Permeation As the Rate-Limiting Step in the Phosphorylation of Uridine and Choline and Their Incorporation into Macromolecules by Novikoff Hepatoma Cells. Competitive Inhibition by Phenethyl Alcohol, Persantin, and Adenosine*

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ABSTRACT: The incorporation of uridine or choline into intracellular free phosphorylated intermediates by exponentially growing Novikoff rat hepatoma cells in suspension culture follows simple Michaelis–Menten kinetics. The apparent V_m's and K_m's for the incorporation of uridine or choline by whole cells, however, are at least one order of magnitude lower than those for the phosphorylation of the substrates by cell-free extracts. Further, the incorporation of uridine by whole cells is inhibited competitively by adenosine, persantin, and phenethyl alcohol, whereas the phosphorylation of uridine by cell-free extracts is inhibited noncompetitively by phenethyl alcohol and is unaffected by adenosine or persantin. Phenethyl alcohol is also a competitive inhibitor for choline incorporation by whole cells and a noncompetitive inhibitor for choline kinase as measured in cell-free extracts. Both the uridine and choline kinase activities of the cells are located in the soluble portion of the cytoplasm and isolated fragments of plasma membrane are free of kinase activities. The overall results are interpreted to indicate that the transport of uridine and choline into the cell are reactions distinct from phosphorylation and that permeation is the rate-limiting step in the incorporation of these precursors by whole cells into the intracellular pools of phosphorylated intermediates. The inhibition of the transport of uridine or choline into the cells results in a proportional inhibition of the incorporation of the precursors into ribonucleic acid and membrane phosphatidylcholine, respectively.

Thus the rate of incorporation of these precursors into macromolecules is also limited by the rate with which they enter the cells.

The mode of entry of uridine and other nucleosides, or of most other small molecular weight substances, into bacteria or animal cells is little understood, except that in most instances specific permeation reactions are involved (see Rothstein, 1968). An understanding of these processes is of theoretical but also of practical importance since many of these substances have been widely employed as specific precursors in studies on the rates of synthesis of macromolecules by various types of cells, and several reports indicate that low rates of uptake of some of these precursors into the intracellular pool of phosphorylated intermediates may severely limit their incorporation into macromolecules without reflecting a low rate of synthesis of the latter (Edlin and Broda, 1968; Plagemann *et al.*, 1969).

Results from studies on the intracellular degradation of uridine to uracil or the deamination of cytidine to uridine have indicated that nucleosides are taken up by bacteria (Peterson and Koch, 1966; Peterson *et al.*, 1967) or mammalian cells (Jacquez, 1962) by facilitated diffusion (inactive transport). A permeation reaction has also been implicated in the uptake of choline by erythrocytes (Askari, 1966; Martin, 1968) and by various mammalian tissue segments *in vitro* (Sung and Johnstone, 1965; Schuberth *et al.*, 1966; Marchbanks, 1968), but the conclusions vary as to whether the process can be considered inactive or active transport. In these studies nongrowing cell systems were employed in which little choline was further phosphorylated, and transport was measured by determining the intracellular accumulation of free choline or its oxidation product, betaine.

In growing cells, on the other hand, nucleosides and choline are further phosphorylated and utilized for the synthesis of nucleic acids and phosphatidylcholine, respectively, but little is known about the possible interrelationships between these processes and the significance of transport reactions in these cells. Sung and Johnstone (1965) have concluded from their studies that Ehrlich ascites tumor cells lack a transport system for choline although they presented evidence for active transport of choline in kidney cortex slices. Since after exposure to radioactive choline the only labeled acid-soluble component of the Ehrlich ascites cells was phosphorylcholine, these investigators concluded that choline enters these cells by diffusion and is trapped inside the cells by being rapidly phosphorylated.

A number of investigations have shown that the incorporation of uridine into the intracellular free nucleotide pool by *Escherichia coli* (Bremer and Yuan, 1968) or chick embryo cells in monolayer culture (Skehel *et al.*, 1967; Scholtissek, 1968b) follows normal Michaelis–Menten kinetics with K_m's of about 1.3×10^{-6} and 1.0×10^{-5} M, respectively. This finding has led to the suggestion that the uptake and

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conversion of uridine into UMP may be determined by one enzyme, possibly uridine kinase, which phosphorylates uridine in the cell membrane (Bremer and Yuan, 1968; Scholtissek, 1968b). Consistent with this hypothesis, we have found previously that the fluctuations in the rate of incorporation of uridine into the free nucleotide pool by Novikoff rat hepatoma cells during the course of the growth cycle in suspension culture correlates temporarily with variations in the uridine kinase activity of the cells as measured in cell-free extracts (Plagemann *et al.*, 1969). Quantitatively, however, the uridine kinase activity of the cells fluctuates appreciably less than the rate of uridine incorporation by whole cells, and a similar discrepancy was found in the variations of the rate of phosphorylation of choline by whole cells and the choline kinase activity of the cells (Plagemann, 1969). These results suggested that possibly the rate of entry of the substances into the cell (transport) might play an important role in determining the rate of phosphorylation of uridine or choline by these cells. The results of the present investigation support this conclusion and demonstrate that the rate of incorporation of uridine and choline into macromolecules is also limited by the rates of the respective permeation reactions.

Experimental Procedure

Materials

Phenethyl alcohol was obtained from Matheson, Coleman & Bell, East Rutherford, N. J.; [^{3}H]5-uridine, methyl[^{3}H]-choline and unlabeled uridine and adenosine from Schwarz BioResearch; 2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido(5,4-*d*)pyrimidine (persantin) was a gift from Geigy Pharmaceuticals, Yonkers, N. Y. B4 was composed of 10 mM of Tris-HCl (pH 7.4), 10 mM NaCl, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM triethanolamine, and basal salt solution of 136 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 4.2 mM Na_2HPO_4, 0.8 mM KH_2PO_4, and 0.02 mg of phenol red/ml.

Methods

Cell Culture. The cultivation of Novikoff rat hepatoma cells (line N1S1-67) in medium 67 in suspension culture has been described previously (Plagemann and Swim, 1966; Ward and Plagemann, 1969). Cells were collected by centrifugation from cultures in the exponential phase of growth between 1.4 and 2.5 $\times$ 10^6 cells per ml (Ward and Plagemann, 1969) and suspended to the original cell concentration in basal medium 42 (BM42; Plagemann and Swim, 1966) or in choline-free BM42 where indicated.

Incorporation of Uridine or Choline by Whole Cells. Suspensions of cells in BM42 or choline-free BM42 were supplemented with labeled uridine or choline, respectively, as indicated in the appropriate experiments, and incubated on a gyrotory shaker at 37°. Duplicate samples of cell suspension were analyzed as follows. (1) For total radioactivity associated with the cells: The samples were immediately centrifuged at 500*g* for 2 min at 0°. The cells were rapidly washed with 5 ml of cold (0°) basal salt solution and suspended in 0.2 ml of 0.5 N trichloroacetic acid. The mixtures were heated at 70° for 30 min and analyzed for radioactivity (see below). (2) For radioactivity in acid-insoluble material: Other samples were quickly frozen (2 min) in a bath of solid CO_2 in ethanol. Later the samples were thawed, mixed with perchloric acid at 0°, and the precipitates were washed repeatedly with perchloric acid and trichloroacetic acid as described previously (Ward and Plagemann, 1969). The precipitate of each sample was suspended in 0.1 ml of 0.5 N trichloroacetic acid and heated and analyzed for radioactivity as described under 1. The counting efficiency of ^{3}H was approximately 12% for all samples. The radioactivity incorporated into acid-soluble material was estimated by subtracting the values for 2 from those for 1.

Enzyme Assays. Fractions from sucrose density gradients were assayed for cytochrome oxidase, total ATPase, and acid phosphatase activities as described previously (Plagemann, 1968b). Cell-free extracts were prepared from exponentially growing cells and assayed for uridine kinase and choline kinase activities as described by Plagemann *et al.* (1969) and Plagemann (1969), respectively (see also Figure 6). The reaction products were separated from their respective substrates by paper chromatography as described below. One unit of uridine or choline kinase was defined as the amount of enzyme that converted 1 mμmole of substrate into total uridine nucleotides or phosphorylcholine, respectively, at 27°/hr.

Chromatography. Acid extracts from uridine-labeled cells or uridine and choline kinase reaction mixtures were analyzed by ascending chromatography on Whatman No. 3MM paper as described previously (Plagemann *et al.*, 1969; Plagemann, 1969). The paper was developed with a mixture of 1 M ammonium acetate (pH 5.0) and 95% ethanol (3:7, v/v) at 30° for 18 hr. Mixtures of appropriate standards were cochromatographed. The positions of UMP, UDP, UTP, UDP-G,[1] and CDP-choline were located by examining the paper under ultraviolet light, and those of choline and phosphorylcholine by spraying the paper with a 1% (v/v) solution of iodine in methanol. Chromatograms of the experimental samples were cut into 1-cm segments at right angles to the direction of migration, and the segments were rocked each with 1 ml of H_2O for 1 hr and then analyzed for radioactivity. The counting efficiency of ^{3}H was approximately 12% and close to 100% of the radioactivity was recovered from the paper.

Radioactivity Determinations. All samples were transferred quantitatively to bottles containing 15 ml of a scintillator–solvent mixture described previously (Plagemann and Swim, 1966) and the radioactivity was measured with a Packard liquid scintillation spectrometer (Model 2002).

Results

Effect of Phenethyl Alcohol on Incorporation of Uridine into Acid-Soluble Material and RNA by Whole Cells. Results from previous experiments (Plagemann, 1968a) have shown that treatment of N1S1-67 cells with phenethyl alcohol inhibits immediately and to about the same extent the incorporation of labeled uridine, thymidine, and amino acids into acid-precipitable material. The data were interpreted to indicate that the chemical rapidly inhibits the synthesis of RNA, DNA, and protein, respectively, and this was confirmed by chemical analyses of samples of cells. Results from subsequent experiments, however, have shown that the rapid inhibition of the incorporation of the labeled precursors into macromolecules largely results from a failure of phenethyl alcohol

[1] UDP-glucose (UDP-G) refers to those components which migrate in chromatography and electrophoresis identical with authentic UDP-G (Plagemann *et al.*, 1969). We have not attempted further identification.

TABLE I: Distribution of ^3H among Uridine Nucleotides in Acid Extracts of Cells Labeled with Uridine in the Absence and Presence of Phenethyl Alcohol.[a]

Phenethyl Alcohol (mM)	UTP		UDP		UDP-G		UMP		Uridine
	cpm[b]	%[c]	cpm[b]	%[c]	cpm[b]	%[c]	cpm[b]	%[c]	(cpm[b])
0	10,500	66	550	3	1830	12	3050	19	150
8	9,890	67	770	5	1620	11	2520	17	120
16	5,940	66	670	7	960	11	1480	16	160

[a] Details of the experiment are described in the legend to Figure 1. After 120-min labeling with uridine in the absence and presence of the indicated concentrations of phenethyl alcohol, 1.5×10^7 cells were collected by centrifugation, washed twice in basal salt solution, and extracted with perchloric acid as described previously (Plagemann *et al.*, 1969), and the acid extracts were analyzed by ascending paper chromatography as described in Materials and Methods. [b] Total amount of radioactivity associated with the indicated components after chromatography of 25 μl of each acid extract. [c] Per cent of total radioactivity in nucleotides.

treated cells to incorporate the precursors into the acid-soluble cellular pools. A comparison of the data in Figure 1A,B shows that the incorporation of uridine into total cell material was inhibited by various concentrations of phenethyl alcohol to about the same extent as its incorporation into acid-insoluble material. The rate of incorporation of uridine into total cell material is an estimate of the rate of its incorporation into free nucleotides since the latter are obligatory intermediates in the incorporation of uridine into RNA. Throughout the experiment 60–80% of the total cell associated radioactivity was in acid-soluble material (values in Figure 1A minus those in Figure 1B) whether the cells were treated with phenethyl alcohol or not. The composition of the acid-soluble pool was investigated by washing samples of cells free of uridine, extracting them with perchloric acid, and separating the uridine nucleotides by paper chromatography. The data in Table I show that although the total radioactivity recovered in uridine nucleotides was markedly reduced as a result of treating the cells with phenethyl alcohol, its distribution among the various nucleotides was about the same whether 8 or 16 mM of phenethyl alcohol was present in the medium during labeling or not. About 65% of the total radioactivity in nucleotides was present in UTP, 5% in UDP, 11% in UDP-G, and the remainder in UMP. The data, therefore, indicate that phenethyl alcohol interfered with the conversion of extracellular uridine into intracellular UMP by the cells and not with the further phosphorylation of UMP to the triphosphate level. Significant amounts of uridine were not found in any of the acid extracts.

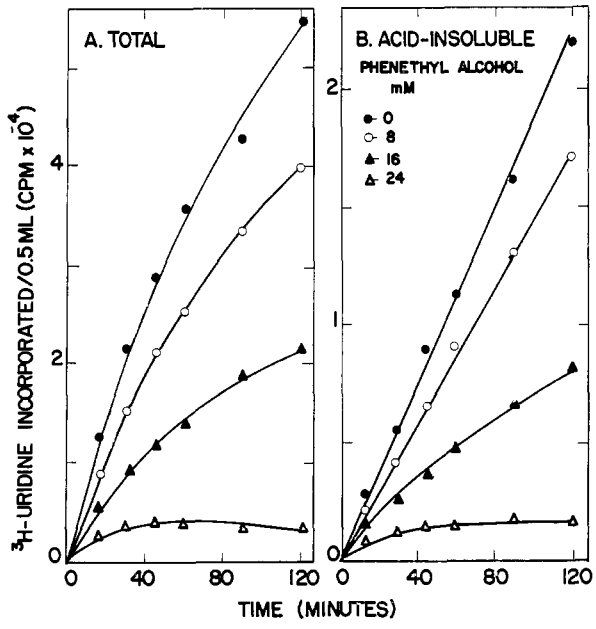

FIGURE 1: Effect of phenethyl alcohol on uridine incorporation into total cell material (A) and acid-insoluble material (B) by whole cells. Samples of a suspension of cells in BM42 (2×10^6 cells/ml) were supplemented with the indicated concentrations of phenethyl alcohol and immediately thereafter (0 time) with 0.01 mM [^{3}H]5-uridine (40 cpm/$\mu\mu$mole). At various times thereafter, duplicate 0.5-ml samples of suspension were analyzed for radioactivity in total cell material (A) or acid-insoluble material (B) as described in Materials and Methods. All points are averages of the duplicate analyses.

The Lineweaver–Burk plots in Figure 2 demonstrate that the inhibition by phenethyl alcohol of the incorporation of uridine into nucleotides by whole cells was of the simple competitive type. From these data it cannot be distinguished whether phenethyl alcohol inhibited the entry of uridine into the cell or its subsequent phosphorylation, but the data presented subsequently indicate that it is the entry reaction that is inhibited by the drug.

Effect of Persantin and Adenosine on the Incorporation of Uridine into Acid-Soluble Material and into RNA by Whole Cells. Scholtissek (1968b) has shown that persantin specifically inhibits the incorporation of nucleosides into the nucleotide pool of chick embryo cells in monolayer culture and that this inhibition is accompanied by a proportional inhibition of nucleoside incorporation into nucleic acids. These findings have been confirmed for the incorporation of uridine by N1S1-67 cells in suspension, although appreciably higher concentrations of persantin were required to achieve a comparable degree of inhibition under the specific conditions of cell and uridine concentrations employed in the present study. Under the experimental conditions specified in the legend to Figure 1, uridine incorporation into total cell

TABLE II: Kinetic Constants for the Inhibition of Uridine and Choline Phosphorylation by Whole Cells and Cell-Free Extracts by Persantin, Phenethyl Alcohol, Adenosine, and Azauridine.[a]

		Whole Cells			Cell-Free Extract		
Substrate	Inhibitor	Inhibition	K_i (M)	K_m/K_i	Inhibition	K_i (M)	K_m/K_i
Uridine	Phenethyl alcohol	Competitive	3.5×10^{-3}	3.7×10^{-3}	Noncompetitive	1×10^{-2}	5×10^{-2}
	Persantin	Competitive	4.0×10^{-6}	3	None		
	Adenosine	Competitive	1.3×10^{-4}	1.0×10^{-1}	None		
	Azauridine[b]	Competitive	8.0×10^{-4}	1.6×10^{-2}	Competitive	6×10^{-3}	8×10^{-2}
Choline	Phenethyl alcohol	Competitive	1×10^{-2}	5.0×10^{-4}	Noncompetitive	2×10^{-2}	3.5×10^{-2}

[a] The types of inhibition were deduced from the Lineweaver–Burk plots in Figures 2, 4, and 5 and from similar plots for the inhibition by persantin and adenosine. The latter data were obtained under experimental conditions identical with those described in the legend to Figure 2, except that 0.01 and 0.05 mM persantin or 0.05 and 0.2 mM adenosine were used instead of phenethyl alcohol. The K_i's were calculated from the slopes of the inhibitor curves assuming simple competitive or noncompetitive inhibition. [b] Data from M. Korbecki and P. G. W. Plagemann, in preparation.

material and into acid-insoluble material was inhibited about 40% by 0.01 mM persantin, 80% by 0.05 mM, and over 95% by 0.2 mM. Further, as reported for erythrocytes (Kübler and Bretschneider, 1964) and chick embryo cells (Scholtissek, 1968b), the inhibitory effect of persantin was of the simple competitive type (see Table II).

Skehel *et al.* (1967) found that adenosine inhibits competitively the incorporation of uridine into the acid-soluble fraction of chick embryo cells in monolayer culture (K_i = 5.4×10^{-4} M), whereas Scholtissek (1968a) reported that it has no effect. The results presented in Figure 3A illustrate that uridine incorporation by N1S1-67 cells into total cell material was little affected by the presence of a twofold higher concentration of adenosine in the medium, but that it was inhibited 30–40% by a tenfold excess of adenosine and about 90% by a 200-fold excess. These effects were accompanied by corresponding degrees of inhibition of the incorporation of uridine into acid-insoluble material (Figure 3B). The inhibition was of the simple competitive type (see Table II). Chro-

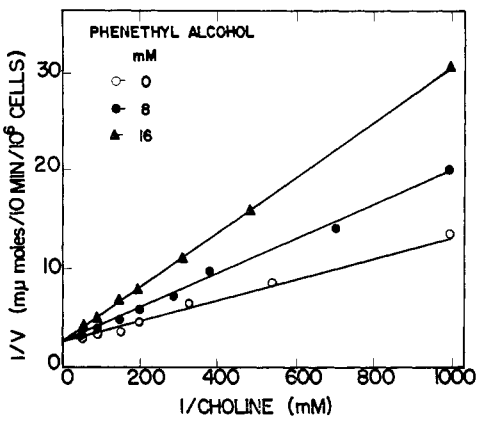

FIGURE 2: Lineweaver–Burk plots of the incorporation of uridine into total cell material by whole cells in the absence and presence of various concentrations of phenethyl alcohol. Portions of a suspension of 2×10^6 cells/ml of BM42 were mixed with phenethyl alcohol to the indicated concentrations and immediately thereafter, 10-ml samples of these suspensions were supplemented with 5, 10, 15, 20, or 50 μM [^{3}H]5-uridine (40 cpm/μμmole) or with 50, 100, or 200 μM [^{3}H]5-uridine (4 cpm/μμmole) and incubated on a gyrotory shaker at 37°. At 2.5-, 5-, and 10-min incubation, duplicate 1-ml samples were analyzed for radioactivity in total cell material as described in Materials and Methods. The initial rates of incorporation were estimated from plots of the amount of radioactivity incorporated as a function of time.

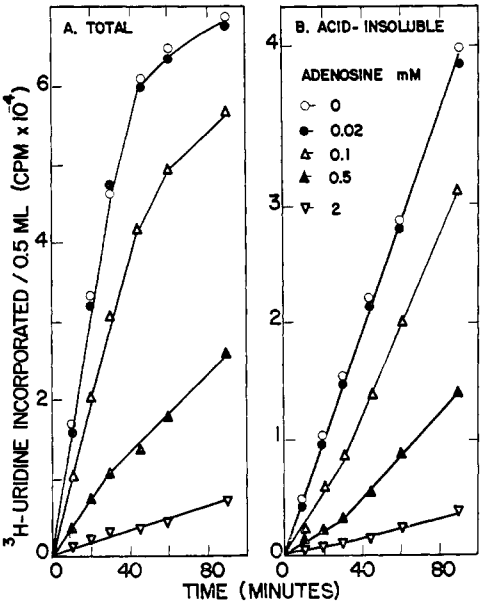

FIGURE 3: Effect of adenosine on uridine incorporation into total cell material (A) and acid-insoluble material (B) by whole cells. At 0 time, samples of cells were suspended to 2×10^6 cells/ml of BM42 supplemented with the indicated concentrations of adenosine and 0.01 mM [^{3}H]5-uridine (40 cpm/μμmole). At various times thereafter, duplicate 0.5-ml samples of suspension were analyzed for radioactivity in total cell material (A) or in acid-insoluble material (B) as described in Materials and Methods. All points represent the average of the duplicate analyses.

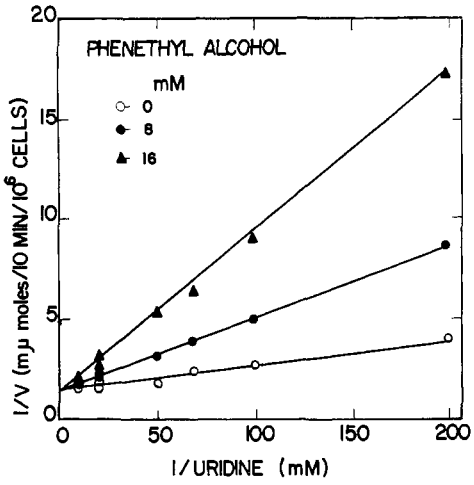

FIGURE 4: Lineweaver–Burk plots of the incorporation of choline into total cell material by whole cells in the absence and presence of phenethyl alcohol. Portions of a suspension of 2×10^6 cells/ml of choline-free BM42 were mixed with phenethyl alcohol to the indicated concentrations and immediately thereafter, 10-ml samples of these suspensions were supplemented with various concentrations (1–20 μM) of methyl[^{3}H]choline (35 cpm/μμmole) and incubated on a gyrotory shaker at 37°. At 10-, 20-, 30-, and 50 min incubation, duplicate 1-ml samples of suspension were analyzed for radioactivity in total cell material as described in Materials and Methods. The rates of incorporation were estimated from plots of the amounts of radioactivity incorporated as a function of time (see Plagemann, 1968b).

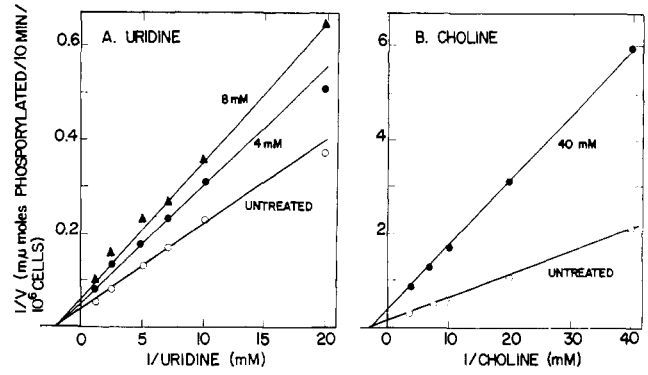

FIGURE 5: Lineweaver–Burk plots of the phosphorylation of uridine (A) or choline (B) by cell-free extract in the absence or presence of phenethyl alcohol. Cell-free extracts were prepared and treated with phenethyl alcohol and then assayed for the phosphorylation of uridine or choline under the conditions described in the legend of Figure 6, except that the individual reaction mixtures contained (A) 50 μCi/ml of [^{3}H]5-uridine (27,000 μCi/μmole) and 50, 100, 150, 250, 400, and 1000 μM unlabeled uridine and (B) 25, 50, 100, 150, or 250 μM methyl[^{3}H]choline (73 μCi/μmole). Reaction rates were estimated from the linear portions of plots of the per cent of substrate phosphorylated as a function of time (see Figure 6 and Plagemann *et al.*, 1969).

matographic analysis of the adenosine employed in this study revealed no detectable contamination with uridine. The phosphorylation of UMP to UTP was probably not affected by either persantin or adenosine since the relative distribution of label among the uridine nucleotides was about the same in acid extracts from treated and untreated cells (see Table I).

Effect of Phenethyl Alcohol on Choline Incorporation by Whole Cells. It has been demonstrated previously (Plagemann, 1968b) that N1S1-67 cells rapidly convert choline into phosphorylcholine which accumulates intracellularly and serves as a precursor for the synthesis of membrane phosphatidylcholine probably *via* the Kennedy pathway (Kennedy, 1957), but that significant amounts of CDP-choline do not accumulate. Label in phosphorylcholine is readily distinguished from that in phosphatidylcholine since phosphorylcholine, although not removed by washing the cells with basal salt solution, is solubilized by extracting the cells with perchloric acid, whereas the phosphatidylcholine remains in the acid-insoluble fraction (Plagemann, 1968b). It was also shown (Plagemann, 1968a) that various concentrations of phenethyl alcohol inhibit the incorporation of [^{3}H]choline into the acid-soluble cellular pool to about the same extent as the incorporation of uridine, thymidine, or amino acids without affecting the synthesis of phosphatidylcholine from phosphorylcholine accumulated in cells. The data in Figure 4 demonstrate that the inhibition of choline incorporation by phenethyl alcohol was also of the competitive type. Results from other studies showed that persantin, at 0.4 mM, had no effect on the incorporation of [^{3}H]choline by N1S1-67 cells suspended in BM42 containing 0.01 mM choline.

TABLE III: Kinetic Constants for the Phosphorylation of Uridine and Choline by Whole Cells and Cell-Free Extracts.[a]

Substrate	Preparation	V_m (mμmoles/10 min per 10^6 cells)	K_m (M)
Uridine	Whole cells	1.0–1.5	1.3×10^{-5}
	Cell free	28.0	5.0×10^{-4}
Choline	Whole cells	0.45	5.0×10^{-6}
	Cell free	14.0	7.0×10^{-4}

[a] The constants were calculated from the data in Figures 2, 4, and 5. For uridine incorporation by whole cells, the maximum range of V_m values observed in a number of experiments is indicated.

Kinetic Analysis of the Incorporation of Uridine and Choline by Whole Cells and of Their Phosphorylation by Cell-Free Extracts. Table III lists the apparent V_m's and K_m's for the incorporation of uridine and choline into the acid-soluble pool by whole cells. Table III also lists the V_m's and K_m's for the phosphorylation of uridine and choline by cell-free extracts as calculated from the kinetic analyses illustrated in Figure 5. The data show that the apparent V_m's (mμmoles of substrate converted per 10 min per 10^6 cells) and the K_m's for the *in vitro* reactions were at least one order of magnitude higher than those for whole cells. These results suggest that the rate-limiting step in the conversion of uridine and choline into phosphorylated compounds by whole cells was not limited by the kinase activities but probably rather by the rate of entry of the substrates into the cell. This conclusion is supported by studies on the effect of phenethyl alcohol, adenosine and persantin on the kinase activities *in vitro*.

Effect of Phenethyl Alcohol on the Phosphorylation of Uridine and Choline by Cell-Free Extracts. As indicated by

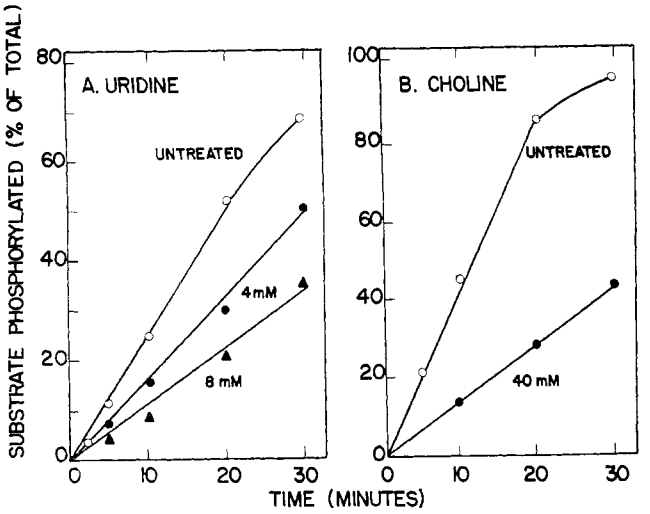

FIGURE 6: Effect of phenethyl alcohol on the phosphorylation of uridine (A) and choline (B) by cell-free extract. Cell-free extracts were prepared from 4×10^7 cells (A) and 1.3×10^8 cells (B) in 2 ml of B4. Samples of 0.2 ml of the cell-free extracts were mixed with 0.01 ml of H_2O or appropriate solutions of phenethyl alcohol and immediately thereafter, with 0.2 ml of a reaction solution containing in mM: ATP, 20; $MgCl_2$, 10; Tris-HCl (pH 8.2), 200; mercaptoethanol, 10; KCl, 100; and (A) [^{3}H]5-uridine (100 μCi/μmole), 0.5, or (B) methyl[^{3}H]choline (73 μCi/μmole), 0.7. The mixtures were incubated at 27° and at the indicated times, 50-μl samples were transferred to tubes in a boiling-water bath and heated for 1 min. The samples were then clarified by centrifugation and 10-μl samples of the supernatant fluids were analyzed chromatographically as described in Materials and Methods. Values represent per cent of total uridine or choline converted into uridine nucleotides (A) or phosphorylcholine (B), respectively. The indicated concentrations of phenethyl alcohol are final concentrations in the reaction mixture.

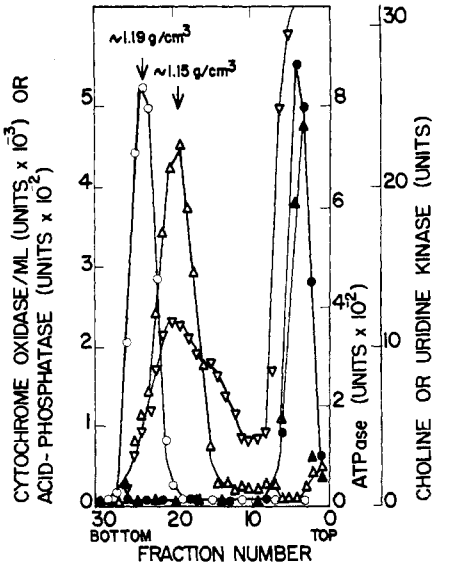

FIGURE 7: Isopycnic centrifugation of cytoplasmic fraction and analysis of gradient fractions for various enzyme activities; 1×10^8 cells were suspended in 3 ml of B4 and allowed to stand for 10 min at 0°. The cells were disrupted by ten strokes with a Dounce homogenizer and the nuclei were removed by centrifuging the lysate at 600g for 5 min. The supernatant fluid (cytoplasmic fraction) was layered over 27 ml of a linear 0.5–1.6 M gradient of sucrose in 1 mM Na_3-EDTA and the gradient was centrifuged at 20,000 rpm, 4° for 18 hr. After centrifugation, 1-ml fractions were collected from the gradient by means of an ISCO gradient fractionator and each fraction was assayed as follows: 0.02 or 0.05 ml for cytochrome oxidase activity (○); 0.3 ml for ATPase activity (Na^+K^+-activated plus Mg^{2+}-activated, Δ); 0.2 ml for acid phosphatase activity (▽); for uridine (▲) and choline (●) kinase activities. Samples of 0.03 ml were mixed with 0.03 ml of the appropriate reaction mixture described in the legend of Figure 6, and incubated at 27° for 30 min.

the data in Figure 6, phenethyl alcohol inhibited both the phosphorylation of uridine and choline by cell-free extracts. Further, the Lineweaver–Burk plots illustrated in Figure 5 suggest that in both cases the inhibition was of the typical noncompetitive type.

Results from additional studies showed that neither 0.4 mM persantin nor 3.5 mM adenosine had any effect on the uridine kinase activity as measured in cell-free extracts under the conditions described in the legend to Figure 6 (0.25 mM uridine). No effect of persantin on uridine phosphorylation could be detected even when the uridine concentration in the reaction mixture was as low as 0.87 μM and the persantin concentration as high as 650 μM, an 800-fold excess. Persantin, at 0.65 mM, also had no effect on the *in vitro* phosphorylation of choline under the assay conditions described in the legend to Figure 6 (0.35 mM choline).

Kinetic Analyses of the Inhibitions of Uridine and Choline Phosphorylation by Whole Cells and Cell-Free Extracts. Table II summarizes the kinetic data for the various inhibitors of uridine and choline incorporation by whole cells and of the *in vitro* kinase reactions. The K_m/K_i ratios indicate that there existed marked differences in the relative effects of the inhibitors on the *in vivo* incorporation and *in vitro* reactions. Phenethyl alcohol was less effective in inhibiting the incorporation of choline and uridine by whole cells than the kinase reactions. Persantin and adenosine inhibited the incorporation of uridine by whole cells at relatively low concentrations, but had no effect on the *in vitro* reaction even at relatively high concentrations. Azauridine, on the other hand, inhibited competitively both the incorporation of uridine by whole cells and the uridine kinase eaction (Korbecki and Plagemanny, 1969), and to a similar degree (Table II).

Distribution of Uridine and Choline Kinase Activities in Cells. If nucleoside kinases play an important function in the uptake of nucleosides from the medium by cells in culture (Bremer and Yuan, 1968; Scholtissek, 1968b), it would be anticipated that the kinases are associated with the plasma membrane. We have previously developed procedures for the fractionation of N1S1-67 cells and the separation of various cytoplasmic membrane structures by isopycnic centrifugation in sucrose density gradients (Plagemann, 1968b, 1969). We employed these techniques to examine the distribution of uridine and choline kinase activities among cellular components of the cells. Upon fractionation of the cells in B4 or in a 0.4 M sucrose solution containing 1 mM Na_3EDTA, 75–85% of the total cellular uridine and choline kinase activities was recovered in the cell sap, 4–10% in the nuclear fraction, and 11–18% in a combined mitochondrial–microsomal fraction. The complete cytoplasmic fraction prepared by fractionating cells in B4 was also analyzed by isopycnic centrifugation in a sucrose density gradient (Figure 7). In this type of gradient the mitochondria of exponentially growing N1S1-67 cells equilibrate at 1.19 g/cm^3 as indicated by the distribution of cytochrome oxidase activity and the fragments of the plasma membrane equilibrate at a density of about 1.15 g/cm^3 as

indicated by peaks of ATPase and acid phosphatase activities (see Plagemann, 1968b, 1969, and Figure 7). All of the choline and uridine kinase activities were recovered in the upper 7 ml of the gradient and thus were not associated with particulate membrane components of the cell.

Discussion

The following lines of evidence indicate that rapidly growing Novikoff hepatoma cells in suspension culture, like nongrowing mammalian cell systems or bacteria, take up uridine and choline by specific transport reactions and that the latter are processes distinct from the phosphorylating reactions. The incorporation of uridine or choline into the intracellular pool of phosphorylated intermediates follows normal Michaelis–Menten kinetics, but the apparent V_m's and K_m's are at least one order of magnitude lower than the corresponding parameters for the *in vitro* kinase reactions (Table III). The incorporation of uridine is competitively inhibited by persantin, adenosine and phenethyl alcohol, whereas persantin and adenosine have no effect on the *in vitro* uridine kinase reaction and phenethyl alcohol acts as a noncompetitive inhibitor (Table II). Similarly, the incorporation of choline is inhibited competitively by phenethyl alcohol and the choline kinase noncompetitively. Further, there exist marked differences in the K_m/K_i ratios for the various *in vitro* and *in vivo* inhibitions (Table II). It seems unlikely that the observed differential effects of the inhibitors are due to technical difficulties in measuring the various reactions, since using the same techniques, azauridine was found to be a competitive inhibitor of both the incorporation of uridine by whole cells and the *in vitro* uridine kinase reaction (Korbecki and Plagemann, 1969). The kinase activities are probably not associated with the plasma membrane (Figure 7) as would be expected if these enzymes play a vital role in the entry of uridine and choline into the cell.

The operation of an uridine transport reaction in Novikoff hepatoma cells is also indicated by the fact[2] that the incorporation of uridine by these cells into the pool of free nucleotides follows normal Michaelis–Menten kinetics only at concentrations below 0.1 mM uridine in the medium. At concentrations between 0.1 and 1 mM, the rate of uridine incorporation into the nucleotide pool is directly proportional to its concentration in the medium. Similar results were obtained by Jacquez (1962) with suspensions of Ehrlich ascites cells in which uridine uptake was determined by measuring the intracellular degradation of externally supplied uridine to uracil. This investigator suggested that the transport system becomes saturated at about 0.5 mM of uridine in the medium and that at higher concentrations uridine enters the cell mainly by simple diffusion. Choline transport by erythrocytes (Askari, 1966), brain slices (Schuberth *et al.*, 1966) and synapsosomes (Marchbanks, 1968) is also a saturable process and simple diffusion is the predominant type of uptake by these cells at higher concentrations. Simple diffusion of choline may not occur in Novikoff cells since previous results (Plagemann, 1968b) have shown that the rate of incorporation of choline by these cells is independent of the concentration in the medium above 0.01 mM.

The overall results for the incorporation of uridine by Novikoff cells are reminiscent of the data by Peterson *et al.* (1967) with *E. coli*. These investigators showed that the apparent V_m's and K_m's for the intracellular conversion of exogenously added uridine to uracil or for the intracellular deamination of cytidine to uridine by whole cells were at least one order of magnitude lower than the corresponding *in vitro* reactions. They concluded that the degradative enzymes are present in excess in the cells and that the rates of the intracellular enzymatic reactions are limited by the rates of entry of the substrates. Therefore, they suggested, the rates of deamination of cytidine and of the degradation of uridine by whole cells are effective measures of the rates of entry of the respective substrates.

Similarly, our results indicate that the rate of phosphorylation of uridine and choline by whole N1S1-67 cells is limited by the rate of transport of the substrates into the cell. Uridine and choline kinase seem to be present in excess in exponentially growing cells, although this may not be the case for stationary phase cells (Plagemann, 1969; Plagemann *et al.*, 1969), and upon entry, uridine and choline are rapidly phosphorylated and effectively trapped in the cells. This conclusion is supported by the finding that little or no uridine (Table I, Plagemann *et al.*, 1969) or choline (Plagemann, 1968b) can be detected in labeled cells that have been washed several times with basal salt solution. Rapid phosphorylation would remove uridine and choline from the intracellular pool and thus would allow entry of the substances at maximum rates. Since our data are similar for two unrelated substances like uridine and choline, this may represent a general mechanism by which cells take up and trap substances which are not taken up by active transport and accumulated against a concentration gradient. Further, these findings explain the fact that N1S1-67 cells accumulate large and expanded pools of uridine nucleotides or phosphorylcholine upon exposure to uridine[2] or choline (Plagemann, 1968b, 1969), respectively. It also follows from these conclusions that the transport of uridine or choline can be measured by determining the rate of their incorporation into total cell material. It is also of interest that the apparent K_m for the incorporation of uridine into intracellular free nucleotides by whole *E. coli* cells (1.3×10^{-6} M, Bremer and Yuan, 1968) is very similar to that for the degradation of uridine to uracil (3.3×10^{-6} M; Peterson *et al.*, 1967). Thus, it seems likely that in *E. coli*, too, both processes measure the same reaction, namely the rate of transport of uridine into the cell.

Although the data do not contribute to an understanding of the molecular mechanism of the transport reactions for choline and uridine, they are of interest in that they demonstrate that these processes are inhibited competitively by a number of structurally related and unrelated substances and that the rates of incorporation of uridine into RNA or of choline into phosphorylcholine are limited by the rates with which the respective precursors are transported into the cells. Thus, any reduction in the rate of entry as is caused by treatment with the various inhibitors will result in a corresponding inhibition of the incorporation of the precursors into macromolecules without necessarily indicating a decrease in the rate of synthesis of the latter. It follows that extreme caution is required in equating low or decreased rates of uridine or choline incorporation into macromolecules with inhibition of RNA or phosphatidylcholine synthesis, respectively (see also Plagemann *et al.*, 1969).

[2] P. G. W. Plagemann, submitted for publication.

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Isolation and Properties of a Protein from Chloroplasts Required for Phosphorylation and H^+ Uptake*

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ABSTRACT: A soluble protein, which is obligatorily required for both H^+ uptake and photophosphorylation in chloroplasts but which contains no trypsin-activated adenosine triphosphatase activity is readily extracted from chloroplasts by ethylenediaminetetraacetic acid. Purification of this protein, CF_e, has been achieved and it appears that a single protein is required both for H^+ uptake and phosphorylation. Addition of the purified factor to ethylenediaminetetraacetic acid treated chloroplasts under hypotonic conditions results in 100% restoration of phosphorylation, complete restoration of the ability of the ethylenediaminetetraacetic acid chloroplasts to accumulate H^+ in light as well as restores the ability of trypsin to activate adenosine triphosphatase in the reconstituted ethylenediaminetetraacetic acid chloroplasts. The factor has been identified as CF_1, but which probably contains bound Mg^{2+} or other divalent cations.

Extraction of spinach chloroplasts with EDTA removes a protein which is required both for H^+ uptake and for photophosphorylation. Addition of a single protein, isolated from the soluble EDTA extract of the chloroplasts, completely restores both phosphorylation and the ability of the chloroplasts to accumulate H^+ during electron flow. Purification of this protein, CF_e, has been achieved. Previous studies (McCarty and Racker, 1965) have indicated that the factor responsible for restoring H^+ uptake to EDTA-treated chloroplasts is CF_1. However, an antibody prepared against CF_1 and Dio 9, agents which inhibit phosphorylation, does not inhibit H^+ uptake (McCarty and Racker, 1965). Treatment of CF_1 with trypsin is known to activate a Ca^{2+}-requiring ATPase (Vambutas and Racker, 1965). Treatment of the purified protein herein reported with trypsin does not activate any ATPase activity. However, CF_e can be converted by removal of cations into a protein which appears to be identical with CF_1, as obtained from Dr. E. Racker. A description of some of the chemical, enzymatic, and functional properties of CF_e is the subject of this report.

Methods

Preparation of Chloroplasts. Intact spinach chloroplasts were prepared as before (Lynn, 1968). EDTA-treated chloroplasts were prepared from 150 g of commercial spinach using the method of McCarty and Racker (1968) except that the pH was 7.2 and the chloroplasts were extracted for only 5 min at room temperature. The treated chloroplasts, after centrifugation, 12,000*g* for 10 min, were resuspended in 0.15 M sucrose.

Isolation of Coupling Factor. All of the following steps were performed at 0–5°.

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