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Uridine Kinase Activities and Pyrimidine Nucleoside Phosphorylation in Fluoropyrimidine-Sensitive and -Resistant Cell Lines of the Novikoff Hepatoma

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Uridine kinase, the rate-limiting enzyme in the activation (phosphorylation) of uridine and the corresponding chemotherapeutic analogues, is present as two isoenzymes localized exclusively in the cytosol of rapidly growing neoplasms, including the S-37 sarcoma, EL-4 leukaemia, HeLa cells (a human carcinoma) and the Novikoff hepatoma. The activities of the isolated isoenzymes are markedly decreased when the concentrations of ATP, phosphate or Mg^{2+} that are optimum *in vitro* are replaced by concentrations approximating to those found *in vivo*. Further, comparisons of the K_m values of isolated uridine kinases with those for cellular uptake of pyrimidine nucleosides and their rate of intracellular phosphorylation suggest that nucleoside-transport systems play a rate-limiting role in nucleoside analogue activation and consequently that it is impossible to estimate the K_m of uridine kinase in the intact cell. During the development of tumour-cell resistance to 5-fluorouracil or 5-fluorouridine *in vivo* there was an early differential increase in the activity of a low-affinity (high-K_m) uridine kinase isoenzyme, as measured in cell extracts, and a 7-fold increase in the K_m values for the uptake of both uridine and 5-fluorouridine into the intact resistant cells.

Two isoenzymes of uridine kinase (ATP–uridine 5′-phosphotransferase, EC 2.7.1.48) have been described in rapidly growing transplantable tumours, which are poorly differentiated (Krystal & Scholefield, 1971; Krystal & Webb, 1971; Keefer *et al.*, 1974). One form resembles the adult (I) and the other the embryonic (II) form found in normal (non-neoplastic) tissues. Transient increases in the activity of either or both isoenzymes early in the development of resistance to 5-azacytidine (Keefer *et al.*, 1974, 1975*a*,*b*) suggested that these changes may be useful in planning therapeutic strategies to reverse or otherwise prevent the development of tumour-cell resistance. Central to this problem is the demonstration that both isoenzymes exist, or can be induced, in the tumour, and understanding the kinetic parameters of pyrimidine ribonucleoside and analogue phosphorylation by the two partially purified isoenzymes of uridine kinase, as well as by intact cells. Thus, if chemotherapy induces a species of uridine kinase with a high affinity for 5-fluorouridine, these cells should synthesize 5-fluorouridine 5′-monophosphate at a faster rate than do untreated tumour cells at all concentrations of 5-fluorouridine, if indeed uridine kinase represents the only rate-limiting step in the activation of this nucleoside analogue.

The development of resistance to 5-fluorouracil in rodent ascites tumours have been studied by several laboratories (Heidelberger *et al.*, 1960; Reichard *et al.*, 1962). Of particular interest was the report by Reichard *et al.* (1962) that uridine kinase was not lost during the early stages of resistance to 5-fluorouracil in several sublines of the Ehrlich ascites carcinoma. However, the physical properties of the enzyme differed significantly in the sensitive and resistant tumour cells (Skold, 1963); no differences in cellular uptake (transport) of the analogue were observed in these studies. On the basis of our present knowledge, it is proposed that these changes, concurrent with the development of resistance, represent changes in the isoenzyme profile of uridine kinase.

To understand more fully the contribution, if any, of changes in the uridine kinase isoenzyme profile and in the total uridine kinase activity in de-differentiated tumours to the development of tumour-cell resistance to pyrimidine nucleoside analogues, the activities and kinetic parameters were compared in preparations of the partially purified enzymes and indirectly in intact tumour cells that were either sensitive or resistant to 5-fluorouracil. These studies necessarily led to an evaluation of nucleoside uptake (transport) in these tumour-cell lines. The relative access of the uridine kinase isoenzymes to cellular

substrate pools was also assessed by establishing their subcellular localization.

Materials and Methods

Tumour cells

Novikoff hepatoma cells were carried intraperitoneally in 120–140g female Sprague–Dawley rats. Animals were injected with 7×10^7 cells, which resulted in a transplant generation time of 7 days. The EL-4 leukaemia was carried intraperitoneally in 6-week-old female C57B1/6 mice and the transplant generation time was 7 days after injection of 2.5×10^7 cells. The S-37 sarcoma was similarly carried intraperitoneally in 25g male Swiss white mice, with a transplant generation time of 7 days after injection of 3.5×10^6 cells. All animals were purchased from Laboratory Supply Co. (Indianapolis, IN, U.S.A.) and were maintained on water and Purina chow *ad libitum*. HeLa cells (Rhino strain) growing in late exponential phase were received from Dr. Martin Evans, Virology Laboratory, The Children's Hospital, Columbus, OH, U.S.A.

A protocol modified from that of Reichard *et al.* (1962) successfully established a 5-fluorouracil-resistant subline. In this modified protocol, which was successful in the production of a resistant subline, 5-fluorouracil in 0.9% NaCl (20mg/kg; Sigma, St. Louis, MO, U.S.A.) was injected on days 2, 4, and 6 after intraperitoneal transfer of 2ml of a 1:3 (v/v) cell suspension. As a routine, at least 80% of these animals yielded 4ml of packed tumour cells on day 7.

Measurement of tumour-cell resistance

Resistance or sensitivity to 5-fluorouracil and 5-fluorouridine was estimated by the effect of a pharmacological dose of the base or nucleoside analogue on the mitotic index of the tumour *in vivo*. Tumour-bearing animals were given intraperitoneal injections of 120mg of 5-fluorouracil/kg (Klubes & Cernas, 1974) or 40mg of 5-fluorouridine/kg. Controls received 0.9% NaCl. Then 16h later the same animals received intraperitoneal injection of 1.6mg of the alkaloid Colcemid (Calbiochem, San Diego, CA U.S.A.)/kg to inhibit mitosis, and after an additional 1.5h the ascites fluid was removed and the cells were repeatedly washed with 0.9% NaCl with centrifugation at 150*g* for 5min to remove erythrocytes. Animals used in these comparisons yielded approx. 4ml of packed cells, with no apparent difference between treated or untreated animals. The saline-washed tumour cells were fixed for chromosome staining in methanol/acetic acid (3:1, v/v). Chromosomes were stained with Giemsa buffer, pH6.8 (GIBCO, Grand Island, NY, U.S.A.). Fixed slides were visually counted with a light-microscope equipped with a counting grid. The percentage of metaphase mitotic figures present in at least 3000 cells is reported as the mitotic index. The values reported are averages of at least two animals.

Enzyme purification and fractionation

Uridine kinase was partially purified and the isoenzymes were separated by the method of Krystal & Webb (1971). Briefly, tumour cells were washed with cold 0.9% NaCl, suspended in 2vol. of 200mM-Tris/HCl, pH7.4 (buffer A), and sonicated. The 105000*g* supernatant was fractionated with streptomycin sulphate, then $(NH_4)_2SO_4$, and a portion of the 30–50%-satd.-$(NH_4)_2SO_4$ precipitate, containing 10mg of protein, was applied to a column (58cm×0.635cm²) of Sepharose 6B; the enzyme was eluted with buffer B [containing 200mM-Tris/HCl (pH7.4), 20% (v/v glycerol and 20mM-mercaptoethanol] and 0.85ml fractions were collected for uridine kinase assay. All procedures were carried out at 4°C.

For subcellular-localization studies, the Novikoff ascites-tumour cells were swollen for 5min in 2vol. of 50mM-Tris/HCl (pH7.5)/10mM-$MgCl_2$/2.0mM-dithiothreitol, before homogenization with 25 strokes of a Dounce homogenizer. The homogenate was fractionated (Jackson & Chalkley, 1974) into subcellular components, and each fraction, except for the cytosol, was sonicated. The 105000*g* supernatant of each fraction was treated with streptomycin sulphate and $(NH_4)_2SO_4$ as outlined above. The 30–50%-satd.-$(NH_4)_2SO_4$ precipitate was resuspended in buffer B and dialysed against buffer B before assay for uridine activity. All protein concentrations were determined by the biuret method (Gornall *et al.*, 1948).

Uridine kinase assay

Uridine kinase activities were measured by a modification of the procedure of Krystal & Webb (1971). The final concentration of all components in the standard assay was 125mM-Tris/HCl (pH7.4)/10mM-$MgCl_2$/10mM-2-mercaptoethanol/10% (v/v) glycerol/1.0mM-ATP/0.4mM-[2-^{14}C]uridine (0.05μCi; New England Nuclear, Chicago, IL, U.S.A.)/0.4mg of enzyme protein/ml. Activities in column fractions were measured as a routine by adding 100μl of each column fraction to an equal volume of complete substrate mixture, and incubating for 20min at 23°C. The reaction was terminated by dilution with 2.0ml of 1.0mM-ammonium formate followed by heating at 95°C for 2min. The mixture was filtered through DEAE-cellulose filter discs (DE-81; Reeve Angel and Co., Clifton, NY, U.S.A.), the discs were washed with 1.0mM-ammonium formate, dried under vacuum, and then counted for radioactivity in a liquid scintillant (Scintisol; Isolab, Akron, OH, U.S.A.). Uridine kinase activity is expressed in units (nmol of uridine nucleotide formed/60min at 23°C).

Kinetics of partially purified isoenzymes

K_m and $V_{max.}$ values for nucleosides (uridine or 5-fluorouridine) and ATP were determined for each isoenzyme after separation on a Sepharose 6B column. Appropriate fractions of each peak free of cross-contamination were pooled (cf. Fig. 1) and diluted with buffer B to give an activity of 6nmol/h, because a preliminary study showed that this amount of enzyme activity had a linear velocity with minimum substrate concentrations (20μM-uridine, 13μM-5-fluorouridine or 30μM-ATP) for up to 20min of incubation at 23°C. Incubations for all subsequent kinetic studies were for 15min.

Nucleoside phosphorylation was studied with the ATP concentration fixed at 1.0mM. Uridine concentrations were varied from 20 to 400μM, with the specific radioactivity of uridine maintained at 600c.p.m./nmol. Fluorouridine concentrations were varied from 13 to 250μM, with a constant specific radioactivity of 5-fluoro[2-^{14}C]uridine (Nuclear Dynamics, El Monte, CA, U.S.A.) of 250c.p.m./nmol.

The K_m for each isoenzyme with respect to ATP was determined with the uridine concentration fixed at 400μM. The Mg^{2+} concentration was kept constant at 5, 10 or 25mM and the ATP concentration was varied from 20 to 1000μM. The effect of phosphate on enzyme activity was determined by the addition of 240mM-KH_2PO_4, pH7.4, to give final concentration from 4 to 33mM. The blanks for these kinetic studies consisted of boiled enzyme added to the appropriate reaction mixture for each kinetic point.

Phosphorylation of nucleosides by intact cells

Saline-washed tumour cells (10^6cells/ml) were preincubated for 15min at 37°C in medium RPMI-1640, pH7.7 (GIBCO) on a gyratory shaker (150rev./min) before measurement of nucleoside phosphorylation in the intact cells (Lee *et al.*, 1975). The [^{14}C]nucleoside concentration was adjusted within the range 5–100μM, with specific radioactivities identical with those specified above, and the cells were reincubated at 37°C for either 10 (uridine) or 15 (fluorouridine)min at 37°C on the rotary shaker. Both uptake and phosphorylation of the respective nucleosides was linear during these time-periods. The 0.9% NaCl-washed cells were extracted with cold 5% trichloroacetic acid to give the total acid-soluble radioactivity. The nucleotide content of the latter (i.e. phosphorylated nucleoside) was estimated by passing the acid-soluble fractions, after neutralization with an equal volume of 0.31M-NaOH in 200mM-Tris/HCl, pH7.4, through DEAE-cellulose filter discs (DE-81). The filter discs were washed with 1.9mM-ammonium formate and dried before radioactivity counting in a liquid scintillant (see above).

Since the rate of nucleoside conversion into nucleotide was a constant percentage of the rate of incorporation of nucleosides into total acid-soluble radioactivity, nucleotide-synthesis rates are expressed as a percentage of the $V_{max.}$ for incorporation of radioactivity into the acid-soluble pool. The K_m for phosphorylation by intact cells was calculated as the K_m for incorporation of radioactivity into the acid-soluble pool. The K_m is defined here as the concentration of nucleoside in the medium at which specific uptake into the acid-soluble pool is half-saturated.

The kinetic parameters for both the purified isoenzymes and the intact tumour cells were determined by a computer-calculated Lineweaver–Burk plot. The data were compiled on a Linc-Eight instrument (Digital Equipment Corp., Malboro, MA, U.S.A.).

Determination of uridine phosphorylase activity

Uridine phosphorylase (EC 2.4.2.1) was measured in the cytosol fraction, after dialysis for 12h against 200vol. of 0.05M-potassium phosphate, pH7.4 at 4°C, by the method of Reichard & Skold (1958). Activity is expressed as nmol of uracil formed/h per mg of protein, at 37°C.

Results and Discussion

The two uridine kinase isoenzymes present in rapidly growing tumours were measured as a routine in a 30–50%-satd.-$(NH_4)_2SO_4$ fraction of the 105000*g* supernatant of the sonicated cells (Krystal & Scholefield, 1971; Krystal & Webb, 1971; Keefer *et al.*, 1974). Because the sonication procedure may solubilize particulate-bound or organelle-bound fractions of enzyme, it was desirable to establish whether or not the two uridine kinase species found in the sonicated cell preparation originated in the same or different cell compartments. As shown in Table 1, the 30–50%-satd.-$(NH_4)_2SO_4$ precipitate of the cytosol fraction prepared by careful homogenization and fractionation of subcellular components contains 97% of the activity measured in whole-cell extracts. Subsequent fractionation on a Sepharose 6B column (results not shown) resolved two peaks of activity which represent 56% (isoenzyme I) and 44% (isoenzyme II) of the cytosol activity respectively. This distribution compares favourably with the distribution of the isoenzymes found (Fig. 1) after Sepharose 6B chromatography of the 30–50%-satd.-$(NH_4)_2SO_4$ fraction of 105000*g* supernatant of whole-cell sonicates (59% species I and 41% species II). It is also apparent that there is essentially no uridine kinase present in nuclei, mitochondria or microsomal fraction. Since the two species of uridine kinase are localized in the cytosol fraction, they must have the same metabolic pool and presumably

Table 1. *Subcellular distribution of uridine kinase isoenzymes I and II in Novikoff hepatoma cells*

The activities were measured on equivalent samples of the 30–50%-satd.-$(NH_4)_2SO_4$ fraction of a supernatant prepared by sonication in buffer of each subcellular fraction, then centrifuging at 105000*g* for 60min. The activity in the cell-free extract prepared from the sonicate was 60nmol/h per mg of protein and was taken as 100%. Values in parentheses indicate the fraction of activity in species I and II.

Cellular fraction	Uridine kinase activity (% of total)
Expt. A. Sonicated whole cells	
1. Cell-free extract	100
2. 30–50%-satd.-$(NH_4)_2SO_4$ fraction	117*
3. Sepharose 6B	
(*a*) Isoenzyme I	(59)
(*b*) Isoenzyme II	(41)
Expt. B. Subcellular fractions	
1. Cytosol	
(*a*) 30–50%-satd.-$(NH_4)_2SO_4$ fraction	97
(*b*) Sepharose 6B	
(i) Isoenzyme I	(56)
(ii) Isoenzyme II	(44)
2. Nuclei	1
3. Mitochondria	<1
4. Microsomal fraction	1

* The increase in activity was due to removal of interfering enzymes.

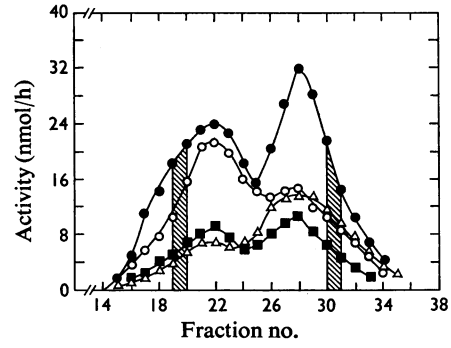

Fig. 1. *Sepharose 6B chromatography of the 30–50%-satd.-$(NH_4)_2SO_4$ fraction of cytosol from the S-37 sarcoma (●), the Novikoff hepatoma (○), the EL-4 leukaemia (■) and HeLa cells (△)*

Columns were loaded with 10mg of the $(NH_4)_2SO_4$ fraction and eluted with buffer B (see the Materials and Methods section), and fractions (0.85ml) were collected for assay. Enzyme activity is expressed as nmol/h per 0.1ml of each fraction. Fractions were pooled as shown (hatched area) and diluted with buffer B (if necessary) to approx. 6 units of activity before kinetic studies. All column profiles were reproducible in separate preparations to within 10%.

metabolic roles. Cytosol rather than membrane localization rules out a role for this enzyme in nucleoside transport, except for facilitated diffusion, whereby nucleoside uptake could be coupled to intracellular phosphorylation.

The Sepharose 6B elution profiles of uridine kinase from the EL-4 leukaemia, Novikoff hepatoma, S-37 sarcoma and HeLa cells are presented in Fig. 1. These rapidly growing tumour cells contain both the adult (maximum activity in fraction 22) and embryonic (maximum activity in fraction 28) forms of the enzyme, although the relative activities of the two isoenzymes vary. For kinetic studies fractions representing each enzyme species were pooled as shown (hatched areas). The incomplete resolution of species I in HeLa cells precluded kinetic studies of this particular isoenzyme. Table 2, which lists the $V_{max.}$ values for each isoenzyme in terms of activity per mg of the 30–50%-satd.-$(NH_4)_2SO_4$ fraction or per 5×10^6 cells, also shows the activities of the two isoenzymes in these cells. The EL-4 leukaemia and the S-37 sarcoma have similar distributions of the isoenzymes, with approx. 40% species I and 60% species II, but the total activity per cell or per mg of protein is significantly lower in the EL-4 leukaemia. In contrast, the Novikoff hepatoma has an isoenzyme distribution that is opposite to that in the EL-4 leukaemia and S-37 sarcoma (i.e. 60% type I and 40% type II); in HeLa cells the distribution is 30% type I and 70% type II.

The data in Table 2 further indicate that both species I and II in the EL-4 leukaemia and the S-37 sarcoma, as well as species II in HeLa cells, have K_m values for uridine of approx. 50μM. This is the same value reported by Krystal & Scholefield (1971) for both peaks of enzyme from the mouse Ehrlich ascites carcinoma. However, the uridine kinase isoenzymes purified from the Novikoff rat hepatoma repeatedly show differences in their affinities for both uridine and 5-fluorouridine (Table 2). Thus the K_m values for uridine phosphorylation by species I and II of the Novikoff hepatoma are 33 and 60μM respectively. With 5-fluorouridine substrate, the respective K_m values are 18 and 53μM. The differences between the mean K_m values of species I and II for four experiments were evaluated by Student's *t* test and were significant ($P<0.01$) for uridine and ($P<0.02$) for 5-fluorouridine.

The existence of two uridine kinase isoenzymes in rapidly growing tumours suggested that this enzyme might serve as a useful model system to determine whether activity of such key isoenzymes fluctuate in a predictive manner during chemotherapy with pyrimidine analogues. However, as shown below, the activities of the uridine kinase isoenzymes as

Table 2. *Kinetic parameters of partially purified uridine kinases*

The $V_{max.}$ values are expressed as nmol/h per 5×10^6 cells or nmol/h per mg of protein from the 30–50%-satd.-$(NH_4)_2SO_4$ fraction (numbers in parentheses). The values for $V_{max.}$ per cell or per mg of protein are based on calculations that 10mg of the 30–50%-satd.-$(NH_4)_2SO_4$ fraction can be isolated from 1×10^8 S37 sarcoma cells, 1×10^8 Novikoff hepatoma cells, 2×10^8 EL-4 leukaemia cells or 2.5×10^8 HeLa cells. K_m values are expressed as μM. Results reported are the means of two to four experiments that varied within 15%.

		Isoenzyme I		Isoenzyme II		
Enzyme source	Substrate	K_m	$V_{max.}$	K_m	$V_{max.}$	Total $V_{max.}$ (I+II)
Novikoff hepatoma	Uridine	33	70 (140)	60	47 (94)	117 (234)
	5-Fluorouridine	18	74 (147)	53	49 (99)	123 (246)
EL-4 leukaemia	Uridine	52	11.5 (47)	53	16.5 (67)	28 (114)
	Uridine+10mM-phosphate	52	7.8 (32)	40	7.1 (29)	15 (61)
HeLa cells	Uridine	—	9 (43)	46	19 (97)	28 (140)
	Uridine+10mM-phosphate	—	—	38	10.6 (54)	—
S-37 sarcoma	Uridine	48	70 (140)	59	110 (220)	180 (360)
	Uridine+10mM-phosphate	56	43.5 (87)	70	60 (120)	104 (207)

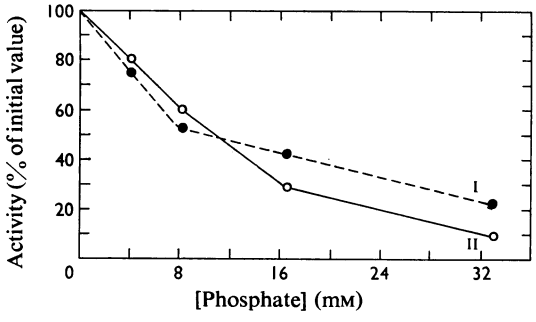

Fig. 2. *Effect of increasing phosphate concentration on the activities of uridine kinase species I (●) and II (○) isolated from the S-37 sarcoma*

Portions of enzyme obtained as described in legend to Fig. 1 were assayed in the presence of 0, 4.1, 8.3, 16.5 and 33mM-KH_2PO_4, pH7.4. The total activity of species I and II in the absence of phosphate was 140 and 220nmol/h per mg of protein.

measured *in vitro* under optimum conditions may not truly reflect their activities within the cell.

Fig. 2 shows that both forms of uridine kinase in the 30–50%-satd.-$(NH_4)_2SO_4$ fraction of the S-37 sarcoma cytosol are equally sensitive to inhibition by increasing phosphate concentration. As the phosphate in the assay medium is increased above 11.0mM, which is within the intracellular range (Levinson, 1970), species II is inhibited to a greater extent than is species I. This phosphate inhibition was non-competitive with respect to uridine, as indicated by the similar K_m values for both isoenzymes determined in the presence and absence of 10mM-phosphate (Table 2). In contrast, the $V_{max.}$ values were decreased by 40–50%.

In related experiments, the K_m for ATP was determined in the presence or absence of 10mM-phosphate for both species of uridine kinase isolated from the S-37 sarcoma. As shown in Table 3, the K_m for ATP at 10mM-Mg^{2+} does not change significantly in the presence of 10mM-phosphate, but the $V_{max.}$ for both species is decreased by 40–50%. Therefore phosphate was again a non-competitive inhibitor with respect to ATP. This conclusion was further supported by experiments (results not shown) which indicated that the activities of both species of uridine kinase from the Novikoff hepatoma were decreased rather than enhanced by the addition of excess of ATP in the presence of phosphate. Thus the activities of species I and II were 45 and 30% lower at 10mM-phosphate when the ATP concentration is 1.0mM.

The data in Table 3 also rule out the possibility that the inhibitory effect of phosphate is related to a decrease in the effective concentration of Mg^{2+}. Although 10mM-Mg^{2+} is the optimum concentration in the assay (cf. the Materials and Methods section), 20mM is the concentration normally found in the

Table 3. *Effect of Mg^{2+} concentration on the K_m for ATP of uridine kinase isoenzymes I and II*

Final ATP concentrations were 20, 50, 100, 200 and 1000μM with the uridine concentration kept at 0.4mM. The K_m values are expressed in μM, and $V_{max.}$ values are expressed as nmol/h. Values represent the mean of two experiments that varied within 10%.

	Species I			Species II		
$[Mg^{2+}]$ (mM)	K_m	$V_{max.}$	$K_m/V_{max.}$	K_m	$V_{max.}$	$K_m/V_{max.}$
5	66	6.3	10.5	118	7.2	16.4
10	81	6.5	12.5	90	6.7	13.4
25	106	6.1	17.4	37	5.4	6.9
10 (+10mM-phosphate)	95	3.3	28.8	91	3.7	24.6

cell (Webb, 1963). Increasing Mg^{2+} from 5 to 25mM increases the K_m of species I for ATP from 66 to 106μM; in contrast the K_m for species II falls from 118 to 37μM. The $V_{max.}$ values vary only slightly or not at all, and the $K_m/V_{max.}$ values vary in parallel with the K_m values. These results indicate that phosphate and Mg^{2+} act by independent mechanisms, since the former affects the $V_{max.}$, whereas the latter affects the K_m for ATP. The absence of a change in the $V_{max.}$ while the Mg^{2+} concentration is varied is consistent with the report by Krystal & Scholefield (1971) that both species of uridine kinase show a broad optimum range for bivalent cation concentrations.

The existence of two isoenzymes in the soluble cytoplasmic fraction of rapidly growing tumour cells (which in the Novikoff hepatoma had different substrate affinities), together with the demonstration that adjustment of the concentrations of phosphate, Mg^{2+} or ATP in the enzyme assay to those found in the cell markedly and in some instances differentially changed the isoenzyme activities, suggested that the assays *in vitro* may not give a true indication of the intracellular capacity of the cells to phosphorylate uridine or its analogues. Attempts were therefore made to develop 5-fluorouracil- (or 5-fluorouridine)-resistant cell lines in order to determine whether there was any correlation between tumour-cell resistance and activity of the isolated enzymes.

Fig. 3 shows the Sepharose 6B elution profile of the uridine kinase isoenzymes from the Novikoff hepatoma cells after growth of cells for one and two generations in the presence of subtherapeutic concentrations of 5-fluorouracil, i.e. 20mg/kg doses given on days 1, 3 and 5 after transplantation; the profile (with standard errors) of uridine kinase from the untreated (control) Novikoff hepatoma is shown for comparison. In the first transplant generation there is a 3.7-fold increase in the activity of species II, accompanied by a 45% decrease in the activity of species I. At the second transplant generation, the activities of species I and II are 40 and 230% respectively of those in the control. Thus the uridine kinase isoenzymes do fluctuate in activity during

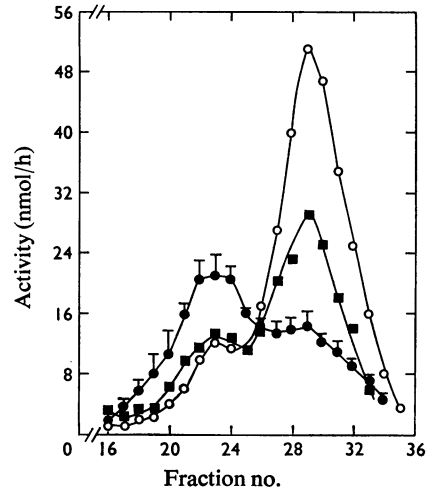

Fig. 3. *Sepharose 6B chromatography of uridine kinase preparations from first* (○) *and second* (■) *generations of the Novikoff hepatoma in the presence of 5-fluorouracil according to the protocol given in the Materials and Methods section*

The profile for the normal Novikoff hepatoma (●) ±S.E.M. (nine different preparations) is shown for reference. In all cases, a 10mg sample of protein from the 30–50%-satd.-$(NH_4)_2SO_4$ fraction was applied to a column (58cm×0.635cm²) of Sepharose 6B equilibrated with 200mM-Tris/HCl (pH7.4)/20mM-2-mercaptoethanol/20% glycerol. Fractions (0.85ml) were collected and assayed for activity, which is expressed as nmol of uridine nucleotide formed/h per 100μl of each fraction.

chemotherapy with 5-fluorouracil, as was previously demonstrated (Keefer *et al.*, 1974, 1975*b*) for the Novikoff hepatoma cells during treatment with subtherapeutic doses of 5-azacytidine (Keefer *et al.*, 1974, 1975*b*).

By carrying the Novikoff hepatoma cells for nine transplant generations in the presence of subtherapeutic doses of 5-fluorouracil by the protocol outlined in the Materials and Methods section, a

Table 4. *Mitotic indices of cells with or without therapeutic doses of 5-fluorouracil and 5-fluorouridine*
Animals weighing 160 and 180g received 0.5ml of 0.05% Colcemid, and were killed 1.5h later. Cells were fixed, stained and counted. The values reported represent the means of two animals, with variations less than 10% between animals. At least 3000 cells were counted in each preparation. The activity of isoenzymes I and II was measured with uridine and represents the peak activity (cf. Fig. 3). Activity is given as nmol of uridine formed/h per 100μl of the Sepharose 6B column fraction with the peak activity. Further details are given in the legend to Fig. 3.

Cells	Treatment (mg/kg)		Mitotic index (%)	Activity (uridine) Species I	Species II
Novikoff hepatoma	None		2.3	22	14
	5-Fluorouracil	(120)	0.5		
	5-Fluorouridine	(40)	0.7		
Nov/fluorouracil	None		2.2	18	24
Generation 5	5-Fluorouracil	(120)	1.03		
Nov/fluorouracil			2.2	18	17
Generation 6	5-Fluorouracil	(120)	2.05		
Nov/fluorouracil	None		1.9	17	16
Generation 7	5-Fluorouracil	(120)	1.7		
Nov/fluorouracil	None		1.9	24	15
Generations 7–9	5-Fluorouracil	(120)	1.9		
Generation 12	5-Fluorouridine	(40)	1.9		

resistant cell line was developed. This subline, denoted Nov/FU, was shown by mitotic indices to be fairly resistant to 5-fluorouracil as early as transplant generation 6 (Table 4). Despite the fluctuations in the relative isoenzyme activity measured after isolation, there was no clear-cut correlation between uridine kinase activity and the development of resistance. This lack of correlation could not be attributed to a loss of uridine phosphorylase, since the cells were also cross-resistant to 5-fluorouridine. Of interest, however, was the early increase in isoenzyme II, which has the lower affinity for the nucleoside analogues.

Attempts were made to correlate changes in isoenzyme profile during the development of tumour-cell resistance with changes in the rate of intracellular nucleoside phosphorylation. It was expected, on the basis of a previous study with intact cells (Lee *et al.*, 1975), that measurements of nucleotide synthesis by intact cells would provide a reasonable estimate of total uridine kinase activity and would also indicate the changes in intracellular uridine kinase activity.

As shown by the inset in Fig. 4, uridine phosphorylation in intact cells follows simple Michaelis–Menten kinetics. The rate of nucleotide synthesis was also a constant percentage of the rate of nucleoside uptake (i.e. increase in the acid-soluble radioactivity). Since both saturation curves had the same shape, the corresponding Lineweaver–Burk plots (Fig. 4) yield similar K_m values, i.e. 21μM for uridine uptake and 26μM for nucleotide synthesis. The saturation curves for 5-fluorouridine were comparable (N. Greenberg, unpublished work).

Table 5 lists the kinetic parameters for nucleoside uptake and phosphorylation by intact 5-fluoro-

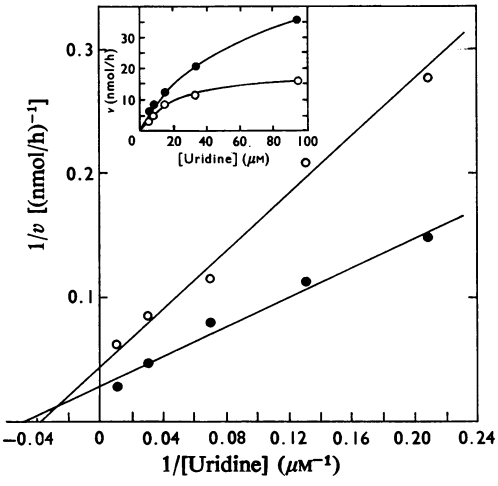

Fig. 4. *Lineweaver–Burk plot of the incorporation of uridine into total acid-soluble material* (●) *and acid-soluble nucleotides* (○) *in intact Novikoff hepatoma cells*
Inset is the v-versus-[S] plot of the same data. Cells were incubated at a concentration of 1×10^6/ml in medium RPMI-1640 at 37°C in the presence of increasing concentrations of uridine (final uridine concns. 4.8, 7.7, 14.4, 33.6 and 96μM). After incubation an equivalent volume of ice-cold medium was added before washing the cells and precipitating with 5% trichloroacetic acid. Activity (v) was expressed as nmol/h per 5×10^6 cells.

uracil-sensitive and -resistant Novikoff hepatoma cells and for the EL-4 leukaemia and S-37 sarcoma. In the untreated (sensitive) cell lines the K_m values for uridine ranged from 3.9μM for the EL-4 leuk-

Table 5. *Kinetic parameters for nucleoside phosphorylation by intact cells*

The $V_{max.}$ values for uptake of nucleosides into total acid-soluble material and for synthesis of nucleotides by intact cells are expressed as nmol/h per 5×10^6 cells. The percentage of material phosphorylated (% phosphorylated) in the acid-soluble pool is the mean for all concentrations measured in the substrate-saturation curve. The K_m values are expressed in μM. Results represent the means of three experiments that varied within 15%.

Cells	Substrate	K_m	$V_{max.}$ (uptake)	% phosphorylated	$V_{max.}$ (nucleotide synthesis)
(I) Sensitive					
(*a*) Novikoff hepatoma	Uridine	20	22	56	12
	5-Fluorouridine	74	28	52	15
(*b*) S-37 sarcoma	Uridine	13	46	—	—
(*c*) EL-4 leukaemia	Uridine	4	8	56	4
	5-Fluorouridine	23	6	68	4
(II) Resistant					
Nov/fluorouracil	Uridine	145	51	48	24
Generations 7, 8, 9	5-Fluorouridine	532	67	68	45

aemia to 20μM for the Novikoff hepatoma, a range comparable with that reported for other cell lines (Plagemann, 1971; Stambrook *et al.*, 1973). The K_m values for 5-fluorouridine uptake in both the Novikoff hepatoma and the EL-4 leukaemia were 4–6-fold higher than those values for uridine uptake. Since the affinities of the partially purified isoenzymes were similar for uridine and fluorouridine as substrates (Table 2), the results in Table 5 suggest that the affinity constants measured in the intact cell represent some other parameter, most probably the affinity of the membrane-transport system for the substrates. In fact, the K_m values are consistent with those for transport for substrates reported elsewhere (Plagemann, 1971; Stambrook *et al.*, 1973). Finally, a comparison of the $V_{max.}$ for nucleoside phosphorylation (per 5×10^6 cells) by the various untreated cell lines (Table 5) correlated poorly with the amount of uridine kinase recovered from these cells (cf. Table 2); in fact, the $V_{max.}$ for phosphorylation in the intact cell represents only 10–20% of the maximum extractable kinase activity as measured under optimum conditions. Thus the uridine kinase appears to be functioning far below its potential optimum in the tumour cells, and nucleoside transport, which precedes phosphorylation, is a limiting factor in pyrimidine nucleoside phosphorylation. This possibility is also reported by studies on the 5-fluorouracil- (or 5-fluorouridine)-resistant cell line, discussed below.

A comparison of the appropriate values in Table 5 indicates that the $V_{max.}$ for nucleoside uptake and subsequent phosphorylation for both uridine and 5-fluorouridine in the fluorouracil-resistant line, i.e. Nov/FU generations 7, 8 and 9, are at least 2-fold greater than the corresponding $V_{max.}$ values for the sensitive Novikoff hepatoma cells. Further, this increase in apparent $V_{max.}$ does not correlate with the extractable uridine kinase activity (cf. Table 4). However, the percentage of nucleoside that becomes phosphorylated subsequent to transport is similar in both the sensitive and the resistant line for both uridine and 5-fluorouridine. This change in the K_m is significant despite the change in apparent $V_{max.}$, since calculations using the rate equation for first-order processes $\{v = (V_{max.} \cdot [S])/(K_m + [S])\}$ show that the rate of 5-fluorouridine transport into the Nov/FU cells at extracellular 5-fluorouridine concentrations of 100, 50 and 10μM are 65, 50 and 33% of the corresponding rates of transport into normal Novikoff hepatoma cells; less than 10μM-fluorouridine is known to inhibit rRNA synthesis and maturation (Wilkinson *et al.*, 1975). These data further support the possibility that the rates of pyrimidine nucleoside synthesis by intact cells are regulated at a step before uridine kinase; this step must necessarily include nucleoside transport.

Because of recent suggestions that nucleoside transport may be linked to nucleoside phosphorylase activity (Li & Hochstadt, 1976) and studies indicating its loss in certain 5-fluorouracil-resistant tumour-cell sublines (Reichard *et al.*, 1959), this enzyme was assessed in the sensitive and Nov/FU cells. The uridine phosphorylase activities were 653 ± 14 and 680 ± 21 nmol/h per mg of cytosol protein in the sensitive and resistant cells respectively, indicating no significant difference.

Since uridine kinase plays a key role in the synthesis of pyrimidine nucleotides and nucleotide analogues from the corresponding nucleosides in rapidly growing tumours (Anderson & Brockman, 1964) and the intracellular concentration of substrate is probably well below the K_m (cf. Almersjo *et al.*, 1975), moderate adaptive changes in the concentration of uridine kinase could have a very significant effect on the intracellular rate of phosphorylation. Because transport, or a very closely related parameter before nucleoside activation by uridine kinase, was

rate-limiting, accurate measurements of intracellular uridine kinase activity are impossible. However, because the K_m value for transport of 5-fluorouridine into the tumour cells was 4–6-fold higher than that for uridine uptake, whereas the K_m values for the phosphorylation of both substrates by the isolated uridine kinases are similar, as for thymidine kinase and thymidine transport (Freed & Mezger-Freed, 1973), uridine kinase activity is distinct from nucleoside transport. Finally, the decreased nucleoside transport observed in Nov/FU cells re-emphasizes the importance of this parameter, shwon by Fisher (1962) to be a factor in the development of resistance to methotrexate.

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