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MODE OF ORTHOPHOSPHATE UPTAKE AND ATP LABELING BY MAMMALIAN CELLS

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SUMMARY

Incubation of HeLa cells with [^{32}P]orthophosphate results in more rapid labeling of the γ-phosphorus of ATP than of the intracellular pool of orthophosphate. The specific radioactivity of ATP equals that of extracellular orthophosphate after 2 h of incubation. A similar pattern of labeling is seen with human erythrocytes when incubated at physiological concentrations of orthophosphate (2 mM) and pH 7.4–7.8. At lower pH, 6.8–7.2, the rate of orthophosphate uptake increases and exceeds the rate of labeling of ATP. These data are explained by the existence of a primary system for ATP uptake which involves the mediation of membrane-bound glyceraldehyde-3-phosphate dehydrogenase. Phosphate first enters the cell as 1,3-diphosphoglyceric acid, is then transferred to ATP, and then enters the intracellular orthophosphate pool. At lower pH monovalent orthophosphate also enters the erythrocyte by a process not involving glyceraldehyde-3-phosphate dehydrogenase.

INTRODUCTION

The mechanism of orthophosphate transport into mammalian cells is a point of considerable controversy. Several investigators [1–7] have interpreted ^{32}P-labeling patterns in which the specific activity of ATP exceeds that of intracellular orthophosphate as demonstrating the involvement of glyceraldehyde-3-phosphate dehydrogenase in phosphate uptake, as shown in Eqns. 1–3.

$$\text{Glyceraldehyde-3-P} + {}^{32}\text{P}_i \text{ (extracellular)} + \text{NAD}^+ \rightleftarrows \text{1,3-[}^{32}\text{P]-diphosphoglycerate} + \text{NADH} + \text{H}^+ \quad (1)$$

$$\text{1,3-[}^{32}\text{P]-Diphosphoglycerate} + \text{ADP} \rightleftarrows \text{3-Phosphoglyceric Acid} + [{}^{32}\text{P}]\text{ATP} \quad (2)$$

$$[{}^{32}\text{P}]\text{ATP} + \text{H}_2\text{O} \rightarrow \text{ADP} + {}^{32}\text{P}_i \text{ (intracellular)} \quad (3)$$

The most comprehensive studies of phosphate transport have been made with human

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erythrocytes in which the labeling patterns are complicated by the large pool of 2,3-diphosphoglycerate which enters the pathway as shown by Eqns. 4 and 5.

$$1,3\text{-}[^{32}P]\text{-Diphosphoglycerate} + 3\text{-Phosphoglyceric Acid} \rightarrow 2,3\text{-}[^{32}P]\text{-diphosphoglycerate} + 3\text{-Phosphoglyceric Acid} \quad (4)$$

$$2,3\text{-}[^{32}P]\text{-Diphosphoglycerate} + H_2O \rightarrow 3\text{-Phosphoglyceric Acid} + {}^{32}P_i \text{ (intracellular)} \quad (5)$$

Erythrocytes also contain a high level of adenylate kinase, thus the β- and γ-phosphorus atoms of ATP and ADP rapidly reach isotopic equilibrium.

The pathway described above is by no means universally accepted, however, as other workers [8–10] have found that under other incubation conditions the specific activity of intracellular orthophosphate equals or exceeds that of ATP. In an attempt to resolve some of these discrepancies and to elucidate the physiologically significant mechanism of orthophosphate transport, we have studied the incorporation of radioactivity from extracellular $^{32}P_i$ into intracellular orthophosphate and into the β- and γ-phosphorus atoms of ATP, under conditions designed to mimic physiological states. Parallel experiments were conducted with human erythrocytes and with HeLa cells in suspension culture. Preliminary accounts of this work have been presented (11, 12).

MATERIALS AND METHODS

Preparation and incubation of cells

Human erythrocytes were collected by venipuncture from a single individual (WGN) into lithium oxalate-containing Vacutainer tubes. The erythrocytes were washed by centrifugation from isotonic buffer at 4° C, the buffy coat was removed, and the cells were incubated at a hematocrit of 0.1 in isotonic buffer within 1 h of collection. The pH and extracellular orthophosphate concentration do not change during the incubation period at this hematocrit. HeLa cells were grown in suspension culture in Eagles minimal medium, Joklik-modified (Grand Island Biological Co.), to a cell density of about 5×10^5 per ml. The cells were collected by centrifugation at room temperature and were resuspended in fresh growth medium to a cell density of $2–5 \cdot 10^6$ per ml and incubated immediately. The isotonic incubation medium (290 m*Os*M) used for erythrocytes contains 107 mM NaCl, 4 mM KCl, 2 mM sodium phosphate, 2 mM $MgCl_2$, 1 mM NH_4Cl, 0.003 % streptomycin sulfate, 0.005 % penicillin G, and is buffered at the desired pH with 30 mM endonorbornene-2,3-dicarboxylic acid and its sodium salt [13, 14].

Erythrocytes were incubated in a shaking water bath at 37 °C. HeLa cells were incubated at 37 °C with constant agitation by a magnetic stirring bar. Radioactive labeling was initiated after a preliminary equilibration period of 45–60 min by the addition of carrier-free [^{32}P]orthophosphate in 0.01 M HCl (New England Nuclear) in a volume not exceeding 10 μl per ml of incubation mixture.

Determination of the specific radioactivity of orthophosphate

At intervals after the addition of [^{32}P]orthophosphate, samples of erythrocytes or HeLa cells were removed from the incubation mixtures, diluted 5–10 fold

with 0.15 M NaCl, and centrifuged in an International Clinical Centrifuge at $1000 \times g$ for 1 min. A portion of the supernate was removed for determination of the specific radioactivity of extracellular orthosphosphate. The remainder of the supernate was removed by aspiration, the cells were resuspended and recentrifuged twice. The final washed pellet was dispersed in 1 ml of 5 % trichloroacetic acid and after standing in the cold for 30 min the denatured protein was removed by centrifugation. Orthophosphate was extracted and quantitated from the initial supernate and from the trichloroacetic acid extract by the method of Martin and Doty[15], scaled down to accommodate 1-ml aqueous samples. A portion of the organic extract of phosphomolybdate was removed for radioactivity measurement by liquid scintillation spectrometry. A scintillation mixture containing 0.5 % 2,5-diphenyloxazole in toluene Triton X-100 (2:1) was used with a Beckman LS 200 B instrument. The counting efficiency in an adjustable window set from 2 to infinity was greater than 99 %, and was not altered by inclusion of 10 % water in the sample.

Determination of the specific radioactivity of γ[^{32}P]ATP

At intervals after addition of [^{32}P]orthophosphate, samples of cell suspensions were pipetted into 4 vols. of boiling water to inactivate enzymes and release nucleotides. Samples were clarified by centrifugation and ATP was concentrated and partially purified by chromatography on DEAE-Sephadex [16]. Samples were then lyophilized, NH_4HCO_3 was removed under reduced pressure with the aid of a heat lamp, and the specific radioactivity of the γ-phosphorus of ATP was determined by the method of Hammerstedt and Niehaus [17]. This method involves incubation of the ATP sample with galactokinase (Sigma) and [^{3}H]galactose (New England Nuclear; 10–80 μCi/μmol). The [^{3}H]galactose-1-[^{32}P]phosphate thus formed is purified by chromatography on QAE-Sephadex and the specific radioactivity of the phosphorus is determined from the ^{32}P/^{3}H ratio. The counting efficiency for ^{3}H, as determined by use of an internal standard of [^{3}H]toluene, is 10 %. The counting efficiency for ^{32}P is greater than 99 %.

In parallel incubations employing no ^{32}P, ATP was quantitated by the luciferase assay, as modified by Hammerstedt [18].

Determination of the specific radioactivity of β[^{32}P]ATP and ADP

ATP was extracted and partially purified as described above, which procedure did not separate ATP from ADP. The nucleotides were then incubated with hexokinase (Sigma) (1 unit/ml) and glucose (5 mM) to remove the γ-phosphorus from ATP to glucose 6-phosphate. Less than 0.5 % of the original ATP remained after 30 min, as determined by the luciferase assay. The reaction mixture was then boiled for 5 min to inactivate hexokinase and incubated with myokinase (Sigma) (1 unit/ml) plus galactokinase and [^{3}H]galactose as described above. Subsequent chromatography of the reaction mixture on QAE-Sephadex completely resolved [^{3}H]galactose-1-[^{32}P]-phosphate from all other radioactive compounds, including glucose 6-phosphate.

RESULTS AND DISCUSSION

Erythrocytes

Erythrocytes were incubated in the isotonic buffer containing 5 mM glucose and

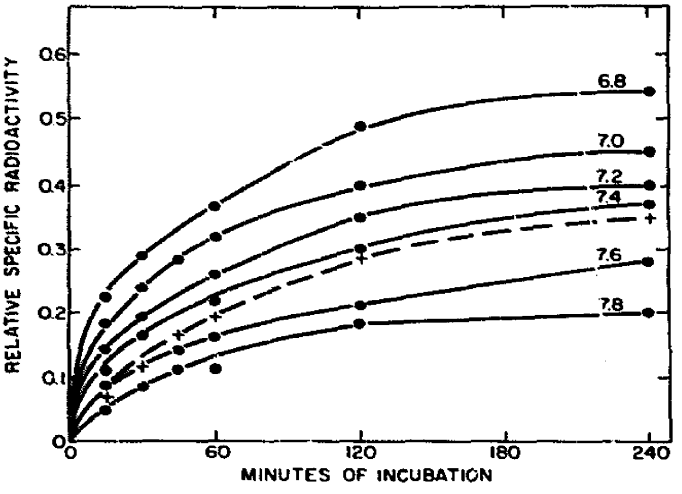

Fig. 1. Labeling pattern of orthophosphate and the γ-phosphorus of ATP within human erythrocytes incubated at varying pH values. Washed human erythrocytes were preincubated for 45 min at 37 °C in buffered NaCl plus glucose, the pH of the solution was readjusted to the desired value and carrier-free [^{32}P]orthophosphate added to a final specific radioactivity of 100 μCi per μmol. Samples were removed and processed at intervals as described in the text. The specific radioactivity of intracellular orthophosphate (●—●) and the γ-phosphorus of ATP (+- - -+) are presented relative to the specific radioactivity of extracellular orthophosphate. The relative specific radioactivity observed for orthophosphate at each pH and the mean ATP relative specific radioactivity for all pH values are presented. The ranges for ATP relative specific radioactivity were 0.04–0.07, 15 min; 0.09–0.11, 30 min; 0.14–0.16, 45 min; 0.18–0.20, 60 min; 0.25–0.31, 120 min; 0.33–0.39, 240 min.

2 mM orthophosphate at pH 6.8, 7.0, 7.2, 7.4, 7.6 and 7.8 for 45 min, whereupon the pH was readjusted to the initial value and samples were removed for quantitation of ATP. [^{32}P]orthophosphate was added to the incubation mixtures and samples were removed for determination of the specific radioactivity of orthophosphate and ATP at 15, 30, 45, 60, 120 and 240 min (Fig. 1). The pH of the samples did not change during the incubation. The time course of incorporation of label from extracellular orthophosphate into ATP is independent of the pH of the medium, whereas the labeling of intracellular orthophosphate is strongly pH-dependent. Thus at lower pH (6.8–7.2) the specific radioactivity of orthophosphate exceeds that of ATP, at pH 7.4 the specific radioactivities are nearly identical, and at higher pH (7.6–7.8) the specific radioactivity of the γ-phosphorus of ATP exceeds that of intracellular orthophosphate. The specific radioactivity of the β-phosphorus of ATP was identical to that of the γ-phosphorus, even at the 15-min time point.

Neither intracellular orthophosphate nor the γ-phosphorus of ATP reaches isotopic equilibrium with extracellular orthophosphate during the course of the incubation (Fig. 1). At the various pH values, the specific radioactivity of intracellular orthophosphate reaches a final value between 20% and 60% that of extracellular orthophosphate. The specific radioactivity of ATP reaches 35% that of extracellular orthophosphate after 4 h of incubation. The reason for this lack of isotopic equilibration is not immediately obvious, but our findings are not without precedent. Gourley [1] incubated [^{32}P]orthophosphate with whole blood at a hematocrit of 0.5. Under these incubation conditions, the specific radioactivity of extracellular ortho-

phosphate decreases during the course of the incubation. After 3 h the specific radioactivity of ATP has reached its maximum absolute value, which was only about 20% that of extracellular orthophosphate. Intracellular orthophosphate, which had not yet reached its maximum value by 3 h had a specific radioactivity about 10% that of the extracellular pool and did not reach isotopic equilibrium even after 8 h. Latzkovits et al. [19] have also found a lack of isotopic equilibrium between extracellular orthophosphate and either intracellular orthophosphate or ATP; although under their incubation conditions (12 mM orthophosphate) both intracellular pools achieve about 70% the specific radioactivity of the extracellular pool. Till et al. [10] incubated [^{32}P]orthophosphate with human erythrocytes in plasma and found that from the third to the eighth hour the specific radioactivities of intracellular orthophosphate and of ATP remained at 20% that of plasma orthophosphate without any tendency to equilibrate further. They concluded that about 64% of the intracellular pool of orthophosphate, ATP, and ADP does not participate in tracer exchange, probably because of being bound to hemoglobin. Hamasaki and Rose [20], however, have measured the binding of phosphorylated compounds to purified hemoglobin and concluded that less than 10% of the ATP in the cell is bound to hemoglobin. Therefore, although intracellular compartmentation is probably responsible for the non-equilibration of intracellular orthophosphate and ATP with the extracellular precursor, the precise nature of the compartmentation remains obscured.

The specific radioactivities of intracellular orthophosphate and of ATP were determined by alternate methods to exclude the possibility of systematic errors in our values. Erythrocyte suspensions were labeled with ^{32}P and filtered through columns of Sephadex G-75 as described by Till et al. [21]. The specific radioactivity of the orthophosphate present in the filtered cells was not significantly different ($P > 0.05$) from that present in the centrifuged cells. The specific radioactivity of the γ-phosphorus of ATP was determined by the method of Till et al. [10], using hexokinase and [^{3}H]2-deoxyglucose. The double labeled 2-deoxyglucose 6-phosphate was isolated by paper chromatography and the specific radioactivity of the phosphorus was determined from the isotope ratio. The values obtained by this method agreed within 5% with the values obtained using galactokinase and [^{3}H]galactose. Furthermore, there can be no appreciable contribution of ITP to the synthesis of galactose 1-phosphate in our system since ITP is less than 10% as effective a substrate for galactokinase as is ATP, and ITP is almost quantitatively destroyed during the heating which is used to remove NH_4HCO_3 from the isolated ATP fraction.

No radioactivity was found in the γ-phosphorus of ATP when an unlabeled erythrocyte suspension was added to boiling water containing $^{32}P_i$. Therefore, it is apparent that enzymes such as phosphoglycerate kinase and pyruvate kinase are inactivated and ATP labeling does not occur after the cells are broken.

The quantity of ATP and P_i within the erythrocyte is also pH-dependent (Table I) as has been previously noted [22]. The concentration changes occurred during the preincubation period, however, and ATP and orthophosphate levels remained constant during the incubation with ^{32}P. The initial more rapid labeling of intracellular orthophosphate seen at lower pH (Fig. 1) may therefore be a result of a higher rate of monovalent [^{32}P]orthophosphate exchange during incubation.

The effect of the extracellular orthophosphate concentration on the rate of ATP labeling was examined at pH 7.2 and 7.6. Cell suspensions containing 7, 9, 13

TABLE I

EFFECT OF EXTRACELLULAR pH ON THE INTRACELLULAR CONCENTRATION OF ERYTHROCYTE ORTHOPHOSPHATE AND ATP

Portions of the erythrocyte suspensions used in the experiments described in Fig. 1 were removed before the addition of ^{32}P for determination of orthophosphate and ATP levels, as described in the test. The mean value of duplicate determinations at each pH are reported. Individual determinations differed by less than 10 %. n.d. = not determined.

pH of incubation	μmol per ml packed cells	
	Orthophosphate	ATP
6.8	2.0	1.7
7.0	1.9	n.d.
7.2	1.7	1.8
7.4	1.4	n.d.
7.6	1.2	1.3
7.8	0.8	n.d.
8.0	0.3	1.1

and 20 mM extracellular orthophosphate were preincubated for 45 min and the pH was readjusted. [^{32}P]orthophosphate was added and samples were withdrawn into boiling water after 5 min, the extent of ATP labeling having been determined to be linear with time over approximately 10 min. The apparent K_m of extracellular orthophosphate for ATP labeling is about 80 mM (Fig. 2), a value which is consistent with that found by Ho and Guidotti [23] for phosphate uptake under very different incubation conditions.

Attempts to confirm the role of glycolytic enzymes in orthophosphate trans-

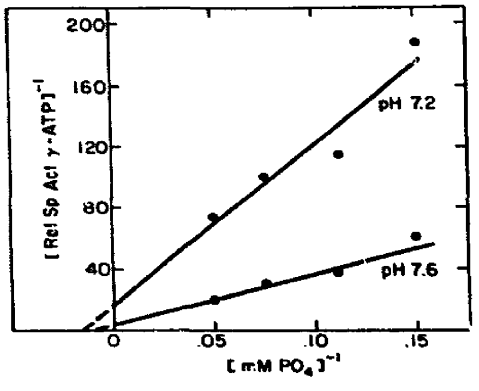

Fig. 2. Determination of the apparent K_m of extracellular orthophosphate for the labeling of the γ-phosphorus of ATP in human erythrocytes. Erythrocyte suspensions in 7, 9, 13 and 20 mM orthophosphate at pH values 7.2 and 7.6 were preincubated at 37 °C for 45 min; the pH of the solution was readjusted and carrier-free [^{32}P]orthophosphate added to a final specific radioactivity of 100 μCi per μmol. Samples were removed at 5 min and the relative specific radioactivity of the γ-phosphorus of ATP determined as described in the text.

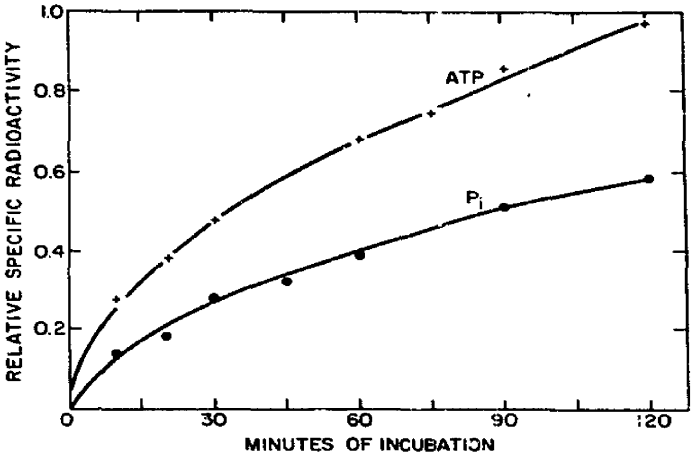

Fig. 3. Labeling pattern of orthophosphate and the γ-phosphorus of ATP within HeLa cells incubated at pH 7.4. HeLa cells were incubated in growth medium and carrier-free orthophosphate added to a final specific radioactivity of 100 μCi per μmol. Samples were removed and processed as described in the text. The specific radioactivity of intracellular orthophosphate (● — ●) and the γ-phosphorus of ATP (+ — +) are presented relative to the specific radioactivity of extracellular orthophosphate.

TABLE II

EFFECT OF EXTRACELLULAR pH ON THE LABELING OF INTRACELLULAR ORTHOPHOSPHATE AND γ-ATP OF HeLa CELLS

HeLa cells were preincubated for 45 min in growth medium which had been adjusted to the desired pH. [^{32}P]orthophosphate was added and samples were removed and processed as described in the text. The specific radioactivity of intracellular orthophosphate and the γ-phosphorus of ATP are given relative to the specific radioactivity of extracellular orthophosphate. P_i refers to total orthophosphate without regard to ionic species.

pH of Incubation	Relative Specific Radioactivity					
	30 Min			60 Min		
	P_i	γ-ATP	γ-ATP / P_i	P_i	γ-ATP	γ-ATP / P_i
6.8	0.22	0.39	1.8	0.41	0.76	1.9
7.0	0.23	0.43	1.9	0.38	0.66	1.7
7.2	0.27	0.47	1.8	0.37	0.73	2.0
7.4	0.26	0.47	1.8	0.41	0.72	1.8
7.6	0.24	0.43	1.8	0.37	0.65	1.8
7.8	0.23	0.38	1.7	0.35	0.64	1.8

port by the use of specific enzyme inhibitors have been unsuccessful. Our analytical system depends upon the cell being in a metabolic steady state, in which orthophosphate and ATP concentrations remain constant during the incubation period. Inhibition of glyceraldehyde-3-phosphate dehydrogenase by iodoacetic acid results in a precipitous drop in ATP concentration, so that energy-dependent membrane barriers no longer exist. Chloroacetol phosphate (a gift of Dr. F. C. Hartman) which specifically inhibits phosphoglycerate isomerase, does not enter the erythrocyte and hence is inactive as an inhibitor of whole cell glycolysis.

HeLa cells

HeLa cells were incubated with constant stirring in growth medium (5% serum, 8 mM orthophosphate, pH 7.4) and samples were removed at 10, 20, 30, 45, 60, 90 and 120 min for determination of the specific radioactivity of intracellular orthophosphate and of the γ-phosphorus of ATP. The uptake of orthophosphate and the labeling of ATP were both considerably more rapid by HeLa cells (Fig. 3) than by erythrocytes (Fig. 1). At all time points the specific radioactivity of ATP greatly exceeds that of intracellular orthophosphate, and reaches the specific radioactivity of extracellular orthophosphate (RSA = 1) by 120 min of incubation. The pH of the medium has no effect on orthophosphate uptake or ATP labeling in HeLa cells (Table II). Cells were preincubated for 45 min in growth medium which had been adjusted to pH 6.8, 7.0, 7.2, 7.4, 7.6 and 7.8. No change in pH occurred during this period. [^{32}P]orthophosphate was added and samples were removed at 30 and 60 min for determination of the specific radioactivity of intracellular orthophosphate and of the γ-phosphorus of ATP. These values were independent of pH, and identical to the values found in the previous experiment (Fig. 3). The quantity of intracellular orthophosphate and of ATP was also independent of the pH of the medium.

Inclusion of 5 mM sodium azide to inhibit mitochondrial respiration had no effect on orthophosphate uptake or ATP labeling, indicating that the incorporation of orthosphosphate into ATP is not dependent on oxidative phosphorylation under these incubation conditions.

CONCLUSIONS

Based on the data presented here and on pertinent observations from the literature, we propose the following model for orthophosphate transport into mammalian cells.

Human erythrocytes take up orthophosphate from extracellular fluids by two different mechanisms. At physiological phosphate concentration (1–2 mM) and at pH greater than 7.4, the principal pathway of orthophosphate entry involves the participation of glyceraldehyde-3-phosphate dehydrogenase, so that label from [^{32}P]-orthophosphate first appears within the cell as 1,3-diphosphoglyceric acid. The labeled phosphate is transferred to ATP by phosphoglycerate kinase, or to 2,3-diphosphoglyceric acid by diphosphoglycerate mutase, prior to its appearance in the intracellular orthophosphate pool. The initial binding of extracellular orthophosphate probably involves the major glycoprotein of the membrane, which has been shown by Ho and Guidotti [23] and by Cabantchik and Rothstein [24] to be involved in the transport of several anions, including phosphate. The transport of anions is inhibited

by covalent modification of this protein by various reagents, including the transport substrate pyridoxal phosphate, which appears to interact at the same rate as do nonpermeant inhibitors [25]. This evidence, along with the specific inhibition of phosphate transport observed by Ho and Guidotti [23] strongly support a role for this major glycoprotein in orthophosphate uptake by erythrocytes under the incubation conditions we employed. Phosphate is presumably then transferred from this transport protein to glyceraldehyde-3- phosphate dehydrogenase, which has been shown by Shin and Carraway [26] to be localized on the inner surface of the erythrocyte membrane, and by Yu and Steck [27] to be bound to the cytoplasmic aspect of the anion transport glycoprotein. The physiological significance of this involvement of glyceraldehyde-3-phosphate dehydrogenase in phosphate uptake is not immediately apparent, but our results with HeLa cells indicate that this phenomenon is not restricted to the erythrocyte and may be common to many mammalian cells.

At lower pH or higher phosphate concentration, monovalent orthophosphate may also directly enter the erythrocyte by a process not involving glyceraldehyde-3-phosphate dehydrogenase. Differences in pH and extracellular orthophosphate concentrations may well explain the large variations seen in the previous literature as to the relative labeling of intracellular orthophosphate and ATP [1–10].

In HeLa cells, the pattern of labeling of intracellular orthophosphate and ATP is independent of pH (Table II) and of orthophosphate concentration in the range 2–10 mM (unpublished data). This labeling pattern is not dependent on oxidative phosphorylation. Therefore we propose that phosphate enters the HeLa cell by a glyceraldehyde-3-phosphate dehydrogenase-mediated process, similar to that seen in erythrocytes, and that no secondary process of diffusion occurs under the conditions we examined (pH 6.8–7.8; 2–10 mM P_i).

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