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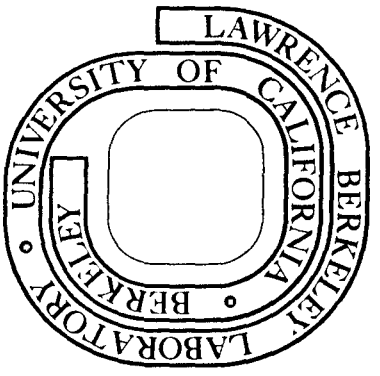

DISCLAIMER

A SIMPLE TECHNIQUE FOR DETECTION AND QUANTITATION OF LACTOSE SYNTHESIS AND SECRETION

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Running Title: Detection and Quantitation of Lactose

ABSTRACT

A combination of high specific activity ^{14}C-glucose, two-dimensional chromatography, and autoradiography can be utilized to follow the flow of glucose carbon into lactose and various intermediary metabolites of the mammary gland. The technique described requires a minimum amount of tissue manipulation, is quantitative, utilizes μg quantities of sample, and can be used for tissue pieces, single cell suspensions, and cells in culture. Clean separation of lactose from intermediates of glucose metabolism in a single chromatogram will aid in our ability to study the regulation of mammary-specific functions.

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The utilization of glucose by the mammary gland is important for both intermediary metabolism and mammary-specific differentiation. The initiation and maintenance of milk synthesis involves alterations in the glucose metabolic pathways. A large portion of glucose is required for the synthesis of lactose, a process which is unique to the mammary gland (1). In order to study the regulation of mammary-specific functions, it is therefore important to have a simple technique for measuring lactose synthesis and alterations in glucose carbon flow.

There is extensive literature on enzyme activities associated with one pathway relative to another (2). However, enzyme activities in tissue homogenates do not necessarily reflect the behavior of these enzymes in vivo. In the rabbit mammary gland, lactose synthetase activity is present by day 15 of pregnancy, but lactose is not detected until day 24 of pregnancy (3). A similar situation is found in the rat, where lactose production occurs at parturition but the enzymes catalyzing lactose synthesis undergo only small changes in activities at this time (4).

Radioactive tracers have been employed to identify substrate requirements for milk production (5,6) and to determine the glucose metabolic pathways operative in the mammary gland (7-10). A combination of paper chromatography and autoradiography, based on the same principles used in the present study, has been utilized previously and has provided both qualitative and quantitative data on the metabolic pathways of various substrates (10-14). However, the relatively low specific activity of the radioactive isotopes used in these experiments did not permit detection of many metabolites including lactose. In the reports where lactose synthesis has been measured using radioactive glucose as a substrate, the procedures have required extensive concentration and desalting (10,13). In addition to being time consuming, the procedures require mg quantities of tissue.

The technique for measuring lactose synthesis and secretion that we report in this paper utilizes high specific activity ^{14}C-glucose, two-dimensional chromatography, and autoradiography which has been used previously in both plant (15,16) and animal cell studies (17,18). The technique allows detection of many metabolites of glucose and clean separation of radioactive lactose from other metabolites. We have thus eliminated the need for extraction and desalting procedures. Furthermore, the technique requires only μg quantities of tissue, which permits this method to be used on mammary cells in culture or on human tissue, where the amount of available material is usually very small.

METHODS

Dissociation and Incubation Procedures

Mature virgin and lactating Balb/cCrgl mice (kindly supplied by Dr. D.R. Pitelka at the Cancer Research Laboratory, University of California at Berkeley) were killed by cervical dislocation. The number 4 mammary glands were removed and either cut into 1-2 mm^2 pieces or dissociated to single cells with collagenase (Worthington Biochemical Corp; CLS III, 125-170 U per mg) according to Emerman and Pitelka (19). The mammary gland pieces and 1 x 10^6 cells of the cell suspension were put into 5 ml tubes with 0.5 ml of Ham's F-12 medium containing high specific activity [U-^{14}C]-glucose (New England Nuclear; final specific activity - 30-60 Ci/mole). The glucose concentration in the medium was 5.5 mM. The samples were incubated at 37°C in 95% air, 5% CO_2 for 1, 2 and 3 hr periods. The single cell suspensions were centrifuged at 1,000 rpm for 3 min to pellet the cells. The medium was removed from all samples and frozen for later analysis. The cells and tissues were rapidly washed twice with Hanks' balanced salt solution (H-BSS) containing unlabeled glucose, once with diluted H-BSS (1:2 with H_2O), and killed with 1 ml of 80% methanol (vol/vol) in 0.01N NaOH containing 0.1% SDS. To avoid pelleting the cells between washes, the cells were collected on a Millipore

filter after the first wash and then washed twice under vacuum. The filter was immersed in the methanol. The pieces of tissue were homogenized after killing.

Separation and Identification Procedures

The methanol was evaporated under a stream of nitrogen to 0.2 ml. The extract was brought to 0.4 ml with 0.1% SDS in 0.01N NaOH, and sonicated briefly to disrupt any protein aggregates. An aliquot of each sample was removed for protein determination by the method of Lowry et al. (20) using an Autoanalyzer II system (Technion), another aliquot was measured for total radioactivity using liquid scintillation spectrometry, and aliquots were applied to Whatman #1 paper (22" x 18") for two-dimensional chromatographic analysis of labeled compounds. To analyze the glucose metabolites excreted by the cells, an aliquot of medium was applied to another paper. The chromatography procedures have been reported previously (17,21). The papers were first run in a descending solvent system of phenol:water:acetic acid (84:16:1) for 24 hr. After drying they were turned 90° and run with butanol:water:propionic acid (50:28:22) for 24 hr. For a better separation of the phosphorylated region, duplicate papers were run for 48 hr in each direction. The location of labeled compounds was determined with autoradiography using X-ray film. The labeled areas were cut from the paper and the radioactive content of metabolites was quantitated with an automated Geiger-Muller apparatus (22). The results were expressed as nanomoles of ^{14}C/mg protein applied to the paper using a computer printout (23).

The identification of labeled spots was carried out by eluting and rechromatographying the spots with pure standards visualized by the appropriate chromogenic spray. Identification was considered positive when the labeled unknown and the standard were superimposable. The spots corresponding to the phosphorylated sugars were eluted in H_2O and incubated with 0.1 mg phosphatase purified from Polidase S in 0.4 ml of 0.01 M acetate

buffer (pH 5) and 0.001 M $MgCl_2$ for 2 hr to free the sugars (17,18). The sugars were then chromatographed and identified as described above.

RESULTS

Good chromatographic separation of labeled metabolites from ^{14}C-glucose incorporation into mammary gland tissue and cells is obtained using the method described. Intermediates of the Embden-Meyerhof pathway, hexose monophosphate shunt, and tricarboxylic acid cycle and ^{14}C-lactose are identified in a single chromatogram. Qualitative differences in the glucose metabolic patterns between lactating and nonlacting tissue are observed (Fig. 1), the most significant difference being the absence of labeled lactose in the autoradiogram of mammary tissue from nonlacting mice. Lactating tissue utilizes more glucose than nonlactating tissue as determined by measuring the amount of ^{14}C-glucose depleted from the medium. The major portion of the increased glucose utilization is directed to lactose and lactate synthesis (Fig. 2 & 3). Glucose is also a major substrate for lipid synthesis in the mammary glands of nonruminant animals (24). Analysis of the data shows greater incorporation of ^{14}C into α-glycerol phosphate, a precursor of triglycerides, in tissue from lactating mice than from nonlacting mice (data not shown, see also Fig. 1).

By sampling tissue exposed to ^{14}C-glucose at different time intervals, we can study the dynamics of glucose carbon flow in mammary tissue. The intracellular ^{14}C-lactose pool is saturated by 1 hr, the earliest time point in these experiments, but there is a steady increase in the extracellular lactose pool, indicating the constant synthesis and secretion of lactose (Fig 2).

DISCUSSION

This technique, combining high specific activity ^{14}C-glucose, paper chromatography, and autoradiography, offers several advantages over similar techniques used to measure lactose synthesis (10,13). Laborious procedures

required to extract labeled metabolites and the resulting loss of radioactivity are avoided. Minute quantities of material can be used, permitting this method to be applicable to the study of tissues which are difficult to obtain, in particular, normal and malignant human mammary epithelial tissue.

The dynamics of carbon flow can be followed by determining the level of ^{14}C in various glucose metabolites and lactose as a function of time. At any given time the amount of ^{14}C incorporation into the different pathways indicates the relative use of the pathways. This comprehensive technique which measures lactose synthesis and secretion while at the same time determines metabolite patterns will add to our ability to study the complex mechanisms involved in the regulation of mammary-specific functions.

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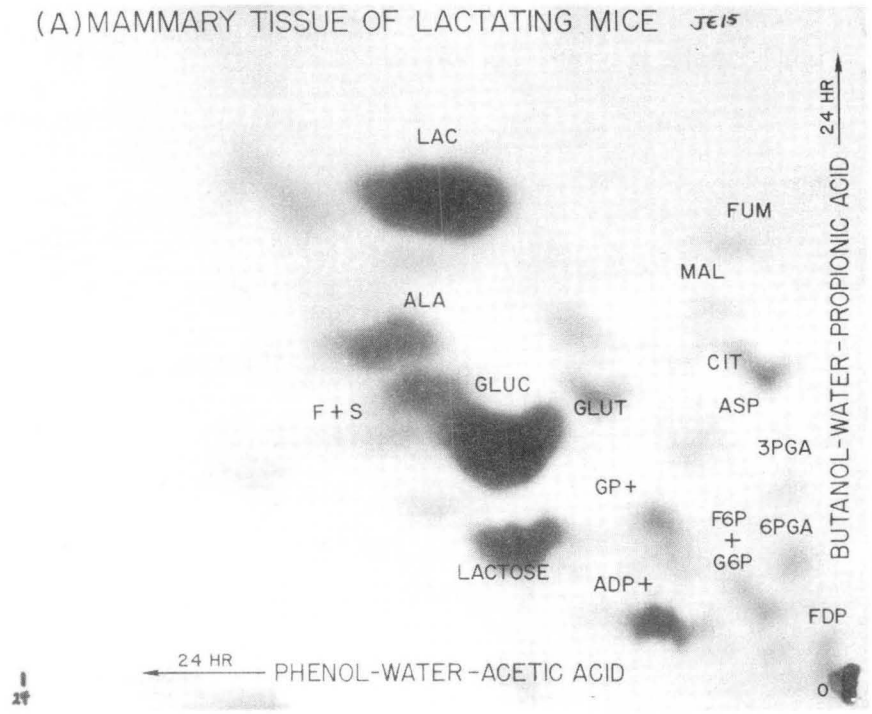
FIGURE LEGENDS

Fig. 1. Autoradiograms of labeled glucose metabolites separated by paper chromatography. Mammary tissue from lactating mice (A) and from nonlactating mice (B) was incubated in 0.5 ml of 5.5 mM [U-^{14}C]-glucose (final specific activity 30 Ci/mol) for 1 hr. Note the lactose spot in A and its absence in B. Abbreviations: O, origin; FDP, fructose-1,6-diphosphate; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; 6PGA, 6-phosphogluconate; 3-PGA, 3-phosphoglycerate; GP, α-glycerol phosphate; ASP, aspartate; CIT, citrate; MAL, malate; FUM, fumerate; GLUT, glutamate; GLUC, glucose; S, sorbitol; F, fructose; ALA, alanine; LAC, lactate.

Fig. 2. Incorporation of ^{14}C into lactose im mammary tissue given 0.5 ml of 5.5 mM [U-^{14}C]-glucose (final specific activity 30 Ci/mol) for the times indicated. After the cells were rapidly killed, the radioactive content of lactose was determined as described in the text. Open circles and triangles - intracellular lactose; closed circles and triangles - extracellular lactose; (▲Δ) - lactating tissue; (●○) - nonlactating tissues.

Fig. 3. Incorporation of ^{14}C into lactate in mammary tissue given 0.5 ml of 5.5 mM [U-^{14}C] glucose (final activity 30 Ci/mol) for the times indicated. After rapid killing, the radioactive content of lactate was determined as described in the text. Open circles and triangles - intracellular lactate; closed circles and triangles - extracellular lactate; (▲Δ) - lactating tissue; (●○) - nonlactating tissue.



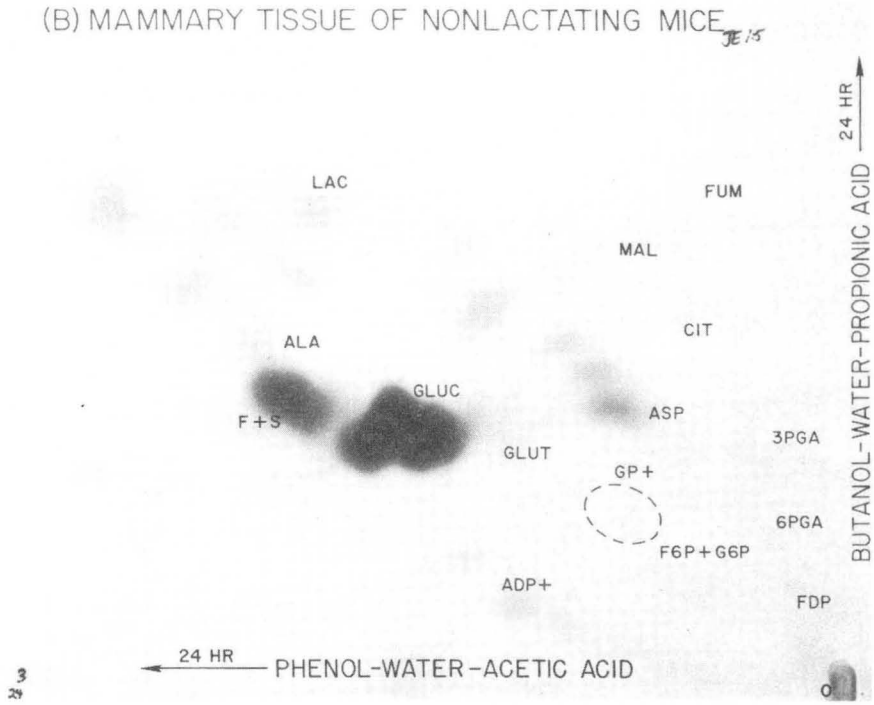


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Fig 1.

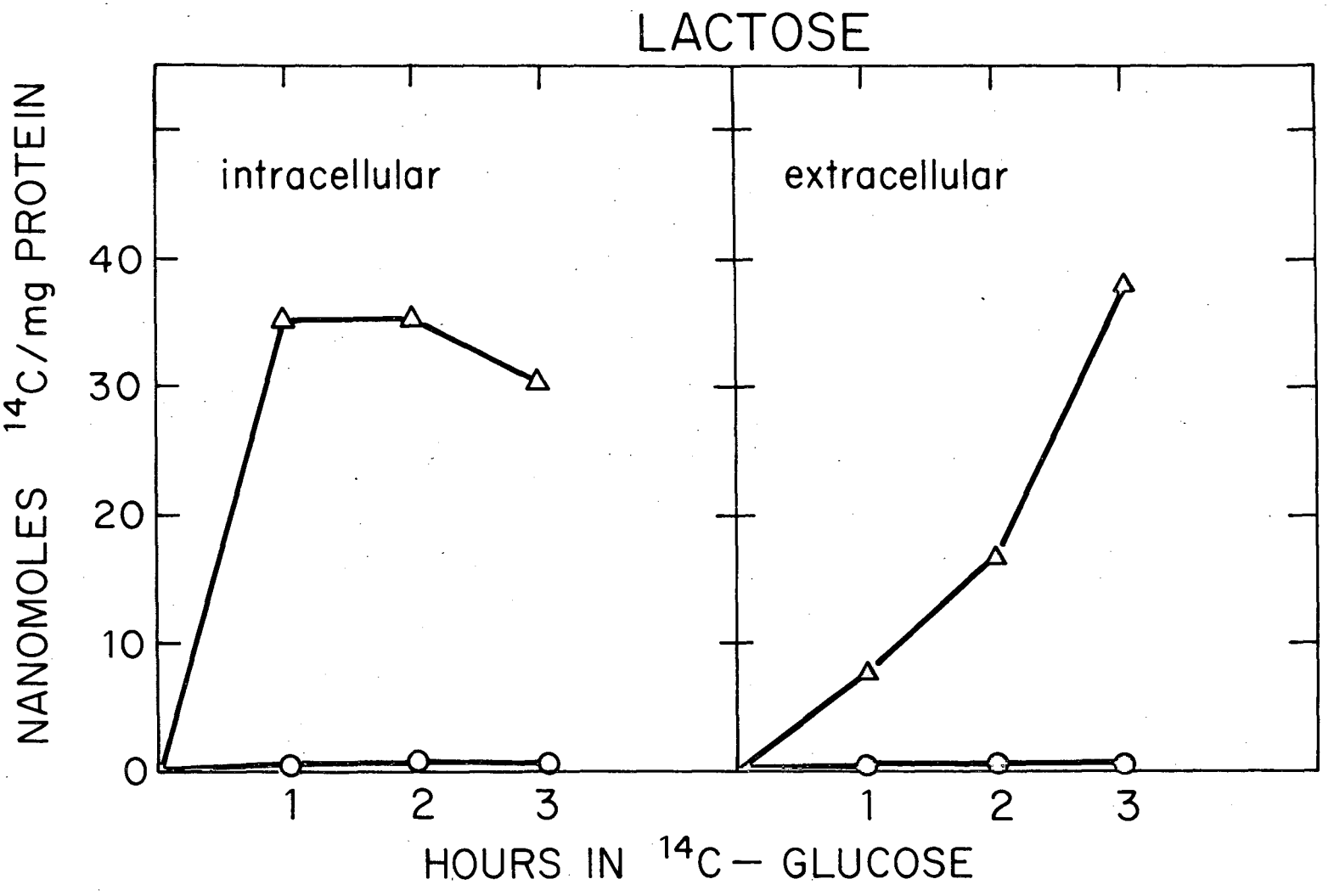

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Fig. 2

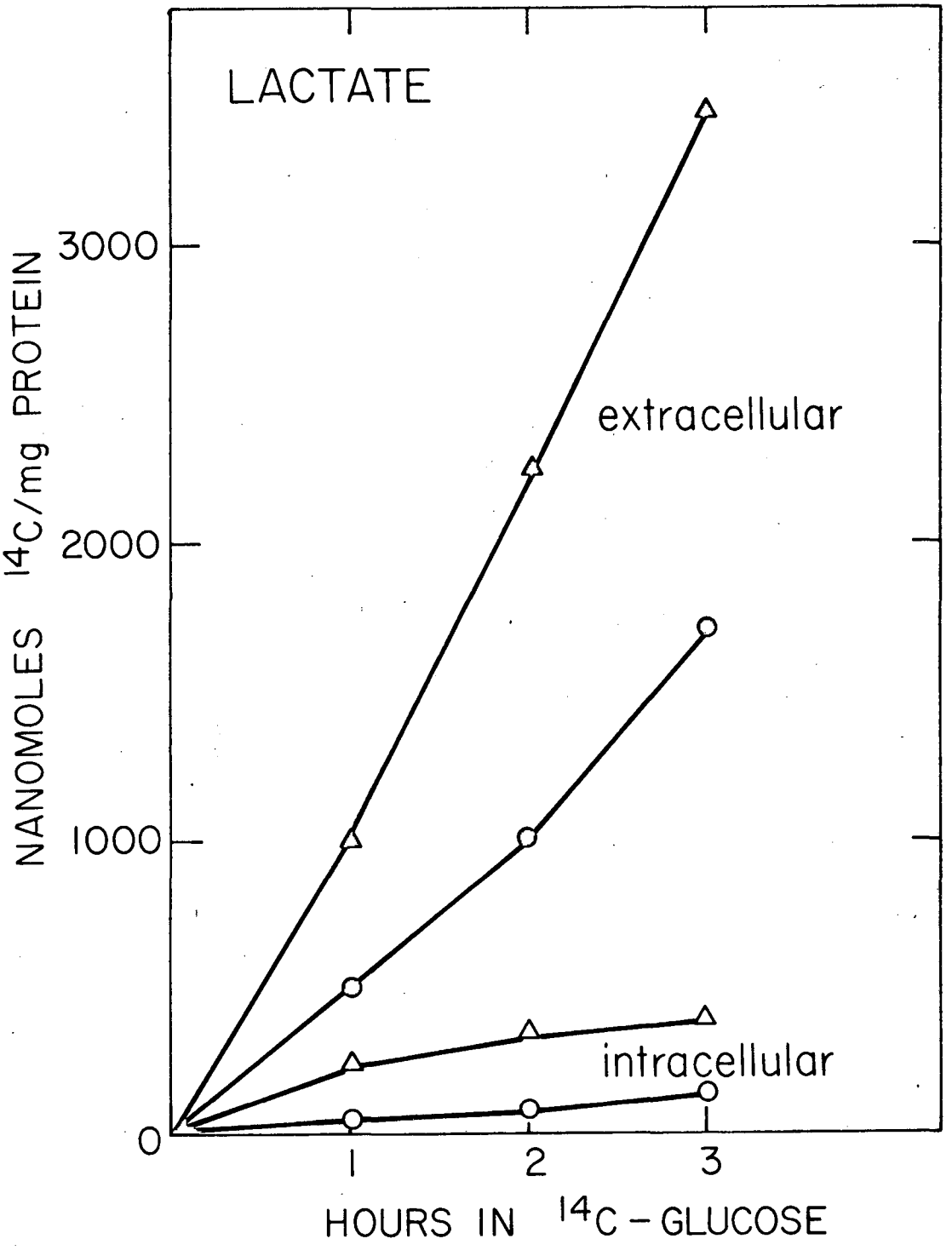

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Fig. 3

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