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Authors

Bissell, M.J.
White, R.C.
Hatie, C.
et al.

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M. J. Bissell, R. C. White, C. Hatie,
and J. A. Bassham

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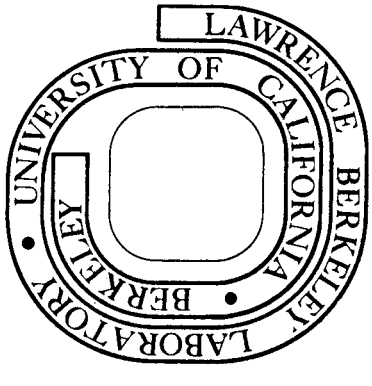

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The Dynamics of Metabolism of Normal and Virus-transformed Chick Cells in Culture

Key words: Steady-state metabolism, ^{14}C-glucose, two-dimensional chromatography, autoradiography

M. J. BISSELL, R. C. WHITE, C. HATIE AND J. A. BASSHAM
Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720

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ABSTRACT: Application of steady-state tracer technique to normal and transformed cells in tissue culture allows quantitation of intracellular pool sizes of many metabolites and the determination of rate of carbon flow along diverse paths. Using a unique apparatus to control the environmental conditions, it is shown that the rate of carbon flow into TCA cycle intermediates and amino acids is unchanged upon transformation. The increased glycogen formation and glycolysis varies with the level of glucose concentration in the medium, and correlates with the faster glucose transport of transformed cells, and cannot be explained by a difference in growth rate alone.

The widespread use of animal cells growing in tissue culture as models in the investigation of properties of normal and malignant cells increases the importance of a clear understanding of the dynamics of metabolism in such cells. By dynamics we mean the rates of uptake and conversion of substrates, quantitative aspects of metabolism including pool sizes of intermediary metabolites, flow of material along diverse paths, and the regulation of these flows in response to the external environment. The contradictory literature dealing with transport and metabolism of glucose, for example, (1,2,3; 4,5,6,7; 8,9) suggests that there is a great need to obtain quantitative information about the metabolism of these cells under very carefully controlled conditions.

Many variables have been inadequately controlled at the time of the comparative experiments. These include the composition of the medium, days in culture, growth rate, population density, pH and temperature in tissue culture incubators, and the nutritional state of normal and transformed cells. The growing cells deplete their medium of many essential nutrients, e.g., glucose, in a short time (see Results). A difference in glucose concentration would trigger an entirely different pattern of metabolism (10,11) and would alter the intracellular pool sizes and the enzyme activities. Some transformed cells leak growth factors (12) which further modifies the composition of the medium. Thus when normal and transformed cells are started in identical conditions, even with daily changes of the medium, they are in essentially very dissimilar environmental conditions at the time of the experiment. A comparison of the pool sizes (13) or membrane carbohydrate components (14) after prolonged labeling could therefore be misleading, and perhaps the cause of directly

contradictory results (14,15). Another possible reason for the lack of more definitive studies is the fact that the pool sizes of some metabolites is very small, and a prohibitably large number of cell samples are needed for accurate measurements. Furthermore, the steady-state concentration of some metabolites can vary several-fold during conventional sampling and killing procedures.

Many of these difficulties can be circumvented by the use of steady-state radioactive tracer analysis, which has been successfully employed in the study of dynamics of metabolism in photosynthetic cells (16,17). We have devised a unique "steady-state apparatus" for use in tissue culture cells which allows us to carefully control the environmental conditions during the course of our labeling experiments. Using kinetic tracer technique, the present report describes the flow of glucose carbon along the metabolic pathways in normal and Rous-transformed chick cells in culture. It is shown that the rate of glycogen formation and the pool sizes of glycolytic and pentose shunt intermediates increases with increasing transformation while the rate of carbon flow through the TCA cycle and amino acids is essentially unchanged and is independent of the degree of transformation.

MATERIALS AND METHODS

a. Growth of Cell Cultures. Primary cultures were prepared from 10-day old C/O or C/B type SPF chick embryos (18) free of resistance inducing factor as described previously (19,9). The cells were seeded in Medium 199, which was supplemented with tryptose phosphate broth (10%), calf serum (4%), and heated chicken serum (1%). Secondary cultures were prepared 4 days after the primary seeding by trypsinization of primary

cultures and were seeded at the desired cell concentration in 35-mm petri dishes. An additional 1 mg/ml of glucose was added to the medium at this time. For studies with transformed cultures, half the cells of a single embryo were usually infected 4 hours after primary seeding with 0.2 ml of Schmidt-Ruppin strain of Rous-sarcoma virus and secondary cultures were prepared as above. Assays of virus stocks have been described (9).

b. The Steady-state Apparatus. This consists of an enclosed chamber of 1/2 inch plexiglas containing a rotating table with 2 rows of indentations capable of holding 35-mm culture dishes. The table is placed over a specially designed circulating water bath. The chamber can be flushed continually with any given gas (5% CO_2 in air in the case of experiments here) which is warmed by passage through a copper coil in the bottom of the water bath. Plates are introduced through a large opening with a gas tight seal. Two 20-mm openings directly over each row of plates are covered with gas tight rubber septums and allow injection or removal of medium. Two 50-mm sliding ports covered with rubber stoppers, allow isolation of one plate at a time for removal so as not to disturb the environment of the chamber. At each opening there is a lever which, when pressed, tips the dishes for complete removal of the medium and may also be used as a mixing device. The rubber stoppers in the sliding parts may be replaced with those holding a pH meter or a thermometer for continual monitoring of the pH and temperature.

c. Kinetic and Steady-state Experiments. After 48 hours the secondary cultures were removed from the incubator and placed in our steady-state apparatus. The medium was changed to fresh Medium 199 (containing no tryptose phosphate or serum). After temperature and pH equilibration

(1 and 1/2 hours) without opening the system or removing the cells, the plates were washed 3 times with glucose-free Hank's buffer. Medium containing high specific activity ^{14}C-glucose (New England Nuclear, final specific activity 53.5 mC/mM) was added to each culture. The cells were permitted to take up the radioactive substrate for various lengths of time in order to establish the rates of labeling of the various metabolic pools. The medium was then removed and frozen for later analysis, the cells were washed rapidly with cold unlabeled glucose-containing Hank's buffer, and killed by addition of cold 80% methanol (less than 15 sec after removal of medium). The killed cells were then scraped with a rubber policeman, disrupted by sonic oscillation and applied to filter paper for analysis by two-dimensional paper chromatography (20). The killing is almost instantaneous, as no change in metabolite pattern was observed when the cells were left in methanol for various times. The samples were first run with phenol:water:acetic acid (84:16:1) for either 24 or 48 hours. After drying, the paper was turned 90° and run with butanol:water:propionic acid (50:28:22) for another 24 or 48 hours. After drying, the location of the labeled metabolites was detected by radioautography and the content of ^{14}C was determined as described (20). The unknown spots were eluted and the procedure was repeated after addition of unlabeled known compounds which later were localized with appropriate chemical reactions. When good separation of the phosphorylated compounds was not achieved even in 48-hour chromatograms, these regions were eluted and treated with phosphatase [purified from Polidase S (Schwarz Laboratories) by ammonium sulfate precipitation; 25 µg/0.3 ml]. The samples were left at 37°C overnight, taken up in 80% methanol, and rechromatographed as previously.

d. Column Chromatography and Chemical Determination of Glucose and Lactate. ^{14}C components of the medium were isolated by column chromatography on a Bio-gel P-2 (200-400 mesh) column (1.5 x 120 cm). A ^{14}C-activity elution profile was obtained and each radioactive peak was further purified by two-dimensional chromatography after the volume was reduced by lyophilization. The pure lactate and glucose were eluted and analyzed. Glucose was measured by the glucose oxidase method (Worthington Biochemical) and lactate by the use of reagent kits of Boehringer & Mannheim.

e. Measurement of the Rate of DNA Synthesis and Glucose Uptake. The rate of incorporation of [^{3}H]thymidine into DNA was used as a measure of overall rate of DNA synthesis. The procedure employed here has been shown to be an accurate measure of the rate of DNA synthesis in chick cells when compared with autoradiography or the level of mitosis (21). The rate of glucose uptake was measured by using [^{3}H] 2-deoxy-D-glucose (2). Protein concentrations were measured by the method of Lowry et al. (22). Duplicate samples were placed in appropriate scintillation fluid and counted in a Packard scintillation counter (9).

RESULTS

To insure that the observed metabolic patterns of the cells were reflections of their normal states in tissue culture, the usual growth medium (Medium 199) was employed in all experiments except that ^{14}C-labeled glucose was substituted for unlabeled glucose in the same concentration. A typical radioautograph of a two-dimensional paper chromatogram (Fig. 1) shows the separation and identification of a large number of labeled compounds derived from the catabolism of ^{14}C-glucose. Most of the phosphorylated metabolites seen close to the origin in this chromatogram were

separated more completely on another chromatogram made from the same sample and developed 48 hours in each direction.

To insure steady-state metabolism during the course of the labeling experiment, it is important to maintain a reasonably constant level of glucose before and during the period of metabolism with labeled substrate. Preliminary experiments showed that when a fresh medium was given following 48-hour growth of cultures, the glucose concentration of the medium decreased very rapidly during the first minutes for both normal and transformed cells, and then more slowly as a more nearly steady-state metabolism was achieved (transformed cells depleted up to 50% of the glucose in the medium by 1 hour). Large changes in glucose concentration (and consequent metabolic changes) were avoided by providing the cells with fresh medium (containing unlabeled glucose) 1 and 1/2 hours prior to the beginning of the labeling experiment.

After 30 min metabolism with ^{14}C-glucose in fresh medium, all of the glycolytic intermediate metabolites measured had reached a steady-state level of labeling in both normal and transformed cells (Fig. 2). The increase in the steady-state levels of these compounds from normal to transformed cells varied with the degree of transformation and was around 3- to 5-fold. Fructose-1,6-diphosphate (FDP), however, rose from 10- to 15-fold (Fig. 2).

To test whether or not these steady-state labeling levels were equivalent to actual pool sizes, two kinds of experiments were performed. In one, the lactate excreted into the medium was separated from glucose on a Bio-gel P-2 column, the lactate and glucose were further purified by chromatography, and the isolated compounds were assayed for ^{14}C and

total quantity, allowing the specific radioactivity to be calculated. The specific radioactivity of lactate was found to be comparable to that of glucose (within 10%). It therefore may be concluded that the measured pool sizes of glycolytic intermediates (which are on the pathway from glucose to lactate) are close to the actual pool sizes.

As another test for possible dilution of labeled pools by carbon from unlabeled precursors, a "chase" experiment was performed. Normal and transformed secondary cells were seeded in labeled 5.5 mM glucose in the usual medium and grown for 48 hours. The labeled cells were then given fresh medium containing 5.5 mM unlabeled glucose, and periodic samples were taken as usual. In less than 30 min the level of ^{14}C in glycolytic intermediate compounds had fallen to zero. However, significant ^{14}C label remained after 1 hour in nucleotide and glutamate pools, and some label was still present in citrate and aspartate.

In the normal experiments (^{14}C-glucose present during the period of sampling) the labeled pools of tricarboxylic acid cycle intermediates and amino acids did not reach steady-state within 1 hour (Fig. 3). In part, this is due to the presence of unlabeled amino acids in the medium which contribute to the intracellular pools of these amino acids (unpublished results). Moreover, the pools are large and the turnover of carbon from glucose is relatively slow, as demonstrated in the "chase" experiment. Nevertheless, the rate of flow of ^{14}C from glucose into the amino acids and citrate and malate is the same for normal and transformed cells (Fig. 3).

In two other pathways of glucose metabolism, the pentose phosphate shunt and glycogen synthesis, the rates of carbon flow were increased

in transformed cells as compared with normal cells. The evidence for increased pentose phosphate shunt was that the levels of 6-phosphogluconate and ribose-5-phosphate increased about 3-fold, and reached steady-state levels by about 30 min.

To measure glycogen formation, the origin of the paper chromatograms (which contains glycogen along with proteins and other macromolecules) was hydrolyzed, and the glucose in the hydrolysis products was separated by a second chromatography. Under the usual experimental conditions, the transformed cells formed about 10 times more glycogen than the normal cells, and a higher proportion of the radiocarbon at the origin was found in glycogen (Fig. 4). It is noteworthy that when transformed cells were analyzed for glycogen prior to the infusion of fresh medium and after 48-hour growth, little or no glycogen was found, indicating that the cells had utilized their supply of glycogen after exhausting the supply of glucose in the medium.

The level of uridine diphosphoglucose (UDPG) was only slightly higher in transformed cells than in normal cells (Fig. 4). This is probably the result of a higher level of glucose-6-phosphate and glucose-1-phosphate (which would make the UDPG pool higher) and the greater activity of glycogen synthesizing step (which would make the UDPG pool smaller).

Glucose concentration (10,11,9), rate of growth and population density (9) all contribute to the regulation of glucose metabolism in cells in culture. The effect of population density was eliminated by comparing normal and transformed cells at more or less the same density (500-550 μg protein per 35-mm dish at the time of the experiment). When normal cells were induced to grow at a comparable growth rate with

transformed cultures by addition of 5% serum (23,2), the steady-state pool sizes of the glycolytic intermediates were still 2- and 3-fold lower than in transformed cultures (Table I). Moreover, addition of serum to transformed cultures increased the glycolytic pool sizes without any change in growth rate (Table I).

The effect of glucose concentration was demonstrated by labeling the cells in both 5.5 mM and 0.55 mM ^{14}C-glucose. At the lower glucose concentration, there was a pronounced decrease in the glycolytic pools for both normal and transformed cells without any effect on the rate of carbon flow through the TCA cycle (Table I). The differences between normal and transformed cells, however, were even more pronounced at 0.55 mM glucose concentration, although the more rapid disappearance of glucose from the medium of transformed cells at this low glucose concentration did not allow the maintenance of steady-state conditions.

The rate of glycogen formation and the flow of carbon through the pentose shunt were also much lower at 0.55 mM glucose for both normal and transformed cells.

DISCUSSION

By using steady-state tracer techniques, we have measured and compared the pool sizes of glycolytic intermediates in normal and transformed cells and followed the rate of flow of carbon through other metabolic paths. In any comparison of normal and malignant cells, variations due to differences in growth rate must be considered. When added serum or trypsin treatment are used to induce normal density-inhibited chick cells to grow, glucose uptake increases immediately (23). At identical growth rates, however, the rate of glucose uptake is still nigher in the

transformed cells (2, and Table I). The increase in glucose uptake after malignant transformation was reported as long ago as 1925 by Cori and Cori (24) and the increase in aerobic glycolysis is Otto Warburg's classical theory of carcinogenesis stated in 1930 (25). The extent and reason for such differences, and the relation between biochemical manifestations of "normal growth" and tumor growth are still the subject of many controversies. The faster growth rate of transformed cells per se could not account for the extent of the increase in flow of carbon from glucose through the pentose phosphate shunt, via glycolysis, and into glycogen synthesis observed in our studies (Table I and unpublished results). However, since the pattern and direction of such changes can be reproduced by varying the glucose concentration of the medium, it is conceivable that the increased glucose uptake of the transformed cells may explain some or all of the changes observed. The data from Table I points to a correlation between the pattern of glucose metabolism and the glucose concentration in the medium, as well as to the rate of glucose transport.

That the glucose concentration in the medium is critical to enzyme levels has been shown in HeLa cells (10). The interrelationship between glucose transport and metabolism has also been demonstrated recently in Ascites tumor cells (11). HeLa or Ascites tumor cells in suspension do not have "normal" counterparts, however. Our findings with monolayer culture, where normal and transformed cells derived from the same embryo may be compared under controlled conditions, suggest that an increased glucose transport may also be responsible for the differences observed between normal and transformed cells. Other factors, such as population density (9), pH (26,27), and temperature being equal, anything that

increases the glucose entry into the cells seems to cause an increased flow of carbon into glycogen and through the pentose phosphate and glycolytic pathways. Thus, a rise in glucose concentration of the medium, addition of growth-stimulating factors, or viral transformation can all cause such an increase. With changes due to any of these agents, however, the flow of carbon through the TCA cycle--at least under tissue culture conditions--is essentially unchanged. The mechanism by which an increased glucose transport gives rise to a change in metabolite patterns remains unknown.

The steady-state tracer technique may further be used to determine the site and mechanism of regulation as demonstrated in studies with photosynthetic cells (17). The increase in FDP/F6P ratio found after transformation, for example, implicates phosphofructokinase as an important site of regulation in glucose metabolism, as is well known in other systems (28). The many postulated theories on integration of glycolysis and respiration (28) may be tested by use of $^{32}P_i$ as a second tracer. Once the steady-state condition has been reached, the system may be perturbed and the interrelationship between the adenylate charge, inorganic phosphate concentration and other metabolites may be determined.

The application of these techniques to animal cells, however, does raise a number of difficulties which were absent in the case of plant cells. For example, the use of a complex organic medium means that while the cells use the ^{14}C-labeled glucose as the principal source of carbon for metabolism, other organic compounds in the medium can contribute carbon to the metabolism, thereby diluting the labeled carbon in the metabolic pools. The extent to which other sources of carbon (besides the labeled

substrate) contribute to the metabolism may be monitored by the use of other labeled substrates in parallel experiments, by dual tracer experiments, and by measurement of the specific radioactivity of certain products of metabolism. We have demonstrated that for glycolytic and pentose shunt intermediates, the ^{14}C-levels measured represent the actual pool sizes. In the instances where the ^{14}C-carbon is diluted, as is the case for TCA cycle intermediates and amino acids, it is still of interest to determine the rate of flow of carbon from the specific labeled substrate into metabolic pools.

There has been a recent surge of interest in glucose metabolism in cells growing in tissue culture. The testing of such ideas as Racker's intriguing hypothesis connecting the increased glycolysis and tumor growth to a shift in intracellular pH (29) can be facilitated by careful quantitative studies in a strictly controlled and well defined system. Such control seems not to have been achievedpreviously in tissue culture studies.

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TABLE 1. The relation between the rate of growth, glucose concentration and transport and the ^{14}C-pool sizes of normal and transformed cells

Glucose conc. mM	Type of cell	Lactate	FDP	Citrate	Tdr incorporation†	2-DG uptake‡
		nmoles of ^{14}C / mg protein				
0.55	N	4.5	2.0	1.6	205.8	40.1
	T	25.0	21.5	1.5	705.3	196.3
5.5	N	16.8	9.2	1.5	198.2	36.7
	N+5% serum	38.3	29.8	1.7	713.8	95.8
	T	62.0	82.3	1.4	786.9	209.5
	T+5% serum*	108.3	116.9	1.1	759.2	Variable

* The additional serum causes considerable detachment of well transformed cells and thus causes higher variability.

† ^{3}H-thymidine incorporation into DNA used as a measure of rate of DNA synthesis (21) (DPM/µg protein).

‡ 2-deoxy glucose uptake used as a measure of rate of glucose transport (2,5,6) (DPM/µg protein).

Cells were grown as in Methods. Fourteen hours prior to the experiment, 5% chicken serum was added as indicated. The cells were preincubated in either low (0.55 mM) or high (5.5 mM) glucose for 1 and 1/2 hours prior to the experiment. Legend as in Figs. 1 and 2. These are the 30-min points where the glycolytic intermediates have reached steady-state. Average of two experiments.

FIGURE LEGENDS

Fig. 1. Glucose metabolites in chick cells. The cells were exposed to 1.0 ml of 5.5 mM uniformly labeled ^{14}C-glucose in Medium 199 for 1 hour (final specific activity 53.6 mC/mM). After removal of medium and washing, half of the mixture of methanol-extracted pools and sonicated cells were applied to paper, as described in Methods. The radioautograms were developed after one week. Abbreviations used: Gluc, glucose; Glut, glutamate; Asp, aspartate; Mal, malate; Lac, lactate; Cit, citrate; Ala, alanine; Gln, glutamine; PEP, phosphoenolpyruvate; DHAP, dihydroxyacetone phosphate; Gp, glycerol phosphate; 3PGA, 3-phosphoglyceric acid; FDP, fructose 1,6-diphosphate; PMP, pentose monophosphate region; HMP, hexose monophosphate region; UDPG, uridine diphosphate glucose; NDP, diphosphonucleotide region; O, origin.

Fig. 2. ^{14}C-pool sizes of the glycolytic intermediates in normal and transformed cells. Procedure was as in Fig. 1. Replicate plates were removed at indicated times. The spots were removed from the chromatograms and counted. The FDP, HMP and PMP regions were further eluted, treated with phosphatase and rechromatographed as described in Methods. Average of 5 experiments. The values for normal cells were within the 10% of the mean; the values for transformed cells varied with degree of transformation.

o——o transformed cells

o——o normal cells

Fig. 3. The rate of ^{14}C-glucose carbon flow through the TCA cycle and amino acids. Procedure was as in Figs. 1 and 2.

o——o transformed cells

o——o normal cells

Fig. 4. The rate of glycogen formation and macromolecular synthesis and ^{14}C-pool of uridine diphosphate glucose. Procedure was as in Figs. 1 and 2. The dashed lines are the total ^{14}C content of the origins. The solid lines are glycogen measured as ^{14}C-glucose. The origins were cut and hydrolyzed in 1 N trifluoroacetic acid (1 hour at 121°C). This procedure allows the selective hydrolysis of the carbohydrate polymers. The hydrolytic products were chromatographed as in Methods.

o——o transformed

o——o normal

LAC
ALA
MAL
CIT
GLUT
PEP
GLUC
ASP
GLN
3PGA
AMP
GP +
DHAP
PMP
HMP
NAD+
ATP
FDP
ADP
NDP
UDPG
O

Fig. 1

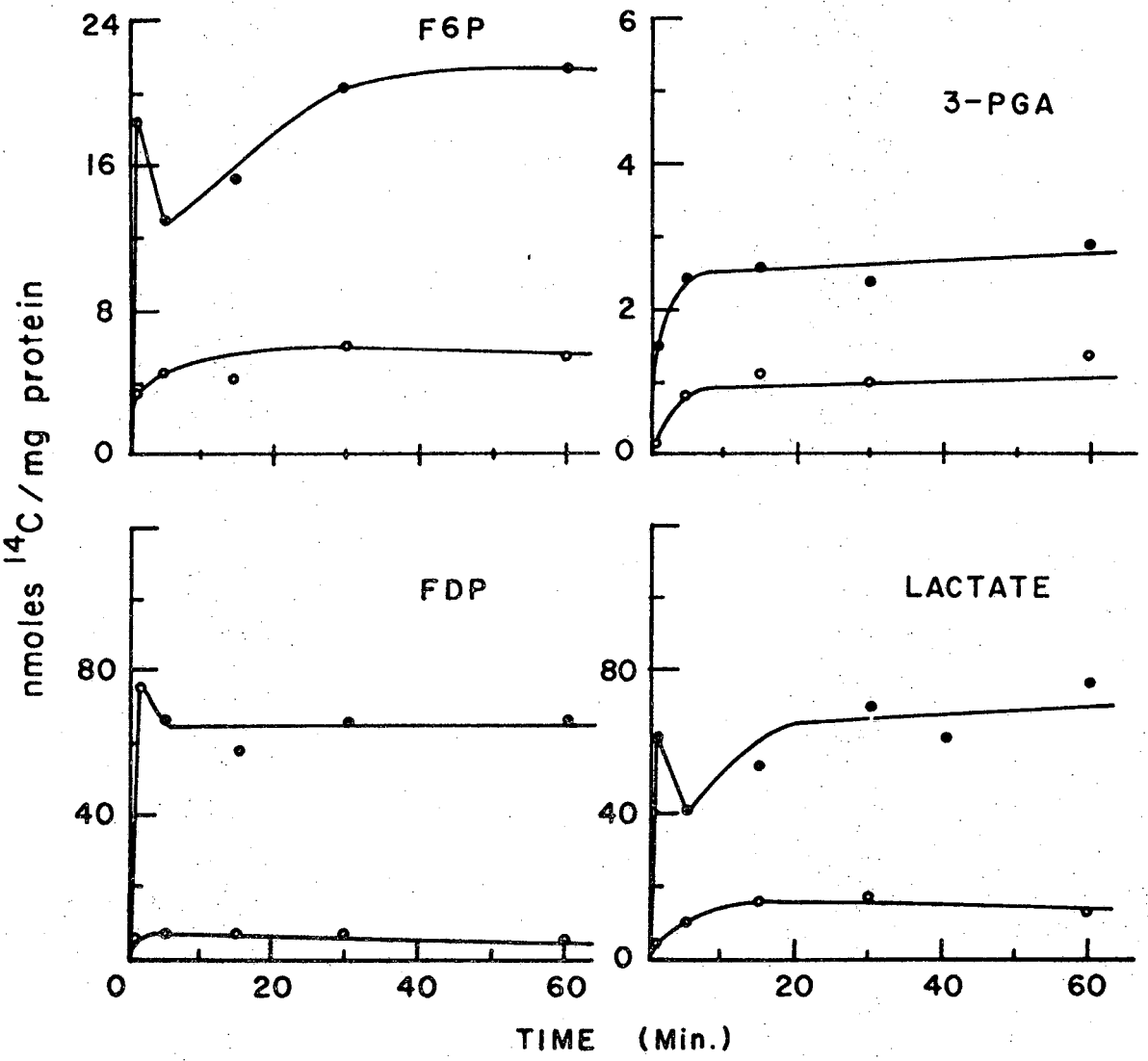
F6P
3-PGA
FDP
LACTATE
nmoles ^{14}C/mg protein
TIME (Min.)
24
16
8
0
6
4
2
0
80
40
0
20
40
60
XBL736-4832

Fig. 2

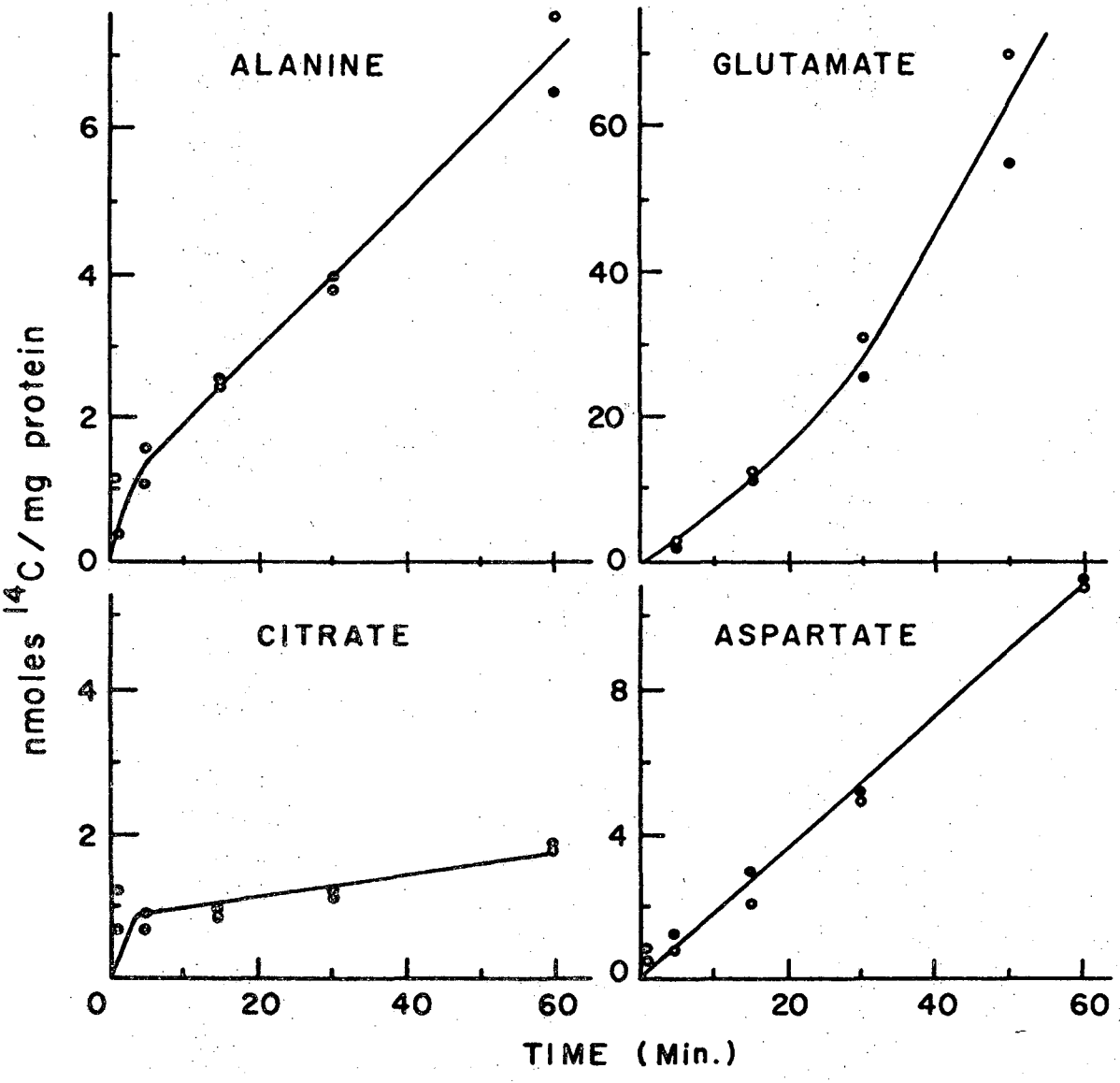
ALANINE
GLUTAMATE
CITRATE
ASPARTATE
nmoles ^{14}C / mg protein
TIME (Min.)
0
2
4
6
20
40
60
8
XBL736-4833

Fig. 3

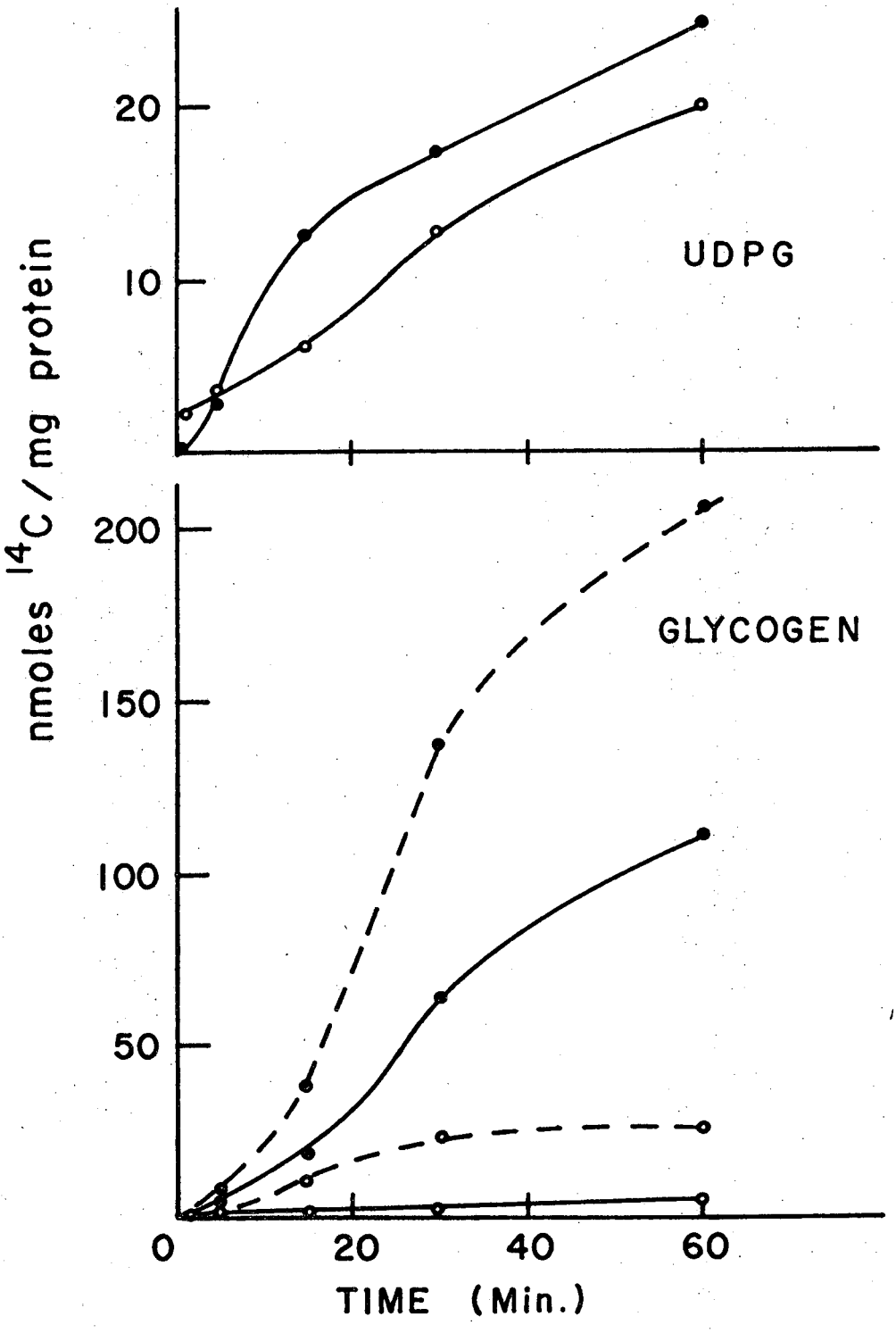
20
10
UDPG
200
150
100
50
GLYCOGEN
nmoles ^{14}C / mg protein
0
20
40
60
TIME (Min.)
XBL736-4831

Fig. 4

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BERKELEY, CALIFORNIA 94720