Biochem. J. (1975) **148**, 295–302
Printed in Great Britain

Thymidine Transport in Phytohaemagglutinin-Stimulated Pig Lymphocytes

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(*Received* 16 *December* 1974)

Thymidine and uridine transporters in peripheral pig lymphocytes have structural features in common, but are not identical. Accelerated entry of [^{3}H]thymidine begins 12h after the addition of phytohaemagglutinin. The increased thymidine uptake into the cells is characterized by an increase in $V_{max.}$ without alteration of the apparent K_m (0.6±0.08μM). Thymidine kinase activity is increased 12h after stimulation. Both the increased thymidine uptake and the increased thymidine kinase activity are inhibited in cultures incubated with puromycin: rates of degradation of the two systems are unchanged after phytohaemagglutinin addition, and indicate similar half-lives of about 2h. Thymidine kinase is rate-limiting for thymidine entry up to 18h after phytohaemagglutinin addition; increase in its synthesis is detectable about 6h before net incorporation of thymidine into DNA is significantly promoted.

Addition of phytohaemagglutinin to lymphocytes elicits a number of transport and synthetic events associated with the transition of cells from their resting G_0 state and subsequent passage through the cell cycle (Hausen & Stein, 1968; Kay, 1968; Peters & Hausen, 1971). This includes an immediate increase in uridine uptake, owing to a rise in the apparent $V_{max.}$ for the process (Peters & Hausen, 1971). Thymidine entry is not immediately promoted (Hausen & Stein, 1968), but has been found in pig lymphocytes to rise 12–18h after the addition of phytohaemagglutinin (Barlow & Ord, 1974) but before the onset of significant DNA synthesis at 20h. Increased amounts of thymidine kinase can also be detected after 12h. The concomitant phosphorylation of thymidine has therefore been investigated, since thymidine kinase activity may be rate-limiting for thymidine uptake (Hare, 1970; Schuster & Hare, 1971). The specificity of the thymidine-transporter system was also examined.

Experimental

Methods

Preparation of leucocyte cultures. Whole pig blood was mixed with heparin (2000i.u./litre) on collection at the slaughterhouse. The blood was left for 1h at room temperature (20°C), after which time the upper half was decanted and diluted 9:1 with 6% (w/v) Dextran solution (Dextraven 150; Fisons Ltd., Loughborough, Leics., U.K.). The mixture was allowed to sediment for a further 2h at 37°C, after which the leucocyte-rich plasma fraction was decanted from the agglutinated erythrocytes and centrifuged at 100g for 5min in a BTL bench centrifuge. The supernatants were discarded and the cell pellets treated by the method of Fallon *et al.* (1962) to remove contaminating erythrocytes, except that all solutions were supplemented with heparin (2i.u./ml) to prevent coagulation of cells. Leucocytes were retrieved by centrifugation at 100g for 5min, the pellets washed once in iso-osmotic saline (0.9% NaCl) and finally resuspended in warm (37°C) medium. The cell concentration was adjusted to 2×10^6/ml, and the cultures were incubated in an atmosphere of air+CO_2 (95:5) for 24h at 37°C before further treatment.

Nigrosin-exclusion testing after this time showed viability was greater than 95%, falling to 90% by 48h.

Culture medium. Cells were cultured in Parker 199 tissue culture medium (Gibco Bio-Cult Biochemicals Ltd., Paisley, Renfrew, U.K.) supplemented with 5% (v/v) foetal bovine serum (Gibco), 2.2g of $NaHCO_3$/litre, 100mg of glutamine/litre, 100i.u. each of penicillin and streptomycin/ml (Glaxo Laboratories Ltd., Greenford, Middx., U.K.) and 2i.u. of heparin/ml (Evans Medical Ltd., Speke, Liverpool, U.K.).

Preparation of acid-soluble extracts. Batches of 2×10^7 cells were harvested by centrifugation at 1000g for 2min. The supernatants were discarded and the tubes immediately set on ice. The pellets were washed twice in ice-cold iso-osmotic saline; 0.5ml of the second wash was removed for scintillation counting and all but 0.2ml of the remainder discarded. Then 0.2ml of 10% (w/v) trichloroacetic acid was added, the pellet resuspended and the radioactivity in the acid-soluble extract determined. The radioactivity in the last wash contributed not more than 5% of the radioactivity in the acid-soluble extracts;

corrections were made for this in calculating radioisotope uptakes.

Preparation of DNA extracts. Acid-precipitated material was washed twice in ice-cold 5% (w/v) trichloroacetic acid. The pellet was then resuspended in 0.4ml of acid and the nucleic acids were extracted after incubation at 70°C for 20min; 0.2ml of extract was taken for scintillation counting.

Preparation of protein extracts. Material from which the nucleic acids had been removed was washed twice in water and then digested in 0.4ml of 1M-NaOH at 60°C for 2h. The digest was then neutralized with 0.1ml of 5M-HCl before withdrawal of 0.1ml for scintillation counting.

Preparation of cytoplasmic extracts for enzyme analysis. Cells (2×10^8) were washed three times in iso-osmotic saline, then lysed by three freeze-thaws in 1ml of a buffer containing 0.15M-KCl, 3mM-β-mercaptoethanol and 10mM-Tris–HCl, pH8.0. The lysate was separated from the cell debris by centrifugation at 1000000*g* for 1h in a Spinco model L centrifuge. The clear supernatant was used as the source of the enzyme. If not immediately used for assay it was stored at −20°C, but never for more than 24h.

Assay of thymidine kinase. The method of Ives *et al.* (1963) was used. The standard assay contained the following in a volume of 1.0ml: 0.2ml of nucleoside, 0.2ml of enzyme and 0.6ml of stock assay solution. Final concentrations were: ATP 9.0mM, $MgCl_2$ 9.0mM, 3-phosphoglycerate (potassium salt) 7.5mM, KCl 0.154M, and Tris–HCl 50mM, pH8.0. The concentration range for thymidine was 0.1–0.5μM in kinetic experiments or 0.1μM for single concentration measurements, with a radioactive concentration of 10μCi/ml of assay.

The reaction was started by addition of enzyme to the assay mixture in a shaking water bath at 37°C. After 10min the tubes were removed and heated for 2min in a boiling-water bath. The heated samples were stored at −20°C to await chromatographic separation.

Column chromatography. Thymidine and dTMP were separated on Dowex-1 (formate form) columns (1.5cm×0.5cm, void volume 0.3ml; Dowex-1 (Cl^- form; 8% cross-linked, dry mesh 200–400; Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.) by the method of Hurlbert *et al.* (1954) by using stepwise elution. Enzyme assay volumes of 1.0ml were mixed with 1.0ml of a carrier solution containing 1mM-thymidine and 1mM-dTMP.

Paper-chromatographic analysis of acid-soluble fractions. Trichloroacetic acid was extracted from the solutions with ether and the method of Lane (1963) was used to separate thymidine and its nucleotides.

Incorporation of radioactive isotopes and measurement of radioactivity. Nucleoside uptake was determined by measurement of the incorporation of [5-^{3}H]-uridine (27.9Ci/mmol) and [6-^{3}H]thymidine (25.4Ci/mmol) into the acid-soluble fraction. Cultures, prepared as described above, were exposed to the tritiated precursor (2μCi/ml) together with a range of concentrations of unlabelled nucleoside. In kinetic experiments duplicate measurements, made 5.0, 7.5 and 10.0min after addition of label, allowed calculation of the initial velocity of uptake. Protein synthesis was determined from the incorporation of L-[4,5(*n*)-^{3}H]lysine (8.7Ci/mmol) by exposing cultures for 2h to a tracer radioactive concentration of 4μCi/ml. All radioactivity measurements were made in a Beckman liquid-scintillation counter model no. CPM 2000. Volumes of 0.5ml or less were counted for radioactivity in a scintillation fluid containing 0.8% (w/v) butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] and 8% (w/v) naphthalene in 1,4-dioxan. Volumes in excess of 0.5ml were counted for radioactivity in a scintillation fluid containing 70% (v/v) toluene, 30% (v/v) Triton X-100 with 0.35% (w/v) butyl-PBD. Sufficient counts were recorded to give an accuracy of ±3% and the efficiency of ^{3}H counting was approx. 50%.

Materials

Phytohaemagglutinin (purified: Wellcome Research Laboratories, Beckenham, Kent, U.K.) was reconstituted in water and added to cultures at a final concentration of 3.0μg/ml. Puromycin hydrochloride was obtained from Sigma. Nitrobenzylthioguanosine

Table 1. *[^{3}H]Thymidine uptake and incorporation into pig lymphocyte cultures 12–24h after phytohaemagglutinin*

Trichloroacetic acid (5%, w/v)-soluble and -insoluble fractions were prepared as described under 'Methods'. Each result represents the average of five separate determinations of uptake and incorporation from 2×10^7 cells labelled with 0.1μM-[^{3}H]thymidine at 2μCi/ml for 10min: S.E.M. values are indicated.

Time after phytohaemagglutinin addition (h)	$10^3\times$Thymidine uptake (pmol/10min per 10^6 cells)	
	Acid-soluble fraction	Acid-insoluble fraction
0	4.0±0.52	1.4±0.32
12	4.9±0.26	2.6±0.10
14	5.7±0.52	3.0±0.21
16	6.2±0.33	3.6±0.07
18	7.1±0.46	4.2±0.25
20	11.2±0.33	7.5±0.20
22	11.7±0.43	8.7±0.20
24	19.9±1.40	19.2±1.90

Table 2. [^3H]*Thymidine distribution in the acid-soluble fractions of phytohaemagglutinin-treated cells*

Ice-cold trichloroacetic acid (5%, w/v) extracts were prepared from 10^8 cells exposed to 0.1 μM-[^{3}H]thymidine at 2 μCi/ml for 10 min. Thymidine and its nucleotides were separated by ascending chromatography (Lane, 1963). Chromatograms were treated as described under 'Methods'. Recovery was approx. 80%.

Time after phytohaemagglutinin addition (h)	Distribution of acid-soluble radioactivity (% of total)			
	Thymidine	dTMP	dTDP	dTTP
0	48.8	3.9	12.7	34.6
12	43.6	8.2	17.1	31.1
14	28.0	7.3	24.0	40.7
16	24.3	3.3	30.1	42.3
18	13.6	12.0	33.5	40.9
20	9.4	10.5	36.9	43.2
24	8.2	11.4	33.4	47.1

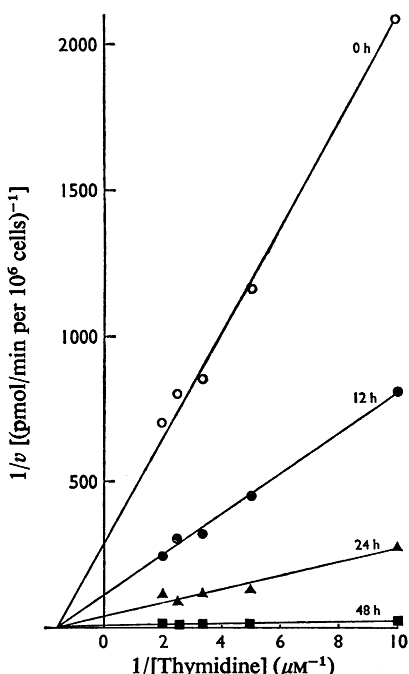

Fig. 1. *Lineweaver–Burk plot of* [^3H]*thymidine uptake* 0, 18, 24 *and* 48 *h after phytohaemagglutinin addition to pig lymphocyte cultures*

Initial velocities of uptake were determined from duplicate samples of 2×10^7 cells withdrawn 5, 7.5 and 10 min after addition of various concentrations of [^{3}H]thymidine at 2 μCi/ml, as described under 'Methods'. Regression lines were plotted by the method of least squares. $K_m = 0.62 \pm 0.07$ μM.

was a gift from the National Institutes of Health, Bethesda, Md., U.S.A. Radioactive compounds were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Thymidine uptake and incorporation into DNA 0–24 *h after phytohaemagglutinin*

Earlier reports (Forsdyke, 1968; Goldberg *et al.*, 1969) have shown that S phase for phytohaemagglutinin-stimulated pig lymphocytes begins between 24 and 48 h after the addition of the mitogen. To determine the relation between the increased influx of thymidine and the onset of DNA synthesis, [^{3}H]-thymidine uptake into trichloroacetic acid-soluble and -precipitable material was measured 0–24 h after stimulation with phytohaemagglutinin in cultures exposed to a constant amount of thymidine and ^{3}H radioactivity (Table 1). Increased uptake of thymidine was detectable at about 12 h; if incorporation into DNA is calculated relative to that into the acid-soluble fraction, increased incorporation into DNA was evident from about 20 h. No measurements were made of intracellular concentrations of thymidine nucleotides; the distribution of ^{3}H radioactivity between the acid-soluble thymidine derivatives (Table 2) is discussed below.

Kinetic investigations of uridine uptake after phytohaemagglutinin stimulation have shown this to be associated with an increase in apparent $V_{max.}$ without alteration of the K_m (Peters & Hausen, 1971). A similar relationship applies after serum activation of mouse cells (Lemkin & Hare, 1973), and the same kinetic principles have been applied to Novikoff hepatoma and chick embryo cells (Plagemann, 1971; Scholtissek, 1968).

In the present study an investigation into the kinetics of thymidine uptake showed that the transport system was saturated at about 2.0 μM external thymidine. Entry of nucleoside was linear with time for 10–20 min. Initial velocities of uptake at various external thymidine concentrations 0, 18 and 24 h after the addition of phytohaemagglutinin were used in the construction of Lineweaver–Burk (1934) plots

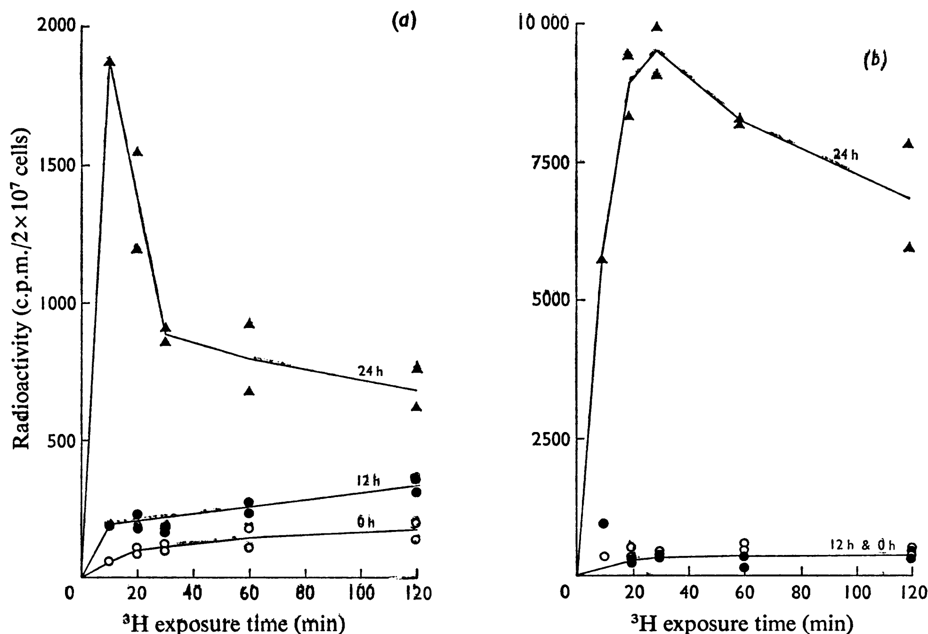

Fig. 2. *Time-course of (a) dTMP and (b) dTTP production in phytohaemagglutinin-treated cells compared with controls*

Duplicate samples (10ml) were withdrawn from 100ml cultures containing 2×10^6 cells/ml, at various times after addition of [^{3}H]thymidine. Acid-soluble extracts were prepared for paper-chromatographic analysis as described under 'Methods'. Nucleotide spots made visible by u.v. light were cut out and the eluted radioactivity determined by scintillation counting.

(Fig. 1). The apparent $V_{max.}$ for uptake increased without alteration of the apparent K_m ($0.62\pm0.07\,\mu$M). This value for K_m is similar to others reported in different cell types (Mizel & Wilson, 1972; Plagemann & Erbe, 1974).

Thymidine phosphorylation 12–24h after phytohaemagglutinin

Thymidine kinase (EC 2.7.1.75) is required for utilization of exogenous thymidine. It was therefore necessary to determine whether the transport or phosphorylation of thymidine was rate-limiting in its entry into the cell.

Analysis of the distribution of ^{3}H among thymidine nucleotides in the acid-soluble fraction of cells subsequent to phytohaemagglutinin treatment showed that at all times the radioactivity in dTMP was less than that in dTDP which was in turn less than that in dTTP (Table 2), suggesting that the equilibrium of the phosphorylation sequence was well to the right. The greatest change appeared to be in the increased proportion of dTDP, which doubled between 12 and 20h. At none of the times studied was any thymidine catabolism detectable.

When the time-course of dTMP and dTTP production during a 2h incubation with thymidine in stimulated cells was compared with untreated controls, rapid conversion of dTMP into dTTP was shown by 24h, at the start of DNA synthesis (Fig. 2). Even in cultures at 24h, less than 10% of the thymidine in the medium was utilized, so that the rapid fall in uptake into dTMP in these cultures 30min after incubation is unlikely to be due to exhaustion of extracellular thymidine. Intracellular concentrations of dTTP after 30min incubation cannot be less than $0.5\,\mu$M. Contributions to the dTTP pool from endogenous synthesis between 12 and 24h after phytohaemagglutinin stimulation require investigation.

When thymidine kinase activity was assayed in the 100000g supernatants, the apparent $V_{max.}$ of the enzyme was found to increase after phytohaemagglutinin stimulation without detectable alteration of the K_m of $4.3\,\mu$M (Fig. 3). This K_m is comparable with estimations in other cell types (Bresnick & Thompson, 1965; Berk & Clayton, 1973).

Under the conditions of the kinase assay, conversion into dTDP and dTTP was undetectable. The accuracy of the method is limited by the extent of conversion into dTMP, which did not exceed 10% of

the [^{3}H]thymidine initially present. Small changes in K_m would not therefore be detected. In other cell systems, under conditions of rapid growth, changes in the proportion of thymidine kinase isoenzymes have been reported (Adler & McAuslan, 1974).

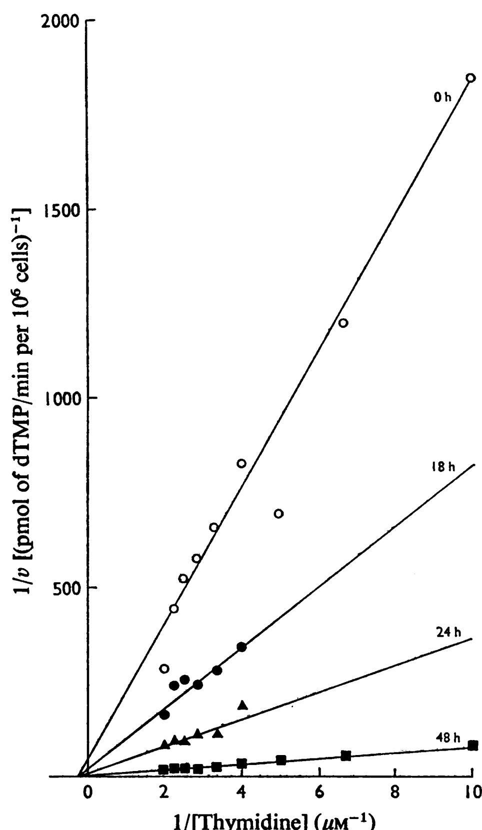

Fig. 3. *Lineweaver–Burk plot of thymidine kinase* (*EC* 2.7.1.75) *activity* 0, 18, 24 *and* 48*h after phytohaemagglutinin addition*

The enzyme was assayed as described under 'Methods'. Regression lines were plotted by the method of least squares. $K_m = 4.3 \pm 2.1\,\mu$M.

Thymidine phosphorylation was calculated per 10^6 cells and the increase in apparent $V_{max.}$ could be due either to an increased amount of enzyme or to an increase in its activity. Comparison of the ratio of the apparent $V_{max.}$ in the presence of phytohaemagglutinin with that in its absence, for thymidine uptake and for thymidine kinase, showed them to be approximately equal up to the time of the earliest rise in DNA synthesis, after which the relative increase in uptake was considerably greater than that of kinase activity (Table 3). The marked increase in thymidine uptake 24h after the addition of phytohaemagglutinin was therefore no longer solely attributable to the availability of the phosphorylating enzyme. In human lymphocytes the peak in thymidine kinase activity after phytohaemagglutinin occurred between 50 and 70h, after the peak of DNA synthesis (Pegoraro & Bernengo, 1971); detailed analyses were not made before the onset of DNA synthesis at about 20h.

Effect of puromycin on thymidine uptake and phosphorylation

Thymidine uptake 15, 18 and 24h after the addition of phytohaemagglutinin was inhibited by prior exposure to puromycin (10μg/ml) (Table 4). Lysine incorporation into the soluble proteins of the cells was completely prevented (Table 5). Thymidine kinase activity was also decreased. Treatment with puromycin decreased thymidine uptake below that found in control cells analysed at the time when the inhibitor was added to the experimental cultures. This suggested that the system for thymidine transport might have a relatively high turnover in lymphocytes. The half-lives for the transport system for thymidine and for thymidine kinase were both approx. 2h (Fig. 4) and were unchanged in cultures after phytohaemagglutinin stimulation. Possible changes in rates of degradation after puromycin treatment (see Schimke & Doyle, 1970) were not examined.

Table 3. *Extent of stimulation of* [^{3}*H*]*thymidine uptake and thymidine kinase* 0–48*h after phytohaemagglutinin addition*

$V_{max.}$ is expressed as pmol of thymidine or dTMP/min per 10^6 cells.

Time after phytohaemagglutinin addition (h)	Uptake		Kinase	
	$V_{max.}$	$\frac{V_{max.}}{V_{max.}\text{ (control)}}$	$V_{max.}$	$\frac{V_{max.}}{V_{max.}\text{ (control)}}$
0	3.45	—	24.0	—
18	8.94	2.6	53.6	2.2
24	26.1	7.6	120.4	5.0
48	340.0	98.6	525.4	21.8

Table 4. *Thymidine uptake and kinase activity in phytohaemagglutinin-treated cells exposed to* 10 μg *of puromycin/ml*

Cells were exposed to 10 μg of puromycin/ml for 2h before harvest at various times after phytohaemagglutinin addition. In uptake measurements [^{3}H]thymidine was added 10min before withdrawal of samples of 2×10^7 cells. In kinase experiments sufficient cells were harvested for duplicate or triplicate determinations. Results are means±S.E.M. for the numbers of determinations given in parentheses.

Time after phytohaemagglutinin addition (h)	$10^3\times$Thymidine uptake (pmol/10min per 10^6 cells)		$10^3\times$Thymidine kinase (pmol of dTMP/10min per 10^6 cells)	
	−Puromycin	+Puromycin	−Puromycin	+Puromycin
0	4.7±0.56 (11)	3.2±0.34 (6)	7.0±0.77 (7)	5.4±0.75 (4)
13	7.0±0.83 (11)	—	—	—
15	9.1±1.32 (6)	4.2±0.26 (6)	—	—
16	9.7±0.42 (6)	—	—	—
18	10.3±0.62 (11)	4.5±0.39 (11)	7.4±0.80 (6)	5.3±0.40 (7)
22	34.4±5.91 (5)	—	—	—
24	43.5±2.87 (11)	19.3±1.76 (11)	12.2±2.26 (7)	2.4±1.24 (7)

Table 5. L-[^{3}*H*]*Lysine incorporation into cytoplasmic and total protein in the presence and absence of* 10 μg *of puromycin/ml*

Uptake into cytoplasmic (supernatant) protein was determined with cultures used for the assay of thymidine kinase (see Table 4) which were incubated with L-[^{3}H]lysine for 2h. Incorporation was measured as described in the text and is expressed as counts/2h per 10^6 cells.

Time after phytohaemagglutinin addition (h)	^{3}H radioactivity	
	−Puromycin	+Puromycin
Cytoplasmic protein		
0	15.1, 11.7	11.1
18	63.2, 57.5	8.6, 5.0
24	80.6, 98.8	15.3
Total protein		
0	2482±291 (3)	2960±317 (3)
18	4129±521 (3)	2927±484 (3)
24	3779±502 (3)	1807±69 (3)

Specificity of the thymidine-transport system

The increased uptakes of thymidine and uridine after phytohaemagglutinin show distinctly different time-courses (Ling, 1971). In erythrocytes (Cass & Paterson, 1972) the nucleosides are believed to enter on the same transporters, but in liver competition between uridine and thymidine was not found (Ord & Stocken, 1973). The pig lymphocyte cultures showed a higher affinity for thymidine than for uridine (Table 6), and thymidine transport was easily inhibited by uridine, which, however, was markedly less effective against thymidine kinase. Conversely thymidine was a poor inhibitor of uridine uptake.

The mutual inhibition suggested some structural similarities in the two transporters. To test this, the effect of two further inhibitors on the uptake of the nucleosides was investigated. The nucleoside analogue nitrobenzylthioguanosine and *p*-chloromercuribenzoate competitively inhibited thymidine and uridine uptake with approximately the same K_i against the two nucleosides (Table 6). Thymidine kinase was again relatively less affected, suggesting that the two inhibitors were combining with structurally related sites on the two transporters.

Discussion

The competition studies suggested that the transporters for uridine and thymidine had structures in common but were not identical, and that both were present in resting lymphocytes. In contrast with the fate of thymidine, little free uridine is present intracellularly in unstimulated lymphocytes. It is believed (Kay & Handmaker, 1970) that the capacity for uridine phosphorylation is sufficient to allow the immediately increased entry of this nucleoside after stimulation with phytohaemagglutinin.

In cells other than from the liver it has commonly been observed (see Everhart & Rubin, 1974; Plagemann *et al.*, 1974) that [^{3}H]thymidine is only taken up in association with DNA synthesis. In the pig lymphocyte cultures used here, kinetic studies indicated that initially thymidine kinase was a rate-limiting step for thymidine uptake and that the increase in uptake detected as early as 12h after the addition of phytohaemagglutinin was due to greater kinase activity. The sensitivity of both uptake and kinase to inhibition by puromycin confirmed the close association between the two events. The unchanged rate of disappearance of the kinase from puromycin-inhibited cultures suggested that the increase in phosphorylating activity from 12h was caused by increased synthesis of the enzyme rather than by any change in the rate of degradation of the

Table 6. *Inhibition of [^{3}H]uridine and [^{3}H]thymidine uptake, and of thymidine kinase*

K_i values for inhibition of uptake were calculated from Lineweaver–Burk plots by using 0.1–0.5 μM-[^{3}H]thymidine and 10–50 μM-[^{3}H]uridine. Calculation of initial velocities of uptake were as described under 'Methods'. The K_i values for inhibition of thymidine kinase were estimated from graphs of initial rate of phosphorylation of thymidine versus inhibitor concentration. Preliminary experiments were made to determine suitable concentrations of inhibitors for use in construction of the reciprocal plots. These were as follows: *p*-choromercuribenzoate 20.0 μM, nitrobenzylthioguanosine 1.0 μM. Uridine versus [^{3}H]thymidine, 0.50 μM; thymidine versus [^{3}H]uridine, 150.0 μM. K_m and K_i values are expressed as μM.

	[^{3}H]Uridine uptake	[^{3}H]Thymidine uptake	Thymidine kinase
K_m uptake or kinase	28.7	0.63	4.3
K_i versus uridine	—	0.7	>10.0
K_i versus thymidine	122.0	—	—
K_i versus *p*-chloromercuribenzoate	64.5	61.9	200.0
K_i versus nitrobenzylthioguanosine	1.1	1.3	>10.0

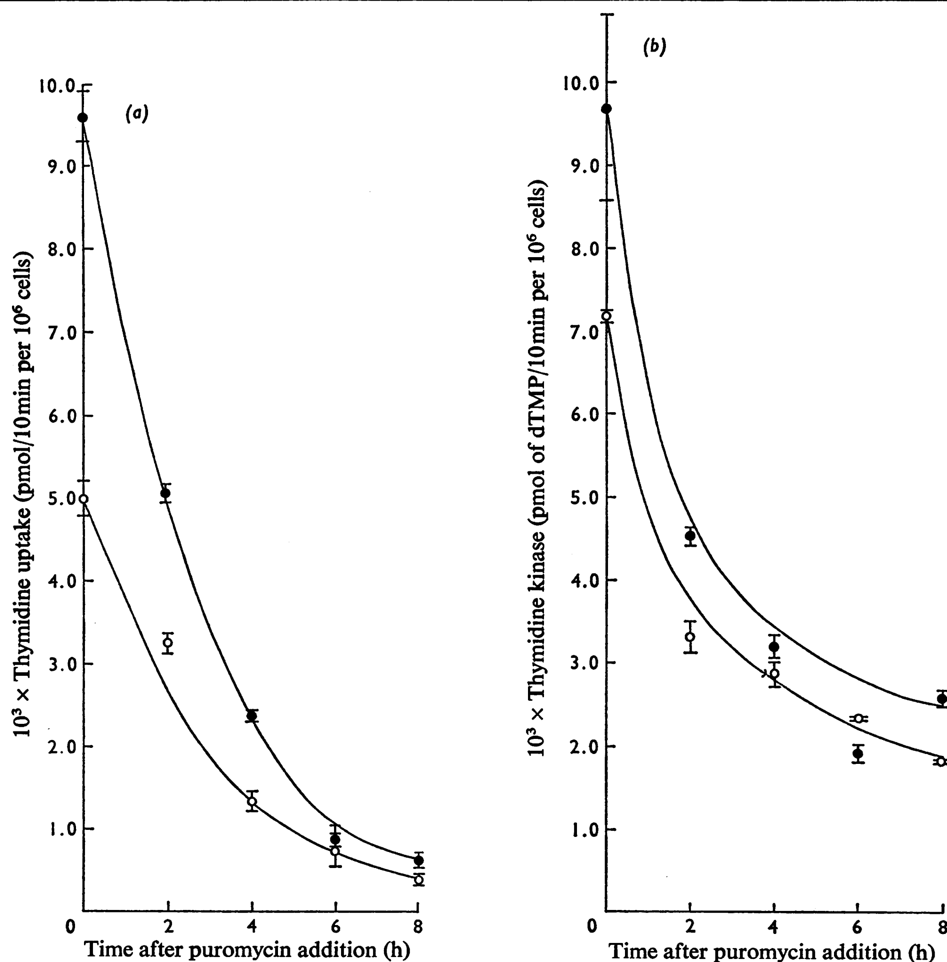

Fig. 4. *Half-life determination of thymidine uptake* (*a*) *and kinase activity* (*b*)

Estimations of half-lives were made by measuring [^{3}H]thymidine uptake and phosphorylation at intervals of 2h over an 8h period of exposure to puromycin at 10 μg/ml, which corresponded to the interval 12–20h after phytohaemagglutinin addition. The times at which uninhibited values were decreased by 50% were then determined. Uptake measurements were made on triplicate samples of 2×10^7 cells exposed to [^{3}H]thymidine for 10min. Kinase assays in duplicate were made on cells harvested in parallel. Half-life estimates were checked by construction of semi-log plots. Rate of decay in activity was approximately first-order in both cases. Thymidine uptake: half-life of control, 2.2h; phytohaemagglutinin-stimulated cultures, 2.2h. Thymidine kinase: half-life of control, 2.3h; phytohaemagglutinin-stimulated cultures, 2.0h. ○, Control; ●, +phytohaemagglutinin.

protein. The signal eliciting the greater synthesis remains to be established.

dTMP stabilizes thymidylate kinase (Hiatt & Bojarski, 1960). This may account for the twofold increase in proportion of [^{3}H]dTDP in the acid-soluble nucleotides between 12 and 18h. The increase in thymidine incorporation into DNA through this period seemed principally attributable to increased amounts of the nucleoside phosphates. Not until after 20h did other, unidentified, factors appear to be involved, promoting both DNA synthesis and thymidine uptake into the cells, without affecting the amounts of dTMP and dTDP.

A relatively short half-life for thymidine kinase is appropriate for its classification in that group of proteins whose concentrations show periodic fluctuations within the cell cycle (Mitchison, 1971). It also raises the possibility that in regenerating liver, where thymidine kinase activity is undetectable until the start of the S phase, the enzyme may normally be present in low concentrations in the G_0 state, although its detection is precluded because of the very high rate of catabolism of thymidine in these cells.

S. D. B. has a Research and Training Scholarship from the Medical Research Council. Financial assistance from the Cancer Research Campaign is also gratefully acknowledged.

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