Evidence for Thymidine Diphosphate as the Precursor of Thymidine Triphosphate in Tumor

TRANSFER OF THE TERMINAL PHOSPHATE OF ADENOSINE TRIPHOSPHATE TO THYMIDYLATE*

DAVID H. Ives

From the Department of Agricultural Biochemistry, The Ohio State University, Columbus, Ohio 43210

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The phosphorylation of thymidine and thymidylate to thymidine triphosphate, a proximal precursor of DNA, has been demonstrated repeatedly in extracts from a large variety of growing cells (2–7). A salient feature of a number of these studies was the surprising failure of one presumed intermediate, thymidine diphosphate, to accumulate in the reaction mixtures. Indeed, in several experiments where the time course of thymidine phosphorylation was examined, thymidine diphosphate could not be detected in the reaction mixtures until well after the appearance of thymidine triphosphate, the terminal product of the phosphorylation sequence (2, 3, 5). On the other hand, the appearance of thymidine triphosphate, the terminal product of the phosphorylation sequence (2, 3, 5). On the other hand, recent investigations by Grav and Emellie (6) have revealed measurable amounts of dTDP formed by Landschutz ascites-tumor cells in the first few minutes of reaction, before the appearance of dTTP. On this basis, they concluded the following sequence of reactions obtains

\[ \text{dTMP} \rightarrow \text{dTDP} \rightarrow \text{dTTP} \]

Thus far, two general approaches have been used to examine the possible role of dTDP, and both suffer certain critical deficiencies. First, the kinetics of product formation from labeled thymidine or thymidylate (2–6), while providing much useful information, may have little meaning in the interpretation of the mechanism of dTTP biosynthesis. If the relative activities of the enzymes catalyzing the formation or degradation of thymidine products are very dissimilar, the accumulation of a given phosphorylated fraction might be due to large differences in the relative rates of certain enzymic reactions, rather than to the order in which the various products are formed. Thus, a highly active nucleoside diphosphokinase might remove dTDP formed by thymidylate kinase of lower activity, thereby preventing the accumulation of diphosphate in the reaction mixture. Conversely, triphosphatase in the enzyme mixture might degrade dTTP to dTDP, even if the mechanism of triphosphate synthesis did not involve dTDP. A second approach has involved the use of diphosphate pools or "sinks" to trap the dTDP as it formed (7, 8). As applied, this method had two drawbacks. First, it did not rule out the possible formation of dTTP by pyrophosphorylation, followed by dephosphorylation to dTDP, the mechanism proposed by Bianchi et al. (3).

Secondly, the diphosphate pool itself could have been phosphorylated to triphosphate within the first minute or two of the reaction (2, 6).

The purpose of this report is to distinguish between the sequential phosphorylation mechanism and the pyrophosphorylation mechanism for the biosynthesis of thymidine triphosphate in extracts of mammalian tumor cells. In the present case the multienzyme mixture was extracted from the Novikoff hepatoma, and dTTP synthesis was examined by an experimental technique different from those used in any of the previous reports. The transfer of ATP phosphate moieties to the thymidylate acceptor-molecule was studied by means of ATP labeled specifically in the \( \gamma \) or \( \beta \)-phosphate positions, as shown in Fig. 1. The sequential mechanism of nucleoside triphosphate synthesis (I) would be expected to produce doubly labeled dTTP from ATP-\( \gamma-\text{P} \) or from ATP-\( \beta-\text{P} \), while the pyrophosphorylation mechanism (II) would produce dTTP containing only one labeled phosphate moiety, either from ATP-\( \gamma-\text{P} \) or from ATP-\( \beta-\text{P} \). Conceivably, this rather unorthodox mechanism might take one of two forms: (IIa) the \( \beta \)-phosphate of ATP could become the \( \beta \)-phosphate of the product, or (IIb) the \( \beta \)-phosphate of ATP could become the terminal phosphate of dTTP. This isotope-transfer technique, along with chemical degradation and analysis of the dTTP, should distinguish between these three reaction mechanisms, and, moreover, it can be expected to be independent of the relative rates of the individual reactions.

**EXPERIMENTAL PROCEDURE**

**Materials**—Crystalline pyruvate kinase, adenylyl kinase, and hexokinase were obtained from Sigma Chemical Company, and crystalline glyceraldehyde phosphate dehydrogenase and 3-phosphoglycerate phosphokinas were purchased from California Corporation for Biochemical Research. Thymidine-\( 2-\text{C} \) (25 to 30 \( \mu \)c per \( \mu \)mole) was purchased from New England Nuclear Corporation, and the thymidine nucleotides were synthesized from this stock with the kinases in Novikoff hepatoma extracts. Carrier-free \( \text{P} \)-orthophosphate designated for oral use was obtained from Volk Radiochemical Company. Phosphoenolpyruvate (tricyclohexylammonium salt), NAD, AMP, ADP, and ATP were supplied by Sigma Chemical Company, while cysteine-HCl and the thymidylic acid were purchased from California Corporation for Biochemical Research. The Sephadex G-25 was a product of Pharmacia Fine Chemicals, Inc.

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Preparation of ATP-γ-32P and ATP-β-32P—A combination of the glyceraldehyde phosphate dehydrogenase and phosphoglycerate kinase systems served to incorporate orthophosphate-32P into the terminal position of ATP. In a final volume of 3 ml the reaction mixture contained: Tris-HCl, pH 7.5, 90 μmoles; cysteine-HCl, 11 μmoles; EDTA, 2.5 μmoles; NAD, 6 μmoles; ADP, 10 μmoles; MgCl₂, 15 μmoles; orthophosphate-32P, 0.5 μmole, 2 mc. After adding 0.1 mg (6.5 enzyme units) of glyceraldehyde phosphate dehydrogenase and 0.1 mg (17 enzyme units) of phosphoglycerate kinase, the reaction was started by adding 6 μmoles of di-glycerol phosphate and allowed to react for 10 minutes at 25°. The reaction mixture was diluted and passed onto a 0.6-× 6-cm column of Dowex 1-CI. Unreacted orthophosphate was eluted with 0.01 N HCl, ADP with 0.01 N HCl-0.02 M KCl, and ATP with 0.01 N HCl-0.2 M KCl. The ATP fraction, which generally contained 30 to 50% of the total radioactivity, was neutralized immediately with 1 N Tris base and stored at −15° until use. This method permitted the preparation of essentially pure ATP-γ-32P, with less than 0.1% of the label appearing in the β-phosphate.

The procedure for producing high specific activity ATP-β-32P was to incubate some of the ATP-γ-32P preparation with an excess of adenyl acid in the presence of rabbit muscle adenylate kinase, thereby producing terminally labeled ADP. The adenylate kinase reaction mixture contained in a final volume of 10 ml: Tris-HCl, pH 7.5, 400 μmoles; adenyl acid, 50 μmoles; MgCl₂, 100 μmoles; ATP-γ-32P, 50 μc; and adenylate kinase (myokinase, Sigma), 1 mg. The reaction mixture was incubated for 20 minutes at 37°, diluted to 100 ml, and the ADP-β-32P was isolated by selective elution from a Dowex 1-CI column, as above.

Approximately 97% of the radioactivity added to the reaction mixture as ATP-γ-32P was isolated in the ADP fraction. This fraction (9 ml) was neutralized and converted to ATP-β-32P with the following system: 200 μmoles of Tris-HCl, pH 7.5; 10 μmoles of phosphoenolpyruvate (tricyclohexylamine salt); 50 μmoles of MgCl₂; and 1 mg of pyruvate kinase (130 enzyme units), incubating for 20 minutes at 37° in a volume of 10 ml. The resulting ATP-β-32P was isolated as above.

Preparation of Novikoff Hepatoma Enzyme—Rats of the Holtzman strain were inoculated with Novikoff hepatoma cells, strain N1-S1, out of suspension tissue culture (kindly donated by Dr. V. R. Potter). After re-establishment of the tumor culture in the rats, the tumor line was maintained by weekly intraperitoneal transplantation of 100 to 200 mg of minced tumor. In order to obtain maximal levels of thymidylate kinase in the resulting extracts the rats were given 40 mg of thymidine (9) 20 minutes before being killed for enzyme preparation. The solid tumor, weighing 5 to 10 g, was excised from the mesenteric membrane and homogenized in 3 volumes of 0.154 M KCl. The homogenate was centrifuged for 30 minutes at 30,000 × g, and then the supernatant was centrifuged for 60 to 90 minutes at 30,000 r.p.m. in the No. 30 rotor of the Spinco model L ultracentrifuge. This high speed supernatant fraction contained substantial levels of thymidine kinase, thymidylate kinase, nucleoside diphosphokinase, as well as a variety of phosphatases, and was used without further fractionation in the experiments described below.

Thymidylate Kinase Reaction—After a series of preliminary experiments (not shown) reaction flasks were set up to study the incorporation of radiophosphate from ATP-γ-32P into the thymidine triphosphate fraction in the presence, or in the absence, of a phosphoenolpyruvate-pyruvate kinase ATP-regenerating system. In an identical experiment ATP-β-32P was employed to determine if pyrophosphate transfer between ATP and dTMP could occur.

The complete thymidylate kinase reaction mixture, in a volume of 10 ml, consisted of: Tris-HCl, pH 7.5, 500 μmoles; dTMP, 100 μmoles; ATP-γ-32P or ATP-β-32P, 5.5 × 10⁶ c.p.m. or 3.5 × 10⁶ c.p.m., respectively; phosphoenolpyruvate, 100 μmoles; MgCl₂, 180 μmoles; KCl, 674 μmoles (from the enzyme and isotope solutions); pyruvate kinase, 1 mg; and high speed supernatant of 25% Novikoff hepatoma homogenate, 2.5 ml. This reaction mixture was incubated at 37°, and 0.5-ml aliquots were withdrawn at 0, 30, and 60 minutes to permit determination of the specific activity of the various phosphate moieties of ATP. At the end of the 60-minute incubation period the remainder of the reaction mixture was heated for 2 minutes in a boiling water bath and saved for radiochemical analysis of dTTP.

Analysis of ATP Phosphate Moieties—After heating for 2 minutes to coagulate proteins, the 0.5-ml aliquots were centrifuged, diluted with water, and chromatographed on Dowex 1-CI columns as described above. The ATP fraction was neutralized with Tris base. One-fifth of this fraction was wet-ashed in H₂SO₄ for determination of total phosphate specific activity, and the remainder was quantitatively converted to ADP and glucose 6-phosphate with crystalline yeast hexokinase, according to the method of Berger et al. (10). This reaction mixture was chromatographed on 0.6-× 6-cm Dowex 1-CI columns. Glucose 6-phosphate, which was eluted from the columns with 0.01 N HCl, was ached and taken as the terminal phosphate moiety of the ATP. The ADP fraction was eluted with 10 ml of 0.02 N HC1-0.02 M KCl. To release the acid-labile β-phosphate moiety, the ADP was made to 1 N HCl and boiled for 15 minutes in a tube containing 200 mg of Nuchar C-190 activated charcoal. After removal of the charcoal by centrifugation, the supernatant was found to be free of material absorbing at 260 μm. It was

1 A large excess of thymidylate was used in the reaction system to provide a pool which would dilute any labeled thymidylate formed when dTMP is dephosphorylated by phosphatase and rephosphorylated by thymidine kinase. Under these conditions the specific activity of this thymidylate pool never exceeded 3% of the specific activity of the dTDP and dTTP produced in the reaction mixture. Similarly, the rather high ATP concentration used was to dilute the contribution of exchange reactions involving ATP.
involved in this series of reactions were determined as shown in Table I. The major portion of the thymidylate kinase reaction mixture was chromatographed on a 1-×7-cm column of Dowex 1-formate. ATP and the less tightly bound ionic components were eluted with 65 ml of 4 N formic acid-0.4 M ammonium formate. This fraction was saved and examined for possible labeling in the thymidylate pool (see below). After rinsing the column with water until neutral, dTTP was eluted with 20 ml of 1.25 M ammonium formate. This fraction was passed through a Dowex 50W-H⁺ column to remove the ammonium ions, and lyophilized to dryness. The ultraviolet spectra of this product closely resembled those of authentic thymidylate, at both acidic and basic pH values. This material also yielded a positive Ceriotti test for deoxyribose (12), although the amount of nucleotide used did not afford accurate quantitative analysis.

An aliquot of the labeled thymidine triphosphate solution was subjected to selective hydrolysis of the terminal phosphate by incubating in 0.5 n perchloric acid at 70° for 30 minutes. The resulting dTDP was freed of orthophosphate and a trace of unhydrolyzed dTTP, by chromatography on Dowex 1-formate columns. The dTDP was ashed, and the specific activity of its combined phosphate moieties was determined.

Thymidylate from the pool in the original reaction mixture was isolated by column chromatography, adsorbed on charcoal to free it from orthophosphate contamination, eluted from the charcoal, and ashed. Its specific activity was an expression of the degree of labeling of the thymidylate pool resulting from hydrolysis of thymidylate to thymidine and rephosphorylation by thymidine kinase.

**Enzyme Assays**—Comparative assays for thymidine kinase, thymidylate kinase, nucleoside diphosphokinase, and the corresponding phosphatases were carried out with 14C-labeled thymidine, dTMP, dTDP, and dTTP, as described previously (2). The enzyme extract was freed of small molecule contamination by gel filtration through Sephadex G-25, and radioactive reaction products were separated by chromatography on strips of DEAE-cellulose ion exchange paper (Whatman DE-81) and quantitated on a Vanguard model 880 ADE autoradioscanner. Nucleoside diphosphokinase activity was also determined by the spectrophotometric method of Ratliff et al. (13). This method permitted an approximate comparison of the specificities for the various pyrimidine nucleoside triphosphates in the direction of the back reaction. Since purine nucleoside triphosphates tended to react directly with the coupled assay system, their specificities could not be assessed by this method.

**RESULTS AND DISCUSSION**

**Relative Activities of Various Soluble Enzymes**—In order to gain a clearer understanding of the process by which thymidine or thymidylate is converted to thymidine triphosphate in extracts of Novikoff hepatoma, the relative activities of enzymes involved in this series of reactions were determined as shown in Table I. Direct assay, with 14C-labeled thymidine, dTMP, and dTDP showed kinase activities for all three substrates. Similarly, labeled dTMP, dTDP, and dTTP were employed to determine the activities of phosphatases capable of attacking these nucleotides. It is interesting to note that while phosphomonosterase and triphosphatase were fairly active, thymidine diphosphatase was barely detectable under these conditions. In this connection, it should be pointed out that the kinase activities presented actually represent net phosphorylation capacity over and above the competing phosphatases in the reaction mixture, rather than the total potential kinase activity.

Novikoff hepatoma extracts appear to contain no enzyme system analogous to adenylate kinase involving dTTP and dTMP. Thus, radioactive dTMP is phosphorylated by ATP but gives no evidence of reacting directly with dTTP. It appears likely then, that dTDP cannot be readily degraded to the monophosphate either by the action of phosphatase or by an adenylate kinaselike disproportionation reaction.

Perhaps the most striking feature of Table I is the exceedingly high nucleoside diphosphokinase activity in the Novikoff hepatoma extract, being at least three orders of magnitude more active than the other enzymes capable of reacting with thymidine derivatives. This may be taken to mean that any dTDP produced by thymidylate kinase in these reaction mixtures should attain equilibrium with dTTP very quickly indeed. However, the mere presence of an active nucleoside diphosphokinase in a crude cell-free system certainly does not prove that the enzyme phosphorylates dTDP in the intact tumor cell. It is possible that this dTDP-phosphorylating enzyme of tumor is analogous to the nucleoside diphosphokinase found in porcine liver mitochondria by Chiga and Plaut (14), and such a particulate enzyme might be unavailable to the soluble system in the intact cell.

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A highly active enzyme of this type was also detected in Landshutz ascites tumor cells by Grav and Smellie (6).

The excessively high concentration of the diphosphate-phosphorylating enzyme might suggest that it is a multifunctional enzyme of fairly broad specificity. Table II reveals that this enzyme (or enzymes) has the ability to react with both ribonucleotides and deoxyribonucleotides with the same order of efficiency. This is in general agreement with the properties of a crystalline enzyme isolated from brewers' yeast by Ratliff et al.
It is not within the scope of the present report to characterize the nucleoside diphosphokinase, or thymidylate kinase for that matter. Rather, the mechanism of their concerted action in the multienzyme supernatant system is to be analyzed below.

Labeled Phosphate Transfer Studies—Fig. 2 depicts the transfer of the labeled terminal phosphate of ATP to thymidylic acid. Initially all of the radioactive phosphorus resided in the $\gamma$-phosphate of ATP, such that the specific activity of this moiety was exactly three times that of the whole ATP. With time, ATPases in the enzyme mixture caused a gradual loss of the $\gamma$-phosphate, with the formation of ADP and orthophosphate-$^{32}$P (not shown). In the absence of an ATP-regenerating system this factor alone should not have produced any decrease in the $\gamma$-phosphate specific activity. The slight but steady decrease which was observed can be explained by the action of adenylate kinase on the accumulating unlabeled ADP. Evidence for the presence of this enzyme in the tumor extract is also seen in the gradual appearance of label in the middle phosphate of ATP, amounting to 10% of the $\gamma$-phosphate after 60 minutes of reaction time.

At the end of 60 minutes the accumulated thymidine triphosphate, totaling more than 1.5 $\mu$moles per flask, was isolated. It was not considered practical to attempt isolation of this product at 30 minutes, due to the relatively small quantities available. After identification, an aliquot was ashed for determination of the specific activity. The slight but steady decrease which was observed can be explained by the action of adenylate kinase on the accumulating unlabeled ADP. Evidence for the presence of this enzyme in the tumor extract is also seen in the gradual appearance of label in the middle phosphate of ATP, amounting to 10% of the $\gamma$-phosphate after 60 minutes of reaction time.

By assuming a constant rate of thymidylate phosphorylation throughout the 60-minute period (confirmed in unpublished experiments with $^{14}$C-labeled thymidylate), and a comparison might be made between dTTP and the ATP--$^{32}$P/ATP--$^{32}$P (not shown). The specific activity of the combined $\gamma$- and $\beta$-phosphates of dTTP. It can be seen that nearly all of the radioactivity in this derived dTTP resided in its $\beta$-phosphate, because the thymidylate pool which would contribute the $\alpha$-phosphate still had a very low specific activity at 60 minutes. Thus, the presence of substantial labeling in the $\beta$-phosphate of dTTP implies that thymidylate was phosphorylated in two sequential steps, with dTDP as the probable intermediate. A comparison of the specific activity of the dTTP with that of whole ATP shows it to be approximately twice that of ATP, again implying a two-step transfer of ATP--$\gamma$-phosphate to thymidylate. Because of the increment of label in the middle phosphate of the ATP, however, a more valid comparison might be made between dTTP and the ATP--$\gamma$-phosphate, directly. By assuming a constant rate of thymidylate phosphorylation throughout the 60-minute period (confirmed in unpublished experiments with $^{14}$C-labeled thymidylate), and assuming a negligible intermediate pool of dTDP, the dTTP

Table II

<table>
<thead>
<tr>
<th>Nucleoside Diphosphokinase from Novikoff Hepatoma</th>
<th>Corrected activity</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>UTP</td>
<td>0.14</td>
<td>100</td>
</tr>
<tr>
<td>dTTP</td>
<td>0.12</td>
<td>86</td>
</tr>
<tr>
<td>CTP</td>
<td>0.11</td>
<td>79</td>
</tr>
<tr>
<td>dCTP</td>
<td>0.10</td>
<td>71</td>
</tr>
</tbody>
</table>

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specific activity should be 67% of the average specific activity of the ATP-γ-phosphate moiety. The nearly linear decline in the specific activity of the precursor phosphate allows direct comparison between the final specific activity of dTTP, and ATP-γ-phosphate taken at 30 minutes, the midpoint of the reaction. On this basis the average specific activity of dTTP was 68% of the 30-minute ATP-γ-phosphate value, i.e. 86% of the value predicted for a two-step phosphorylation mechanism. This discrepancy probably can be explained in terms of some unlabeled thymidine polyphosphate in the enzyme extract prepared from tumor-bearing rats given thymidine by injection shortly before death. Any dialysis or gel filtration techniques carried out in absence of substrate tended to destroy thymidylate kinase, so undialyzed extract was used in this experiment. However, since contribution of nucleotide from undialyzed enzyme would tend to lower the dTTP specific activity, rather than raise it, dTTP specific activities nearly twice those predicted for the pyrophosphorylation mechanism (Fig. 1, Mechanism II) certainly can be regarded as evidence for the sequential mechanism (Mechanism I).

Similarly, the dTDP (derived from dTTP) should have a specific activity of exactly one-half that of its presumed precursor, the γ-phosphate moiety of ATP. Indeed, this value averaged 48% of ATP-γ-phosphate at 30 minutes, although it should be noted that determination of the specific activity of dTTP was subject to somewhat greater error than that of dTTP, due to the smaller quantities isolated. In any case, labeling of this magnitude must point again to Mechanism I, since the most probable pyrophosphorylation mechanism would not be expected to contribute any label to dTTP so derived.

It might be argued that the increment of label in the β-phosphate of ATP shown in Fig. 2 could compromise the interpretation just presented. Therefore, the experiment was repeated with a phosphoenolpyruvate-pyruvate kinase ATP-regenerating system. This had the desired effect of converting ADP back to ATP as rapidly as it formed, largely eliminating ATP-AMP phosphatase transfer catalyzed by adenylate kinase (Fig. 3). On the other hand, this rephosphorylation of ADP by unlabeled P-enolpyruvate resulted in a slightly more rapid decrease in the specific activity of ATP-γ-phosphate than observed in Fig. 2. Qualitatively, the results in Fig. 3 resemble those in the previous experiment, with specific activities of dTTP and dTDP well in excess of the average whole ATP value, while the thymidylate pool contained only a trace of radioactivity. Relative to the 30 minute ATP-γ-phosphate specific activity the dTTP isolated after 60 minutes is 78% of the predicted value. Thus, the specific activity of dTTP is relatively lower in the experiment utilizing P-enolpyruvate than when this phosphate donor was omitted. The explanation for this additional discrepancy is unclear. It is possible that under the conditions of the experiment, P-enolpyruvate donated unlabeled phosphate directly to thymidylate or dTDP.

Although Davidson's (15) experiments with purified pyruvate kinase seem to eliminate this possibility with various ribonucleoside diphosphates, deoxyribonucleoside diphosphates might react directly with P-enolpyruvate in the crude enzyme mixture used here. The dTDP also is about 72% of the value predicted, probably for the same reasons. Despite these discrepancies, however, these data clearly indicate that thymidylate phosphorylation occurs in a sequential manner.

Finally, to eliminate any possibility of a pyrophosphorylation mechanism, an experiment identical with those shown in Figs. 2 and 3 was carried out, except that ATP labeled in the middle phosphate was employed as the tracer, as shown in Fig. 4. It is apparent that the β-phosphate of ATP cannot contribute either to the β- or to the γ-phosphate of dTTP. Quantitatively, as much dTTP was produced in this experiment as in the previous one, except that it was virtually unlabeled by the ATP. The small amount of label which did appear in the dTTP can be explained by the 1% contamination appearing in the γ-phosphate of the ATP. Thus, in the tumor system, at least, this experiment should dispel the notion that pyrophosphorylation plays a role in the biosynthesis of dTTP.

The radioisotope-transfer experiments just described lend strong support to the theory that enzyme extracts from Novikoff hepatoma tissue catalyze the phosphorylation of dTMP to dTTP via the intermediate formation of dTDP, and therefore are in agreement with the sequence of reactions proposed by Weissman, Smellie, and Paul (4), by McAuslan and Joklik (8) and by Grav and Smellie (6).

\[
\text{dTMP} = \text{dTDP} = \text{dTTP}
\]

The pattern beginning to emerge is that thymidine kinase and thymidylate kinase are produced upon demand by cells preparing for DNA synthesis, in amounts which appear to regulate the rate of dTTP synthesis. As dTTP is formed by thymidylate kinase it probably is brought very rapidly into equilibrium with dTTP by high levels of a nonspecific nucleoside diphosphokinase (Tables I and II). It is interesting that Potter and Nygaard (16) have demonstrated such rapid equilibration \textit{in vivo}, in thymus and spleen of rats treated with injection with thymidine-2-14C. These authors also measured the approximate pool sizes of dTDP and dTTP in these organs and found that in spleen the concentration of dTDP was actually larger than that of dTTP, while in thymus the two pools were of approximately the same size. It is likely that the relative concentrations of dTDP and dTTP in the intact cell are regulated by the ADP to ATP ratio, as is the equilibrium constant of the nucleoside diphosphokinase reaction is close to unity (17).

The actual intracellular site of nucleotide phosphorylation is still open to question. Kielley (18) reported that substantial levels of thymidylate kinase could be released from a particulate fraction (presumably mitochondria) of resting mouse liver by alternate freezing and thawing. If this particulate-bound kinase is the functionally active form of the enzyme \textit{in vivo}, it is conceivable that the two enzymic reactions by which dTMP is converted to dTTP could both occur in the mitochondria, rather than in the cell sap. The appearance of thymidylate kinase and nucleoside diphosphokinase in the high speed supernatant extract (Table I) could be explained by rupture of mitochondria during homogenization in KCl.

The confusion as to the mechanism of dTTP synthesis may have been due, in part at least, to lack of uniformity in the use of ATP-regenerating systems in various laboratories. Thus, experiments which employed ATP plus 3-phosphoglycerate and followed the time course of thymidine phosphorylation generally showed the appearance of dTDP considerably later than dTTP in the time course of the reaction (2, 3, 5). The ultimate appearance of dTDP was probably due to exhaustion...
of the ATP-regenerating system, with concomitant formation of ADP, which would have the effect of shifting the nucleoside diphosphokinase equilibrium toward an increasing dTDP to dTTP ratio. Weissman, Smellie, and Paul (4), who did not employ an ATP-regenerating system, fractionated and counted the phosphorylated products of thymidine-32P after 90 minutes of incubation in the presence of regenerating liver extracts. They determined the percentage distribution of total radioactivity appearing in dTMP, dTDP, and dTTP, and regarded this distribution as reflecting thymidine kinase, thymidylate kinase, and “TDP kinase,” respectively. In a comparison of liver extracts prepared at various times after partial hepatectomy, maximum radioactivity occurred in the dTTP fraction (believed to represent “TDP kinase” activity) a few hours later in the course of liver regeneration than the peak of dTDP + dTTP (representing thymidylate kinase). These authors interpreted their data as evidence for the sequential induction of thymidine kinase, thymidylate kinase, and “TDP kinase.”

However, since the formation of dTTP by diphosphokinase in their system depended on the provision of dTDP by thymidylate kinase, an alternative explanation could be that the equilibrium dTDP to dTTP ratio was changed by a shift in the ADP to ATP ratio. It is quite possible that variations in ATPase activities during the course of liver regeneration may have altered the relative amounts of ADP and ATP in the reaction mixtures at the 90-minute termination point.

At the present juncture it appears that further detailed understanding of the thymidylate kinase-nucleoside diphosphokinase system can best be approached by careful cell fractionation and enzyme isolation and characterization. It can be anticipated that such studies will define the subcellular interrelation and enzyme isolation and characterization. It can be anticipated that such studies will define the subcellular interrelation and regulation of enzymes catalyzing the phosphorylation of deoxyribonucleotides.

SUMMARY

The mechanism of synthesis of thymidine triphosphate from thymidylate was studied in high speed supernatant extracts of Novikoff hepatoma solid-tumor explants. Such extracts were found to have comparable levels of thymidine kinase, thymidylate kinase, phosphomonoesterase, and nucleoside diphosphatase. Only marginal nucleoside diphosphatase activities could be detected, and the extracts appeared to contain no enzyme capable of transferring phosphate from thymidylate to thymidine monophosphate. Taken together, these two facts might suggest that thymidine diphosphate, once formed, is not readily degraded to thymidylate, either by hydrolysis or by disproportionation of phosphate residues.

Contrary to the rather low levels of thymidine kinase and thymidylate kinase activities, exceedingly active nucleoside diphosphokinase was observed in Novikoff hepatoma extracts. This enzyme, more than 1000 times as active as thymidylate kinase under these conditions, was found to be relatively nonspecific in its substrate requirements. It appeared to phosphorylate ribonucleoside diphosphates as well as deoxyribonucleoside diphosphates and probably serves a multifunctional role in the cell.

Adenosine triphosphate labeled specifically in the terminal phosphate (ATP-β-32P), or in the middle phosphate residue (ATP-β-32P), was utilized to determine if thymidylate is phosphorylated by a sequential mechanism involving two molecules of ATP, or if a single pyrophosphorylation reaction converts thymidylate to the triphosphate. The specific activity time course of whole ATP, ATP terminal phosphate, and ATP middle phosphate was observed throughout a 60-minute reaction period. The product of the reaction, thymidine triphosphate, was isolated after 60 minutes and compared with these possible precursors. When ATP-γ-32P was used as the precursor, the thymidine triphosphate had a specific activity about twice the average specific activity of the ATP, or about two-thirds the specific activity of the terminal phosphate of ATP. This evidence strongly indicates that dTTP is formed by the sequential addition of two molecules of ATP terminal phosphate to thymidylate, with thymidine diphosphate as an intermediate product. Further supporting evidence for thymidine diphosphate as an intermediate was obtained by chemical degradation of thymidine triphosphate to the diphosphate, which was found to have a specific activity of approximately one-half that of the terminal phosphate of ATP. Thus, two of the three phosphates of thymidine triphosphate appear to have been donated by the terminal phosphate of ATP, presumably in a sequential manner.

In identical experiments with ATP-β-32P, substantial quantities of thymidine triphosphate were formed, but virtually no label was incorporated into this product. It is apparent, therefore, that thymidine triphosphate synthesis does not involve the transfer of a pyrophosphate moiety from ATP in extracts of Novikoff hepatoma. It is concluded that thymidylate acid is sequentially phosphorylated, first to thymidine diphosphate, then to thymidine triphosphate.

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