THYMIDYLATE KINASE ACTIVITY DURING SYNCHRONOUS GROWTH OF *CHLORELLA PYRENOIDOSA*

by

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INTRODUCTION

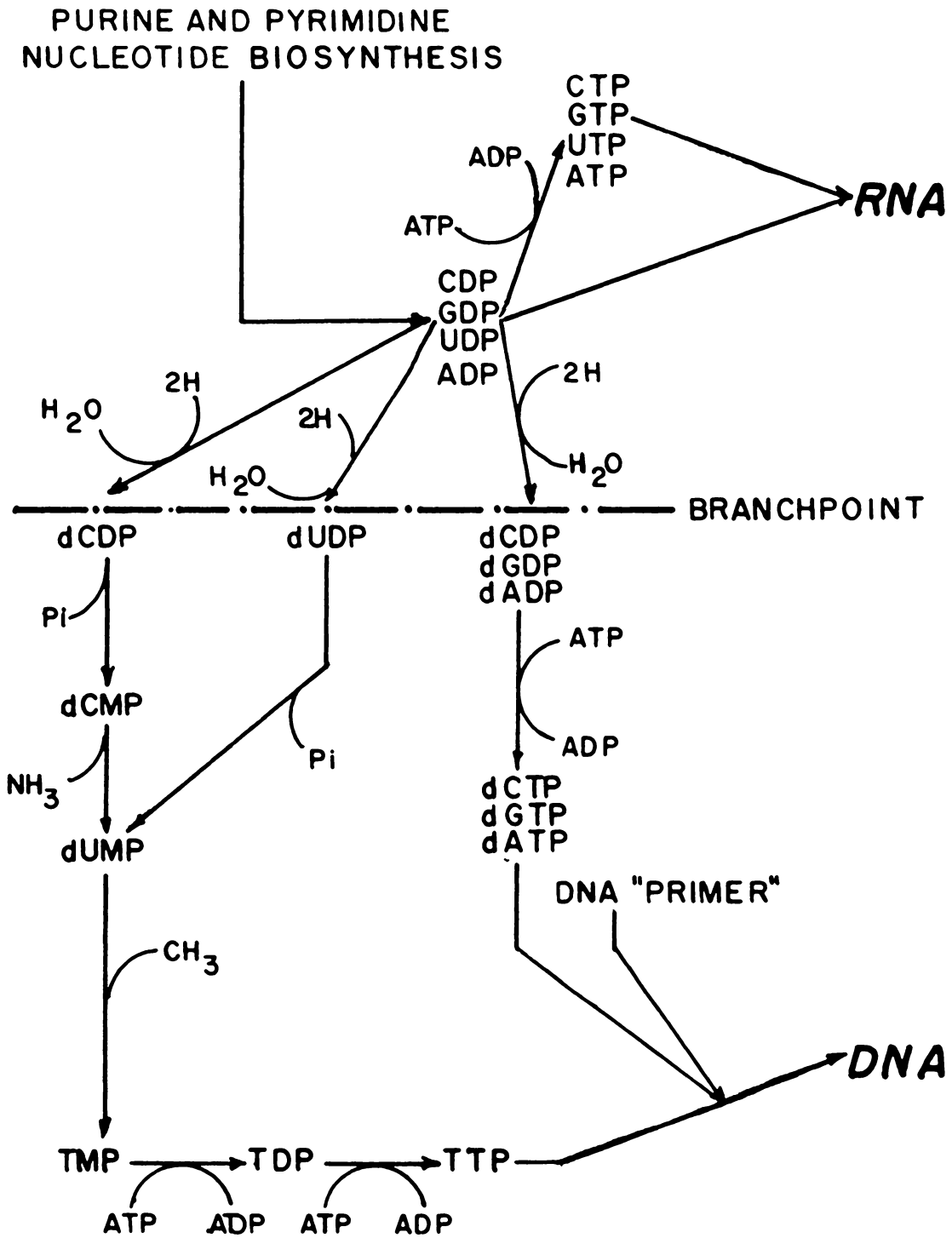
The biosynthesis of deoxyribonucleic acid (DNA) has been found to be a periodic event during the growth and division cycle of most cells[10-16]. Before a cell can duplicate itself, its DNA must be replicated. The period during which this replication takes place varies considerably in duration among cells from different organisms, but generally it occurs only during or immediately prior to the period of nuclear division.

Herrmann and Schmidt[16] have observed periodic synthesis of DNA in synchronous cultures of Chlorella pyrenoidosa. During early cellular development in this organism, the level of DNA (as percent of total cellular-P or cellular dry weight) remained essentially constant. At the onset of nuclear division, however, the level of DNA increased markedly. In contrast, the ribonucleic acid (RNA) level increased at a nearly constant rate throughout these same periods of cellular development. The differential rates of accumulation of these nucleic acids are of interest from the standpoint of the biochemical mechanisms which control their synthesis and catabolism. Comprehensive reviews by Heidelberger[40], Changeux[32], Jacob and Monod[33], Lark[34], and Umbarger[35] provide excellent discussions of control phenomena which may regulate periodic shifts in the levels of these and other macromolecular metabolites during the course of cellular maturation.

It seems probable that the accumulation of DNA reflects only its synthetic rate, since the turnover or catabolism of DNA has been found to be negligible during normal development of most organisms[34]. Current evidence indicates that the synthesis of DNA may be regulated by the level of deoxynucleoside triphosphates (the substrates for the DNA polymerase reaction), or the DNA polymerase reaction, or the availability of pre-existing DNA as a primer[30,31]. However, the availability of deoxynucleoside triphosphates seems to be a likely means of regulation of the rate of DNA synthesis[3,8,12]. The terminal enzymatic step in DNA biosynthesis, the DNA polymerase reaction, cannot take place unless all four deoxynucleoside triphosphates are present simultaneously[31]. DNA polymerization, therefore, would be limited if any of the four deoxynucleoside triphosphates were absent or limited. Thus, control of the production of any one of these nucleotides could result in control of the rate of synthesis of DNA.

A search during recent years to determine the main pathway of deoxyribonucleotide biosynthesis has provided evidence that the ribonucleoside diphosphates are enzymatically converted to the corresponding deoxynucleoside diphosphates[18-21]. Thus, it appears that the nucleotide precursors for the biosynthesis of both RNA and DNA may have a common pathway which branches at the diphosphate level (Fig. 1). Fluctuation in the levels of the ribonucleoside diphosphate pool, therefore, would affect the rates of synthesis of both DNA and RNA. Thus, DNA synthesis could not be limited by the level of nucleotides in the nucleoside diphosphate pool without a corresponding

Fig. 1. Scheme for the biosynthesis of DNA and RNA.


PURINE AND PYRIMIDINE
NUCLEOTIDE BIOSYNTHESIS
CTP
GTP
UTP
ATP
ADP
ATP
RNA
CDP
GDP
UDP
ADP
2H
H2O
2H
H2O
2H
H2O
BRANCHPOINT
dCDP
dUDP
dCDP
dGDP
dADP
Pi
ATP
dCMP
ADP
NH3
Pi
dCTP
dGTP
dATP
dUMP
DNA "PRIMER"
CH3
DNA
TMP
TDP
TTP
ATP
ADP
ATP
ADP

limitation in the rate of RNA synthesis. Since the level of DNA remains constant while RNA is accumulating during most of pre-mitotic growth in C. pyrenoidosa, the control of DNA synthesis would appear to take place somewhere beyond the nucleoside diphosphate branch point.

The sequence of reactions beyond the branchpoint leading to the formation of the four deoxynucleoside triphosphates, deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and thymidine triphosphate (TTP), is illustrated in Fig. 1. dATP, dCTP and d GTP are formed through the phosphorylation of the corresponding diphosphates, while TTP is formed from deoxyuridine monophosphate (dUMP) by a sequence of reactions which includes methylation of dUMP to form deoxythymidine monophosphate (TMP)[28] and phosphorylation of TMP in two steps to form TTP[3,29]. dUMP may be formed either through dephosphorylation of deoxyuridine diphosphate (dUDP) or deamination of deoxycytidine monophosphate (dCMP)[27]. Each of these reactions represents a possible point of control by which deoxynucleoside triphosphate production could be regulated. Control of the activity of one or more of these reactions could regulate the synthesis of one of the deoxynucleoside triphosphates, and, consequently, the synthesis of DNA.

The enzyme system which converts TMP to thymidine diphosphate (TDP) and TTP (hereafter referred to as the TMP kinase system or TMP kinase activity) would seem a likely point of control. It appears

that TMP kinase activity can be closely correlated with the rate of proliferation of mammalian cells[4-7]. Adult normal rat liver, for example, does not contain a detectable level of the enzymes necessary to phosphorylate TMP. However, following partial hepatectomy, thymidylate kinase activity increases markedly in the rapidly regenerating liver. TMP kinase activity is also detectable in the actively growing tissue of newborn mammals and is lost as the animals grow older[7]. Moreover, Keilley[3] has demonstrated that all the deoxyribonucleotide monophosphates except TMP are actively phosphorylated in normal mouse liver; whereas, in mouse ascites hepatoma, a very actively regenerating tissue, TMP is extensively phosphorylated. These observations suggest that TMP kinase activity may be regulating the rate of DNA synthesis.

The present study is one facet of a research program to evaluate the hypothesis that DNA biosynthesis may be regulated by one or more specific enzymatic steps beyond the nucleoside diphosphate branch point. Specifically, it is an attempt to determine the relationship of TMP kinase activity to the periodic replication of DNA and to gain insight into the cellular mechanisms controlling the activity of this enzyme system during synchronous growth of C. pyrenoidosa.

MATERIALS AND METHODS

Organism and culture conditions

The high temperature strain 7-11-05 of Chlorella pyrenoidosa described by Sorokin and Myers[1] was used in these studies. Synchrony was induced by the intermittent illumination procedure of Baker and Schmidt[2] and the resultant synchronized cells were cultured in the 35 l Plexiglass chamber under the same conditions as previously described. Actively growing cultures of asynchronous cells were used as an enzyme source for preliminary enzyme characterization studies.

Enzyme assay

Apparent TMP kinase activity was determined by measuring the conversion of TMP-2-^{14}C to TDP and TTP. All enzyme assays were carried out in 6 ml graduated hematocrit tubes. The incubation mixtures (0.8 ml), pH 8.4, contained 50 μM of Tris-HCl buffer, 20 μM of $MgCl_2$, 8 μM of ATP, 1 μM of TMP-2-^{14}C (2.95 X 10^5 counts per min) and 0.3 ml of enzyme. After incubation at 38.5°, the reactions were stopped by heating for two min in a boiling water bath. The tubes were then cooled immediately by immersion in an ice bath and frozen until separations on ion-exchange columns could be made.

Ion-exchange separations of TDP and TTP from TMP

Separation of TDP and TTP from TMP was accomplished by ion-exchange chromatography on a 1 x 6 cm column of Dowex-1 Cl^- (200-400 mesh, 4 percent cross-linkage). 0.5 ml of the assay supernatant was

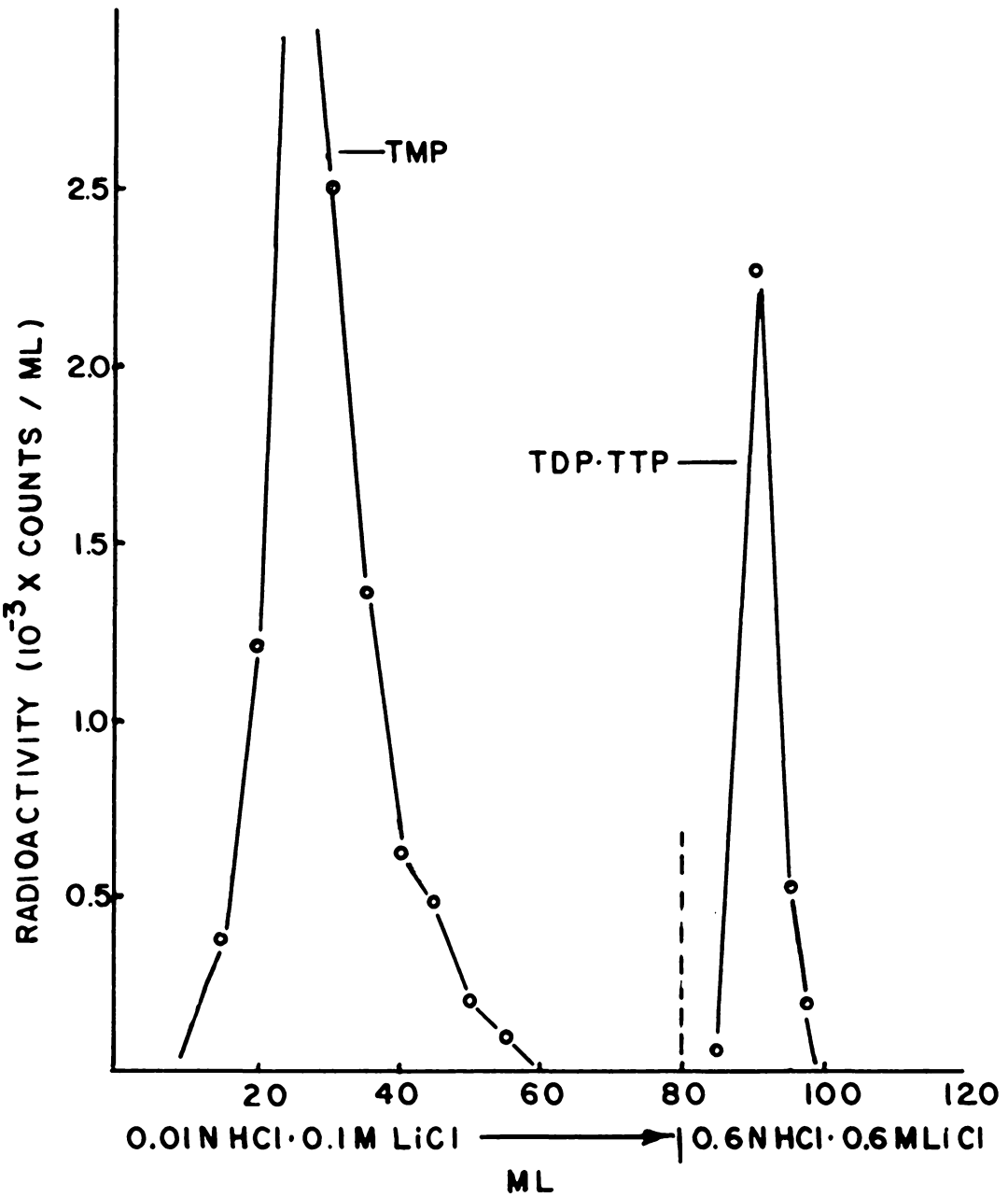
added to the column and TMP was removed by washing with 75 ml of 0.01 N HCl-0.1 M LiCl. TDP and TTP were eluted simultaneously with 25 ml of 0.6 N HCl-0.6 M LiCl. The typical elution pattern for this column is illustrated in Fig. 2. Identity of the radioactive components of the column eluates was established by paper chromatography in isobutyric acid/ NH_4OH/ water (66:1:33). The radioactive spots were located with a Nuclear-Chicago Strip Counter. No TDP was detected in the 25 ml eluate. All the radioactivity in this fraction was associated with a single spot which was identical to authentic TTP. The absence of detectable amounts of TDP could be explained on the basis of a more rapid conversion of TDP to TTP than of TMP to TDP[3].

Enzyme preparation and characterization

Asynchronous cells were harvested by continuous-flow centrifugation using a Sorvall RC-2 refrigerated centrifuge, washed three times with 0.05 M Tris-HCl buffer (pH 8.4), and sonicated in a Raytheon 10 KC sonic oscillator for 30 min at maximum amperage. Under these conditions, virtually complete cell rupture was obtained[37].

When a comparison was made of apparent activity in the whole sonicate, low-speed supernatant (25,000 xg for 30 min), and high-speed supernatant (100,000 x g for two hr), it was found that the high- and low-speed supernatants contained essentially the same enzyme activity, while whole sonicates contained only 68 to 74 percent of this activity. This difference in apparent activity could possibly be due to the presence of either TTP and TDP phosphatases or TTP

Fig. 2. Typical elution pattern of thymidine nucleotides from a Dowex-1 Cl^- column.


TMP
TDP·TTP
RADIOACTIVITY (10^{-3} X COUNTS / ML)
2.5
2.0
1.5
1.0
0.5
20
40
60
80
100
120
0.01 N HCl · 0.1 M LiCl
0.6 N HCl · 0.6 M LiCl
ML

pyrophosphatase in the particulate fraction of the sonicates. By removing the reaction products, these enzymes could reduce the apparent activity of the kinase system. It is also conceivable that the high- and low-speed centrifugations are removing a TMP phosphatase which could compete with the kinase system for substrate. The clear low-speed supernatant was used, therefore, as the source of crude enzyme for the following studies.

Freezing and thawing of washed freshly harvested cells resulted in variable losses of activity (23–36 percent); whereas, freezing and thawing the high- or low-speed supernatants of sonicated cells resulted in complete loss of enzyme activity. It was found, however, that the low-speed supernatant could be stored at 0–3° for at least 5 hr with no apparent loss of activity. Moreover, storage for 18 hr resulted in less than 10 percent loss of activity.

A typical plot of enzyme concentration (ml of crude preparation) vs. product accumulation is shown in Fig. 3. The pH optimum for TMP kinase activity in the low-speed supernatant was found to be 8.4 (Fig. 4). No temperature optimum was demonstrated since enzyme activity continued to increase almost linearly with increasing temperature over the range tested (28–52°). Consequently, all further assays were run at 38.5°, the optimum growth temperature for the organism.

Fig. 3. Enzyme concentration vs. product accumulation for Thymidylate Kinase activity from C. pyrenoidosa.

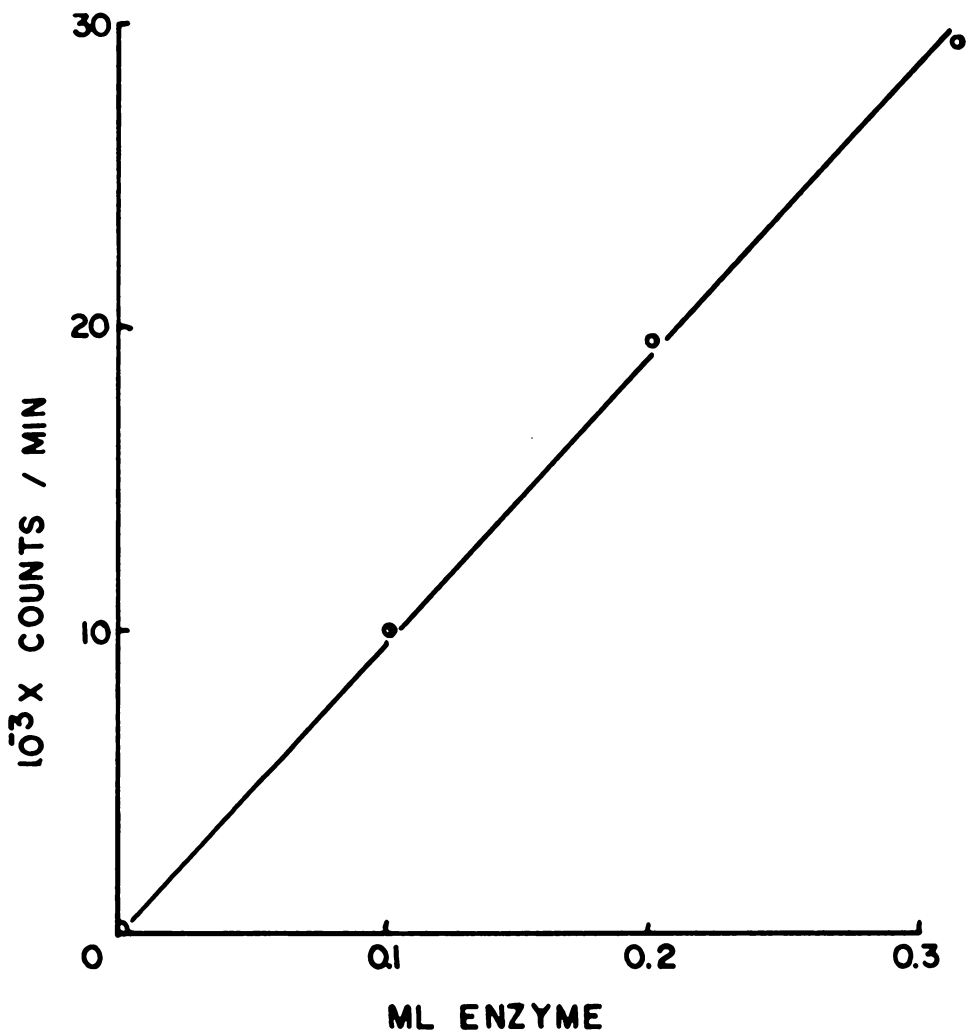
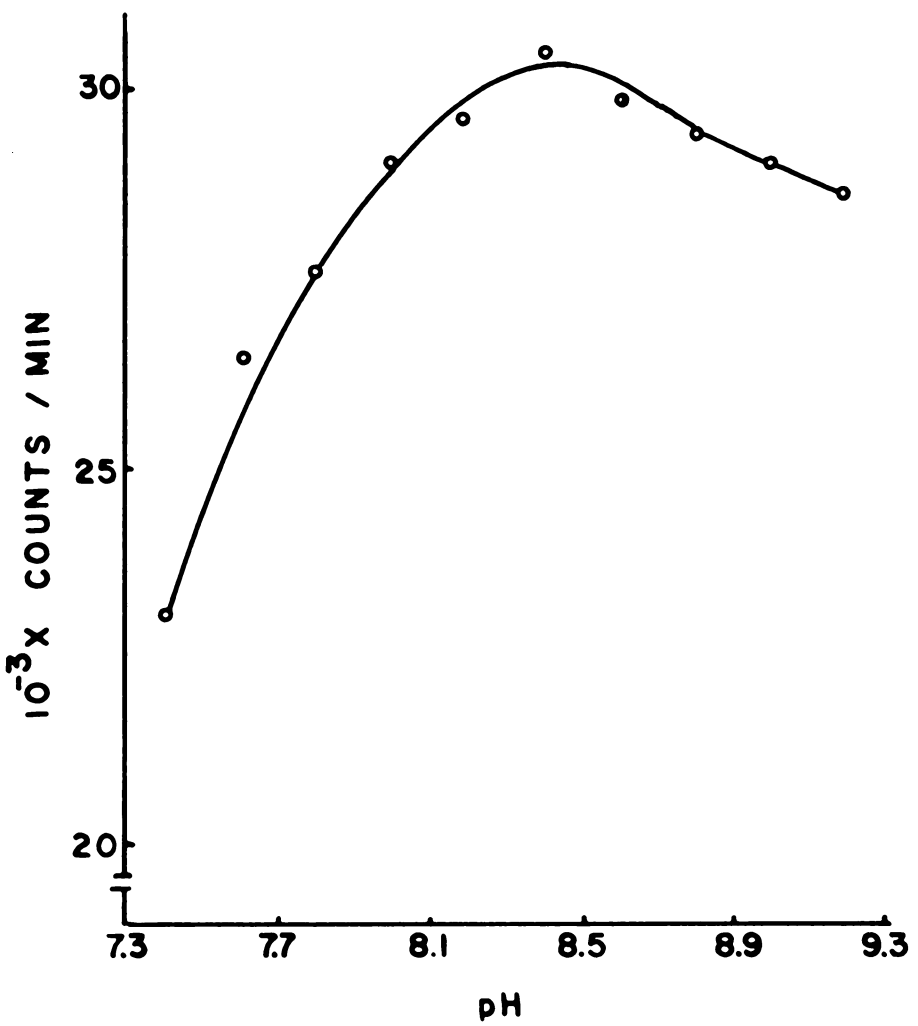

30
20
10
0
0.1
0.2
0.3
10^{-3} X COUNTS / MIN
ML ENZYME

Fig. 4. pH optimum of Thymidylate Kinase activity from C. pyrenoidosa.


30
25
20
10^{-3} X COUNTS / MIN
7.3
7.7
8.1
8.5
8.9
9.3
pH

Synchronous growth studies

In two separate experiments, cells were harvested at one or two hr intervals for 14 hr of synchronous growth. Cell number per ml of culture (using a Levy-Hausser hemacytometer) and total cellular-P per ml of culture (using the method of Fiske and SubbaRow[9]) were determined at each harvest time on suitable aliquots of the culture. The remaining cells, approximately two ml packed-cell-volume at each sampling period, were harvested by continuous-flow centrifugation, washed three times with Tris-HCl buffer and suspended in 15 ml of the same buffer for sonication. Total cellular-P per ml of this 15 ml suspension was determined on suitable aliquots and used to relate enzyme units per ml of 15 ml cell suspension to units of enzyme per ml of growing culture. The remainder of the 15 ml cell suspension was sonicated and centrifuged at low speed as described above. 0.3 ml aliquots of the resulting supernatant were assayed immediately under the conditions described above.

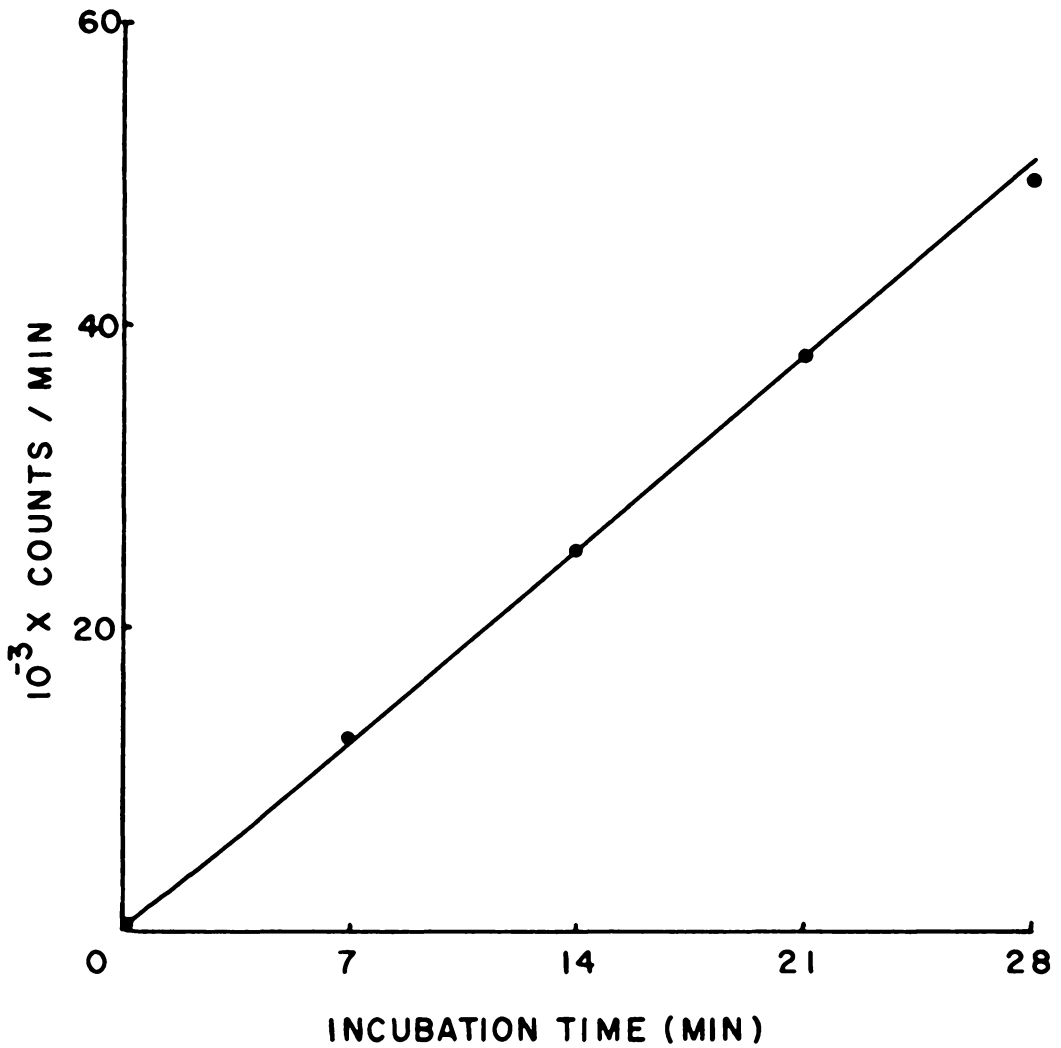
A special preliminary study was undertaken to assure that substrate concentration would not become limiting during the course of the assays performed during the synchronous growth studies. Since DNA synthesis appears to be maximal during nuclear division, this was anticipated to be the period of highest TMP kinase activity. Thus, the low speed supernatant from cells (approximately 2 ml packed-cell-volume, to duplicate the sample size for synchronous growth studies) of 9 hr synchronous culture was assayed for various incubation times up to 28 min. Fig. 5

represents the results of this study. It can be seen that product accumulation increased linearly with time, indicating that substrate concentration did not become limiting within 28 min of incubation. Therefore, enzyme assays during synchronous growth studies were run for 20 min to provide a margin of safety.

Inhibition studies

Aliquots from the low-speed supernatants from each sampling period were boiled for two min, cooled in ice, and centrifuged to sediment coagulated protein. The resulting supernatants were the source of heat-treated enzyme preparations used in the inhibition study. 0.3 ml aliquots of these preparations were substituted for buffer in the enzyme assay mixtures.

Fig. 5. Product accumulation vs. incubation time for Thymidylate Kinase activity from C. pyrenoidosa.


60
40
20
0
7
14
21
28
10^{-3} X COUNTS / MIN
INCUBATION TIME (MIN)

RESULTS AND DISCUSSION

To reveal different facets of periodism in apparent TMP kinase activity during synchronous growth, enzyme activity has been plotted on three different bases, μ units per μg total cellular-P (Fig. 7), μ units per cell (Fig. 8), and μ units per ml of growing culture (Fig. 9).

While total cellular-P increased at an exponential rate (Fig. 6) similar to those observed for cellular dry weight and total cellular-S[2,16,36,37] apparent TMP kinase activity showed marked periodism during cellular development (Fig. 7). When expressed as percent of total cellular-P or cellular dry weight, apparent TMP kinase activity increased only slightly during the early stages of cellular development. Immediately before nuclear division[41] (7th hour), however, there was a marked increase in apparent enzyme activity which reached a maximum when the culture contained the greatest percentage of multinucleate cells. At the onset of cell division (daughter cell release), apparent enzyme activity decreased, reaching a minimum at the end of this cellular event (Figs. 6 and 7).

The possibility that TMP kinase activity may be regulating the rate of DNA accumulation (synthesis) is suggested by the observation that periods of increased or decreased DNA accumulation (Fig. 7, replotted from Herrmann and Schmidt[16]) are preceded by corresponding periods of increased or decreased apparent TMP kinase activity (Fig. 7),

Fig. 6. Cell number (o—o) and total cellular-P (•—•) per ml of culture during synchronous growth of C. pyrenoidosa.

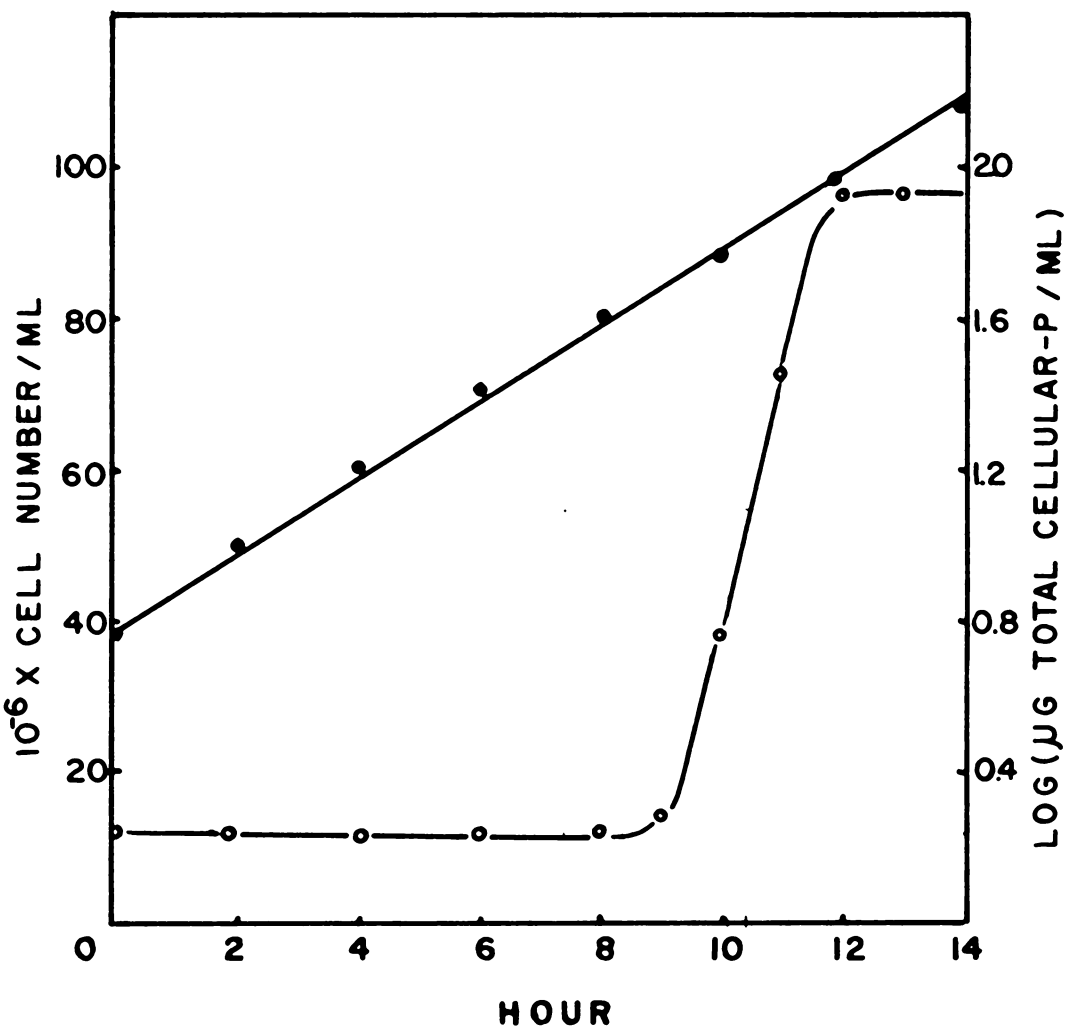
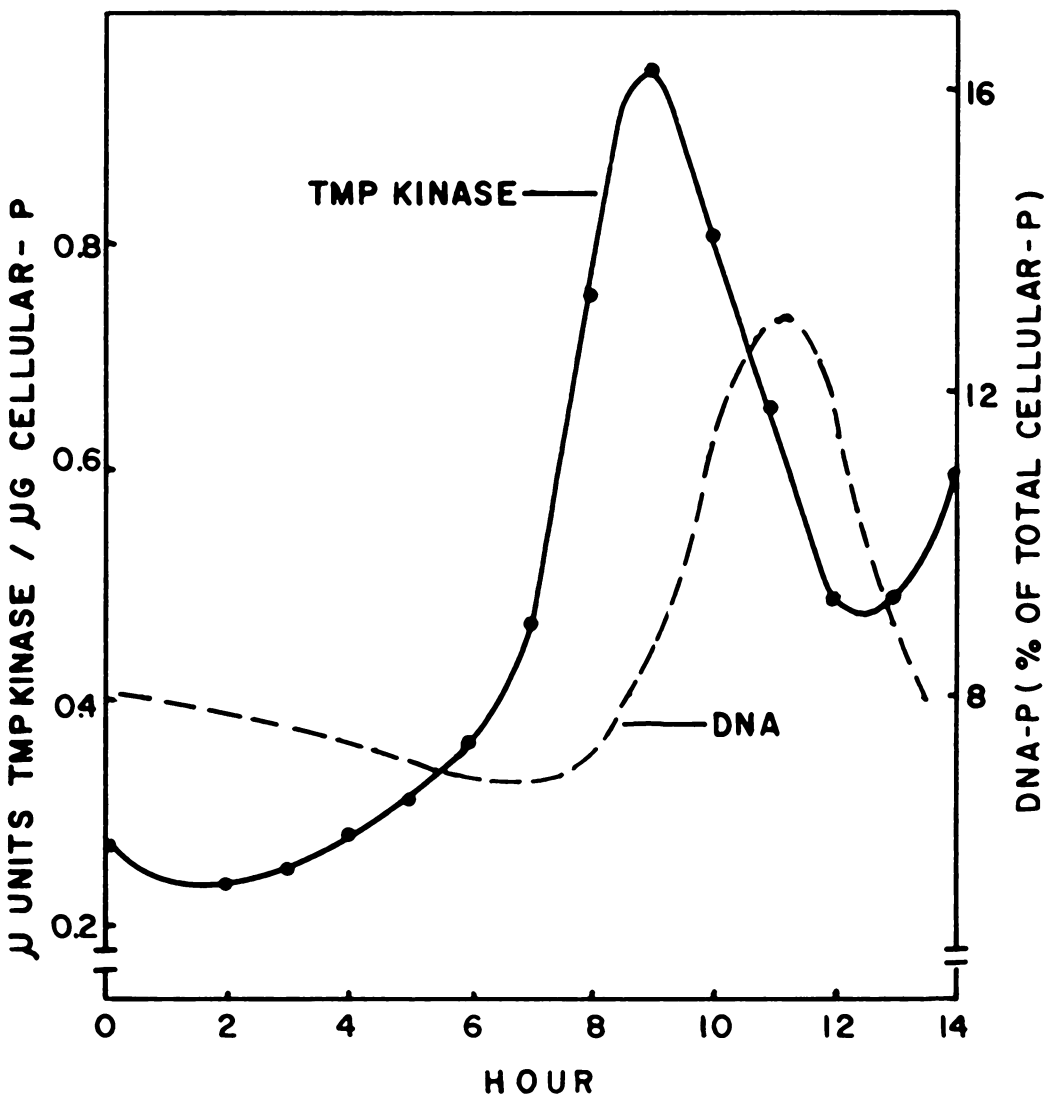

100
80
60
40
20
0
2
4
6
8
10
12
14
HOUR
10^{-6} X CELL NUMBER / ML
2.0
1.6
1.2
0.8
0.4
LOG (μG TOTAL CELLULAR-P / ML)

Fig. 7. Apparent Thymidylate Kinase activity (o—o) and DNA level (——) during synchronous growth of C. pyrenoidosa.


TMP KINASE
DNA
μ UNITS TMP KINASE / μG CELLULAR-P
DNA-P (% OF TOTAL CELLULAR-P)
HOUR
0.2
0.4
0.6
0.8
8
12
16
0
2
4
6
8
10
12
14

respectively. However, before it can be unequivocally stated that the availability TTP is regulating DNA synthesis, the intracellular levels of the other deoxyribonucleoside triphosphates, their turnover rates, and the activities of the other deoxyribonucleotide kinases must be measured during cellular development. Furthermore, if the intracellular level of TTP is found to be regulating the rate of DNA synthesis, the activity of the TMP kinase system cannot be stated to be the enzyme system which is controlling the synthesis of TTP until the active levels of the enzymes involved in the synthesis of TMP (the substrate of the TMP system) are measured. For example, one or more of the enzymes preceding the TMP system may be regulating the availability of TMP to the final kinases. Thus, the observed periodism in apparent TMP kinase activity could prove to be merely the result of induced enzyme synthesis responding to availability of its substrate.

It should be noted, in view of Keilley's[8] observations, that the in vitro enzyme assay procedure precludes evaluation of the effects of subcellular localization on the regulation of TMP kinase activity. Keilley[8] has found that TMP kinase is present in normal mouse liver, but its activity is suppressed by a reversible binding of the enzyme with subcellular particles. Freezing and thawing of the mouse liver homogenates in the presence of TMP released the enzymes from their bound state and restored activity. However, in the present study,

attempts to increase apparent TMP kinase activity by freezing and thawing in the presence of TMP resulted in losses of enzyme activity. It is doubtful, therefore, that the enzyme system is particulate bound under the preparation and assay conditions used in the present study. Thus, the fluctuations in enzyme activity observed in the present study are probably not a reflection of reversible inactivation by particulate binding.

Since there is an 8-fold increase in cell number (Fig. 6), it is noteworthy that there is an 8- to 9-fold increase in apparent enzyme activity per cell just prior to cell division (Fig. 8).

Fig. 9 illustrates a different aspect of the periodicity in enzyme activity. When the enzyme activity is expressed in terms of μ units per ml of culture, an apparent loss of activity between the 10th and 12th hr of growth can be seen. This phenomenon would indicate either an inhibition of TMP kinase activity (or absence of an activator) or an actual loss or inactivation of the enzymes after the period of nuclear division and active DNA synthesis. Again, these observations reinforce the hypothesis that TMP kinase activity may be controlling the rate of DNA synthesis.

Cole and Schmidt[37] have demonstrated that the control of aspartic transcarbamylase activity during cellular development is due, at least partially, to small molecule inhibition by Norit-A adsorbable compounds in boiled supernatant preparations. To determine if similar small molecule inhibition or activation is operative in controlling TMP

Fig. 8. Apparent Thymidylate Kinase activity of C. pyrenoidosa.

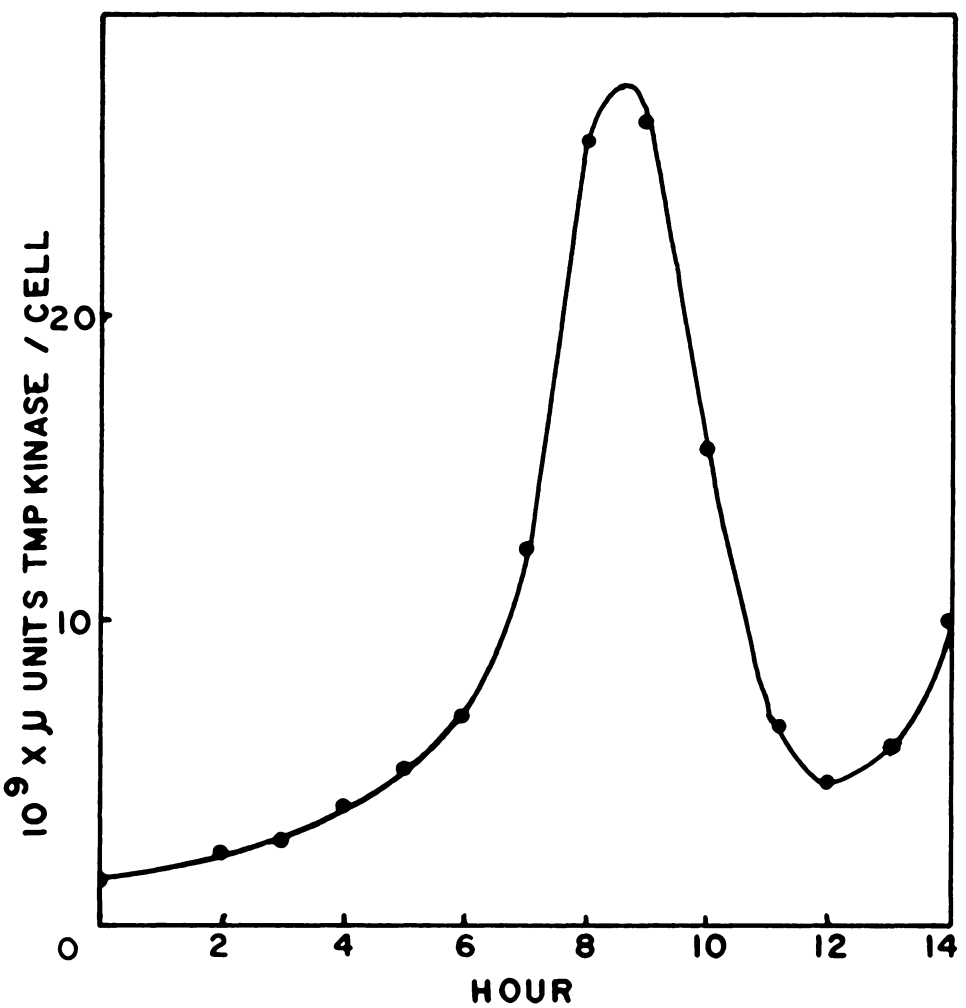
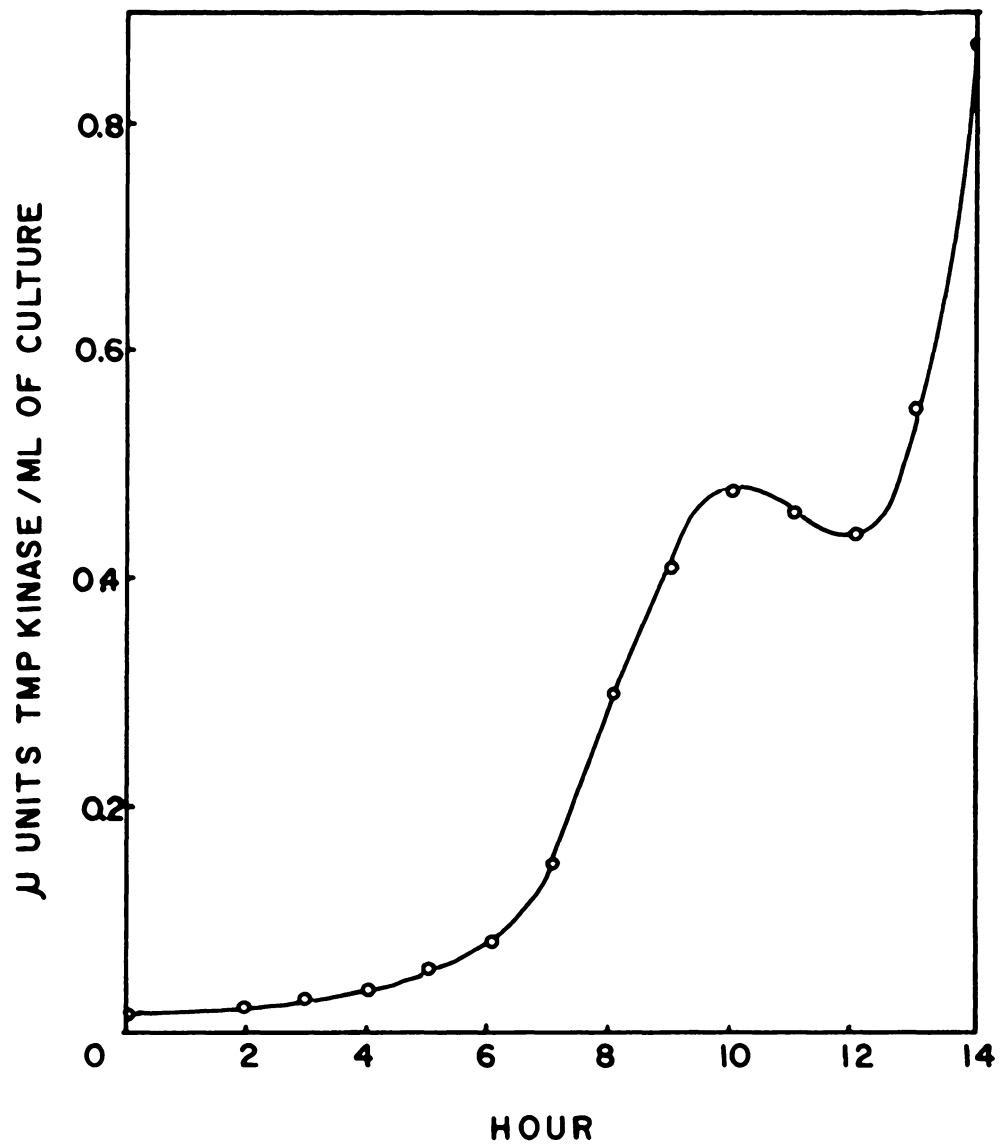

10^9 x μ UNITS TMP KINASE / CELL
20
10
0
2
4
6
8
10
12
14
HOUR

Fig. 9. Apparent Thymidylate Kinase activity per ml of culture during synchronous growth of C. pyrenoidosa.


μ UNITS TMP KINASE / ML OF CULTURE
0.8
0.6
0.4
0.2
0
2
4
6
8
10
12
14
HOUR

kinase activity, boiled supernatant preparations from various periods of cellular development were added to enzyme assay mixtures as follows: 5 hr boiled preparation to 5th hr assay, 8 hr to 5 hr, 7 hr to 7 hr, 12 hr to 7 hr, 11 hr to 11 hr, 13 hr to 13 hr, and 11 hr to 13 hr. Figs. 7, 8 and 9 indicate that inhibition, if operative, would be expected at hr 0-6 or 11-13, since these are periods of low enzyme activity. Similarly, activation would be most likely at hr 6-8 or 13-14 when enzyme activity is apparently increasing at an accelerated rate.

With the exception of the 5 hr to 5 hr additions, no statistically significant alterations of enzyme activity were observed. The addition of 5 hr boiled supernatant to 5 hr enzyme assay resulted in a 5 percent inhibition, which is significant at the one percent level. Addition of 11 hr boiled preparations to 5 hr assays, however, produced no similar effect. Thus, the loss of apparent enzyme activity between the 10th and 12th hr is probably due to a loss or inactivation of enzyme, since no inhibition or activation phenomena could be demonstrated during this period. It appears, therefore, that very little, if any, small molecule inhibition or activation is operative in controlling TMP kinase activity. It would seem more likely that the dramatic shifts in apparent TMP kinase activity are a reflection of variation in rates of enzyme synthesis.

SUMMARY

Synchronous cultures of a high temperature strain of Chlorella pyrenoidosa have been used to determine the apparent activity of the TMP kinase system during cellular development. During the early stages of cellular growth the apparent enzyme activity increased only slightly. However, just before nuclear division there was a rapid increase in enzyme activity which preceded the increased rate of DNA synthesis. At the onset of cell division (daughter cell release) there was a loss of apparent enzyme activity followed by a decreased rate of DNA synthesis. These observations tend to support the hypothesis that TMP kinase activity may be a factor controlling the rate of DNA biosynthesis.

It appears that the dramatic shifts in the level of TMP kinase activity were a result of variations in the rates of enzyme synthesis rather than control of enzyme activity by small molecule inhibitors or activators.

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He is also indebted to Dr. R. W. Engel for providing a departmental atmosphere conducive to academic and scientific achievement and to Dr. K. W. King for his helpful advice and criticisms.

Dr. F. E. Cole's "pioneering achievements in the field of cell rupture techniques" which made this study possible are also worthy of grateful acknowledgement.

The author also wishes to thank Emile, Ted and Mickey for their assistance during the synchronous growth studies.

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TMP KINASE ACTIVITY DURING SYNCHRONOUS GROWTH OF *CHLORELLA PYRENOIDOSA*

ABSTRACT

Synchronous cultures of a high temperature strain of *Chlorella pyrenoidosa* have been used to determine the activity of the TMP kinase during cellular development.

It was observed that the apparent enzyme activity was closely correlated with the rate of DNA biosynthesis. During the period of nuclear division, when DNA synthesis is at its maximum apparent TMP kinase activity is highest. The maximum in enzyme activity, however, slightly precedes the peak in DNA synthesis. These results support the hypothesis that TMP kinase activity is a factor controlling the rate of DNA biosynthesis.

It appeared that the dramatic shifts in the level of TMP kinase activity were a result of variations in the rate of enzyme synthesis rather than control of enzyme activity by small molecule inhibitors or activators.