Deoxycytidine Transport and Pyrimidine Deoxynucleotide Metabolism in Phytohaemagglutinin-Stimulated Pig Lymphocytes

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Increased entry of deoxy[^3H]cytidine begins at about 12h after addition of phytohaemagglutinin to peripheral pig lymphocyte cultures, and is accompanied by a parallel stimulation of deoxycytidine kinase up to the beginning of DNA synthesis at 24h. The increased deoxycytidine uptake is characterized by an increase in V_{max} without alteration of the apparent K_m ($0.7 \pm 0.11 \mu\text{M}$). Although the entries of both nucleosides are promoted at the same time, the stimulation of deoxycytidine uptake is less than that of thymidine, and the two nucleosides are transported by separate systems. In addition to deoxycytidine kinase, the synthesis of deoxycytidylate deaminase and thymidylate synthetase are stimulated after addition of phytohaemagglutinin, but to a lesser extent than that of thymidine kinase. The importance of the latter enzyme in forming dTMP, and of thymidylate kinase in providing dTTP, is discussed.

Our previous investigations (Barlow & Ord, 1975) showed that a specific increase in thymidine transport occurred before the S phase in phytohaemagglutinin-stimulated pig lymphocytes. Since the major deoxynucleoside in circulating blood is deoxycytidine (Schneider, 1955; Schneider & Brownell, 1957), the utilization of this large pool by the stimulated lymphocyte was examined. Like all the other nucleosides, deoxycytidine is only of use to the cell in the phosphorylated form. The restriction of deoxycytidine kinase to predominantly lymphoid tissue (Durham & Ives, 1969) suggested that the enzyme may have a salvage function in these cells, which are exposed to relatively high concentrations of deoxycytidine. Also, in view of the earlier findings with thymidine kinase (Barlow & Ord, 1975), the phosphorylation of deoxycytidine may also limit its uptake.

There are numerous reports of elevated activity of enzymes associated with the synthesis of deoxynucleotides, after the stimulation of quiescent cells to active growth (Bollum & Potter, 1959; Hiatt & Bojarski, 1960; Maley & Maley, 1960). Since the appearance of dTMP may be a rate-limiting step in DNA synthesis, the extent to which endogenous routes and salvage pathways contributed to the total cell content of dTMP were investigated. In addition to the deoxynucleoside kinases, the activities of deoxycytidylate deaminase, thymidylate synthetase and thymidylate kinase were examined after the stimulation of lymphocytes by phytohaemagglutinin.

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Experimental

Methods

Preparation of leucocyte cultures. Cultures were prepared from whole pig blood as described previously (Barlow & Ord, 1975). Serum was dialysed against 0.9% NaCl before use in the culture medium.

Extraction of DNA and the acid-soluble material. Acid-soluble and DNA extracts were prepared as described previously (Barlow & Ord, 1975).

Preparation of cytoplasmic extracts for enzyme analysis. Cells (10^9) were washed three times in 0.9% NaCl, then lysed by three freeze-thaws in 1 ml of buffer. The lysate was separated from the cell debris by centrifugation at 1000g for 5 min in a BTL bench centrifuge, followed by centrifugation at 100000g for 1 h in a Spinco model L centrifuge. The clear supernatant was used as the source of enzyme. Extracts were kept on ice and enzymes were assayed within 2 h of their preparation.

Enzyme assays. All assays contained the following in a volume of 100 μl : 20 μl of nucleoside or nucleotide, containing 2 μCi of ^3H -labelled substrate; 20 μl of enzyme; 60 μl of appropriate assay solution. After 10 min at 37°C the reactions were terminated by heating for 2 min in a boiling-water bath, followed by cooling on ice. The procedure for stopping thymidylate synthetase is given below. Observed initial velocities were directly proportional to the amounts of added extract for each of the enzyme assays.

Assay of deoxycytidine kinase. Cells were lysed in a buffer containing 0.15 M-KCl, 3 mM-2-mercaptoethanol and 10 mM-Tris/HCl, pH 8.0. The enzyme was assayed by the method of Ives *et al.* (1969).

Final concentrations were: 10mM-ATP; 10mM-MgCl₂; 7.5mM-3-phosphoglycerate (potassium salt); 50mM-Tris/HCl, pH 8.0. The concentration of deoxycytidine used was 1.0–10.0 μ M.

Assay of deoxycytidylate deaminase. Cells were lysed in a buffer containing 20mM-Tris/HCl, pH 7.4, 2mM-2-mercaptoethanol, 1.5mM-MgCl₂ and 0.3mM-dCTP. The enzyme was assayed by the method of Maley & Maley (1964) as modified by Gelbard *et al.* (1969). Final concentrations were: 2mM-MgCl₂; 0.1mM-dCTP; 40mM-Tris/HCl, pH 7.9. The concentration of dCMP used was 0.25–2.0mM for kinetic studies and 1.0mM for single concentration measurements.

Assay of thymidylate synthetase. Cells were lysed in the same buffer as was used for the extraction of deoxycytidylate deaminase, dCTP being omitted. The enzyme was assayed by the method of Lomax & Greenberg (1967). The released ³H was separated from unchanged [³H]dUMP by adsorption of the latter by Norit A (Kammen, 1966). The reaction was stopped by addition of 500 μ l of Norit A solution (100mg/ml of 1.0mM-NaH₂PO₄·2H₂O/1.0mM-Na₂HPO₄·12H₂O) to 100 μ l of assay. The charcoal was washed with two 500 μ l samples of phosphate buffer, and the pooled washes were syringed through a 0.45 μ m Millipore filter and the filtrates counted for radioactivity in 10ml of Triton/toluene scintillant (Barlow & Ord, 1975). Background counts accounted for approx. 10% of the total measured. Final concentrations used in the assay were: 50mM-Tris/HCl, pH 7.4; 20mM-MgCl₂; 15.0mM-formaldehyde; 100mM-2-mercaptoethanol; 0.5mM-DL-tetrahydrofolate. The tetrahydrofolate was dissolved in 2-mercaptoethanol and added to formaldehyde before mixing with the remaining constituents. The concentration of dUMP used was 1.0–10.0 μ M for kinetic experiments and 0.2mM for single concentration measurements.

Assay of thymidylate kinase. Cells were lysed in a buffer containing 0.15M-KCl, 10mM-Tris/HCl, pH 7.4, 0.4mM-2-mercaptoethanol, 1.0mM-MgCl₂

and 2 μ M-TMP. The enzyme was assayed by a modification of the method of Valotaire & Duval (1972). Final concentrations were: 8.0mM-ATP; 16.0mM-MgCl₂; 32.0mM-3-phosphoglycerate (potassium salt); 0.15M-KCl; 0.32mM-2-mercaptoethanol; 40mM-Tris/HCl, pH 7.4. The concentration of TMP used was 1.0–10.0 μ M for kinetic experiments and 0.2mM for single concentration experiments.

Chromatographic methods. In the deoxycytidine kinase assay, deoxycytidine was separated from its monophosphate by t.l.c. Polyethyleneimine-cellulose-coated plastic sheets (Camlab Ltd., Cambridge, U.K.) were developed by ascending chromatography in water (Randerath & Randerath, 1964a). Deoxycytidine, dCMP, dUMP and dCTP were separated by a two-dimensional method (Randerath & Randerath, 1964b). Nucleotides were recovered as described by Randerath & Randerath (1966). Samples (0.8ml) from total volumes of 1.0ml of eluate were taken for radioactivity counting.

In the deoxycytidylate deaminase assay, dCMP and dUMP were separated by ascending chromatography on DEAE 81 cellulose paper (Northern Media Ltd., Brough, N. Humberside, U.K.) developed in 4M-formic acid/0.1M-ammonium formate (Ives *et al.*, 1963). This method was also used in the separation of deoxycytidine with dCMP, dUMP and dCTP in acid-soluble extracts. Radioactivity was eluted with 1.0ml of 0.1M-HCl/0.2M-KCl (Ives *et al.*, 1969) and was determined *in situ* after addition of 10ml of Triton/toluene scintillant.

dTMP and dTTP from the thymidylate kinase assay were separated by the method of Lane (1963).

Incorporation of radioactive isotopes and measurement of radioactivity. Kinetic measurements of nucleoside uptake and radioactivity-counting procedures were as described previously (Barlow & Ord, 1975). The specific radioactivities of the nucleosides and nucleotides used were as follows: deoxy[5-³H]-cytidine (21.2 Ci/mmol); [5-³H]dCMP (3.8 Ci/mmol); [Me-³H]dTMP (25.6 Ci/mmol); [5-³H]dUMP (11 Ci/mmol).

Table 1. Deoxy[³H]cytidine uptake and incorporation into pig lymphocyte cultures 0–24 h after addition of phytohaemagglutinin

Trichloroacetic acid (5%, w/v)-soluble and -insoluble fractions were prepared as described previously (Barlow & Ord, 1975). Each value represents the mean ($\pm$ S.E.M.) of three separate determinations of uptake and incorporation from 2×10^7 cells. In uptake experiments, cells were labelled with 0.1 μ M-deoxy[³H]cytidine at 2 μ Ci/ml for 10 min. In incorporation experiments, cells were labelled with 5.0 μ M-deoxy[³H]cytidine at 2 μ Ci/ml for 60 min.

Time after phytohaemagglutinin addition (h)	$10^3 \times$ Deoxycytidine uptake (pmol/10 min per 10^6 cells)	Deoxycytidine incorporation (pmol/60 min per 10^6 cells)
0	7.9 ± 0.73	0.21 ± 0.02
12	8.7 ± 0.35	0.17 ± 0.05
16	9.2 ± 0.54	0.40 ± 0.01
20	12.5 ± 0.66	0.41 ± 0.04
24	23.5 ± 1.72	0.69 ± 0.06

Materials

Purified phytohaemagglutinin (Wellcome Research Laboratories, Beckenham, Kent, U.K.) was reconstituted in water and added to cultures at a final concentration of $3.0 \mu\text{g/ml}$. Puromycin hydrochloride and DL-tetrahydrofolate were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Radioactive compounds were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Deoxycytidine uptake and incorporation into DNA 0–24 h after phytohaemagglutinin treatment

In preliminary investigations of the relationship between the uptake of deoxycytidine and its incorporation into DNA, cells were exposed to a constant amount of deoxy ^3H cytidine at intervals between 0 and 24 h after addition of phytohaemagglutinin (Table 1). As with ^3H thymidine, studied previously (Barlow & Ord, 1975), the increased entry of deoxycytidine was detectable at 12 h after addition of the mitogen,

Fig. 1. Lineweaver-Burk plot of deoxy ^3H cytidine uptake 0, 18 and 24 h after addition of phytohaemagglutinin 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 deoxy ^3H cytidine at $2 \mu\text{Ci/ml}$, as described previously (Barlow & Ord, 1975). A linear regression procedure was used to obtain three values of K_m . Since the estimates were not significantly different, convergent lines were drawn in agreement with the calculated mean value of $K_m = 0.70 \pm 0.11 \mu\text{M}$.

Fig. 2. Lineweaver-Burk plot of deoxycytidine kinase (EC 2.7.1.74) activity 0, 18 and 24 h after addition of phytohaemagglutinin

The enzyme was assayed as described under 'Methods'. Lines were constructed as described in Fig. 1 for a K_m value of $4.5 \pm 1.4 \mu\text{M}$.

but the rate of increase with elapsed time was less: a threefold stimulation of deoxycytidine uptake at 24 h compared with a fivefold stimulation of thymidine transport. The stimulation of incorporation of deoxy- ^3H cytidine into DNA was also noticeably less than that of ^3H thymidine.

The deoxycytidine-transport system was saturated at about $1.0 \mu\text{M}$ external nucleoside, and the rate of entry was linear for 10 min. Measurements of initial velocities of uptake were used in the construction of Lineweaver-Burk (1934) plots (Fig. 1). The apparent V_{max} for uptake increased without alteration of the apparent K_m ($0.70 \pm 0.11 \mu\text{M}$). This value was similar to those previously reported (Cunningham & Remo, 1973; Plagemann & Erbe, 1974), and approximated to that of thymidine uptake ($0.62 \pm 0.01 \mu\text{M}$) in pig lymphocytes (Barlow & Ord, 1975).

Deoxycytidine phosphorylation 0–24 h after phytohaemagglutinin treatment

When deoxycytidine kinase (EC 2.7.1.74) activity was assayed in 100000g extracts the apparent V_{max} of the enzyme was found to increase after phytohaemagglutinin stimulation, without alteration of the K_m ($4.5 \pm 1.4 \mu\text{M}$) (Fig. 2). Since activity was calculated per 10^6 cells, the increase in apparent V_{max} could have been due either to increased synthesis of

Table 2. *Extent of stimulation of deoxy[³H]cytidine uptake and deoxycytidine kinase 0–24h after addition of phytohaemagglutinin* $V_{\max}$ is expressed as pmol of deoxycytidine or dCMP/min per 10^6 cells.

Time after phytohaemagglutinin addition (h)	Uptake		Kinase	
	$V_{\max}$	$V_{\max}$	$V_{\max}$	$V_{\max}$
		$V_{\max}$ (control)		$V_{\max}$ (control)
0 (control)	2.56	—	36.0	—
18	3.92	1.53	49.6	1.38
24	7.08	2.77	113.0	3.14

Table 3. *Deoxy[³H]cytidine 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 $0.1 \mu\text{M}$ -deoxy[³H]cytidine at $2 \mu\text{Ci/ml}$ for 10 min. Deoxycytidine, its nucleotides and dUMP were separated by two-dimensional chromatography on polyethyleneimine-cellulose-coated plastic sheets by the method of Randerath & Randerath (1964b). Chromatograms were treated as described under 'Methods'. Recovery was approx. 90%.

Time after phytohaemagglutinin addition (h)	Distribution of acid-soluble radioactivity (% of total recovered)			
	Deoxycytidine	dCMP	dUMP	dCTP
0	9.2	14.3	18.2	58.3
12	9.0	11.3	21.4	58.4
16	8.0	8.0	24.4	59.5
20	5.8	7.4	29.0	58.0
24	4.8	11.6	32.6	51.0

enzyme or to an increase in its activity. To distinguish between these two possibilities, the rates of decay of the enzymes, prepared from resting cells or cultures 12h after addition of phytohaemagglutinin, were compared in the same way as for thymidine kinase investigated previously (Barlow & Ord, 1975). After complete inhibition of protein synthesis by $10 \mu\text{g}$ of puromycin/ml, estimation of the half-lives gave values of approx. 4h in the presence and absence of phytohaemagglutinin.

The stimulation of deoxy[³H]cytidine uptake and deoxycytidine kinase were compared by calculating the relative stimulation of the two processes from $V_{\max}$ values determined at 18 and 24h after addition of phytohaemagglutinin (Table 2). At each time, the stimulation of both processes was approximately equal.

Specificity of deoxycytidine uptake

Plagemann & Erbe (1974) reported that deoxy-nucleosides are transported by different systems in Novikoff hepatoma cells. In view of the similarity of the apparent K_m values for thymidine and deoxycytidine uptake obtained in pig lymphocytes, mutual inhibition of transport of the two nucleosides was tested. Neither thymidine nor deoxycytidine affected the uptake of the heterologous nucleoside when up

to 1000-fold excess (below $100 \mu\text{M}$) of potential inhibitor over natural substrate was used.

Composition of the acid-soluble pool

Early work by Maley & Maley (1962) showed that deoxy[2-¹⁴C]cytidine was more efficiently incorporated into the thymidine of DNA than into the deoxycytidine. Initial steps in the utilization of deoxycytidine involve either a deamination to deoxyuridine or a phosphorylation to dCMP. Subsequent reactions of these compounds yield dUMP, either by a deamination of dCMP catalysed by deoxycytidylate deaminase, or by a phosphorylation of deoxyuridine by thymidine kinase. With deoxy[³H]cytidine either of these steps would eventually result in the loss of the radioactive marker on conversion of dUMP into dTMP by thymidylate synthetase. Incorporation of dTTP derived from dTMP synthesized by this route would not therefore be detectable radioisotopically.

Since in this work the stimulation of incorporation of deoxy[³H]cytidine into DNA was less than that of [³H]thymidine at 24h, it was decided to determine the relative activities of the two routes from thymidine and deoxycytidine to TMP. The first part of this investigation involved examination of the composition of the acid-soluble pool after exposure of cells to

deoxy[^3H]cytidine. This was followed by analysis of the enzymes concerned in the conversion of deoxycytidine into dTMP.

Two-dimensional chromatography of acid-soluble extracts of cells prepared at various times after addition of phytohaemagglutinin showed that less than 10% of deoxy[^3H]cytidine entering the cell remained as free nucleoside. The rest of the intracellular label

was accounted for in dCMP, dUMP and dCTP (Table 3). No other derivatives were detectable radioisotopically.

At all times after addition of phytohaemagglutinin, dCTP was the most heavily labelled derivative, and this proportion remained fairly constant up to 24h. There was also little variation in the appearance of label in dCMP over this period, although studies of

Fig. 3. Time-course of ^3H incorporation into (a) dCMP, (b) dUMP, (c) dCTP and (d) DNA 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 deoxy[^3H]cytidine. Acid-soluble extracts were prepared for DEAE-cellulose paper-chromatographic analysis as described under 'Methods'. Nucleotide spots made visible by u.v. light were cut out and the eluted radioactivity was determined by scintillation counting.

Fig. 4. Lineweaver-Burk plots of (a) deoxycytidylate deaminase (EC 3.5.4.12), (b) thymidylate synthetase and (c) thymidylate kinase (EC 2.7.4.9) activity 0, 18 and 24 h after addition of phytohaemagglutinin

The enzymes were assayed as described under 'Methods'. Lines were constructed as described in Fig. 1 with the K_m values given in Table 5.

deoxycytidine kinase activity *in vitro* had shown that dCMP production was increasing steadily. The accumulation of label in dUMP rose uniformly between 0 and 24h, suggesting a progressive increase in the activity of deoxycytidylate deaminase.

Time-course of the appearance of ³H in dCMP, dUMP, dCTP and DNA in phytohaemagglutinin-treated cells compared with controls

The production of dCMP, dUMP and dCTP was measured during a 2h exposure of cells to deoxy[³H]-cytidine (Fig. 3). Deoxycytidine and dCMP were not distinguished by the chromatographic system used here. However, a parallel experiment in which dCMP with dUMP were separated from deoxycytidine by another system confirmed that the appearance of label in the deoxycytidine-dCMP spot shown in Fig. 3(a) is representative of the accumulation of dCMP.

Table 4. *Half-lives of thymidine kinase, deoxycytidine kinase, deoxycytidylate deaminase, thymidylate synthetase and thymidylate kinase prepared from cells at 0 and 12h after phytohaemagglutinin treatment*

The enzymes were assayed in 100000g supernatants prepared from resting and stimulated cells 12h after phytohaemagglutinin treatment, harvested at intervals of 2h after addition of 10 µg of puromycin/ml to the cultures. Half-lives were estimated from plots of log (enzyme activity) versus period of exposure to puromycin. The concentrations of substrate used in the enzyme assays were: thymidine 0.1 µM; deoxycytidine 1.0 µM; dCMP 1.0 mM; dUMP 1.0 µM; TMP 1.0 µM.

Enzyme	Half-life (h)	
	Control	+Phytohaemagglutinin
Thymidine kinase	2.1	2.0
Deoxycytidine kinase	5.1	5.0
Deoxycytidylate deaminase	3.0	3.0
Thymidylate synthetase	10.0	3.6
Thymidylate kinase	2.6	2.7

The time-course of dCMP production 24h after addition of phytohaemagglutinin contrasted markedly with that of dTMP (Barlow & Ord, 1975), where maximum production occurred after 10min of exposure to [³H]thymidine. At all times dUMP accumulated more slowly than dCMP after the first 10min of exposure to deoxy[³H]cytidine (Fig. 3b). The initial rate of production of dCTP was faster than that of the other derivatives, and was greatest 24h after addition of phytohaemagglutinin (Fig 3c). The incorporation of ³H into DNA remained linear over the 2h period of exposure to deoxy[³H]cytidine (Fig. 3d), so the fall in production of dCTP at 60min was not the result of an inhibition of DNA synthesis, and is not readily explained.

Kinetic analysis of deoxycytidylate deaminase, thymidylate synthetase and thymidylate kinase 0-24h after addition of phytohaemagglutinin

To compare the relative stimulation of these three enzymes, activity at 0, 18 and 24h was normalized by calculating apparent *V*_{max.} values from Lineweaver-Burk plots (Fig. 4). In all cases the apparent *V*_{max.} of the enzyme increased after phytohaemagglutinin stimulation, without detectable alteration of the *K*_m.

An investigation of the time-course of stimulation of the three enzymes, by using single saturating concentrations of substrate (not shown), indicated a steady rise in activity from the time of addition of the mitogen, but with a progressively faster rate of change. Pegoraro & Bernengo (1971) and Loeb *et al.* (1970), studying deoxycytidylate deaminase and thymidylate kinase respectively in phytohaemagglutinin-stimulated lymphocytes, did not observe changes in activity until after 24h, but no detailed measurements were made before the onset of DNA synthesis. In this work thymidylate kinase showed a unique form of behaviour, corroborated by determinations of *V*_{max.} at 18 and 24h, in reaching a peak of activity at 16h, followed by a decline.

Enzyme activity was calculated per 10⁶ cells. Determination of half-lives before and after addition of

Table 5. *Extent of stimulation of thymidine kinase, deoxycytidine kinase, deoxycytidylate deaminase, thymidylate synthetase and thymidylate kinase at 18 and 24h after addition of phytohaemagglutinin*

*V*_{max.} is expressed as pmol of product/min per 10⁶ cells. The stimulation ratio is *V*_{max.}/*V*_{max.} (control).

Enzyme	<i>K</i> _m (µM)	<i>V</i> _{max.}			Stimulation ratio	
		0h (Control)	18h	24h	18h	24h
Thymidine kinase	4.3 ± 2.1	24.0	53.6	120.4	2.2	5.0
Deoxycytidine kinase	4.5 ± 1.4	36.0	49.6	113.0	1.4	3.1
Deoxycytidylate deaminase	300 ± 30	2540	3410	6940	1.3	2.7
Thymidylate synthetase	2.6 ± 0.06	9.2	16.8	24.8	1.8	2.7
Thymidylate kinase	2.1 ± 0.4	14.8	34.0	30.0	2.3	2.0

phytohaemagglutinin confirmed that, in each case, increased activity was the result of enzyme synthesis *de novo*, since the rates of decay were not decreased after stimulation (Table 4). Thymidylate synthetase was unusual in that its half-life decreased from 10 to 3 h after phytohaemagglutinin treatment.

The stimulation ratios for enzyme activity at 18 and 24 h calculated from values of $V_{\max}$ for each reaction are shown in Table 5; with the exception of thymidylate kinase, the activity of all other enzymes studied increased up to 24 h after addition of phytohaemagglutinin. The greatest stimulation was for thymidine kinase; deoxycytidine kinase, deoxycytidylate deaminase and thymidylate synthetase were all stimulated to approximately the same extent.

Discussion

The timing of the stimulated uptake of thymidine and deoxycytidine differs from that of other compounds previously reported (see Ling & Kay, 1975). The increases in transport, beginning at about 12 h after phytohaemagglutinin stimulation, were primary events, and not the result of an increased demand for nucleoside phosphates after major DNA synthesis had begun at 24 h. As for thymidine uptake studied previously (Barlow & Ord, 1975), kinetic experiments showed that the nature of the deoxycytidine-transport system remained unchanged after phytohaemagglutinin treatment.

During the period before the onset of DNA synthesis, the stimulation of both thymidine and deoxycytidine phosphorylation was equal to that of their respective uptakes. It therefore appeared that the entry of the deoxynucleosides at this time was controlled by their rate of phosphorylation. Since there was no mutual inhibition of uptake, the two deoxynucleosides presumably utilized separate transport systems.

The four enzymes studied in this work, together with thymidine kinase reported previously (Barlow & Ord, 1975), were all found to be unstable. The increases in activity were the result of synthesis of enzymes *de novo*, as opposed to decreases in their rate of degradation.

Measurement of the $V_{\max}$ of deoxycytidylate deaminase showed that at all times the concentration of dUMP (its product) would potentially be far in excess of that required to saturate thymidylate synthetase. Nevertheless, dUMP did not accumulate. Intracellular concentrations of dCMP [estimated at less than 0.02 mM in rat thymus by Ord & Stocken (1958)] are likely to be far below the value of the K_m of deoxycytidylate deaminase. Possibly this enzyme operated *in vivo* well below its maximal activity.

The synthesis of the three enzymes concerned with the conversion of deoxycytidine into dTMP were

stimulated equally 24 h after addition of phytohaemagglutinin, but thymidine kinase appeared to be of prime importance for the formation of dTMP. Both routes to dTTP synthesis converge at the thymidylate kinase stage. It is well known that this enzyme is stabilized by its substrate dTMP, and regulation of its activity at this level has been suggested (Weissman *et al.*, 1960; Hiatt & Bojarski, 1961; Kielley, 1970). The lability of thymidylate kinase in the absence of its substrate may provide a means by which thymidine kinase could control the concentration of dTTP in the intact cell.

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