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QUANTITATIVE TRACER STUDIES OF METABOLIC DYNAMICS OF ANIMAL CELLS GROWING IN TISSUE CULTURE

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Quantitative Tracer Studies of Metabolic Dynamics of Animal Cells Growing in Tissue Culture

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Running Title: Metabolic Dynamics of Animal Cells

ABSTRACT

Metabolic dynamics of animal cells, growing in tissue culture, can be determined quantitatively by allowing the cells to metabolize radioactively labeled substrates under carefully controlled steady-state conditions. In order to avoid artifacts resulting from uncontrolled changes in physiological conditions, a steady-state apparatus for animal cells (SAFAC) has been constructed. In this device cells in up to 30 cell dishes can be given radioactive substrate and incubated for various periods without disturbing the steady-state metabolism prior to killing. Subsequent analysis by two-dimensional paper chromatography and radioautography shows that metabolites are labeled rapidly and subsequently are maintained at constant levels of radioactivity, as expected for steady-state metabolism.

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Quantitative Tracer Studies of Metabolic Dynamics of Animal Cells Growing in Tissue Culture

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Animal cells, growing in tissue culture and attached to a flat surface, are now widely used as model systems for the study of the properties of animal cells *in vivo*. Such cells have many advantages for the study of metabolic dynamics, which include the rates of uptake and conversion of substrates, pool sizes of intermediary metabolites, rates of flow of material along diverse metabolic pathways, and the regulation of these flows in response to physiological conditions and external variables. For example, radioactive carbon-labeled substrate of constant concentration and specific radioactivity cam be administered under carefully controlled conditions until a steady state of labeling of intermediary pools has been achieved. Then a single controlled condition may be altered. Following subsequent analysis of the metabolites and their labeling before and after the perturbation, one can observe the effects of the changed condition. Even the measurement of pool sizes under a constant condition provides information about sites of regulation and their relative importance (1).

Steady-state and perturbed steady-state studies of cells using labeled substrates have provided much information about metabolism and its regulation in the case of photosynthesizing plant cells (2,3). Adaptation of this method to the study of animal cells growing on a flat surface and using complex substrate mixture requires a number of modifications in

equipment and techniques. A brief description of the general method as applied to animal cells has appeared (4). The purpose of the present report is to describe the techniques, and particularly the steady-state apparatus for animal cells (SAFAC).

METHODS

Steady-state apparatus for animal cells (SAFAC)

a. Dish carrier. SAFAC (Fig. 1) is built around a 19.2 cm diameter, 6 mm thick, disc which serves as the cell dish carrier and which can be rotated during the experiment. This dish carrier has 30 holes each 36 mm in diameter in which the 35 mm cell culture dishes (Falcon plastic petri dishes) are carried. The holes are arranged in two concentric circles (Fig. 2) of radii 13.1 cm and 17.0 cm. To prevent the dishes falling through the disc, flat circular bands of plastic are glued to the bottom of the disc in such a way as to cover all but the outer 5 mm of bottom of the holes. This 5 mm space is required for the operation of the dish tilters, described later. The outer edge of the dish carrier is notched in line with each hole for positioning.

b. Chamber. The dish carrier is housed in a closed chamber (fabricated from 10 mm plastic by gluing a vertical decagonal wall to the flat base) with a wall 41 mm high (inside) and enough clearance at the outside of the dish carrier to allow for the dish-tilting mechanisms. The dish carrier is supported above the floor of the chamber by the pointed end of the center shaft resting in a depression in the chamber floor, and by 4 small nylon rollers whose axes are held by plastic attached to the dish carrier, and which roll along the floor of the chamber at a radius

of 13.5 cm. The top of the chamber is also 1 cm thick clear plastic and is removable but is normally attached to the walls of the chamber by screws into holes threaded into the walls. Stopcock grease is also used to make a seal between top and walls. The 10 mm diameter steel shaft, which is rigidly attached to the dish carrier, extends through the top of the chamber to a removable round handle. The dish carrier can be rotated by turning this handle. A flexible strip of plastic mounted vertically, extending from one side to an adjacent side of the decagonal wall, and backed at the center by a spring compressed between the corner and the plastic strip, carries a plastic V-shaped ridge which presses against the outer rim of the dish carrier and drops into a notch in the rim when a given set of culture dishes are in position under the various ports described below. This positioning holds the dish against inadvertent movement, but a moderate twist on the handle forces the ridge out of the notch, and the dish carrier can be turned to the next position.

c. Ports. For the loading of the SAFAC with tissue culture dishes from the incubator, a 13 cm diameter port is provided in the chamber top, positioned so that both rings of dish holes can be reached. This port is closed by a removable round plate with a compression disc which forces an O ring out against the sides of the port when it is tightened with a screw.

Two small ports, one over each circle of dish holes, are fitted with vertical 20 mm diameter plastic tubes glued into the chamber top so that the bottom of the tubes is even with the inside of the top and the tops of the tubes extend upward 15 mm. These are tightly fitted with large serum caps. Hypodermic needles can be inserted through these caps

to the dishes for removal and addition of medium to the cells.

A second pair of ports is positioned over the circles of dish holes about 150 degrees from the first pair. Each of these ports is fitted with a fixed O-ring and a movable plastic tube 45 mm in diameter and 40 mm long. These tubes are closed with removable rubber stoppers. When the tubes are pushed all the way in, they seat against the surface of the dish carrier, forming an air lock around the dish hole and the cell culture dish. The stopper can then be removed, and the cell dish can be removed from the chamber without disturbing the atmosphere of the chamber.

At one of the decagonal corners of the chamber wall, inlet and outlet ports are provided for the flow of gas through the chamber. These are made of 6 mm I.D. plastic tubing glued into the walls horizontally and pointing in nearly opposite directions along the inside walls of the chamber. Thus the inflowing gas tends to circulate around the circumference of the chamber before exiting. From the change in color of indicator dye (Phenol Red) in plates on the dish carrier after admission of 5% CO_2 in air (the medium goes slightly alkaline during transfer from incubator to SAFAC), it is clear that gas mixing is rapid and uniform, when the gas flow rate is adjusted to 400 cc/min.

d. Dish tilters. For steady-state kinetic experiments, rapid removal of medium is essential. Such operations should take no longer than about 5 sec. The medium is in contact with the cells as a 2 mm deep layer during metabolism. For rapid removal of medium, the dishes are tilted so that the medium can be sucked out from one side of the dish through a hypodermic needle. Two pairs of dish tilters are provided in

such positions that the dishes under the two pairs of ports (for medium change and for killing) may be tilted. One part of each tilter is a plastic lever arm which pivots in a vertical direction around a horizontal pin mounted in a bracket attached to the floor of the chamber. Near the chamber, one end of the arm is attached with a pin to a vertical rod which extends out through the top of the chamber to a handle. A spring compressed between the handle and chamber top keeps the rod and outer end of the arm up until the handle is pushed down. When the handle is pushed down, a plastic "finger" on the end of the arm under the dish rises and elevates one side of the dish about 10 mm. When the handle is released, this finger drops down through the open space at the outer edge of the dish hole, permitting the dish carrier to be rotated.

e. Temperature control. The chamber is immersed in a water bath also constructed of clear plastic. Water is recirculated through this bath from a reservoir which is temperature controlled at 40°C in the case of studies with chicken cells. This water first passes through a shallow closed chamber (6 mm deep) attached to the bottom side of the chamber top and covering all of the top area except that occupied by the ports. This chamber is made of 3 mm clear plastic glued to 6 mm strips of plastic which are in turn glued to the chamber top. Inlet and outlet tubes are provided through the chamber top. Use of this temperature control prevents condensation of water vapor on the top which would obscure the worker's view of the dishes during experiments conducted in a room at 22°C.

The stream of 5% CO_2 in air used to gas the chamber is first passed through about 12 m of 6 mm O.D. copper tubing which is wound in a flat coil and immersed in the bath under the chamber. The gas then passes

through a water bubbler immersed in the 38°C reservoir in order to saturate the gas with water vapor. The gas then passes through the chamber via the inlet and outlet ports. During preliminary studies, it was found that with a flow rate of 400 cc/min, a uniform temperature of 38°C could be maintained in all parts of the chamber.

Animal Cells

The procedures for preparation of chick embryo cells are essentially similar to those previously described in detail (5). Ten-day old C/O or C/B type SPF chick embryos are removed from eggs. After decapitation and evisceration, they are minced and washed with tris-saline buffer, then stirred with a magnetic stirrer in 0.25% trypsin (Sigma). At 15-min intervals the suspended single cells are decanted into a "stop bath" containing 2/3 cold medium 199 (Grand Island) and 1/3 calf serum (Microbiological Associates). The procedure is repeated two times. The cells are centrifuged and resuspended in medium 199 containing 2% tryptose phosphate broth, 1% calf serum, and 1% heat inactivated chicken serum [referred to as (2-1-1)]. Primary cultures are seeded at 8×10^6 cells per 100 mm petri dish in 12-1/2 ml medium. The cultures are incubated at 38-39°C in an atmosphere of 5% CO_2 in air to maintain pH of 7.3-7.4. The medium is changed to fresh 199 (2-1-1) on the third day. Secondary cultures are prepared 4 days after the primary seeding. After removal of cells by trypsinization, they are seeded at desired cell concentration (usually 1×10^6 cells per 35 mm dish in these experiments) in 199 (2-2-1). An additional 1 mg/ml of glucose is added to the medium at this time, bringing the glucose concentration to 11.0 mM. While penicilin and streptomycin is included, Fungizone is eliminated

from all experiments, as harmful side effects have been observed in this laboratory.

Kinetic and Steady-state Experiments

After 48 hr incubation, the secondary cultures are removed from the incubator and placed in SAFAC. The apparatus has been previously brought to 38°C. The loading port is closed, and a stream of 5% CO_2 in air saturated with water vapor at 38°C is continuously passed through the chamber at a rate of about 400 cc/min for the duration of the experiment. The medium in the culture dishes is about 2 mm deep, and pH equilibration with the gas phase is rapid. When the dishes are removed from the incubator, the color of the pH indicator goes slightly purple as pH rises, and during the first few minutes in SAFAC the color turns once again reddish orange, indicating a pH of 7.2-7.4. During preliminary experiments with SAFAC we measured the pH at various positions in the chamber with pH electrodes and established that the pH of medium in dishes was uniform throughout the chamber after an equilibration period of a few minutes.

After 15 min, a dish is moved under a medium-changing port. Two hypodermic needles are inserted through the rubber serum cap until the needle tips are close to the liquid in the dish near the edge opposite the plate-tilter. One of these needles is attached to a vacuum through a waste bottle, while the other is attached to a syringe containing fresh medium. The plate-tilter handle is pushed down, raising one side of the dish and causing the medium to flow to the other side. The vacuum needle is inserted to the lowest inside edge of the dish, and the old medium is sucked out. The plate-tilter is released and fresh medium is added.

The added fresh medium in this case may or may not be labeled with radioisotope, depending on the nature of the experiment. If a dual-labeling experiment (^{14}C and ^{32}P) is to be done, ^{32}P-labeled inorganic phosphate may have been added 12 hr before while the cells were in the incubator, and is added with each subsequent change of medium. In some experiments, where complete ^{14}C-labeling of nucleotides is desired, ^{14}C-U-glucose may be added to the cells in the incubator and at later medium changes. The remaining dishes are brought in turn under the appropriate medium-changing port and are given fresh medium.

One hour after the first medium change, the first dish is again brought under the medium-changing port, and the old medium is removed. The cells are washed three times with glucose-free Hank's buffer. Each time about 2 ml of buffer is added to the dish in the horizontal position, and the dish is then tilted twice, causing the buffer to flow back and forth across the cells. After the third rinse is carefully removed from the tilted plate, fresh medium is added and the plate is tilted twice. This medium is identical to that used previously except that unlabeled glucose is replaced with ^{14}C-U-glucose (New England Nuclear Corp., final specific activity 22.5 Ci/mole).

The entire process of medium replacement is completed in less than 10 sec, during which time the cells are not exposed to any change in pH, temperature or aeration. Thus we approach closely to the ideal of the steady-state experiment which is to change no physiological condition, and to instantaneously substitute radioactive substrate for unlabeled substrate. After the first dish, each of the other dishes is subjected sequentially to its prescribed medium change. At times, other agents such as inhibitors may be added to some dishes, in the first or second medium change, or both,

or added later after a period of metabolism with labeled substrate. If the cells are to be used for non-radioactive analysis, such as the determination of pyridine nucleotides, some of the dishes may receive only fresh medium with unlabeled glucose.

After the prescribed periods of incubation with labeled substrate, each dish is moved under one of the ports for killing. The plastic tube is pushed down over the dish, forming an air lock, the rubber stopper is removed, and the medium is sucked out, frozen, and saved for future analysis. The cells are quickly washed with cold Hank's buffer containing unlabeled glucose, and then are killed by the addition of 80% methanol (less than 15 sec after removal of medium). The dish is removed with forceps through the plastic tube, the rubber stopper is replaced, and the plastic tube is raised to permit rotation of the dish carrier.

Analysis

The killed cells are scraped with a rubber policeman and disrupted by sonication. Cells and methanol are removed from the dish, which is then washed with methanol. Killing is essentially instantaneous, as no change in metabolic pattern was observed when the cells were left in methanol for various lengths of time. The combined methanolic suspension and methanol wash is reduced in volume under nitrogen to 0.2 ml, and the mixture is subjected to more sonic disruption to give a finely divided suspension.

Analysis of labeled metabolites is by two-dimensional paper chromatography and radioautography (4,6). A 75-μl aliquot sample is dried on the origin of Whatman No. 1 chromatographic paper. Each chromatogram is first developed for 24 or 48 hr in a solvent made up of 840 ml "liquified" phenol (J. T. Baker USP, about 88% phenol, 12% water), 160 ml water, 10 ml

glacial acetic acid, and 1 ml 1.0 M ethylenediaminetetraacetic acid. After it is dried, the paper is turned 90° and run with butanol-water-propionic acid (50:28:22). From each dish sample, two paper chromatograms are made. One is developed 24 hr in each solvent system, while the other is developed 48 hr in each solvent. After the samples are dried, the location of the labeled metabolites is detected by radioautography and the content of ^{14}C and/or ^{32}P is determined either by scintillation counting or through use of the semi-automatic spot counter (7) as modified for ^{14}C and ^{32}P counting and automatic computation of data (8). The unknown spots are eluted, and the procedure is repeated after addition of unlabeled known compounds which later are localized as colored spots on the chromatograms after specific chemical reactions with sprays of chemicals. When the phosphorylated compounds are not well separated even in 48-hr chromatograms, these regions are eluted and treated with phosphatase [purified from Polidase S (Schwarz Laboratories) by ammonium sulfate precipitation; 25 μg/0.3 ml]. The samples are left at 37°C overnight, taken up in 80% methanol, and rechromatographed as previously.

Protein content of another aliquot sample of cells from each dish is determined by the method of Lowry et al. (9).

RESULTS

Excellent chromatographic separation of labeled metabolites from chick cells can be obtained using the methods described (Fig. 3). Sugars, amino acids, and carboxylic acids are best seen with the 24-hr chromatography, while 48-hr chromatography serves better for nucleotide and sugar phosphate separation.

When ^{14}C-U-glucose (5.5 mM) is administered to chick cells under the described conditions, the intermediate compounds of glycolysis are completely labeled with ^{14}C at the same specific radioactivity per carbon atom position within about 5 to 10 min (Fig. 4). The intracellular pool of lactate is "saturated" with ^{14}C by 15 min, while large pools of secondary metabolites (metabolites further from glycolysis) such as glutamate are more slowly labeled and are not saturated with ^{14}C even after 1 hr. In general, the pools, once saturated, remain reasonably constant over the 1-hr time of the experiment, although there is a slow decline in the level of fructose-1,6-diphosphate, indicating the extreme sensitivity of this pool to a decreasing level of glucose in the medium (4).

The pool of glycolytic intermediates are sufficiently well-defined to permit calculation of the steady-state free energy changes and analysis of the rate-limiting steps under these physiological conditions, as has been done for Chlorella pyrenoidosa (1). For example, the molar ratio of fructose-1,6-diphosphate to fructose-6-phosphate (FDP/F6P) is about 1.0 (after 30 min) for cells under the conditions which gave the data shown in Fig. 4. In a parallel experiment, under identical conditions, but with ^{14}C-glucose added 12 hr earlier and replenished as indicated above, the ^{14}C-labeling of ATP and ADP is "saturated" and gives values of 152.3 nanogram-atoms ^{14}C in ATP, 19.6 nanogram-atoms in ADP and 3.9 nanogram-atoms in AMP (all per mg protein). Thus the ATP/ADP ratio is 7.7 [the "energy charge" (10) is 0.92]. For the reaction mediated by phosphofructokinase:

$$F6P + ATP \longrightarrow ADP + FDP, \Delta G^{o\prime} = -4.2 \text{ kcal} \quad (1) \quad \text{and}$$

$$\Delta G^{s} = \Delta G^{o\prime} - RT \ln 7.7 = -4.2 - 1.3 = -5.5 \text{ kcal}.$$

In other studies, SAFAC may be used to obtain samples quickly following the introduction of some physiological change expected to alter metabolism quickly. For example, after the introduction of 2-deoxyglucose to cells grown and exposed to ^{14}C-glucose under the conditions described above, there are rapid changes (less than 30 sec) in the levels of inorganic phosphate, ATP, ADP, and other compounds (11).

DISCUSSION

Analysis by measurement of appearance of radioactivity in metabolites when the cells are given radioactive substrate can provide meaningful information about metabolic dynamics (flow rates, pool sizes, points of regulation, etc.) if the addition of labeled substrate, metabolism of cells, and sampling of cells are all done in such a way as to rule out uncontrolled perturbation of the physiological conditions. Some reported effects of physiological factors on metabolism include the effects of hydrogen ion concentration on glycolysis (in erythrocytes) (12), the effects of glucose concentration on glycogen synthesis (13,4), and on other metabolic pathways (4,14), and the effects of inorganic phosphate concentration on carbohydrate metabolism (15).

Failure to control such factors as these during attempted quantitative metabolic tracer studies could account for some of the conflicting results reported in the literature. For example, if normal and transformed chick cells were grown for 24 hr on 5.5 mM glucose, and then the medium removed and 5.5 mM ^{14}C-U-glucose added, subsequent sampling and analysis of the cells might give very misleading results. Before the start of the incubation with labeled glucose, the transformed cells, which take up more glucose, can deplete the glucose in the medium more rapidly than the normal cells. Thus it can happen that the transformed cells will have

Thus it can happen that the transformed cells will have been starved while the normal cells have not yet depleted the glucose in the medium. Differences observed in the subsequent metabolism of labeled glucose or enzyme levels may be due much more to the fact that the transformed cells were starved than to metabolic differences inherent in normal and transformed cells. In fact, we observed such effects in preliminary experiments.

The use of SAFAC and other techniques described in this report have enabled us to obtain smooth saturation curves for labeling of metabolic pools, without overshoot or serious change in the saturation level once it has been achieved. The method has been used to determine differences in the metabolic dynamics between normal and transformed chick cells (4). It may also be used to determine the requirement of macromolecular synthesis in the regulation of carbon flow (16). Such techniques would appear to have wide applicability to study of other cell cultures and other variabless.

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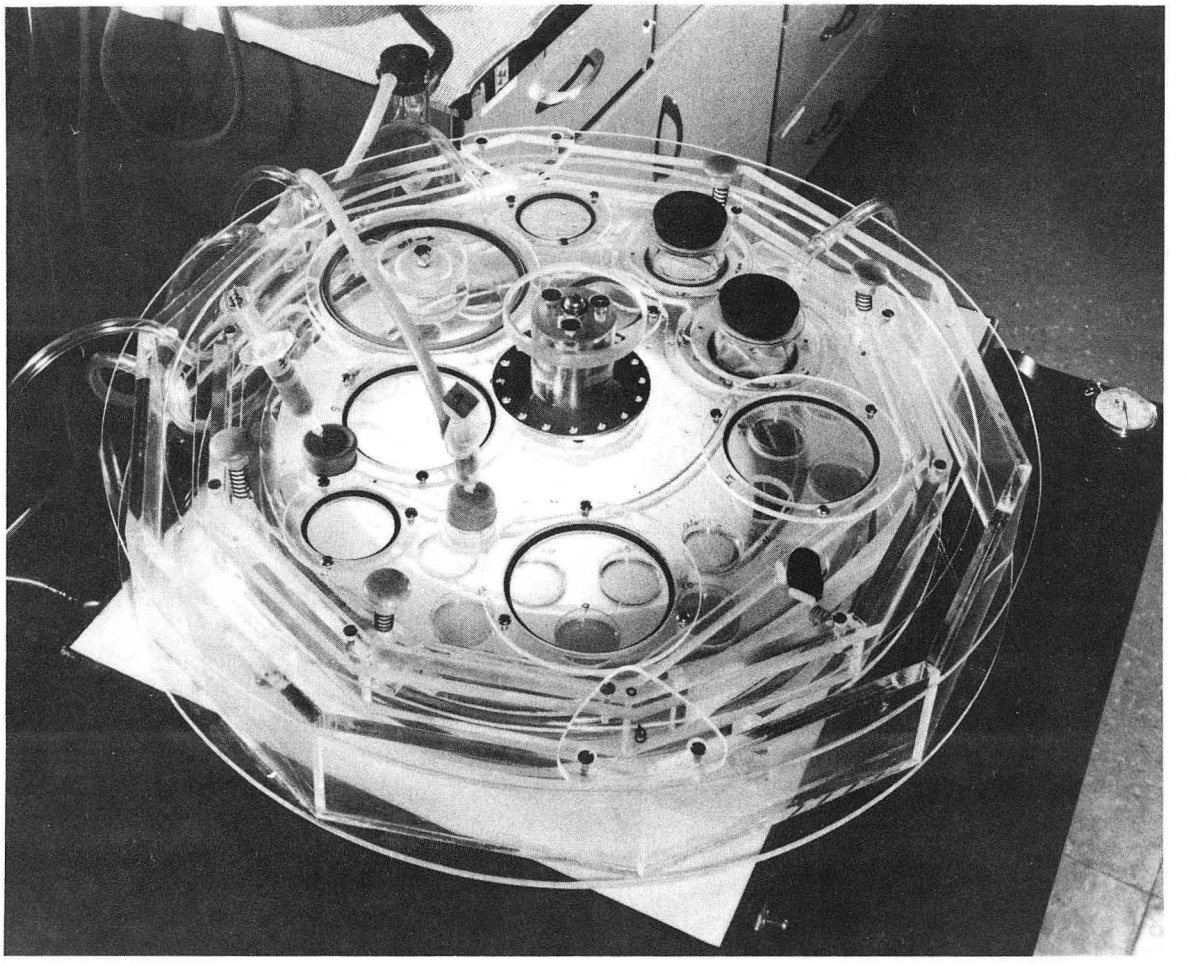
FIGURE CAPTIONS

Fig. 1. Steady-state apparatus for animal cells (SAFAC).

Fig. 2. Dish carrier of the SAFAC.

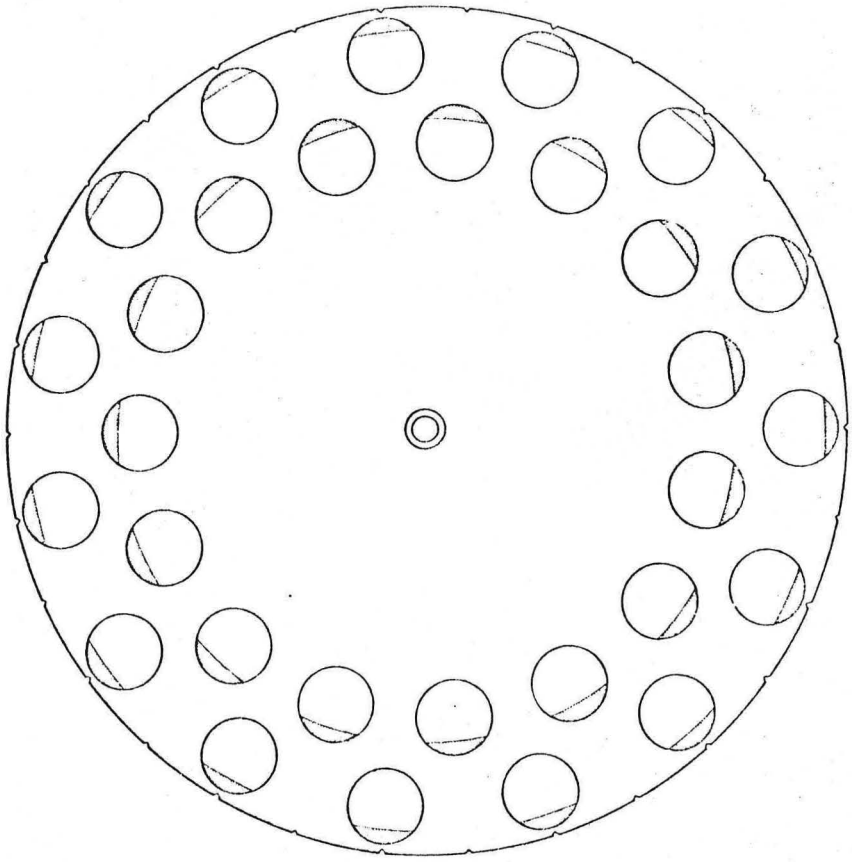
Fig. 3. Radioautographs of two-dimensional chromatogram. a. Chromatographs for 24 hr in each direction. b. Chromatographs for 48 hr in each direction. Abbreviations: LAC, lactate; ALA, alanine; Gln, glutamine; GLUC, glucose; GLUT, glutamate; MAL, malate; CIT, citrate; ASP, aspartate; GolP, α-glycerol phosphate; X_i an unknown, prominently labeled compound; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; PMP, pentose monophosphates; 3PGA, 3-phosphoglycerate; Pi, inorganic phosphate; 6PGlA, 6-phosphogluconate; UDPG, uridine diphosphoglucose; FDP, fructose-1,6-diphosphate.

Fig. 4. ^{14}C Labeling of intermediary metabolites in chick cells given ^{14}C-U-glucose as substrate.



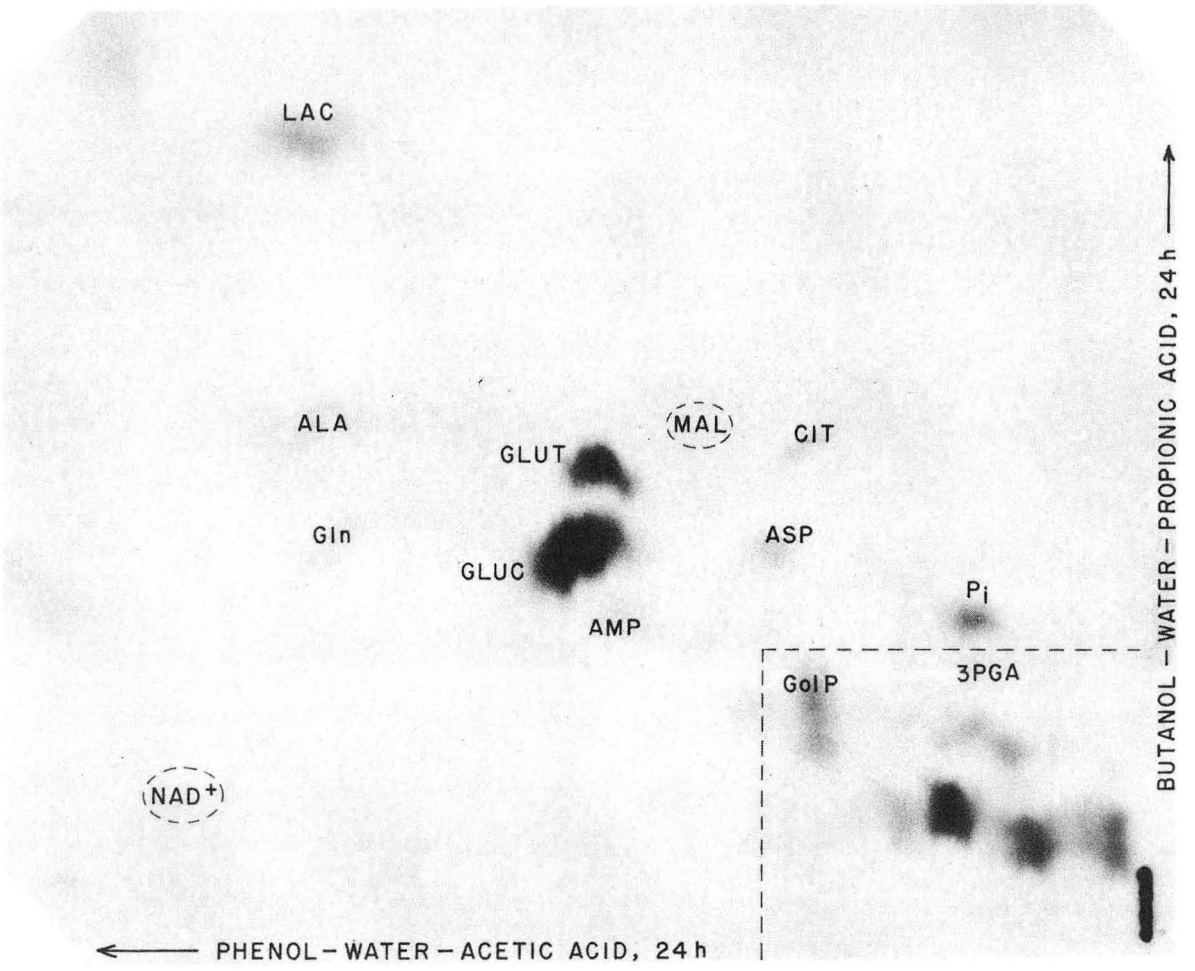
XBB 7310-5795

Fig. 1.



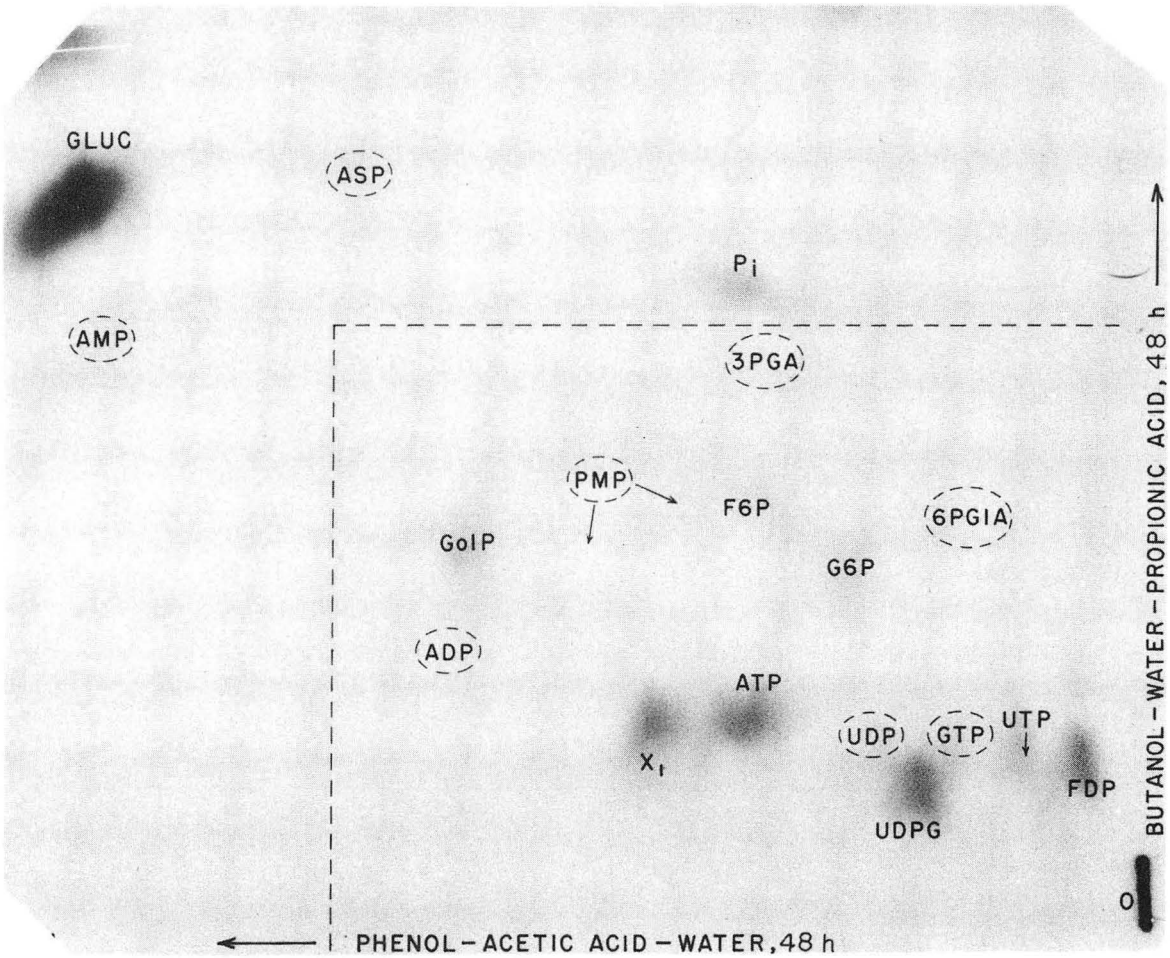
XBL7312-4971

Fig. 2.



XBB 741-376

Fig. 3a.



XBB 741-377

Fig. 3b.

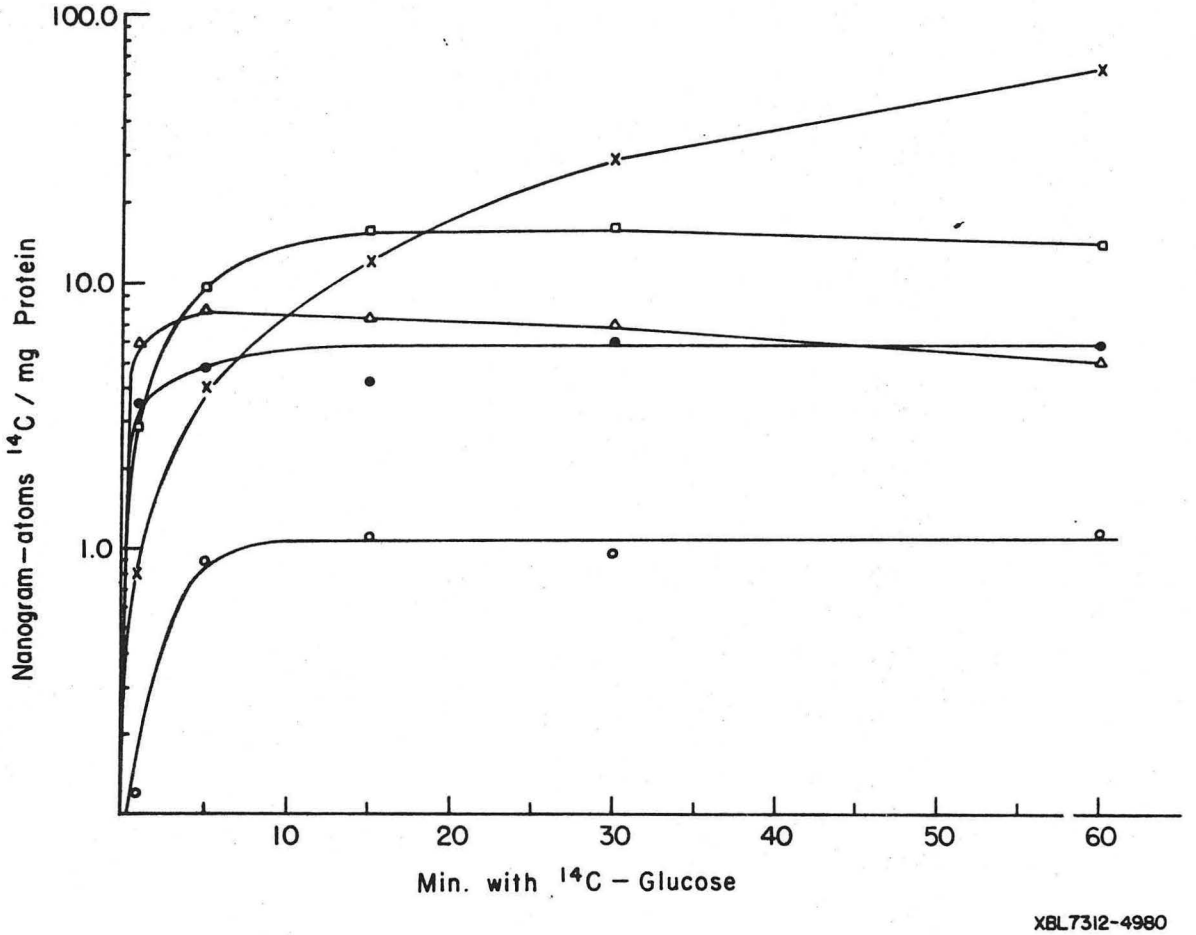

100.0
10.0
1.0
Nanogram-atoms ^{14}C / mg Protein
10
20
30
40
50
60
Min. with ^{14}C – Glucose
XBL7312-4980

Fig. 4.

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