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Carbohydrate Metabolism in Strain L-929 Mouse Fibroblast Cells.*† (27731)

MARY K. JOHNSON AND EMMETT J. JOHNSON (Introduced by Charles C. Randall)
Department of Microbiology, University of Mississippi School of Medicine, Jackson

The L-strain of mouse fibroblast has been used extensively in studies of the mechanism of viral infection. However, little information is available concerning the metabolism of this cell line or the effects of viral infection on its metabolism. It is particularly well suited to biochemical investigation because of its ability to grow rapidly in suspension culture in relatively simple media.

Current work on the biochemistry of cultured mammalian cells has been reviewed by Levintow and Eagle(1). Phillips and Andrews (2) have reported experiments on the effect of various factors on respiratory activity in L-cells. The production of lactic acid by this cell line has been correlated with the growth phase of the cells by Munyon and Merchant (3).

The present study represents a survey of the enzymic constitution of L-cells, with respect to enzymes involved in the intermediary metabolism of carbohydrate, undertaken as a preliminary to an investigation of the effects of vaccinia-virus infection on the carbohydrate metabolism of this cell line.

Methods. The strain L-929 mouse fibroblast cell line was cultured in suspension in YELP medium(4), with 0.12% Methocel, 100 μg/ml streptomycin, and 100 units/ml penicillin. The medium was contained in screw-cap Erlenmeyer flasks and agitated on a rotary shaker at 37°C. New cultures were started by dilution of late log phase cultures (having a cell concentration of between 1.0 and 1.3 $\times$ 10^6 cells per ml) with sufficient fresh medium to reduce the cell count to about 2.5 $\times$ 10^5 cells per ml. When the desired cell concentration had been attained, cultures were harvested by centrifugation at room temperature. Cells used for measurements of respiratory activity (harvested at a cell count of 6-8 $\times$ 10^5 cells per ml) were washed twice with Krebs-Ringer-phosphate solution and then suspended in this solution at a concentration of 5 $\times$ 10^6 cells per ml. Cell extracts used in Warburg experiments were prepared by sonication (as described below) of a suspension containing 2 $\times$ 10^7 cells per ml of Krebs-Ringer-phosphate solution. Conventional manometric technics were employed for measurement of oxygen uptake at 37°C with the Warburg constant volume respirometer. All materials added to Warburg flasks were dissolved in Krebs-Ringer-phosphate solution, and the pH of the solutions adjusted to pH 7.2-7.4.

Cells used for measurements of enzymic activity (harvested at a cell concentration of approximately 1 $\times$ 10^6 cells per ml) were washed twice in Krebs-Ringer-phosphate so-

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lution and suspended in distilled water at a concentration of 2×10^7 cells per ml. The cells were disrupted by treatment for 5 minutes in a 9-kc Raytheon sonic oscillator. Undisrupted cells and debris were removed by centrifugation at 2000 *g* for 10 minutes. The supernatant fluid from this centrifugation is designated as the crude extract. The crude extract was centrifuged in a Model L Spinco ultracentrifuge at 144,000 *g* for 1 hour. The supernatant portion from this centrifugation was the source of enzyme for all assays, except where it is stated that the crude extract was used.

Except as otherwise noted, a unit of enzymic activity was calculated as defined in the method cited for the assay. Specific activity refers to units per mg of protein, the latter determined by the method of Lowry (5).

The methods used for determination of aldolase(6), triose phosphate dehydrogenase (7), isocitric dehydrogenase(8), glucose-6-phosphate dehydrogenase(9), and 6-phosphogluconic dehydrogenase(10) involved measurement of rate of reduction of diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) by determination on the Beckman DU spectrophotometer of changes in optical density at 340 mμ. Phosphofructokinase(11), triose phosphate isomerase(12), and alpha-glycerophosphate dehydrogenase(13) were assayed by following the oxidation of reduced DPN.

Lactic dehydrogenase was assayed by measuring the decrease in optical density at 340 mμ in a reaction mixture containing 1 ml glycylglycine buffer, 0.4 M, pH 7.5; 0.2 ml DPNH (2 mg/ml); 0.1 ml 0.01 M sodium pyruvate adjusted to pH 7.0; 0.1 ml extract; and 1.6 ml distilled water. A unit of enzymic activity was calculated as that amount of enzyme causing an initial change in optical density of 0.100 per minute.

Hexokinase activity was determined by measurement of decrease in reducing sugar concentration after incubation of 1.0 ml 0.01 M glucose, 0.1 ml 0.1 M adenosine triphosphate, 0.1 ml 0.1 M $MgCl_2$, 2.8 ml 0.1 M tris (hydroxy-methyl) amino methane (tris) buffer of pH 8.0, and 2 ml extract at 30° for 1 hour. Protein and phosphorylated sugar were removed by adding 1 ml of incubation mixture to 2 ml 0.3 N $Ba(OH)_2$, followed by addition of 2 ml 0.3 N $ZnSO_4$ and filtration of the suspension. A unit of hexokinase activity is that amount which catalyzes the phosphorylation of 1 μm of glucose in 15 minutes in the above system.

Phosphohexose isomerase activity was assayed by following the appearance of fructose-6-phosphate(14), and pentose phosphate isomerase by following changes in keto-pentose phosphate concentration(15).

Several methods were employed in an attempt to detect transketolase activity in the crude extract and in the fractionated extract, two of these(16,17) involving spectrophotometric determination of glyceraldehyde-3-phosphate formed from ribose-5-phosphate and ribulose-5-phosphate, and another involving detection of the formation of sedoheptulose-7-phosphate from the pentoses(18). The diphenylamine method for determination of fructose(19) was used to detect transaldolase activity in these reaction mixtures.

Utilization of pentose phosphate by the cell extract was tested by incubation of 4 μm ribose-5-phosphate (shown by test for ketopentose to contain some ribulose-5-phosphate), 4 ml 0.05 M tris buffer, pH 7.4, 4 ml extract (containing 3.2 mg protein), and distilled water to a total volume of 10 ml, at 30°C. Aliquots of 1 ml were removed at 5-, 10-, 15-, and 20-minute intervals and added to 1 ml 10% trichloroacetic acid. After centrifugation to remove precipitated protein, 1.0-ml samples were removed for determination of pentose concentration by the Dische modification of the orcinol reaction(19).

Adenosine triphosphatase activity was assayed by measurement of liberation of inorganic phosphate in a reaction mixture containing 0.1 ml 0.1 M adenosine triphosphate, 0.1 ml 0.1 M $MgCl_2$, 3.8 ml 0.05 M tris buffer, pH 8.0, and 1 ml extract, incubated for 1 hour at 30°C. The reaction was stopped by addition of 1 ml 10% trichloroacetic solution, and the inorganic phosphate content determined by the method of Fiske and Subba-Row(20). A unit of enzymic activity was taken as that amount which catalyzed the

TABLE I. Oxygen Consumption by L-Cells on Various Metabolic Intermediates.

Substrate	μl O_2 consumed	% increase over endogenous
Endogenous	45	
Glucose	65	45
Pyruvate	83	85
Lactate	71	58
Acetate	61	36
Citrate	55	22
Alpha-ketoglutarate	55	22
Succinate	58	29
Fumarate	58	29
Malate	54	20

Warburg flasks contained: 1 ml cell suspension (5×10^6 cells); .5 ml Krebs-Ringer-phosphate solution; .5 ml 0.01 M substrate; and .1 ml 10% KOH. Time of run: 210 min.

production of 1 μM of inorganic phosphate per ml of reaction mixture per hour. Pyrophosphatase activity was assayed as described previously(21).

Results and discussion. The results of experiments designed to determine the oxidative activity of whole L-cells suspended in Krebs-Ringer phosphate solution on various substrates are shown in Table I. As evidenced by an oxygen uptake in excess of the relatively high endogenous values, the cells were able to oxidize all of the substrates tested. Hanks' solution, without glucose and containing 0.5% Bactopeptone, was also a satisfactory medium for suspension of cells for measurement of respiration. However, the respiratory activity of cells suspended in either of these solutions showed considerable variation, for reasons as yet undetermined.

When a concentrated suspension of cells was disrupted by sonication, the crude extract retained the capacity to oxidize succinate, 1 ml of extract having an oxygen uptake of 61 μl in 90 minutes, compared with an endogenous value of 9 μl.

These results indicate that the cells have the capacity to utilize the citric acid cycle in the intermediary metabolism of carbohydrates.

Measurements of specific activities of various enzymes in extracts of L-cells are listed in Table II. These values were determined with a relatively crude preparation (the supernatant fraction from the high-speed centrifugation). Consequently they are in many cases probably minimum values, since competing reaction could interfere with the assays. The specific activities are not directly comparable because the units of enzymic activity are calculated in a number of different ways, as defined by the authors of the procedures. The specific activity of alpha-glycerophosphate dehydrogenase is, however, relatively very low, as it is in the case of tumor tissue. Boxer and Shonk(22) reported that the ratio of lactic dehydrogenase to alpha-glycerophosphate dehydrogenase lay between 0.5 and 7 for a group of normal tissues, and between 10 and several hundreds for a group of tumors. If the specific activities of these 2 enzymes shown in Table II are recalculated using the same unit of activity for both, the ratio of lactic dehydrogenase to alpha-glycerophosphate dehydrogenase is 300, a value clearly falling within the tumor tissue group values discussed above.

The presence of the enzymes of the Embden-Meyerhof pathway from hexokinase through glyceraldehyde-3-phosphate dehydrogenase indicates that this pathway can be used for the breakdown of glucose.

Enzymes of the pentose pathway, *i.e.*, glucose-6-phosphate + 6-phosphogluconic dehydrogenases and pentose phosphate isomerase, are also present.

After spectrophotometric assay of 6-phos-

TABLE II. Enzymic Activity of L-Cell Extracts.

Enzyme	Specific activity
Hexokinase	.325
Phosphohexose isomerase	785.0
Phosphofructokinase	.0425
Aldolase	1.52
Triose phosphate isomerase	159.0
Glyceraldehyde-3-phosphate dehydrogenase	6.91
Alpha-glycerophosphate dehydrogenase	.31
Glucose-6-phosphate dehydrogenase	.483
6-Phosphogluconic dehydrogenase	.243
Pentose phosphate isomerase	2.22
Transketolase	0
Transaldolase	0
Lactic dehydrogenase	52.1
Isocitric dehydrogenase (DPN)	0
" " (TPN)	19.2
Adenosine triphosphatase	.8
Pyrophosphatase (crude extract)	
Cells grown with pyrophosphate	22.2
" " without "	21.1

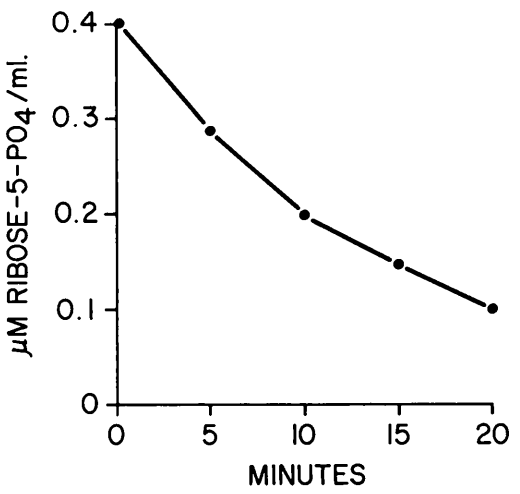

FIG. 1. Utilization of ribose-5-phosphate by L-cell extract.

phogluconic dehydrogenase, analysis of the reaction mixture revealed the presence of keto-pentose, presumably ribulose-5-phosphate.

It appears that the pentose phosphate formed in the operation of this pathway is not metabolized *via* transketolase and transaldolase. Upon incubation of extract with pentose phosphate, no sedoheptulose-7-phosphate, glyceraldehyde-3-phosphate, or fructose-6-phosphate were formed in the various assay systems employed. Pentose phosphate is, however, rapidly metabolized by the extract, as shown in Fig. 1. The breakdown of pentose phosphate in this system was not stimulated by addition of thiamine pyrophosphate or $MgCl_2$, nor inhibited by a 1-hour dialysis of the extract previous to assay. Work on determination of the products of pentose metabolism is now in progress, as are tracer studies designed to quantitate the participation of the various pathways in the metabolism of glucose.

Attempts to induce the formation of increased amounts of pyrophosphatase were unsuccessful, as shown by the fact that the specific activity of this enzyme was not significantly altered in the cells grown in a medium containing 0.01% sodium pyrophosphate.

Summary. Strain L cells, grown in YELP medium in suspension, were found to oxidize glucose, pyruvate, acetate, lactate, citrate, alpha-ketoglutarate, succinate, fumarate, and malate. Crude extracts of these cells retained the ability to oxidize succinate. Extracts of L-cells have been shown to contain: hexokinase; phosphoglucose isomerase; phosphofructokinase; aldolase; triose phosphate isomerase; glyceraldehyde-3-phosphate, alpha-glycerophosphate, isocitric, lactic, glucose-6-phosphate, and 6-phosphogluconic dehydrogenases; pentose phosphate isomerase; adenosine triphosphatase; and pyrophosphatase. Specific activities were determined in each case. No transketolase or transaldolase activity could be detected, although pentose phosphate is utilized by the extracts.

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