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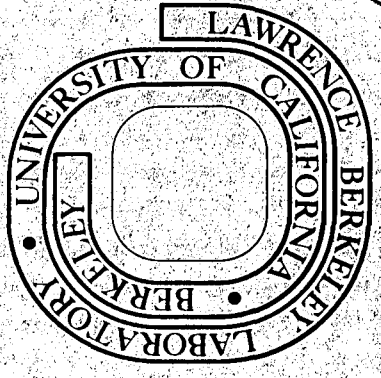

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Transport as a Rate Limiting Step in Glucose Metabolism in Virus-Transformed Cells: Studies with Cytochalasin B

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ABSTRACT

Steady-state kinetic tracer analysis and two-dimensional chromatography and autoradiography were used to examine the relation of glucose transport to its metabolism in cultured chick embryo fibroblasts. Cytochalasin B was added to Rous sarcoma virus-infected cells to bring the rate of glucose uptake to the level of uninfected cells. It is concluded for chick cells in culture that: 1) the transport of glucose, rather than its phosphorylation, is the rate limiting step and 2) the difference in aerobic glycolysis between normal and virus-transformed cells at physiological glucose concentration may be a consequence of the difference in the rates of glucose uptake.

INTRODUCTION

Most cells in the body do not produce lactic acid in the presence of air; by contrast, a majority of tumors produce much lactic acid. The discovery of this distinction between normal and malignant cells is a half century old (Warburg, '30) and has been much debated (Aisenberg, '61; Paul, '65; Shapot, '72; Weinhouse, '70; Wenner, '67) without a consensus as to an explanation for its existence.

When normal cells are placed in tissue culture, they also become exceedingly glycolytic, a fact which led to the notion that growth rather than malignancy is the reason behind increased lactic acid production of tumors (Steck et al., '68). We showed previously that, if population density and environmental conditions were strictly controlled, and if the rate of glucose uptake was taken into consideration, virus-transformed cells in culture were more glycolytic than normal cells irrespective of growth rate (Bissell et al., '72). What caused the additional increased glycolysis still remained to be determined.

Using steady-state tracer analysis and an apparatus designed to control the environmental conditions (Bassham et al., '74) we conducted a series of experiments on the dynamics of glucose metabolism in normal and virus-transformed cells (Bissell et al., '73a, b, '76; Rambeck et al., '75). The results led to the postulate that the increase in glucose transport which occurs after transformation in these cells may be the cause (rather than the result) of increased aerobic glycolysis in these cells.

While many of the questions discussed in this paper have been addressed by others in variety of cell types, I will confine the discussion of the results to the chick cell system where the existence of increased aerobic glycolysis after virus-transformation has been demonstrated under steady-state conditions. I will describe some of the results that led to the above postulate as well as some new data which tend to support it.

METHODS AND MATERIALS

Cell culture and virus infection

Primary cultures were prepared from the body wall of 10-day-old chick embryos (C/B, SPF type eggs, H&N, Inc., Redmond, Washington) essentially as described by Rein and Rubin (1968) and seeded in Medium 199 (Grand Island Biological) supplemented with tryptose phosphate broth and calf and chick serum (Microbiological Associates). Due to harmful side effects, fungizone (and other fungicides) were eliminated entirely from the culture medium (Dolberg and Bissell, '74). For studies with transformed cells, half the cells of a single embryo were infected 4 hr after primary seeding with approximately 10^6 focus forming units of Schmidt-Ruppin strain of Rous-sarcoma virus per 100 mm plate (Falcon). Secondary cultures were prepared 4 days after the primary seeding by trypsinization of primary cultures and were seeded at 1×10^6 per 35 mm dishes (Falcon). Experiments were conducted 48 hr after secondary seeding.

The steady-state apparatus and tracer studies

These have been described in detail (Bassham et al., '74; Bissell et al., '73b). Briefly, the plates were placed in the steady-state apparatus and the medium was changed to fresh medium 199 (containing no serum or tryptose phosphate broth). After temperature and pH equilibration (1 hr), without opening the system the medium was replaced with one containing high specific activity ^{14}C-glucose (New England Nuclear; final specific activity 30-50 Ci/mol). The cells were permitted to take up the radioactive substrate for various lengths of time. The medium was then removed and frozen for further analysis. The cells were washed, killed with 80% methanol, scraped from the plate and disrupted by sonic oscillation. An aliquot was applied to filter paper for analysis by two dimensional paper chromatography (Bassham et al., '74).

Another aliquot was used to determine the level of the protein by the method of Lowry et al. (1951) using Autoanalyzer II system (Technicon). The ^{14}C content of each pool was calculated and was expressed as ng atom ^{14}C per mg protein applied to the paper. When cytochalsin B (CB) (Aldrich Chemical Company) was present, it was added together with the ^{14}C-glucose medium.

Analytical procedures and transport studies

Lactic acid production was measured enzymatically with the use of reagent kit of Boehringer Mannheim. The rate of glucose uptake was measured by use of 2-deoxy-D-[^{3}H] glucose in the presence or absence of CB as described (Dolberg et al., '75). For measurement of protein synthesis a 30 min pulse of ^{3}H-leucine (2 μCi/ml; New England Nuclear) was given to the cells. After removal of the radioactive medium, cells were washed three times with cold Hank's buffer. Cold 5% trichloroacetic acid (TCA) was added. After 15 min the TCA soluble pools were removed and cells were washed three times with buffer. Two ml of 1% lauryl sulfate (SDS) was added to the plates. After solubilization, identical aliquots were used for protein measurement (Lowry et al., '51) and for the level of radioactivity. The samples were counted in a TriCarb Packard Scintillation Counter after addition of scintillation fluid.

RESULTS

A radioautograph of metabolites of virus-transformed cells grown for 60 min in ^{14}C-glucose and developed for 24 hours in each direction is shown in Fig. 1. For a better separation of phosphorylated regions, duplicate chromatograms were developed for 48 hours in each direction. A large number of labeled compounds derived from the catabolism of glucose were separated and identified. Using this system, the steady-state apparatus (Bassham et al., 1974) and a series of kinetic time points, the following questions were

considered in relation to the transformed state:

1) Are all the steps leading to lactic acid production activated or are there only specific steps?
2) Is the TCA cycle functional and, if so, is it comparable to the normal cells?
3) Is energy metabolism altered?
4) Is glycogen synthesis impaired?
5) What is the actual level of oxidative pentose phosphate cycle?
6) Are hydrogen transfer pathways impaired?
7) What is the relation of increased glucose transport to the changes in metabolism?

1) Regulation of glycolytic carbon flow

To our surprise, we discovered that not only the ^{14}C-pool sizes of the product of known rate limiting enzymes of glycolysis (hexokinase, phosphofructokinase, and pyruvate kinase) were elevated, but also the other glycolytic intermediates which were identified and separated in our chromatograms were higher in transformed cells (Fig. 2; Bissell et al., '73b). When glucose concentration in the medium was lowered, these pool sizes in both normal and transformed cells decreased accordingly, although the difference between the two remained. This would indicate that the enzymes of the glycolytic sequence respond to glucose level in the medium in unison and that the overall rate of glycolysis varies with changes in glucose transport including the increased transport in transformed cells. Furthermore, when either normal or transformed cells were retained in low glucose medium and protein synthesis was inhibited with 10 μg/ml cycloheximide (or 20 μg/ml anisomycin), an increase in the concentration of glucose lead to an immediate increase in the glycolytic pools irrespective of the presence or the absence of protein inhibitors (Fig. 3; Bissell et al., '73a). These experiments also indicated that, under our

culture conditions, the glycolytic enzymes existed in excess and were not the primary rate regulators.

2) TCA cycle

In contrast to the glycolytic intermediates where the pool sizes were higher in transformed cells and saturated quickly, the rate of glucose carbon flow through the TCA cycle intermediates and through amino acids were comparable in normal and transformed cells and did not saturate within an hour (Fig. 4). When a steady-state was reached after prolonged labeling in ^{14}C-glucose, the ^{14}C-pool sizes were similar in normal and virus-transformed cells (unpublished results). This indicated that increased glycolysis in these cells after transformation was not at the expense of respiration.

3) Energy metabolites

It had been shown previously that both ribo- and dioxyribonucleotide pool levels were comparable in normal and RSV-transformed chick cells (Colby and Edlin, '70). We confirmed this finding for the adenine nucleotides under our conditions. Prolonged labeling in both $^{32}PO_4^{3-}$ and ^{14}C-glucose and final separation of energy metabolites indicated no difference in either ^{32}P or ^{14}C content of these compounds (White, Bissell and Bassham, unpublished). These results rule out gross changes in ATP synthesis and "energy charge" after transformation, although a change in compartmentalization of the energy metabolites cannot be ruled out.

4) Glycogen synthesis

We found that transformed cells contained little or no glycogen after 48 hours of growth in culture. This, however, proved to be the result of glucose depletion of the medium and not an impairment in glycogen synthesis. The importance of glucose concentration in the level of glycogen synthesis in HeLa cells has been reported previously (Alpers et al., '63). In fresh

medium and physiological glucose concentration, transformed cells not only synthesized glycogen, but did so at a rate far in excess of normal cells (Bissell et al., '73b).

5) Oxidative pentose phosphate cycle

That the ratio of activity of oxidative pentose shunt to that of TCA cycle increases after Rous-transformation has been known for both tumor slices and cells in culture (Ashmore et al., '61; Bissell et al., '72). We measured the total level of shunt using the retention of radioactivity after incubation in tritiated glucose labeled in C-3 position. It was found that, despite the fact that the shunt was twice as high in transformed cells as in normal cells, the ratio of glucose metabolized via shunt to the net flow of that metabolized directly to fructose-6-phosphate is about the same in normal and transformed cells (Rambeck et al., '75). In other words, the increased shunt after transformation was directly proportional to the level of glucose uptake.

6) Pathways of intracellular hydrogen transfer

It had been suggested that the increased glycolysis of most tumor cells may be explained by lack of cytoplasmic glycerol phosphate dehydrogenase (GPD) (Boxer and Devlin, '60). Thus it was postulated that in order to produce the oxidized NAD needed for continuous flow of glucose carbon, the cells oxidize the NADH via conversion of pyruvate to lactate. We compared the existence and the activity of this shuttle in normal and transformed chick cells under steady-state conditions. Based on three lines of evidence, we concluded that GPD is present and active in virus-transformed cells.

a) The GPD level was similar in normal and transformed chick cells.

b) After the cells were grown in uniformly labeled glucose and final separation of intermediates, it was found that transformed cells make 2-3 times more labeled glycerol phosphate from glucose than do normal cells.

c) Examination of tritium content of oxidized NAD following growth in tritiated water (which can be used as an indication of the relative activities of GPD and lactic dehydrogenase showed that the ratio of GPD to LDH activity was comparable in normal and transformed cells (Bissell et al., '76). Thus the increased lactic acid production, at least in culture, could not be explained by a simple impairment of hydrogen transfer pathways.

7) Transport

While there was much evidence suggesting that in chick cells the degree of glucose catabolism via the glycolytic sequence is regulated by the rate of transport, the postulate had not been tested directly. We took advantage of the fact that cytochalasin B (CB), an inhibitor of glucose uptake in cultured cells (Kletzien et al., '72; Estensen and Plagemann, '72; Mizel and Wilson, '72) is reported to affect only the uptake and not the phosphorylation of glucose in these cells (Mizel and Wilson, '72; Kletzien and Perdue, '73). We have shown that CB, at 2.1×10^{-6} M, inhibits only the saturable facilitated component of glucose transport without affecting the non-saturable component (non-saturable at the substrate concentrations used; Dolberg et al., '75). The contribution of the latter mode to overall transport becomes significant at 5.5 mM glucose concentration. Therefore, by varying CB concentration in the medium, one can achieve a situation where the uptake of glucose in transformed cells is lowered to the level of normal cells. One can then ask what happens to the pattern of glucose metabolites in general and to the rate of glucose carbon flow into glucose-6-phosphate (G6P) pool and other glycolytic intermediates. A pattern of glucose metabolites after treatment with 3.0×10^{-6} M CB is shown in Fig. 5. A comparison of Fig. 1 and 5 revealed that the main difference is a quantitative reduction in the level of glycolytic intermediates (near the origin) and in the lactate pool. The analysis of kinetic data (Fig. 6) revealed that both the rate of carbon flow and the final pool

sizes of G6P and other glycolytic intermediates including fructose 1,6-diphosphate (FDP) in CB-treated transformed cells became similar to normal cells. Furthermore, the flow of ^{14}C-glucose carbon into TCA cycle intermediates and the two amino acids derived from it (Fig. 7) was unaffected by CB. It is important to realize that the interpretation of the results with CB is based upon the assumption that CB does not inhibit phosphorylation of glucose. While this has been shown previously for 2-deoxyglucose in chick cells (Kletzien and Perdue, '73), a direct assay of hexokinase activity in chick cells using glucose as a substrate in the presence of CB is essential. It is also important to rule out an additional effect of CB on other metabolic processes of the cells (such as the availability of ATP for phosphorylation, etc.). These experiments are in progress.

DISCUSSION

Previous studies have shown that one of the earliest manifestations of virus-transformation in culture is a change in glucose uptake (for review see Plagemann and Richey, '74; Hatanaka, '74). Using 3-O-methylglucose, it was shown that in chick embryo fibroblasts transformed with Rous sarcoma virus the increase was due to the transport step itself rather than phosphorylation of glucose (Weber, '73; Venuta and Rubin, '73). Nevertheless, some steps in the glycolytic sequence (notably those catalyzed by hexokinase, phosphofructokinase and pyruvate kinase, the three classically rate limiting reactions of glycolysis) were shown to also be activated after transformation. It has been argued, therefore, that the activation of glycolytic pathway is the cause of increased transport (Singh et al., '74). Furthermore, in two transformed variants of 3T3 cells, the activity of hexokinase was directly correlated with the rate of 2-deoxyglucose transport (Romano and Colby, '73). Therefore, the validity of both increased glucose transport after virus-transformation and its relation

to glucose metabolism had been questioned.

The idea of uptake as a rate-limiting step in metabolism of glucose is not entirely novel. In adipose tissue, for example, it was shown by Crofford (1968) [see also Illiano and Cuatrecasas (1970)] that the rate of glucose entry into cells under certain conditions is the rate limiting step. The interrelation between glucose concentration and its metabolism has also been demonstrated in ascites cells (Renner et al., '72). Our results indicate that in chick cells in culture the rate of transport of glucose may also be an important controlling element in the rate of sugar metabolism. In addition, our inability to detect a "defect" in the glucose metabolism of virally transformed chick cells together with inhibition studies with CB presented here, tend to support our previous hypothesis that the increase in aerobic glycolysis after transformation is in part due to the increased transport of glucose.

It should be pointed out, however, that the above conclusion is not in conflict with the fact that some glycolytic enzymes are indeed activated in transformed cells. In chick cells we have shown an appreciable increase in lactic acid dehydrogenase (Bissell et al., '72, '76) level as well as an indication of phosphofructokinase stimulation (Bissell et al., '73b). Others have shown activation of phosphofructokinase (Fodge and Rubin, '73) as well as hexokinase and pyruvate kinase (Singh et al., '74). An increase or decrease in enzyme activity by itself may not be sufficient to account for changes in metabolic flow. Gregory et al. (1976), for example, have recently demonstrated an appreciable increase in hexokinase activity in untransformed 3T3 cells as they become crowded even though glucose transport is much reduced at high density. They have further shown that changes in phosphofructokinase and pyruvate kinase are not necessarily correlated with the transformed state in 3T3 cells. Nevertheless, there is more than one site of regulation in some metabolic pathways. Our results simply indicate that transport of glucose

is an important controlling element in glucose metabolism in virus-transformed chick cells.

An additional point to keep in mind is that these studies do not negate any of the well established conclusions about the regulation of glycolysis--a complex and widely studied field. There is much evidence, for example, that hexokinase and phosphofructokinase are the main glycolytic regulators in red blood cells and muscle respectively. In cells in culture, it seems that all glycolytic enzymes are highly activated in such a way that around and below physiological glucose concentration the rate of glucose uptake across the membrane becomes a limiting factor in glucose catabolism. Since there is an increase in glucose uptake after transformation in chick cells, this in turn is reflected in increased flux across the glycolytic and the oxidative pentose phosphate pathways. It is possible that the increased flux or one of the metabolites could cause yet further activation of the key glycolytic enzymes in transformed cells. What causes the transport change itself is, of course, an active area of research at this time.

ACKNOWLEDGEMENTS

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FIGURE CAPTIONS

Figure 1. Radioautograph of two-dimensional chromatograms of glucose metabolites in chick cells transformed with Rous sarcoma virus. The cells were exposed to 1.0 ml of 5.5 mM uniformly labeled [^{14}C] glucose in medium 199 for 1 hr (final specific activity 30 Ci/mol) as described in Methods. LAC, lactate; ALA, alanine; GLUT, glutamate; GLUC, glucose; MAL, malate; CIT, citrate; ASP, aspartate; 3-PGA, 3-phosphoglyceric acid; GP, glycerol phosphate; DHAP, dihydroxyacetone phosphate; PMP, pentose monophosphate region; HMP, hexose monophosphate region; UDPG, uridine diphosphoglucose; FDP, fructose-1,6-diphosphate; O, origin. For better separation of HMP and PMP regions, duplicate chromatograms were developed for 48 hr in each direction or the spots from 24 hr chromatograms were eluted, treated with phosphatase, and rechromatographed.

Figure 2. ^{14}C pool sizes of the glycolytic intermediates in normal and transformed cells. Procedure was as in Fig. 1 and Methods. The spots were removed from the chromatograms and counted as described. The pool sizes vary with density and growth rate. At comparable growth rates and densities, the difference between normal and transformed cells depends on the degree of transformation. F6P, fructose-6-phosphate; other abbreviations are as in Fig. 1. (●), transformed cells; (o), normal cells. Taken from Bissell et al., '73b.

Figure 3. Normal cells were shifted to medium 199 with 0.55 mM glucose one hr prior to the experiments and protein synthesis was inhibited with addition of either 10 µg/ml cycloheximide or 15 µg/ml anisomycin.

Protein synthesis was inhibited 95% within 15 min as shown by the rate of ^{3}H-leucine incorporation into trichloroacetic acid precipitable material. U-^{14}C-glucose (0.55 mM, specific activity 53.6 Ci/mol) was added at 0 time. After 15 min ^{14}C-glucose was added in such a way as to bring the level to 5.5 mM without changing the specific activity. The first time point taken was 30 sec after increasing the glucose concentration. Further analysis as in Figs. 1 and 2 (Bissell, White and Bassham, unpublished). G6P and F6P were not further separated in this experiment. Circles, normal controls; triangles, normal + cycloheximide; closed symbols, low glucose; open symbols, high glucose.

Figure 4. Rate of glucose carbon flow through the tricarboxylic acid cycle intermediates and amino acids. Procedure as in Figs. 1 and 2. (●), transformed cells; (o), normal cells. Taken from Bissell et al., '73b.

Figure 5. Radioautograph of two-dimensional chromatogram of glucose metabolites in chick cells transformed with Rous sarcoma virus and labeled in the presence of 3.0×10^{-6} M CB. Legend as in Fig. 1. The level of ^{14}C label in ATP, ADP and AMP is reduced because of the lower level of pentose shunt (and thus ^{14}C-deoxyribose). Preliminary results indicate that the total level of energy metabolites is not altered.

Figure 6. ^{14}C pool sizes of the glycolytic intermediates in normal, transformed, and transformed + CB. Procedure was as described in Fig. 1, 2 and Methods. (o), normal cells; (●), transformed cells; (Δ, dashed lines), transformed + CB.

Figure 7. ^{14}C carbon flow into the pools of two amino acids derived from TCA cycle in normal, transformed and transformed + CB. Legend as in Fig. 1, 2 and Methods. (o), normal; (●), transformed; (Δ, dashed lines), transformed + CB.

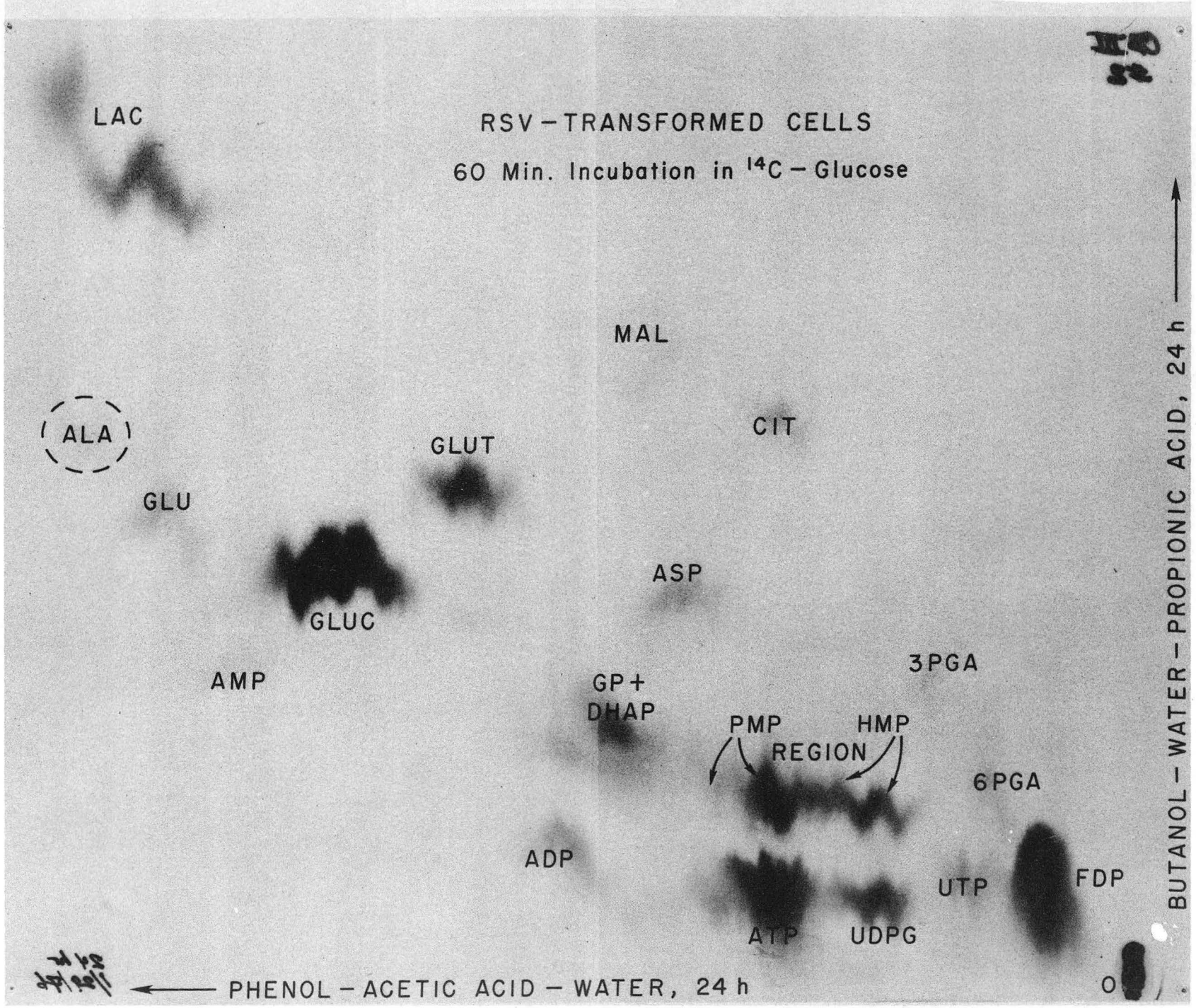

Fig. 1

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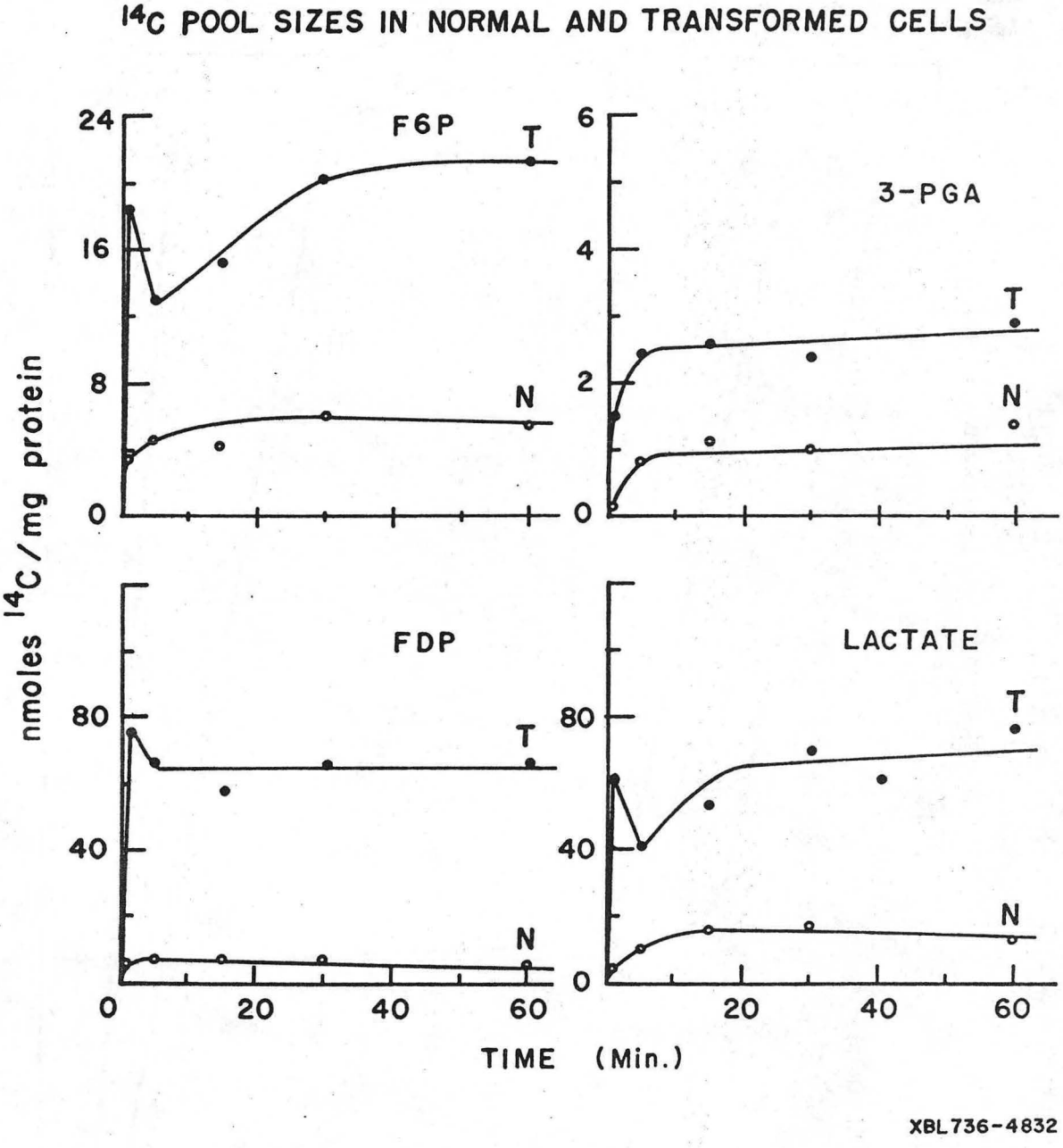

Fig. 2

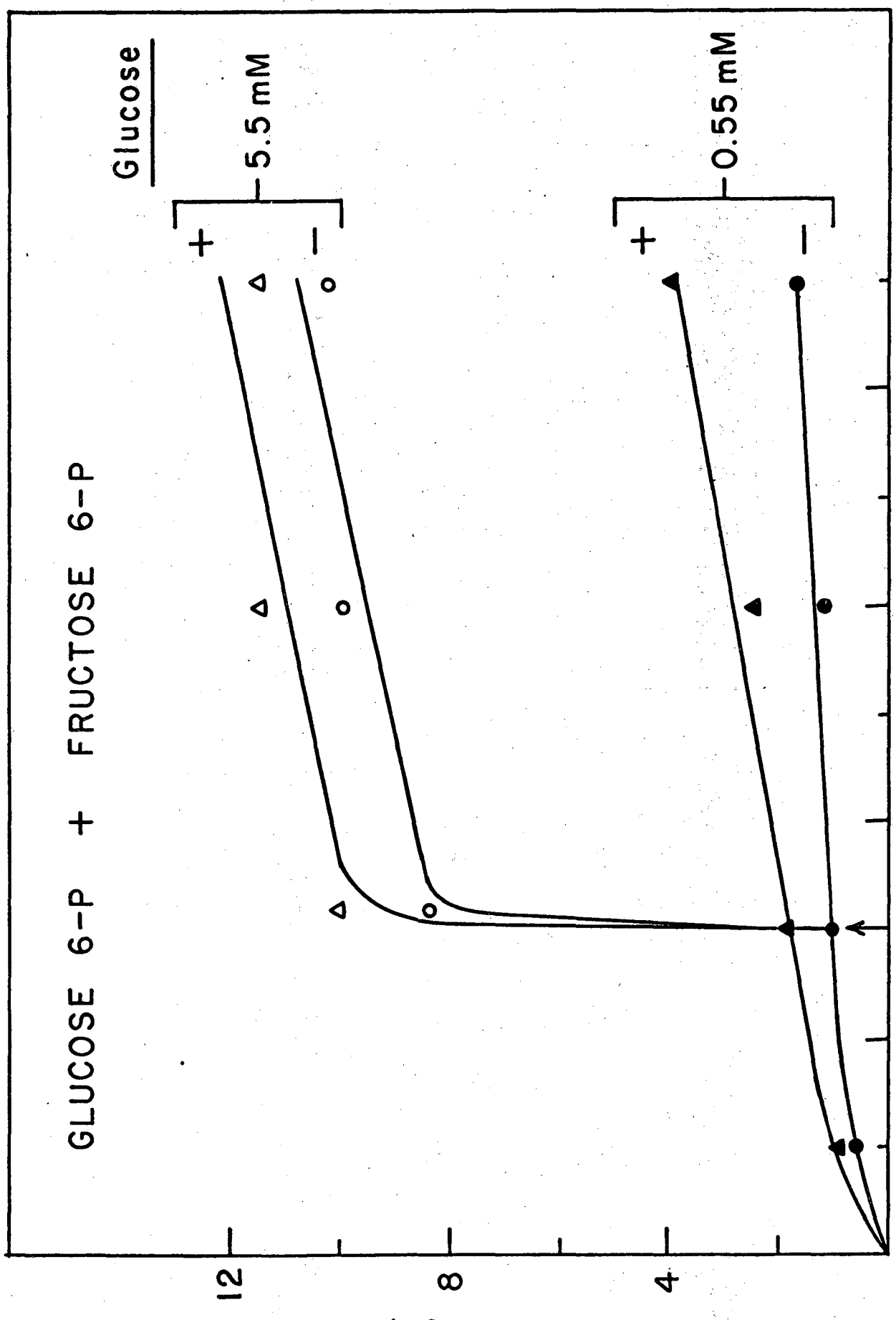

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Fig. 3

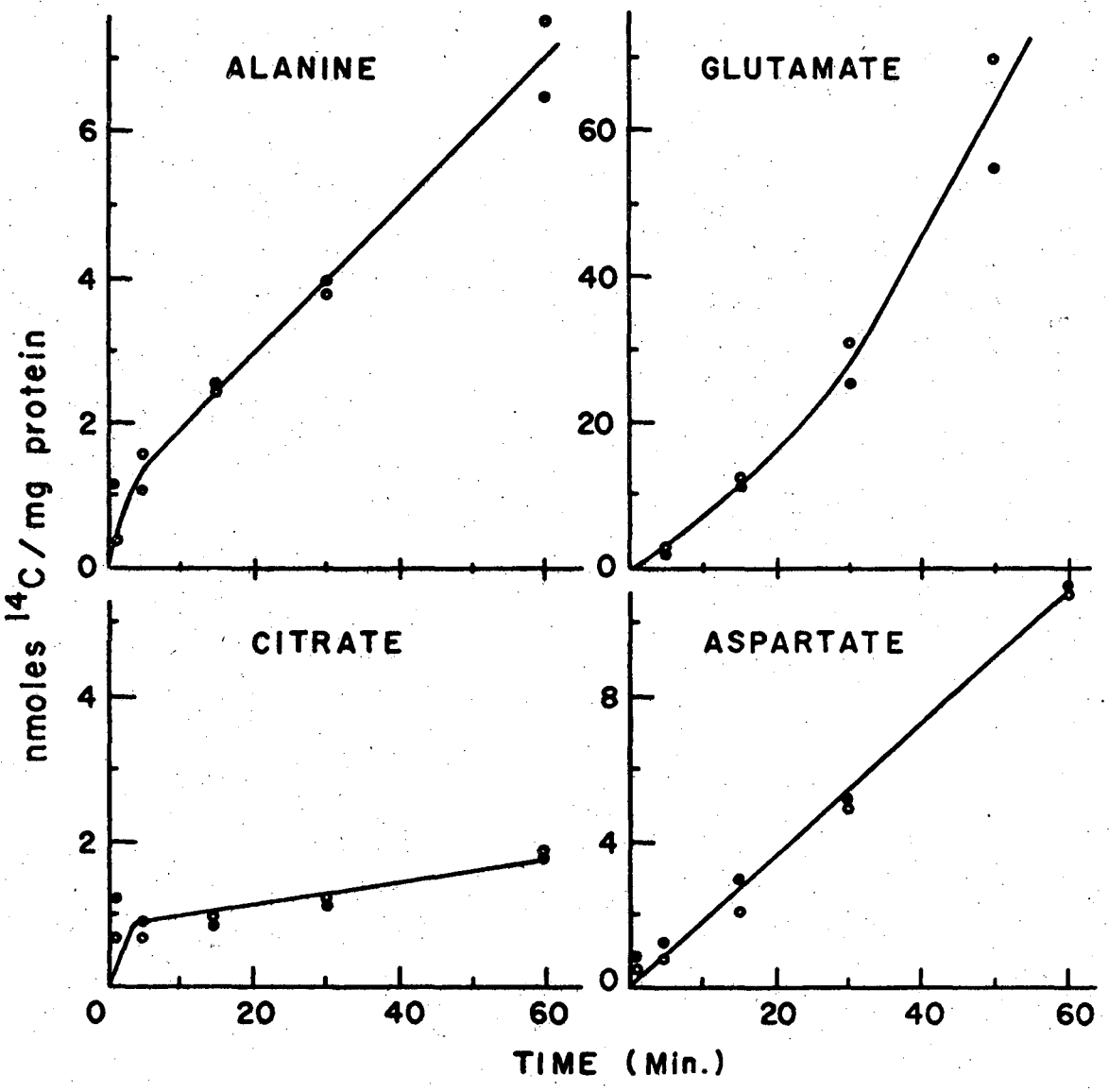


Fig. 4

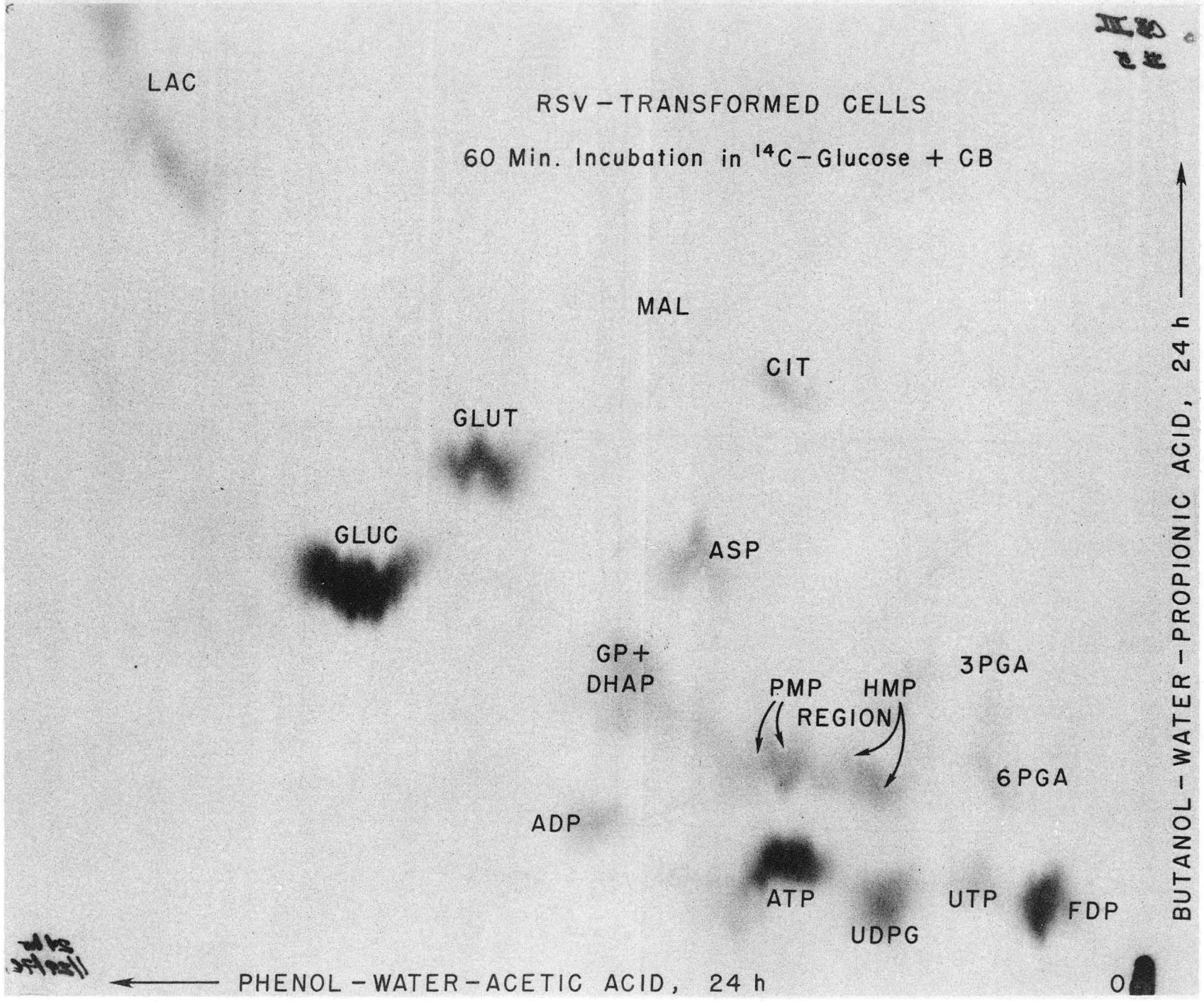
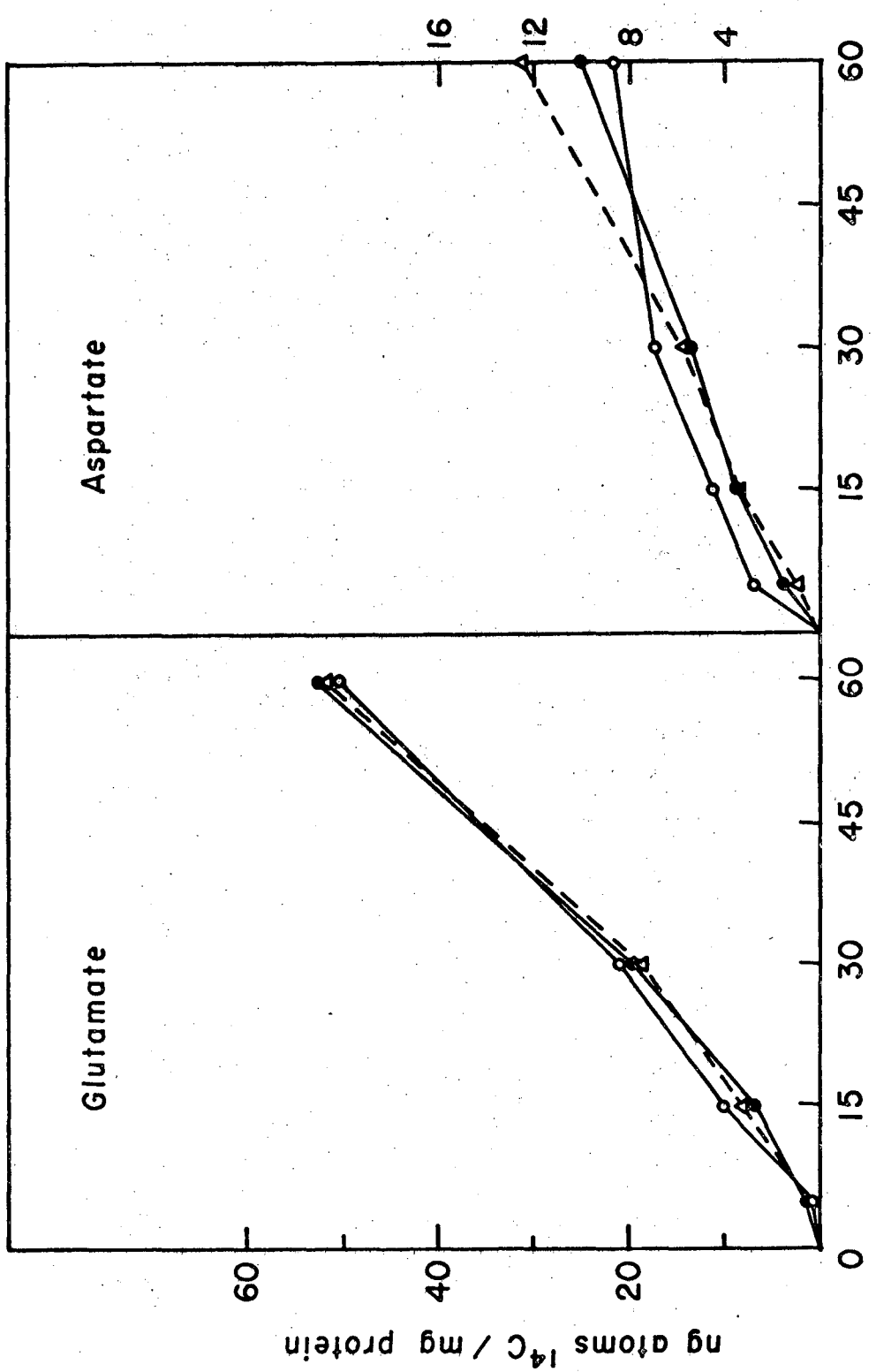


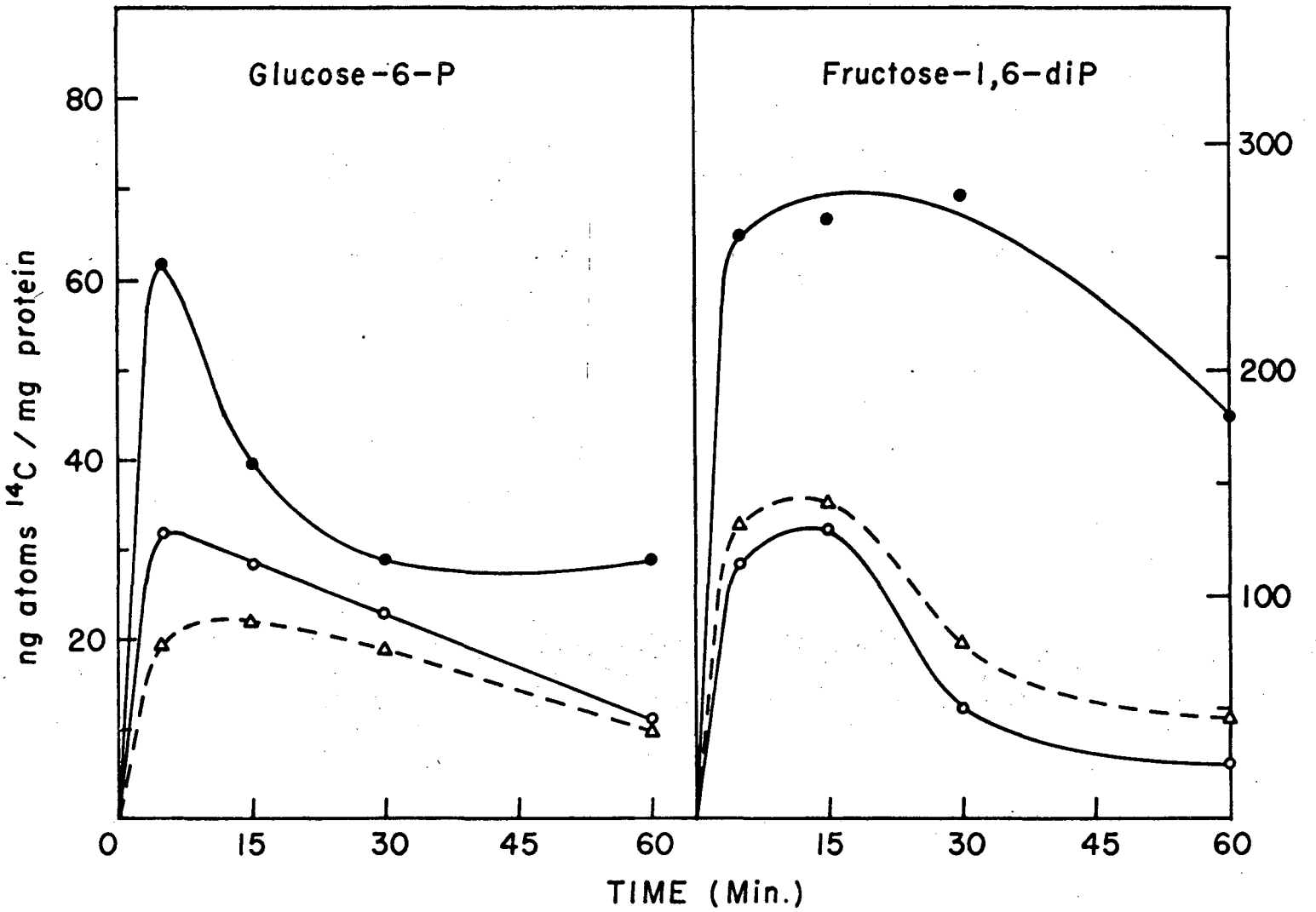
Fig. 5

XBB 764-3533



XBL 764-5839

Fig. 6



XBL 764-5838

Fig. 7

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