experiments, the homogenate was centrifuged for 60 min at 100,000 × *g*. The resultant precipitate was rehomogenized in 2 volumes of the same diluent. The supernatant solution from this centrifugation was sometimes fractionated with ammonium sulfate to concentrate the glycerokinase. Solid ammonium sulfate was slowly added with mechanical stirring to 75% final saturation and the precipitate was collected by centrifugation at 12,000 × *g* for 20 min. This precipitate was dissolved in a small amount of 0.1 M phosphate buffer, pH 7.4, and dialyzed for 12 hr against two changes of the same phosphate buffer. Glycerokinase was assayed in each of the fractions in some experiments.

Assay for Incorporation of Glycerol-C^{14} or Glucose-C^{14} into Glycerides

Incubations were performed as described in the tables. At the end of the incubations the mixtures were extracted with 10 volumes of alkaline aqueous isopropanol–heptane (final proportions by volume were heptane–isopropanol–water–1 N NaOH 40:40:30:1). Aliquots of the heptane phases were evaporated to dryness in a vial under a stream of air and 10 ml of 0.4% diphenyloxazole in toluene were added. Radioactivity was determined by a scintillation spectrometer. Counting efficiency was approximately 80%. Corrections were made for quenching by the addition of internal standards.

Assay for Incorporation of C^{14}-Glycerol into α-Glycerophosphate

Incubation conditions were in general the same as in the experiments on the incorporation of radioactivity into glycerides except for omission of CoA. At the end of the reaction, the incubation mixture was heated in a boiling water bath for 10 min and, after cooling, the tubes were centrifuged at low speed. The deproteinized supernatant solution, and three washes of 1 ml each, were applied to an Amberlite-IR-4B column (formate form, 0.5 × 8 cm). The column was washed with 20 ml of water, and the washings discarded. Total anions were eluted with 10 ml of 1 M NH_4OH. Aliquots (2 ml) of the anion eluate were added to 10 ml of Bray's solution (4) and counted in a liquid scintillation spectrometer. Corrections for quenching were made by the channels ratio method (5).

RESULTS

Precursors of Glyceride Glycerol

Preliminary experiments demonstrated that both glycerol-C^{14} and glucose-C^{14} are incorporated into glycerides in homogenates of lactating mammary tissue (Table 1). The incorporation of radioactivity from both substrates was markedly depressed by the addition of unlabeled α-glycerophosphate, as would be expected if it were an intermediate in the over-all reaction. The incorporation of glycerol-C^{14} into glycerides was slightly depressed by the addition of unlabeled D-glucose to the incubation mixture, but the incorporation of glucose-C^{14} was not depressed by unlabeled glycerol. These results suggest that glucose is more efficient than glycerol as a precursor of α-glycerophosphate. To determine the distribution

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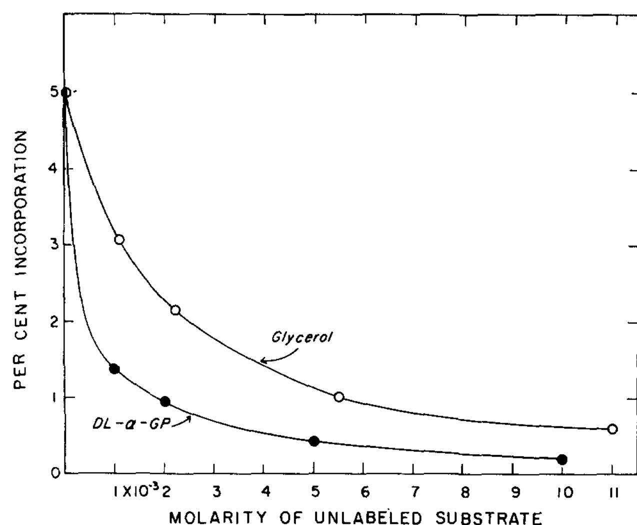

FIG. 1. Effect of unlabeled glycerol and α-glycerophosphate on the incorporation of glycerol-C^{14} into glycerides. The complete system as described in Table 1 was incubated with glycerol-1,3-C^{14} (0.7 μmole, 2.2 × 10^6 cpm) for 1 hr in the presence of varying concentrations of nonradioactive glycerol or DL-α-glycerophosphate.

of radioactivity within the molecule, aliquots of the C^{14}-glycerides were saponified and, following acidification, the fatty acids were extracted into heptane. In the experiments with glycerol-C^{14} and with glucose-C^{14}, all the radioactivity in the glycerides remained in the aqueous phase following saponification, demonstrating that incorporation was into the glycerol moiety. There was no incorporation of radioactivity from either substrate when the homogenate was heated to 80° for 10 min prior to incubation.

Requirements for the Incorporation of Glycerol-C^{14} into Glycerides

There was an absolute requirement for added ATP for the incorporation of glycerol-C^{14} into glycerides and, for maximal incorporation, Mg ions and CoA were also required (Table 2). There was considerably more variability in the effects of cysteine, NaF, and phosphocreatine, although they sometimes appeared to stimulate incorporation.

Route of Incorporation of Glycerol-C^{14} into Glycerides

In order to obtain evidence concerning the route of incorporation of glycerol into glycerides, parallel sets of flasks containing the same amount of homogenate, cofactors, and glycerol-C^{14}, and varying concentrations of unlabeled glycerol or DL-α-glycerophosphate, were incubated (Fig. 1). Unlabeled DL-α-glycerophosphate was three times more effective than an equimolar quantity of unlabeled glycerol in reducing the incorporation of glycerol-C^{14} into glycerides. This suggested strongly

TABLE 2 REQUIREMENTS FOR THE INCORPORATION OF GLYCEROL-1,3-C^{14} INTO GLYCERIDES AND α-GLYCEROPHOSPHATE

Addition or Omission	Glycerides	α-Glycerophosphate
	Relative Incorporation	
None	100*	100†
− ATP	0.3	5
− Phosphocreatine	59	84
− Phosphocreatine, − ATP	0.1	2
− Phosphocreatine, + ATP; 0.0005 M	48	16
− Phosphocreatine, + ATP; 0.002 M	73	37
− Phosphocreatine, + ATP; 0.005 M	—	55
− Phosphocreatine, + ATP; 0.015 M	104	—
− $MgCl_2$	62	46
− CoA	19	—
− L-Cysteine	78	—
+ L-Cysteine, 0.005 M	—	68
− NaF	39	95

* The complete system was identical with that described in Table 1. In the complete system, 13,641 cpm were incorporated into glycerides.

† The complete system contained ATP, 15 μmoles; $MgCl_2$, 1 μmole; NaF, 40 μmoles; phosphocreatine, 3 μmoles; phosphate buffer, pH 7.4, 50 μmoles; glycerol-1,3-C^{14}, 1 μmole, 1.5 × 10^6 cpm, and 0.10 ml of a 1:5 homogenate in a total volume of 1 ml. Incubations were for 2 hr at 37°. In the complete system, 299,000 cpm were incorporated into α-glycerophosphate.

that α-glycerophosphate was an intermediate in the conversion of glycerol to glyceride glycerol.

Incorporation of Glycerol-C^{14} into α-Glycerophosphate

The incorporation of glycerol-C^{14} into α-glycerophosphate was studied directly using the whole homogenate of mammary gland as in the other experiments, but omitting CoA in order to reduce glyceride synthesis and there-

TABLE 3 IDENTIFICATION OF GLYCEROPHOSPHATE BY PAPER CHROMATOGRAPHY

Compound	Solvent System I	II	III
		R_F*	
α-Glycerophosphate	1.02	4.7	0.95
β-Glycerophosphate	0.98	4.7	0.99
2-Phosphoglyceric acid	0.61	2.3	0.83
Glucose-6-phosphate	0.77	2.5	0.50
Fructose-6-phosphate	0.95	3.2	0.63
Fructose-1,6-diphosphate	0.18	1.1	0.32
Inorganic phosphate	1.00	1.00	1.00
Radioactive peak	0.96	4.7	0.95

Solvents I = ethyl acetate–formamide–pyridine 6:4:1.
II = methyl ethyl ketone–methyl Cellosolve–concentrated NH_4OH–H_2O 7:2:0.7:2.3.
III = ethyl acetate–acetic acid–water 3:3:1.

* Relative to inorganic phosphate.

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by increase the accumulation of α-glycerophosphate. In the experiments reported in Table 2, incorporation of C^{14} into the total anion fraction was utilized as a measure of synthesis of α-glycerophosphate. There was an absolute requirement for added ATP, and Mg ions were also required for maximal incorporation. The effects of the other cofactors studied were much more variable and usually of lesser degree.

Identification of α-Glycerophosphate

An incubation was carried out exactly as in the experiment described in Table 2, but on a larger scale. The deproteinized supernatant solution obtained at the end of the incubation was applied to a column of Dowex-50 (H^+ form) which was then eluted with 20 ml of water, thus removing all cations other than H^+ from the solution. This eluate was then applied to a column of Amberlite-IR-4B (formate form) and the initial eluate plus 20 ml of water washes was discarded. The anions were then eluted with 10 ml of 1 M NH_4OH, and the solution was concentrated on a rotary evaporator at 40° under reduced pressure. The radioactive residue was analyzed by paper chromatography in three solvent systems together with standard α- and β-glycerophosphate and several sugar phosphates (Table 3). The radioactive peaks on the developed chromatograms were detected with a paper strip scanner and phosphate-containing compounds were stained with a molybdate spray. In each system the mobility of the radioactive product coincided with the mobility of glycerophosphate. None of these systems, however, separated α-glycerophosphate from β-glycerophosphate. Partial separation of these two isomers was achieved in a solvent system of ethanol–1 M trimethylamine 3:1(v/v). Although separation was not complete, it is certain that at least the great majority of the radioactivity was chromatographically identical with α-glycerophosphate.

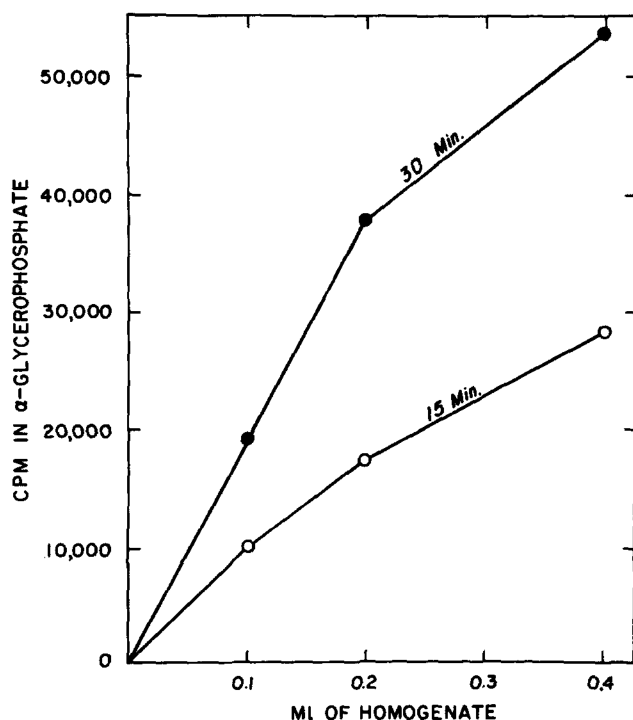

FIG. 2. Incorporation of glycerol-C^{14} into α-glycerophosphate as a function of concentration of homogenate. DL-α-Glycerophosphate, 20 μmoles, was added to trap the radioactivity. Other conditions were as described in Table 2 except for the volume of homogenate.

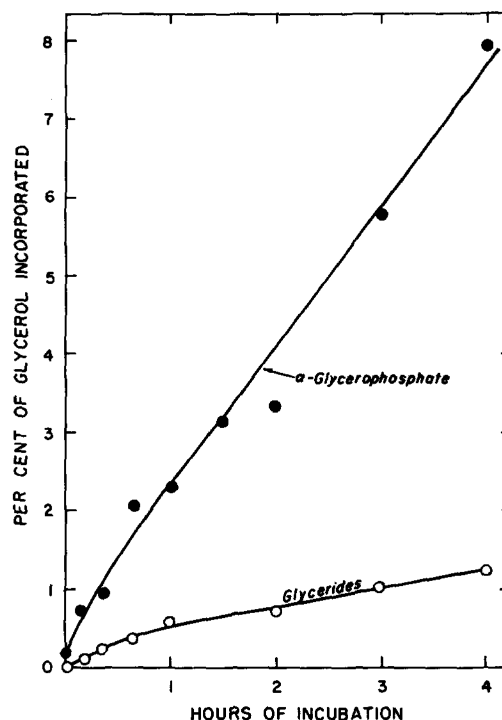

FIG. 3. Comparison of the rates of incorporation of glycerol-C^{14} into α-glycerophosphate and glycerides. Components of the incubation mixture and their concentrations were as in Table 1 except that the total volume was 10 ml. Aliquots of 1 ml were removed at specified times for the determination of radioactivity in α-glycerophosphate and glycerides.

Kinetics of the Incorporation of Glycerol into α-Glycerophosphate

The synthesis of α-glycerophosphate from glycerol was proportional to the amount of enzyme added and to the time of the incubation (Figs. 2 and 3). In addition, Fig. 3 compares the rates of incorporation of glycerol into α-glycerophosphate and glycerides. It can be seen that at all times there was much more radioactivity in α-glycerophosphate than in glycerides. The rates of conversion of glycerol to both compounds were reasonably linear over the period studied. The phosphatides (not shown in the figure) contained approximately the same amount of radioactivity as the glycerides. The phosphatides were

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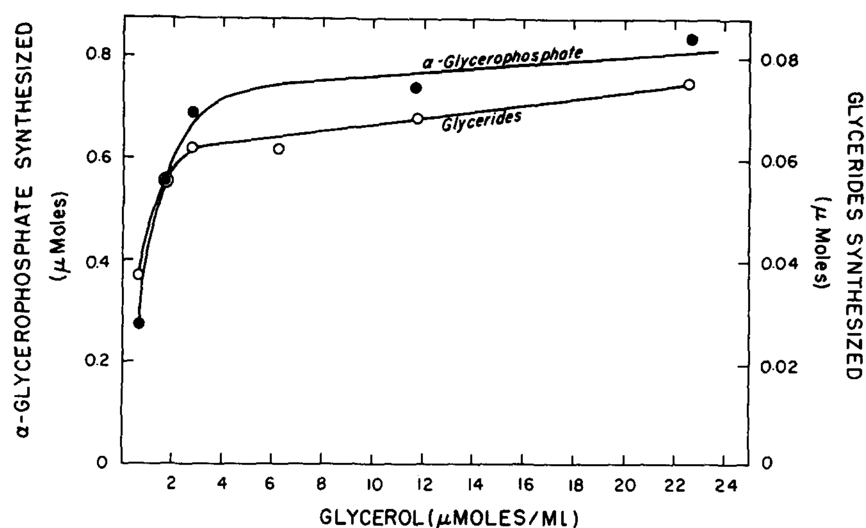

FIG. 4. Effect of the concentration of glycerol on the incorporation of glycerol-C^{14} into α-glycerophosphate and glycerides. Components of the incubation mixture and their concentrations, except for glycerol, were as in Table 1. The volume of each tube was 1 ml. Incubations were for 1 hr.

isolated by silicic acid column chromatography and identified by thin-layer chromatography but were not further studied.

Experiments were performed to determine the maximal conversion of glycerol to α-glycerophosphate and glycerides under conditions of substrate excess (Fig. 4). Approximately ten times as much radioactivity was incorporated into α-glycerophosphate as into glycerides. As nothing is known of the sizes of pools of intermediates in the pathway to glycerides, it is not possible to calculate the relative rates of synthesis of the two compounds from these data. It is reasonable to assume, however, that the added glycerol greatly exceeded the concentration of endogenous glycerol. Therefore, it can be calculated from the specific radioactivity of the glycerol and the radioactivity incorporated into α-glycerophosphate that approximately 10 μmoles of α-glycerophosphate were synthesized per hour by 1 g of tissue.

Distribution of Glycerokinase in the Homogenate of Mammary Tissue

Centrifugally isolated fractions of the whole homogenate were assayed for glycerokinase activity (Table 4).

TABLE 4 DISTRIBUTION OF GLYCEROKINASE IN MAMMARY GLAND HOMOGENATE

Fraction	Activity
	%
Whole homogenate	100
800 × *g* sediment	3.8
12,000 × *g* sediment	2.2
100,000 × *g* sediment	0.3
100,000 × *g* supernatant solution	96.6

Virtually all the activity of the whole homogenate was found in the supernatant solution remaining after centrifugation at 100,000 × *g*. This is in marked contrast to the remainder of the glyceride-synthesizing activity of the homogenate, which was found predominantly in the 100,000 × *g* sediment (6). When the 100,000 × *g* supernatant solution was further fractionated by precipitation with ammonium sulfate at 75% saturation, from 50–85% of the glycerokinase was recovered in the precipitate. The supernatant solution from this fractionation was always completely inactive.

DISCUSSION

Although glycerokinase has been found in a number of microorganisms (7) and plants (8), the enzyme is reported to occur in very few mammalian tissues. It has been found in kidney (9), liver (7, 10), and intestinal mucosa (11–13). In a systematic investigation (not including mammary tissue), Wieland and Suyter (7) were unable to detect glycerokinase, by the usual spectrophotometric assay, in rat heart, brain, lung, small intestine, muscle, or adipose tissue.

The results described in this paper demonstrate that glycerokinase is present in mammary tissue of lactating guinea pigs, and that glycerol is incorporated into glycerides via α-glycerophosphate in this tissue. It is well known that adipose tissue is a constituent of the mammary gland. However, adipose tissue has never been found to contain any glycerokinase when assayed either by the usual enzymatic assay (7) or by an isotopic assay (14) similar to the one used in this study. It therefore appears unlikely that the glycerokinase was

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present in the adipose component of the mammary tissue.

We have been unsuccessful in attempts to demonstrate glycerokinase in guinea pig mammary tissue by the spectrophotometric assay. This one-stage assay is dependent upon observation of the rate of reduction of DPN in the presence of added glycerol, ATP, Mg^{++}, buffer, and 1-α-glycerophosphate dehydrogenase, and the presumed glycerokinase. When using crude tissue preparations, it is necessary to permit a "blank reaction" (any other reaction in which DPN is reduced) to proceed to completion before adding glycerol and following the specific reaction catalyzed by glycerokinase. When either the supernatant solution obtained by centrifugation at 100,000 $\times$ g or the fraction which was precipitable with 75% ammonium sulfate was used, the blank reaction proceeded for several minutes and it was not possible to demonstrate a definite acceleration of this rate by the addition of glycerol. We were also unable to demonstrate phosphorylation of glycerol using the two-stage assay of Wieland (3). The ability of lactating mammary gland to synthesize α-glycerophosphate from glycerol compares favorably with the glycerokinase activity of liver; approximately 10 μmoles/hr per g of mammary gland vs. 18 μmoles/hr per g of liver (7). If one assumes that the intact mammary gland has the same activity as the homogenates, then the mammary gland (about 20 g) could synthesize approximately 5 mmoles of α-glycerophosphate per day which, completely converted, would yield about 3.5 g of triglycerides.

The general properties of the glycerokinase of mammary tissue are quite similar to those of glycerokinase from other sources, including rat and pigeon liver (7, 10), yeast (7), and avocado (8). They all show the same divalent cation and other cofactor requirements and the same distribution by centrifugal fractionation, and all are precipitated by ammonium sulfate.

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