CONCENTRATIVE ACCUMULATION (ACTIVE TRANSPORT) OF 2-DEOXY-D-GLUCOSE IN PRIMATE FIBROBLASTS

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Summary--Incorporation of 2-deoxy-D-glucose into cultured rhesus diploid cells includes transport and subsequent phosphorylation with resultant accumulation of both free and phosphorylated sugar. Accumulation of the free sugar proceeds to a maximal limit of 4-5 mM which is determined by intrinsic cell factors and is independent of medium 2-deoxy-D-glucose up to 5 mM concentration. Concentrative accumulation (active transport) of the free sugar is readily demonstrable on maintaining medium concentrations of 2-deoxy-D-glucose at less than the maximal accumulation potential of the cells (i.e. <4-5 mM). Cellular concentrations of the free sugar in excess of 30-times medium concentrations are demonstrable on incubation of the cells in the presence of <0.05 mM 2-deoxy-D-glucose. In contrast, accumulation of 2-deoxy-D-glucose is not demonstrable in the human erythrocyte.

Sugar transport has been examined in mammalian cell cultures primarily in relation to the demonstration and kinetic analysis of rate changes in sugar uptake associated with cell growth (1-5) and cell transformation by viral agents (6-11). The mechanism of sugar uptake by these cells has not been examined extensively; however, the available data have been taken to indicate that sugar permeation occurs by facilitated diffusion (7,12) at low concentrations and by simple diffusion (12) at high sugar concentrations in accord with a widely held view (e.g. see 13).

In the course of a study of glucose metabolism by diploid cell lines of primate origin, permeability changes were examined with the D-glucose analog 2-deoxy-D-glucose, which has been considered to be the substrate of choice as a model for D-glucose transport (12). Incorporation rates of the analog were found to parallel growth-induced, stimulation of glucose utilization as reported with other mammalian cells. Additional experiments were designed to examine specifically steady-state, intracellular accumulation limits of the free sugar and its phosphorylated product(s). Accumulation of free 2-deoxy-D-glucose in considerable excess of media concentrations by rhesus diploid cells was readily demonstrable under appropriate experimental conditions and forms the basis of this communication.

EXPERIMENTAL

Cells - A diploid cell line (DBS-FRhL-2) derived from rhesus fetal lung

(14) was grown in Eagle's minimal essential medium (MEM) with 2x vitamins and glutamine and 10% fetal calf serum. Growth inhibited, confluent cell cultures grown in 33 mm diameter Petri dishes containing about 5 x 10^4 cells/cm^2 were employed. Variation in protein content within a set of replicate dishes rarely exceeded 10%. Human erythrocytes prepared from freshly drawn blood of a normal donor were employed in comparative experiments.

Incorporation studies - Confluent cultures washed free of growth medium were incubated for specified periods in 1.0 ml modified Hank's medium (minus glucose plus 20 mM Tes buffer pH 7.4) containing [^{14}C]-labeled 2-deoxy-D-glucose. Incubations were terminated by aspiration of the medium. The cells were rinsed rapidly 4 times with cold Hank's medium and extracted with 1.0 ml cold 5% trichloroacetic acid. Following acid removal by ether extraction, aliquots were removed for total counts and for separation of free and phosphorylated forms of the sugar. Separations were carried out routinely on 0.5 ml samples using 0.5 x 4 cm columns of Dowex-1 acetate prepared in disposable capillary pipets (7). The free sugar was collected with the original effluent followed by a water wash in a total 2.5 ml volume. The phosphylated sugar was eluted in 2.5 ml of 0.2 M formic acid plus 0.5 M ammonium acetate. The fractions were counted directly in the presence of 10 ml Aquasol. The reliability of the fractionation method was verified by separation of known mixtures of labeled free and phosphorylated sugar. Separations were also carried out by paper chromatography using several solvent systems, for identifications and comparative analysis. Radioautographs of labeled sugars separated in paper were made with AGFA safety X-ray film. Protein was determined on the trichloroacetic acid extracted residues by the method of Lowry et al (15). Cell volumes were calculated by assuming a 10% protein content by volume and protein specific gravity of 1.1. These calculated values were verified by physical estimates using a Coulter counter. Calculations of intracellular sugar concentrations assumed the total cell volume to be available to the sugar.

Chemicals - [^{14}C] 2-deoxy-D-glucose (50 mCi/mmole) and Aquasol (New England Nuclear); 2-deoxy-D-glucose, 2-deoxy-D-glucose-6-P, and yeast hexokinase (Sigma); and cytochalasin B (I.C.I. Res. Labs., Cheshire, Eng.) were purchased from the indicated sources. [^{14}C 2-deoxy-D-glucose-6-P was prepared as follows: in a final 0.2 ml volume were added 0.01 M Tris buffer pH 8.0, 1 mM MgATP, 1 mM P_i, 0.1 mM [^{14}C] 2-deoxy-D-glucose (0.5 µCi) and 8 units crystalline hexokinase. Incubation was for 1 hour at 37° and the reaction terminated by heating at 100° for 1 minute. A single phosphorylated radioactive product was isolated on Dowex-1 and accounted for 96% of the original radioactivity added. Homogeneity analysis and identification of the product as 2-deoxy-D-glucose-6-P was based on chromatography in paper.

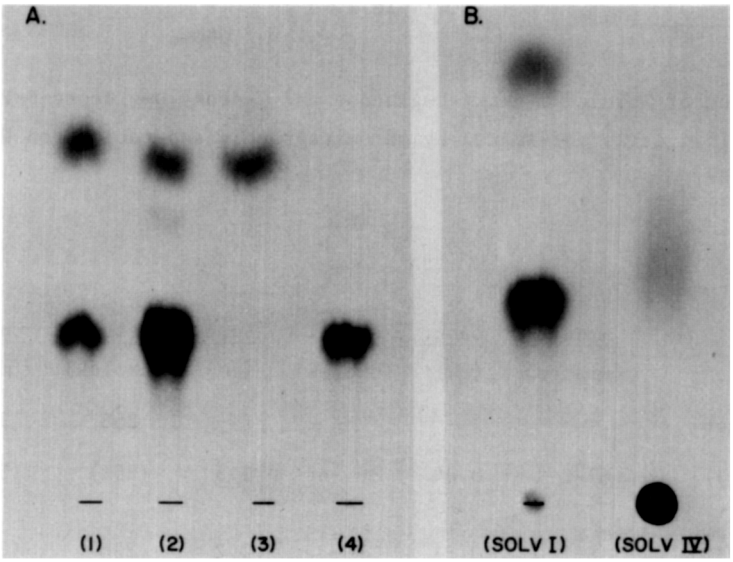


Figure 1. Chromatographic separation and identification by radioautography of labeled 2-deoxy-D-glucose and 2-deoxy-D-glucose-6-P in rhesus diploid cells.

A. Chromatography: Ascending, 16H in Solv I (isobutyric acid/ H_2O/conc NH_4OH, 66/33/1.5). (1) standards: [^{14}C]-2-deoxy-D-glucose (faster) and [^{14}C]-2-deoxy-D-glucose-6-P. (2) cold trichloracetic acid extract of cells incubated with [^{14}C]-2-deoxy-D-glucose (2 hours). (3) cell extract fraction not retained by Dowex-1 (acetate). (4) cell extract fraction retained and subsequently eluted from Dowex-1 (acetate).

B. Chromatographic separations of free and phosphorylated sugar from a cell extract (see Table 1) in two solvents. Composition of solvents: Solv I (as above); Solv IV water saturated n-butanol, phosphorylated sugar remains at the origin, while the free sugar migrates.

RESULTS

Products of 2-deoxy-D-glucose incorporation - Incubation of rhesus diploid cells in the presence of [^{14}C]-labeled 2-deoxy-D-glucose results in intracellular incorporation and accumulation of the label. Chromatographic analysis of the labeled, cell-accumulation products present in a cold-trichloroacetic acid extract is illustrated in the radioautograph shown in Figure 1A, Sample 2. Two major labeled components were resolved. The faster (and lesser) compound was identified as 2-deoxy-D-glucose and the slower as 2-deoxy-D-glucose-6-P. Identification was based on their comparative migration relative to the authentic labeled compounds (Figure 1A, Sample 1). Several minor unidentified

Table 1

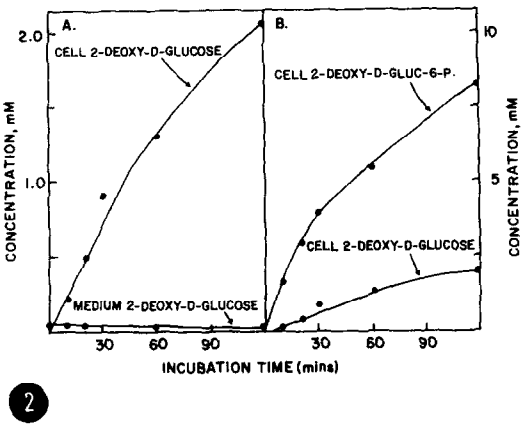
Accumulation of labeled 2-deoxy-D-glucose and 2-deoxy-D-glucose-6-P in rhesus diploid cells determined by chromatographic separations on Dowex-1 and in paper.

	2-DOG		2-DOG-6-P		TOTAL	
	Amount (nmoles)	Conc. (mM)	Amount (nmoles)	Conc. (mM)	Amount (nmoles)	Conc. (mM)
Cell extract	--	--	--	--	258	3.2
Dowex-1 (3)	67±2	0.84	184±4	2.3	(251)	(3.1)
Chromatography						
Solv I (3)	58±1	0.74	178±3	2.2	(236)	(3.0)
Solv IV (3)	55±2	0.69	192±3	2.4	(247)	(3.0)

To a well-rinsed, density inhibited confluent culture of rhesus diploid cells in a T-75 flask were added 4.0 ml modified Hank's medium containing [^{14}C] 2-deoxy-D-glucose at 0.1 mM concentration (2.5 µCi/µmole). After 1 hr incubation at 37°, the cells were washed thoroughly and extracted in 1.5 ml 5% trichloroacetic acid. After acid removal (ether) triplicate 50 µl aliquots were fractionated on Dowex-1 (acetate) and by chromatography in paper. The chromatographic separations are shown in Figure 1. The total protein content of the cells was 8.0 mg. Total cell volume was taken as 80 µl, by assuming a 10% protein content by volume.

compounds were also present. Fractionation of the cell extract on Dowex-1 (acetate) separates effectively free and phosphorylated forms of the sugar as illustrated in Figure 1A, Samples 3 and 4.

Accumulation of 2-deoxy-D-glucose - Intracellular accumulation of 2-deoxy-D-glucose-6-P in rhesus diploid cells following 1 hour incubation in the presence of labeled 2-deoxy-D-glucose at 0.1 mM concentration is shown in Table 1. Comparative measurements of free and phosphorylated sugar are shown following fractionation on Dowex-1 and chromatography in paper employing two separate solvent systems (chromatographic separations are illustrated in Figure 1B). The various fractionation procedures yielded generally similar values for free and phosphorylated sugars, the differences were attributed to distribution differences of minor labeled components. Irrespective of the fractionation procedure employed, a concentrative accumulation of free sugar in the rhesus diploid cells was clearly indicated. Intracellular concentrations of free sugar were 7 to 8-fold greater than the medium concentration employed and accumulation of phosphorylated sugar exceeded free sugar by 3-fold.



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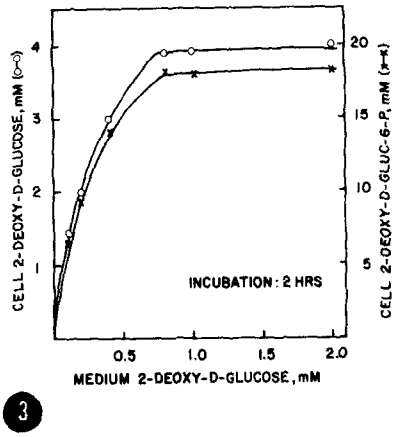

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Figure 2. Accumulation of free and phosphorylated 2-deoxy-D-glucose in rhesus diploid cells as a function of incubation time. Six Petri dishes of confluent cells were incubated at 37° with 0.5 ml modified Hank's medium containing 0.06 mM [^{14}C] 2-deoxy-D-glucose (0.5 µCi/µmole) for 0, 10, 20, 30, 60 and 90 minutes. Free and phosphorylated sugars were separated by Dowex-1. Cell volume was estimated from protein measurements.

Figure 3. Incorporation of 2-deoxy-D-glucose by rhesus diploid cells as a function of medium concentration. Six Petri dishes of confluent cells were incubated with 0.5 ml modified Hank's medium containing labeled 2-deoxy-D-glucose ranging from 0.1 to 2.0 mM (0.02 µCi/µmole). Intracellular free and phosphorylated sugars were separated by Dowex-1. Cell volumes were estimated from protein measurements.

Concentrative accumulation of free sugar in rhesus diploid cells accompanying a 2-hour incubation in medium containing a low level of 2-deoxy-D-glucose is shown in Figure 2A. A continuous accumulation of free sugar occurred. The cellular level exceeded the medium concentration within 10 minutes and the cells contained a 35-fold greater concentration than the medium after 2 hours incubation. Accumulation levels of the phosphorylated product relative to free sugar over the 2-hour incubation period are shown in Figure 2B.

Intracellular accumulation of 2-deoxy-D-glucose proceeds to a maximal, saturable level which is not exceeded by further increases in medium 2-deoxy-D-glucose concentration, at least within the tested range shown in Figure 3. In the experiment shown, maximal accumulation of 2-deoxy-D-glucose (4 mM) occurred within a 2-hour incubation period at medium concentrations $\geq$ 0.8 mM. Parallel accumulation patterns of free and phosphorylated sugar were observed with the latter exceeding the former by 4 to 5-times. The accumulation limits of free and phosphorylated sugar varied somewhat among sets of cell cultures examined,

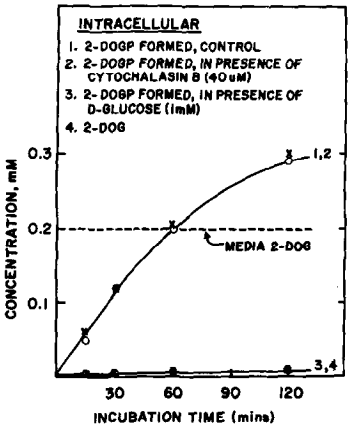

Figure 4. Incorporation of 2-deoxy-D-glucose by human erythrocytes and effect of cytochalasin B and D-glucose. Incubation mixtures contained 8% (v/v) erythrocytes, 0.2mM labeled [^{14}C] 2-deoxy-D-glucose (0.25 μCi/μmole), cytochalasin B and D-glucose where indicated, in 1.0 ml modified Hank's medium. Cells were washed 4 times with 20 ml cold modified Hank's medium and extracted in 1.0 ml 5% trichloracetic acid. Free and phosphorylated sugars were determined following separations by Dowex-1. Cell volume was calculated on the basis of 25% hemoglobin content and 75% of the cell volume was assumed to be available to the external solute.

but remained independent of medium 2-deoxy-D-glucose concentration at least to 5.0 mM.

Incorporation of 2-deoxy-D-glucose by human erythrocytes - In contrast to rhesus diploid cells, human erythrocyte suspensions incubated with labeled 2-deoxy-D-glucose did not accumulate the free sugar as shown in Figure 4, Curve 4. A single, major accumulation product in the erythrocytes was identified as 2-deoxy-D-glucose-6-P (Curve 1). Accumulation of the phosphorylated sugar was not inhibited by cytochalasin B (Curve 2), was effectively blocked by glucose (Curve 3), and was found at a considerably lower concentration than in the rhesus cells (<10%).

DISCUSSION

Demonstration of concentrative accumulation of 2-deoxy-D-glucose required medium concentrations to be less than the accumulation limit of the cells (usually <5 mM) and appropriately extended accumulation periods. The inability to demonstrate a similar concentrative accumulation in other mammalian cells in culture (e.g. see 7,12) may be due to a lack of incorporation of these factors in experimental design. The reliability of the measurements of cell 2-deoxy-D-glucose concentrations was verified with respect to (a) completeness of sepa-

ration of free and phosphorylated sugar (Fig. 1), (b) identity of incorporated free sugar and medium 2-deoxy-D-glucose (Fig. 1) and (c) intracellular concentration computations based on cell volume estimates by protein (comparable volume estimates obtained by Coulter counter). Since rhesus diploid cell sonicates did not dephosphorylate 2-deoxy-D-glucose-6-P, it was assumed that the intracellular free sugar was not a desphosphorylation product.

Incorporation of 2-deoxy-D-glucose by human erythrocytes differs significantly from rhesus cells with little inhibition by cytochalasin B or accumulation of the free sugar evident. Strong competitive inhibition by cytochalasin B of 2-deoxy-D-glucose incorporation (K_i = 1 μM) is demonstrable with the rhesus cells (experiment not shown) as observed with a number of other mammalian cells (9,16). In contrast to the rhesus diploid cell, the human erythrocyte displays expectedly those properties consistent with a facilitated diffusion system (17).

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