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Isotope Inequilibrium of Glucose Metabolites in Intact Cells and Particlefree Supernatants of Ehrlich Ascites Tumor (38671)

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For certain approaches to the quantitative investigation of the various pathways of intermediary metabolism with labelled compounds, the assumption of metabolic steady state, isotope steady state, and isotope equilibrium is of crucial importance.

Recent communications have challenged the presence of isotope equilibrium in liver cells and striated muscle cells (1–6). However, in these cells glucose metabolism is influenced by gluconeogenesis.

In order to investigate the isotope equilibrium of glucose metabolites in a system dominated by glycolysis, we have chosen Ehrlich ascites tumor cells. To further elucidate the relationship of cellular substructure to isotope inequilibrium, we have also determined the relative specific activities of glucose metabolites in particlefree supernatants of Ehrlich ascites tumor cells.

Material and Methods. Experiments with intact cells. The Ehrlich hyperdiploid ascites tumor cells (ELDa) were propagated in (ST/AxD2) F1 hybrids. The ascites cells were collected after 5–7 days, the cells being in the exponential growth phase at that time.

The intact cells were washed twice with 0.15 *M* NaCl and preincubated in Krebs–Ringer phosphate buffer (7) with 50 m*M* Tris, pH 7.4 and 12.5 m*M* glucose in a shaking water bath at 37° for 30 min. The proportion of packed cells to buffer was 1:1 to 1:2. This very high cell concentration was necessary to obtain enough glycolytic metabolites for enzymatic assay as well as for the partial enzymatic degradation. During the 30 min preincubation period the glucose consumption was determined and a sample taken for protein analysis.

The linearity of the glucose consumption and the steady state of the pool sizes were established in preliminary experiments that revealed no significant increase in the content of glycolytic metabolites after 30 min. At 30 min a sample for glucose determination was withdrawn and 20–40 μCi of 1-^{14}C glucose (specific activity 2.8–4.0 mCi/mmole) was added. The amount of added glucose was considered negligible. Samples were withdrawn and added to equal volumes of 6% perchloric acid after 30, 60, 90, and 120 sec, and the specific activity of glycolytic intermediates was determined as described below. Similar analyses were made at 8 and 16 min. The specific activity of lactate was determined at 4 min and 8 min as stated below.

Experiments with particlefree supernatant. The washed cells were homogenized in a glass-glass Potter–Elvehjem homogenizer. The homogenization medium was either distilled water or 0.15 *M* KCl. The homogenate was centrifuged at 100,000 *g* at 4° for 1 hr.

The particlefree supernatant (PFS) was diluted 1:1 with a modified Le Page medium (7), the detailed composition of which is stated in the legend to Fig. 3. The diluted PFS was preincubated for 30 min and the tracer added as for the intact cells. Because of the lower absolute glucose consumption in the PFS it was necessary to extend the incubation time and withdrawing of samples to 64 min. In the same system the specific activity of lactate was determined in samples withdrawn after 15, 30, and 60 min.

Experiments with the mitochondrial fraction. The mitochondrial fraction was prepared according to the method of Schneider

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and Hoogebom (8) and washed in 0.15 *M* NaCl. The activity of hexokinase and phosphohexose isomerase was determined according to (9) and (10).

Determination of the specific activity of intermediates. The specific activity of glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), and 6-phosphogluconate (6-PG) was determined by oxidative decarboxylation of 6-PG with 6-phosphogluconate dehydrogenase. F-6-P and G-6-P were transformed into 6-PG by addition of phosphohexose isomerase, glucose-6-phosphate dehydrogenase, and NADP to the neutralized perchloric acid (PCA) extract of intact cells and PFS. By stepwise omission of glucose-6-phosphate dehydrogenase and phosphohexose isomerase, the activity of 6-PG, 6-PG + G-6P, and 6-PG + G-6-P + F-6-P could be gauged. The activity of the separate metabolites could then be found by subtraction.

The radioactivity determination of carbon atom 1 was performed in two armed Warburg vessels. The metabolites were degraded in PCA extracts of intact cells neutralized to pH 7.2–7.6 with KOH. The contents of the Warburg vessels were as follows:

Center well: 200 μl 2-phenylethylamine (Fluka) + a folded Whatmann 1 filter paper, diam. 23 mm. Main compartment: 1000 μl Tris 0.4 *M*, pH 7.4 + 1000 μl neutralized PCA extract. Side arm 1: $MgCl_2$ 0.1 μmole, NADP 2.0 μmoles, glucose-6-phosphate dehydrogenase 0.02 mg, 6-phosphogluconate dehydrogenase 0.2 mg and Tris 200 μmoles in a total volume of 700 μl. For the determination of F-6-P activity phosphohexose isomerase 0.04 mg was further added to side arm 1. Side arm 2: 500 μl H_2SO_4 6 *N*. Blanks were run in a one armed Warburg vessel with identical composition of center well and main compartment. The sidearm contained 500 μl H_2SO_4 6 *N*.

The vessels were allowed to equilibriate for 15 min and were then closed. The content of side arm 1 was added to main compartment. After 1 hr the content of side arm 2 was added, and 1 hr was allowed for the capture of the CO_2 in the center well. After this the radioactivity of the filter paper was determined by liquid scintillation counting. The experimental points represent mean values of five determinations. The fitted curve has been calculated as described in the section of mathematical treatment.

A similar degradation technique was used by Sato *et al.* to investigate the reversibility of the phosphofructokinase reaction (11).

While this investigation was in progress, an identical procedure for determination of specific activities of glucose and phosphorylated intermediates labelled in position 1 was published by Lange *et al.* (12).

The intermediates were determined as stated in Methods of Enzymatic Analysis (13). Glucose was determined according to the method of Huggett and Nixon (14). The reliability of the decarboxylation procedure was investigated by decarboxylation of a 1-14C-G-6-P standard. The recovery of carbon 1 activity was 85.7% ($s = 1.0, n = 3$). The recovery of the same standard after addition to neutralized perchloric acid extract was the same as for the standard alone. The recovery in the chemical determination of G-6-P was separately found to be 85%. These findings are in line with the results of Lange *et al.* (12).

The specific activity of lactate was determined after isolation and purification. Lactate was extracted for 72 hr as lactic acid with ether from the acidified aqueous extract of intact cells and PFS. After isolation of the ether phase, the ether was evaporated and the remaining substances dissolved in a known volume of redistilled water. The lactate content was determined by the method of Horn and Bruns (15). A known amount of cold sodium lactate (Sigma) was added, and the lactate was further purified on a Dowex-1-formate column (16).

Protein determinations were carried out according to the method of Lowry *et al.* (17).

The radioactivity was determined by liquid scintillation counting. Quench correction was performed by Channels Ratio method. The scintillation mixture was toluene: Triton-X-100 2:1 with PPO 0.5% w/v (18).

All enzymes and cofactors used were purchased from Boehringer, Mannheim, Germany, except for ATP and Tris, which

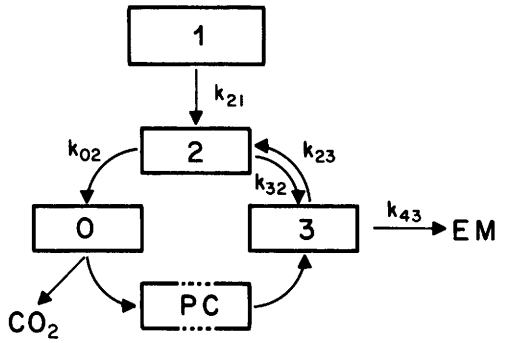

FIG. 1. A model of the pentose cycle and the first steps of glycolysis. Simplifying assumptions: Only passage of glucose-6-phosphate to 6-phosphogluconate and fructose-6-phosphate has been considered, while conversion to glucose-1-phosphate has been disregarded. The pentose cycle has been considered unidirectional at the entry to fructose-6-phosphate. Pool sizes: 1. glucose pool, $Q_1 = 376.0$ n moles/mg protein. 2. glucose-6-phosphate pool, $Q_2 = 1.2$ n moles/mg protein. 3. fructose-6-phosphate pool, $Q_3 = 0.4$ n moles/mg protein. 0. 6-phosphogluconate pool, $Q_0 = 0.4$ n moles/mg protein. Glucose consumption: 7.0 n moles/mg protein/min. PC: pentose cycle. EM: Embden-Meyerhof pathway.

were obtained from Sigma, St. Louis, MO. The remaining chemicals were analytical grade reagents. 1-^{14}C-glucose and U-^{14}C-glucose were purchased from the Radiochemical Center, Amersham, U.K.

Mathematical treatment. 1. Model. The model is illustrated in Fig. 1.

For the specific activity ratios $s_2 = \frac{a_2}{a_1}$ and $s_3 = \frac{a_3}{a_1}$ we have, following the notation of Atkins (19):

$$Q_2\, ds_2/dt = (-k_{02}Q_2 - k_{32}Q_2)s_2 + k_{23}Q_3s_3 + k_{21}Q_1 \quad (1)$$

$$Q_3\, ds_3/dt = k_{32}Q_2s_2 + (-k_{23}Q_3 - k_{43}Q_3)s_3 \quad (2)$$

with initial conditions

$$s_2(0) = s_3(0) = 0 \quad (3)$$

Metabolic steady state gives further

$$k_{02}Q_2 + k_{32}Q_2 - k_{23}Q_3 - k_{21}Q_1 = 0 \quad (4)$$

$$k_{32}Q_2 - k_{23}Q_3 - k_{43}Q_3 + \frac{2}{3}k_{02}Q_2 = 0 \quad (5)$$

It is noted that from (1) and (3) we get

$$\left.\frac{ds_2}{dt}\right|_{t=0} = \frac{k_{21}Q_1}{Q_2}$$

2. *Equilibrium levels.* For large t the left hand sides of (1) and (2) vanish (isotopic steady state). Denoting the limits of s_2 and s_3 by c_2 and c_3 respectively, we solve (1), (2), (4) and (5), obtaining:

$$k_{02}Q_2/k_{21}Q_1 = \frac{3(1 - c_3)}{3c_2 - c_3} \quad (6)$$

$$k_{32}Q_2/k_{21}Q_1 = \frac{2c_3}{c_2 - c_3}\,\frac{1 - c_3}{3c_2 - c_3} \quad (7)$$

$$k_{23}Q_3/k_{21}Q_1 = \frac{1 - c_2}{c_2 - c_3} \quad (8)$$

$$k_{43}Q_3/k_{21}Q_1 = \frac{3c_2 - 1}{3c_2 - c_3} \quad (9)$$

Hence all rate constants can be found, if k_{21}, Q_1, Q_2, Q_3, c_2 and c_3 are determined experimentally. Setting $k_{02}Q_2/k_{21}Q_1 = y_1$ we obtain from (6):

$$c_3 = \frac{3}{3 - y_1}(1 - y_1c_2) \quad (10)$$

which for fixed y_1 is the equation of a straight line in a c_2c_3—coordinate system. For a selection of values of y_1, the corresponding lines are shown in Fig. 4. It should be noted that since $c_2 \leqq 1$ it follows from (8) that $c_3 < c_2$. From (6) and (9) it appears that for $c_3 \to 1$ the limit of y_1 is 0, and the limit of $k_{43}Q_3/k_{21}Q_1$ is 1.

3. *Solution by curve fitting.* We can introduce c_2 and c_3 into (1) and (2), and write out explicitly the solution of this system, with the condition (3). This gives s_2 and s_3 as functions of t, with c_2 and c_3 entering as constants. These constants then can be determined by least squares fitting to the observed series of observations of connected values of s_2 and t. The calculation performed has been based on fixed relative standard deviation.

Results. Figure 2 shows the time-activity relationship for G-6-P and F-6-P, as well as the calculated values for G-6-P in intact ELDa cells. The activity is plotted as relative specific activity, i.e. activity relative to

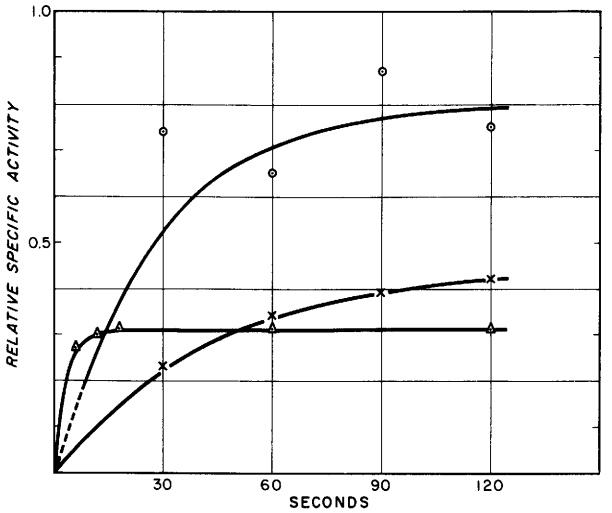

FIG. 2. The relative specific activity-time relationship of glucose-6-phosphate and fructose-6-phosphate in intact ELDa cells. The specific activity of carbon atom 1 of G-6-P and F-6-P was determined by enzymatic degradation in two armed Warburg vessels of the metabolites in perchloric acid extracts of intact cells neutralized to pH 7.2–7.6 (cell suspension: 6% PCA = 1:1). ×——× The relative specific activity of G-6-P. ⊙——⊙ The relative specific activity of F-6-P. △——△ The relative specific activity of G-6-P calculated according to the Methods section. The curve points represent mean values of five determinations.

mother substance. It may be seen that the increase in activities levels off at around 120 sec at values of 0.45 and 0.80 for G-6-P and F-6-P respectively. The experimental curve for G-6-P shows a significant discrepancy from the calculated curve based on glucose consumption, pool sizes, and fitted to the experimental points. The final level comes to be about 0.30. The relative specific activity of G-6-P at 8 and 16 min was 0.45 and 0.41 respectively (means of two determinations).

Figure 3 shows the relative specific activity of G-6-P in PFS of ELDa cells. As seen, the activity of G-6-P approaches a plateau after 64 min at a level of about 0.65. Lactate activity, however, reaches equilibrium with glucose activity after about 60 min.

Table I shows the specific activity of lactate relative to glucose of intact ELDa cells. At 8 min the specific activity relative to glucose is 0.71.

Table II shows the activity of hexokinase and phosphohexose isomerase of the mitochondrial fraction of ELDa cells. As seen, the activity of phosphohexose isomerase relative to hexokinase activity drops from 0.6 to 0.3 after three washings, but an appreciable activity remains.

Finally Fig. 4 shows calculations based on the model in Fig. 1 of the relationship between the activities of G-6-P (c_2) and F-6-P (c_3) to glucose at different rates of the conversion of G-6-P to 6-PG relative to the glucose consumption (y_1). It may be seen that for values of $y_1 < 0.1$, the values for c_2 and c_3 would exceed 0.93.

With the values $c_2 = 0.45$ and $c_3 = 0.80$ suggested by Fig. 2, y_1 would be about 2.0, corresponding to only about 35% of the glucose passing the Embden–Meyerhof pathway.

Discussion. The glucose metabolism of Ehrlich ascites tumor cells is dominated by glycolysis. Lactate production can account for about 75% of the glucose consumption (20). Based on equation (10) and assuming a pentose cycle activity of less than 4% of the glucose consumption, the relative specific activity of G-6-P can be calculated to be >0.9 after 60 sec. However, from Figs. 2 and 3 it appears that relative specific activities of G-6-P higher than 0.65 could not be demonstrated, even in PFS of ELDa cells. At the same time the specific activity

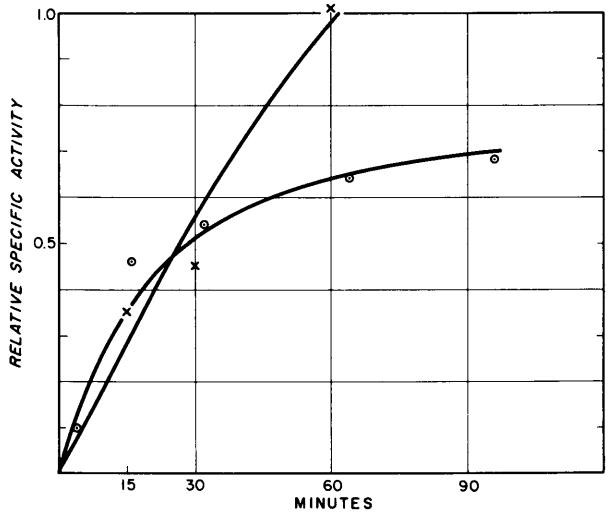

FIG. 3. The relative specific activity-time relationship of glucose-6-phosphate and the specific activity of lactate relative to the specific activity of glucose in PFS of ELDa cells. The experimental points represent median values of 3 determinations, except for lactate 30 min and G-6-P 96 min, which are the mean of two separate determinations. ⊙——⊙: G-6-P. ×——×: lactate. The supernatants were diluted with a modified Le Page medium with the following composition given in final concentrations (PFS/dilution medium = 1:1): $KHCO_3$ 25 m*M*, K_2HPO_4 2.4 m*M*, $MgCl_2$ 6.7 m*M*, Tris 50 m*M*, pH 7.4, ATP 1.0 m*M*, and NAD 0.3 m*M*. The osmolar concentration was adjusted to 0.3 by addition of KCl. Glucose was added to 12.5 m*M*.

TABLE I. THE SPECIFIC ACTIVITY OF LACTATE RELATIVE TO THE SPECIFIC ACTIVITY OF GLUCOSE IN INTACT ELDa CELLS.[a]

		4 min	8 min
Specific activity of lactate / Specific activity of glucose	$\bar{x}$	0.34	0.71
	s	0.09	0.19
	n	4	4

[a] U-^{14}C-glucose was used as tracer.

TABLE II. HEXOKINASE AND PHOSPHOHEXOSE ISOMERASE ACTIVITY IN THE MITOCHONDRIAL FRACTION OF ELDa CELLS.[a]

	First washing		Second washing		Third washing	
	$\bar{x}$	$s(n)$	$\bar{x}$	$s(n)$	$\bar{x}$	$s(n)$
HK	54.8	24.7 (7)	54.2	28.8 (7)	53.1	50.2 (7)
PHI	33.0	9.8 (5)	24.0	5.9 (5)	14.4	3.9 (5)

[a] The mitochondrial fraction was prepared as stated in Material and Methods. For the determination of HK and PHI activity 0.4 *M* Tris buffer, pH 7.4 was used as assay buffer. The determination of HK activity was started by addition of glucose to the reaction mixture. The determination of PHI was started by addition of mitochondrial suspension. Enzyme activity given in munits/mg protein.

of lactate relative to the specific activity of glucose surpasses the relative specific activity of G-6-P, clearly demonstrating compartmentation in the systems investigated. This compartmentation invalidates the model of Fig. 1, and in agreement with this there is initially a lack of fit between the calculated time-activity relationship and the observed relationship for G-6-P in Fig. 2.

Similar observations of isotope inequilibrium have been published for liver by Threlfall and Heath (4) and by Das *et al.* (5), and for diaphragm by Kalant and Beitner (6). In these systems, however, gluconeogenesis plays a definite quantitative role. A possible explanation for the compartmentation found therefore could be differences between glycolytic and gluconeogenetic pools. In ELDa cells such a situation

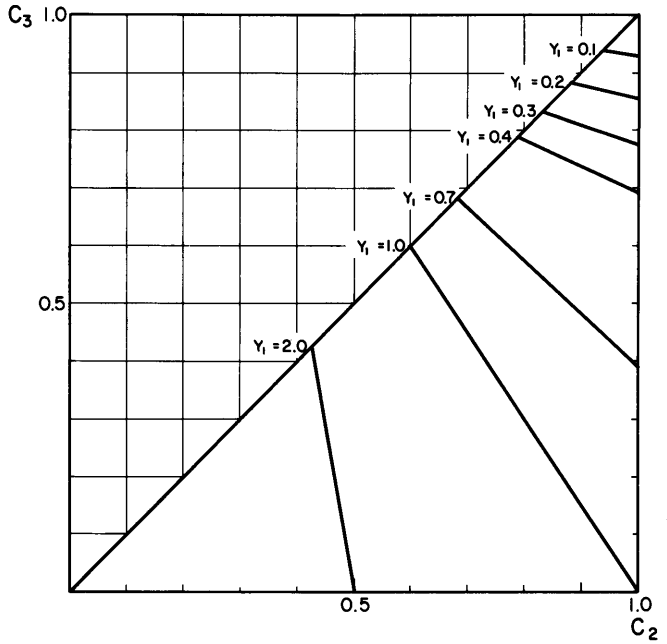

FIG. 4. The relationship of the equilibrium level of the specific activity of glucose-6-phosphate and fructose-6-phosphate relative to the specific activity of glucose (c_2 and c_3), at different rates of the conversion of glucose-6-phosphate to 6-phosphogluconate relative to the glucose consumption. As $c_2 \geqq c_3$ only the part of the graph below the line $c_2 = c_3$ is of interest. $y_1 = k_{02}Q_2/k_{21}Q_1$. The straight lines depict the connected values for c_2 and c_3 at different values of y_1.

is not conceivable, as the glycogen content is at the limit of detection.

The demonstration in Fig. 3 of compartmentation of G-6-P even in PFS of ELDa cells points to a molecular basis for this phenomenon. This finding suggests that inequilibrium is not necessarily caused by spatial subdivision. In fact the inequilibria demonstrated in PFS freely may be ascribed to differential metabolic treatment of molecules of the same pool. Reed had mentioned this as kinetic compartmentation (21). Following this concept, compartmentation is an expression of differential treatment of metabolic molecules. Thus high rates in metabolic pathways compared to the rate of mixing in the pools of metabolites will create compartmentation, demonstrable as isotope inequilibria between consecutive pools of a metabolic pathway.

The demonstration of hexokinase and phosphohexose isomerase activity on the mitochondrial surface gives an example of a multienzyme arrangement, that might cause kinetic compartmentation. This cannot explain the situation in the PFS in which multienzyme complexes nevertheless might occur. Based on simultaneous multisubstrate studies, Moses and Lonberg-Holm (1) has proposed a four compartment model involving glycolytic pathways and the citric acid cycle in ascites tumor cells and Threlfall and Heath (4), and Das *et al.* (5) advocate two G-6-P pools in liver cells. With the kinetic compartmentation concept, however, it becomes increasingly difficult to describe and quantitate the extent of compartments of different pools.

From Fig. 4 in which the inequilibria in the pools of the model in Fig. 1 generated by the action in the pentose cycle are depicted, it appears that an active pentose cycle in principle excludes true equilibrium between glucose and G-6-P. On the other hand the inequilibria reported here for G-6-P and F-6-P hardly may be explained by pentose cycle activity, as this would imply restrictions on the extent of the glyco-

lytic pathway not compatible with previous determinations of the glycolysis in Ehrlich ascites cells (20).

As is also concluded by Kalant and Beitner (6), we think that the occurrence of isotope inequilibrium imposes severe restraints on the applicability of techniques for determination of the extent of metabolic pathway in which isotope equilibrium is an assumption (e.g. 22).

Summary. With an enzyme degradative technique, isotope inequilibrium of glucose metabolites was demonstrated in intact cells and particlefree supernatants of Ehrlich ascites tumor using 1-^{14}C-glucose as tracer. Inequilibrium was found between glucose and glucose-6-phosphate, glucose and fructose-6-phosphate, glucose and 6-phosphogluconate, while glucose-6-phosphate and fructose-6-phosphate were found to be in near-equilibrium within the incubation time investigated. Glucose and lactate were found to be in near equilibrium after 8 min in intact cells. Calculations based on the equilibrium levels found, showed that these inequilibria could not be explained by the effects of the pentose cycle.

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