5. These results are compatible with the view that much of the thyroidal tri-iodothyronine is formed by the coupling of mono- and di-iodotyrosine. The formation of minor quantities of tri-iodothyronine by deiodination of thyroxine is not precluded.

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The Mechanism of Formation of Thymidine 5′-Triphosphate by Enzymes from Landschutz Ascites-Tumour Cells

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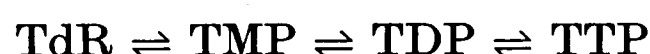
The deoxyribonucleoside 5′-triphosphates of adenine, guanine, cytosine and thymine have been shown to be utilized in the synthesis of polydeoxyribonucleotides by enzymes from Ehrlich and Landschutz ascites-tumour cells (Smellie *et al.* 1960), and the presence of the kinases responsible for the formation of the four deoxyribonucleoside 5′-triphosphates in extracts of ascites-tumour cells has been demonstrated (Keir & Smellie, 1959).

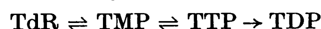
The ability of enzyme preparations from ascites-tumour cells to catalyse the formation of TTP from TdR or TMP is very limited (Keir & Smellie, 1959; Gray *et al.* 1960; Ives, Morse & Potter, 1962), particularly in comparison with their capacity to form dATP, dGTP and dCTP (Keir & Smellie, 1959; Gray *et al.* 1960). Similarly, though extracts of non-proliferating tissues contain active enzymes catalysing the formation of dATP, dGTP and dCTP from the corresponding deoxyribonucleoside 5′-monophosphates, the activities of the kinases responsible for the formation of TTP are exceedingly low (Gray *et al.* 1960).

Fractionation experiments on thymidine kinase and thymidylate kinase led Weissman, Smellie & Paul (1960) to suggest that the formation of TTP from TdR occurred by the stepwise addition of orthophosphate, forming successively TMP, TDP and TTP thus:



$$\mathrm{TdR} \rightleftharpoons \mathrm{TMP} \rightleftharpoons \mathrm{TDP} \rightleftharpoons \mathrm{TTP}$$

However, from experiments on the time-course of phosphorylation of TdR, Bianchi, Butler, Crathorn & Shooter (1961) have suggested that the mechanism involves the addition of orthophosphate to TdR to yield TMP followed by the addition of pyrophosphate to yield TTP directly. TDP observed in the reaction products was considered to arise from the degradation of TTP after prolonged incubation. This mechanism may be illustrated as follows:



$$\mathrm{TdR} \rightleftharpoons \mathrm{TMP} \rightleftharpoons \mathrm{TTP} \rightarrow \mathrm{TDP}$$

The purpose of the present investigation was to distinguish between these two mechanisms, and evidence is presented to show that TDP is an intermediate in the formation of TTP by the enzyme preparations from Landschutz ascites-tumour cells. A preliminary report of this work has already appeared (Grav & Smellie, 1962).

METHODS

Abbreviations. The following abbreviations are used: TdR, thymidine; TMP, thymidine 5′-monophosphate, except where necessary specially to distinguish thymidine 5′-monophosphate from thymidine 3-monophosphate where the abbreviations TMP-5′ and TMP-3′ are used; TDP, thymidine 5′-diphosphate; TTP, thymidine 5′-triphosphate; [α-^{32}P]TTP and [$\beta\gamma$-^{32}P]TTP, thymidine 5′-triphosphate labelled with ^{32}P on the ester phosphate and the terminal pyrophosphate respectively; [α-^{32}P]TDP and

[β-^{32}P]TDP, thymidine 5′-diphosphate labelled with ^{32}P on the ester phosphate and the terminal phosphate respectively; dATP, dGTP and dCTP, the 5′-triphosphates of deoxyadenosine, deoxyguanosine and deoxycytidine.

Biological material. Landschutz ascites carcinoma was maintained by serial transplantation in albino mice of the departmental colony and was harvested 8–9 days after inoculation. The tumour was collected in an equal volume of ice-cold 0·1 M-phosphate buffer, pH 8·1, and the cells were centrifuged down and washed several times with phosphate buffer. Extracts were prepared from the cells after osmotic disruption as described by Smellie, Keir & Davidson, 1959, and were freeze-dried and stored at −60°.

Substrates. [^{32}P]TMP was prepared by the method of Tener (1961) from TdR and cyanoethyl [^{32}P]phosphate. About 60% of the TMP in the reaction products was TMP-5′ and the remainder was accounted for as TMP-3′. In experiments where [^{32}P]TMP itself was used as substrate for the thymidylate kinases, the TMP-5′ was separated from the TMP-3′ as follows. The mixture was adsorbed on to a column of Dowex 1 (Cl^- form), the column was washed with water to remove any nucleosides, and the TMP-5′ and TMP-3′ were eluted by gradient elution (Hurlbert, Schmitz, Brumm & Potter, 1954) with water in the mixing vessel and 0·06 N-HCl in the reservoir. The complete separation of TMP-5′ and TMP-3′ was confirmed by chromatography of the two peaks.

[α-^{32}P]TTP was prepared from [^{32}P]TMP by a modification of the procedure of Smith & Khorana (1958). [$\beta\gamma$-^{32}P]-TTP was prepared from TMP and [^{32}P]orthophosphate by the same method.

TDP was prepared from TTP by partial acid hydrolysis as follows: 200 μmoles of TTP (trisodium salt) and 0·8 ml. of 5 N-HCl in a total volume of 20 ml. were incubated at 50° for 45 min. with continuous shaking, the mixture was cooled rapidly and the pH was adjusted to 7·5 with 10 N-NaOH. Samples of this solution were taken for paper chromatography in ammonium isobutyrate (Keir & Smellie, 1959) to obtain a measure of the extent of hydrolysis, and the bulk of the material was diluted to 1 l. with water and applied to a column (20 cm. × 2 cm.) of Dowex 1 (Cl^- form). The column was washed with water to remove any nucleoside and the nucleotides were eluted by gradient elution (Hurlbert *et al.* 1954) with 2 l. of 0·01 N-HCl in the mixing vessel and 0·2 M-LiCl in 0·01 N-HCl in the reservoir. The flow rate was 60 ml./hr. and fractions of 20 ml. were collected. The elution was followed spectrophotometrically at 267 mμ and tubes from the second peak (tubes 75–100) were combined for the isolation of TDP.

To remove contaminating orthophosphate and pyrophosphate, the pooled TDP fraction was treated with 15 ml. of a suspension of acid-washed charcoal. The mixture was stored for 18 hr. in the cold with occasional shaking and the charcoal was then packed into a column and washed with water until the effluent was free from Cl^- ions. Inorganic orthophosphate and pyrophosphate were removed by elution with 5–8 vol. of 0·01 M-$NaHCO_3$ or until ultraviolet-absorbing material appeared in the effluent, and the TDP was then eluted with 0·14 M-ammonia in aq. 70% (v/v) ethanol. This eluate was concentrated to small volume *in vacuo* at 25° and passed through a column (10 cm. × 1·5 cm.) of Dowex 50 (Na^+ form) to convert the TDP into the sodium salt. Paper chromatography of the product revealed a single ultraviolet-absorbing region corresponding to TDP, and the yield was 40 μmoles.

[α-^{32}P]TDP was prepared from [α-^{32}P]TTP by the above procedure, the product in this case being slightly contaminated with radioactive inorganic orthophosphate and pyrophosphate. No other nucleotides were present and the specific activity of the [α-^{32}P]TDP was approx. 12×10^6 counts/min./μmole when measured in a windowless gas-flow counter.

[β-^{32}P]TDP was prepared from [$\beta\gamma$-^{32}P]TTP by acid hydrolysis as described above. Chromatography of the product in ammonium isobutyrate revealed one major ultraviolet-absorbing region corresponding to TDP; however, subsequent analysis showed that about 25% of the total radioactivity in the preparation was present as [^{32}P]-orthophosphate, presumably as a result of partial degradation of the TDP. The specific activity of the [β-^{32}P]TDP was approx. 4×10^6 counts/min./μmole when determined in a windowless gas-flow counter, and the overall yield was 20%.

TdR and ATP were purchased from the Sigma Chemical Co., TMP was purchased from Schwarz BioResearch Inc. and from the California Corp. for Biochemical Research, TTP was obtained from Pabst Laboratories, [^{3}H]TdR of specific activity 360 mC/m-mole was purchased from Schwarz BioResearch Inc., and cyanoethyl [^{32}P]phosphate of specific activity not less than 50 mC/m-mole was either prepared by the method of Tener (1961) or purchased from The Radiochemical Centre, Amersham, Bucks.

Enzyme experiments. Thymidine kinase and thymidylate kinases were assayed by a modification of the procedure of Weissman *et al.* (1960). Unless otherwise indicated the reaction mixtures contained the following components in a total volume of 3·0 ml.: 15 μmoles of $MgCl_2$, 15 μmoles of ATP, 300 μmoles of tris buffer, pH 7·9, 0·3 μmole of 2-mercaptoethanol, and 3 μc of [^{3}H]TdR or [^{32}P]TMP, [α-^{32}P]TDP or [β-^{32}P]TDP in the quantities indicated in the legends to the Figures. The mixtures were incubated for various times at 37° in a shaking water bath.

The reaction was stopped by adding 1·5 ml. of cold 2·1 N-$HClO_4$ and subsequent manipulations were carried out in the cold. The protein precipitate was centrifuged down and washed three times with 0·5 ml. of 0·5 N-$HClO_4$, and the supernatant fluid and washings were combined. The solution was adjusted to pH 7·8 with 7 N-KOH, allowed to stand overnight in the cold and centrifuged to remove $KClO_4$. The supernatant fluid was diluted to 7·0 ml. with water and, after samples had been taken for the measurement of radioactivity, the remainder was applied to columns of ECTEOLA-cellulose for the separation of TdR derivatives.

ECTEOLA-cellulose (Kodak Ltd., London, or Serva Entwiklungslabor, Heidelberg) was prepared for use by preliminary steeping in twice its own volume of water followed by vigorous agitation with a model E1 Vibro-Mix (A.-G. für Chemie-Apparatebau, Männedorf, Zürich, Switzerland) for 5 hr. The adsorbent was allowed to settle, the supernatant fluid was decanted and this treatment was repeated until the supernatant fluid was clear. The suspension of ECTEOLA-cellulose was transferred to columns and washed twice with 50 ml. portions of water followed by 50 ml. portions of 0·5 N-HCl until the effluent was free from material absorbing in the region 257–267 mμ. Finally, the columns were washed with water until the pH of the effluent returned to neutrality.

Portions (3·5 ml.) of the supernatant fluids from the reaction mixtures were applied to columns (10 cm. ×

1·5 cm.) of ECTEOLA-cellulose and the columns were eluted successively with 100 ml. of water, 50 ml. of 0·01N-HCl, 50 ml. of 0·02N-HCl, 100 ml. of 0·04N-HCl and 70 ml. of 0·1N-HCl. TdR was removed in the water fraction, TMP in the 0·02N-HCl, TDP in the 0·04N-HCl, and TTP in 0·1N-HCl. Fractions (10 ml.) were collected and 0·1 ml. of each was plated on stainless-steel planchets. The time for 400 counts was determined in a Nuclear–Chicago windowless gas-flow counter and the total counts/min. in each portion of the eluate were calculated as a percentage of the total counts/min. recovered from the columns. The pattern of elution of TdR, TMP, TDP and TTP is shown in Fig. 1.

A similar procedure was adopted in the experiments with [^{32}P]TMP, [α-^{32}P]TDP and [β-^{32}P]TDP.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Determination of specific activities. In some experiments with ^{32}P-labelled substrates, the specific activities of the TDP and TTP in the reaction products were determined. Because of the very minute absolute amounts of these compounds normally present in the reaction mixtures, a large excess of TDP and TTP was added in certain experiments to provide sufficient TDP and TTP for accurate spectrophotometric measurement of the amount present.

For spectrophotometric determination of the thymidine nucleotides in the fractions recovered from the ECTEOLA-cellulose columns, further purification was needed to remove adenine nucleotides from the reaction mixture. Portions of the eluates from the columns of ECTEOLA-cellulose corresponding to TMP, TDP and TTP were separately combined in 150 ml. flasks, and 5N-HCl was added to make each solution 0·2N. Flasks containing TMP were heated at 100° for 20 min., and those containing TDP and TTP were heated at 100° for 60 min., after which time the mixtures were cooled and the pH was adjusted to 7·5 with 10N-NaOH. This treatment hydrolysed TDP and TTP to TMP, and adenine phosphates to AMP and adenosine. Each fraction was diluted to give a concentration of NaCl less than 0·01M and applied to columns (12 cm. × 2 cm.) of Dowex 1 (Cl^- form). Adenine and AMP were removed from the columns by washing with water followed by 50 ml. portions of 0·01N-HCl until the extinction of the effluent at 257 mμ approached zero. TMP was finally recovered quantitatively from the columns by eluting with 10 ml. portions of 0·05N-HCl, the eluates were diluted to 50 ml. and the nucleotide concentrations were determined from the extinctions at 267 mμ.

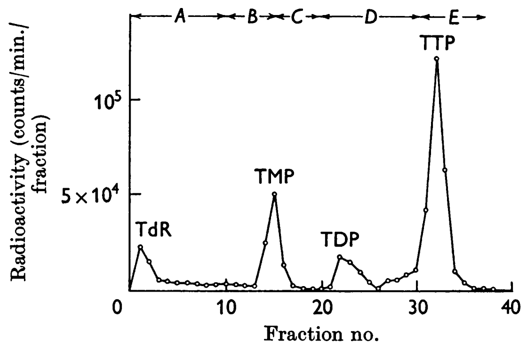


Fig. 1. Separation of ^{3}H-labelled TdR, TMP, TDP and TTP on columns of ECTEOLA-cellulose by stepwise elution with increasing concentrations of HCl: *A*, water; *B*, 0·01N-HCl; *C*, 0·02N-HCl; *D*, 0·04N-HCl; *E*, 0·1N-HCl.

RESULTS

Studies on the time-course of formation of TTP from [^{3}H]TdR such as those illustrated in Fig. 2(*a*) have invariably shown that labelled TMP and TDP appear very shortly after the start of the reaction. The proportion of the total radioactivity in TMP rose very sharply from the start of the incubation and reached a plateau at about 15 min. which was maintained until about 60 min. Thereafter, there was a further slight increase in the proportion of the total radioactivity in TMP. The radioactivity in TDP also began to rise immediately and rapidly reached a steady level that never exceeded 20 % of the total radioactivity. Labelled TTP also appeared early in the course of the reaction, and the proportion rose to a maximum of about 60 % of the total radioactivity in 15–30 min., remained reasonably constant up to 60 min. and then declined slightly, presumably owing to degradation of TTP. On no occasion was labelled TTP detected earlier than labelled TDP.

The results of similar experiments in which the earlier time-intervals were examined in more detail (Fig. 2*b*) showed that, though small amounts of labelled TMP and TDP were produced within the first minute, no radioactivity could be detected in TTP until after 3 min.

The general pattern of these results is consistent with the view that TMP and TDP are intermediates in the formation of TTP from TdR, but, in the absence of further information about the relative rates of the different reactions, cannot be taken as a definite indication of such a reaction sequence.

Other experiments were therefore carried out to determine whether the crude enzyme system could catalyse the formation of TTP directly from TDP.

When [α-^{32}P]TDP was used as substrate it was found that TDP was extensively converted into TTP; more than 60 % of the total radioactivity was recovered as TTP after 1 hr. of incubation (Fig. 3). Moreover, the addition of a tenfold excess of unlabelled TMP to the reaction mixture did not significantly alter the amount of radioactivity appearing in TTP. In these experiments, the specific activities of the TTP formed in the presence and absence of TMP were measured. Though these values were subject to considerable error because of the very small amounts of TTP present, no differences were observed such as would have been necessary had the TTP been formed from TDP via a a large pool of unlabelled TMP.

These experiments clearly demonstrated the ability of the enzyme preparations to form TTP from TDP and provided no evidence for the intermediate formation of TMP. Further, no higher phosphates of TdR than TTP were detected in the reaction mixtures, in contrast with the experience of Bianchi *et al.* (1961).

These conclusions were confirmed by experiments on the time-course of phosphorylation of [β-^{32}P]-TDP shown in Fig. 4. It is clear that the TDP was very rapidly and extensively converted into TTP, more than 60% of the total radioactivity being recovered in TTP within 5 min. About 25% of the total radioactivity at all time-intervals was accounted for as inorganic orthophosphate, but this was due to contamination of the TDP preparation and did not vary significantly during the course of the incubation.

Since the TDP used in these experiments was labelled in the β-phosphate any splitting of the TDP, by for example phosphatase action, would have given rise to unlabelled TMP and [^{32}P]orthophosphate, so that labelled TTP could only have been formed from such TMP if the [^{32}P]orthophosphate had been utilized for the phosphorylation of the TMP or had exchanged with the phosphate of TMP. To exclude these possibilities, experiments were carried out in which unlabelled TDP together with an amount of carrier-free [^{32}P]-orthophosphate corresponding to the amount of ^{32}P in the [β-^{32}P]TDP was substituted for the [β-^{32}P]-TDP. No labelled TTP was formed under these conditions, nor was there any evidence in any of these experiments for the formation of higher phosphates of TdR than TTP.

From these two types of experiment it became clear that the crude preparations of enzyme contain a kinase for the phosphorylation of TDP to TTP and that this reaction occurs very rapidly without

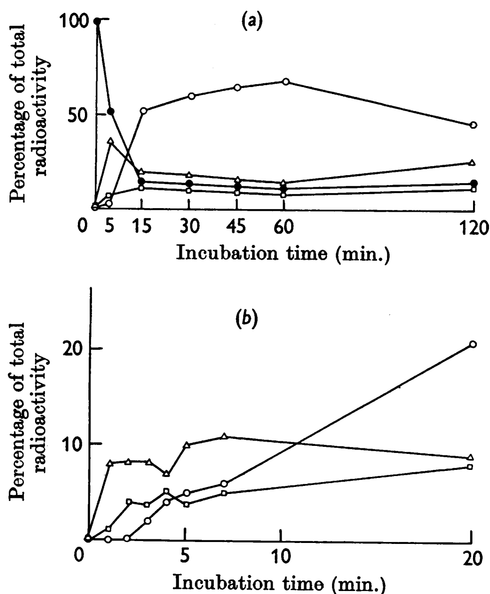


Fig. 2. (*a*) Time-course of phosphorylation of [^{3}H]TdR by extracts of Landschutz ascites-tumour cells. The reaction mixture contained 4·1 mg. of protein. (*b*) Initial time-course of phosphorylation of [^{3}H]TdR by extracts of Landschutz ascites-tumour cells. The reaction mixture contained 4·5 mg. of protein. The results in (*a*) and (*b*) are expressed as percentages of the total radioactivity recovered from the columns of ECTEOLA-cellulose. Different enzyme preparations were used in (*a*) and (*b*). ●, TdR; △, TMP; □, TDP; ○, TTP.

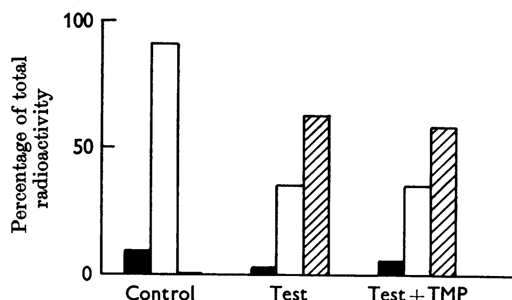


Fig. 3. Phosphorylation of [α-^{32}P]TDP by extracts of Landschutz ascites-tumour cells in the presence and absence of unlabelled TMP. The reaction mixtures contained 24 μm-moles of [α-^{32}P]TDP (specific activity $3{\cdot}5 \times 10^{6}$ counts/min./μmole) and, where indicated, 500 μm-moles of TMP, in addition to 4·5 mg. of protein. Incubation was for 60 min. Solid columns, TMP; open columns, TDP; hatched columns, TTP.

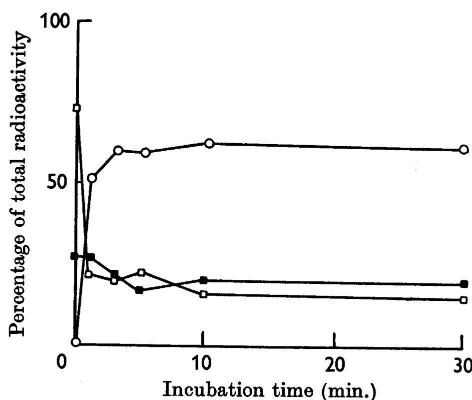


Fig. 4. Time-course of phosphorylation of [β-^{32}P]TDP by extracts of Landschutz ascites-tumour cells. The reaction mixture contained 172 μm-moles of [β-^{32}P]TDP (specific activity 10^{6} counts/min./μmole) and 4·5 mg. of protein. □, TDP; ○, TTP; ■, inorganic orthophosphate.

there being any evidence for the intermediate formation of TMP or of higher phosphates than TTP.

The time-course of phosphorylation of [^{32}P]TMP is shown in Fig. 5. At very early time-intervals labelled TDP appears in the reaction mixture before there is any evidence of labelled TTP, and this is consistent with the results shown in Fig. 2 (*b*). The proportion of the radioactivity in TDP never rises to a high level, but this may be due to the rapid rate at which TDP is converted into TTP and, indeed, the rate of formation of TDP may be the limiting factor in the rate of TTP synthesis.

In other experiments attempts were made to measure the specific activities of the TMP, TDP and TTP at various times during the conversion of [^{32}P]TMP into TTP. Large excesses of unlabelled TDP and TTP were added to the reaction mixtures at the start of the reaction to provide sufficient quantities of TDP and TTP in the reaction products for accurate spectrophotometric measurements. This, however, resulted in marked inhibition of the reaction so that only about 2% of the [^{32}P]-TMP was phosphorylated, and, though the data at least at the earlier time-intervals were consistent with the reaction sequence:

$$\text{TMP} \rightleftharpoons \text{TDP} \rightleftharpoons \text{TTP}$$

the extent of the reaction was so small that the significance of the results seemed doubtful.

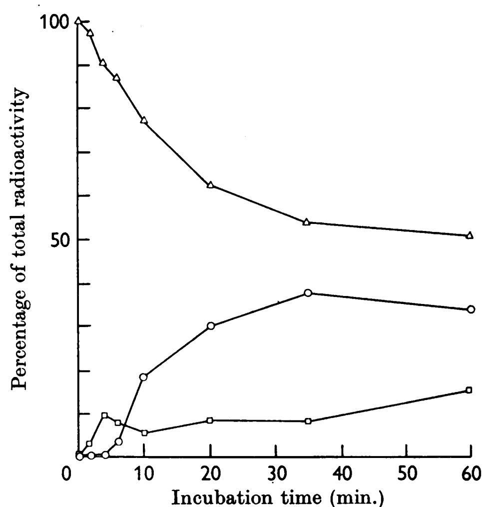


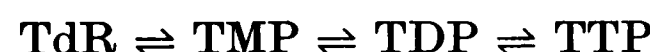
Fig. 5. Time-course of phosphorylation of [^{32}P]TMP by extracts of Landschutz ascites-tumour cells. The reaction mixture contained: 100 μmoles of tris buffer, pH 7·9, 5 μ-moles of ATP, 5 μmoles of $MgCl_2$, 0·1 μmole of 2-mercaptoethanol, 5·5 μm-moles of [^{32}P]TMP (specific activity 36×10^6 counts/min./μmole) and 1·5 mg. of protein, in a total volume of 1 ml. △, TMP; □, TDP; ○, TTP.

DISCUSSION

The kinetic experiments described above provide strong evidence for the role of TDP as an intermediate in the formation of TTP from TdR and TMP by soluble enzyme preparations from Landschutz ascites-tumour cells. Thus, in the time-course experiments with [^{3}H]TdR and [^{32}P]TMP (Figs. 2*a*, 2*b* and 5), labelled TDP was invariably found in the reaction products before labelled TTP, and the shapes of the curves for TDP were consistent with those expected for a transient intermediate. Furthermore, from the experiments with [α-^{32}P]TDP (Fig. 3) and [β-^{32}P]TDP (Fig. 4), it is clear that TDP is very rapidly converted into TTP without the intermediate formation of higher phosphates of TdR than TTP or of TMP.

The view that TDP is an intermediate in this reaction sequence is also supported by the experiments of McAuslan & Joklik (1962). Using enzyme preparations from uninfected HeLa cells and from cells infected with the virus of cowpox, these authors demonstrated that the accumulation of radioactivity in TTP from [^{14}C]TMP is greatly decreased by the presence of an excess of unlabelled TDP.

These observations are clearly consistent with the demonstration by Weissman *et al.* (1960) of three different phosphokinases in extracts of Ehrlich ascites carcinoma, each responsible for one step in the reaction sequence:



$$\text{TdR} \rightleftharpoons \text{TMP} \rightleftharpoons \text{TDP} \rightleftharpoons \text{TTP}$$

and with the subsequent partial purification of TMP phosphokinase and TDP phosphokinase by the centrifuging of extracts of calf thymus in sucrose density gradients (Bojarski, 1962).

These findings are in direct contrast with those of Bianchi *et al.* (1961), who found in experiments with similar enzyme preparations from regenerating rat liver, leukaemic mouse spleen and Ehrlich ascites carcinoma that the formation of TDP was always preceded by the accumulation of TTP. This led these workers to conclude that TTP was formed from TMP by a one-step reaction involving the addition of pyrophosphate and that TDP only arose as a product of degradation of TTP by, for example, phosphatase action. For the following reasons it is our view that this conclusion is not justified by the evidence presented.

(1) The resolution of the radioactivity in the TDP and TTP regions of the paper chromatograms demonstrated by Bianchi *et al.* (1961) is poor by comparison with the resolution we have obtained on columns of ECTEOLA-cellulose, and since at the best we only detected small amounts of TDP it seems quite possible that TDP might not have been detected in all the experiments of Bianchi *et al.* (1961).

(2) In seven out of the nine experiments reported by these authors no data are presented for the radioactivity of TDP at time-intervals of less than 1 hr., despite the observation that in most of the experiments little or no TdR remained in the reaction mixture at the end of this time.

(3) In the two remaining experiments where data for the radioactivity of TDP are presented for the short time-intervals, the early points for both TMP and TDP are so close as to be almost indistinguishable.

(4) Though Bianchi *et al.* (1961) admit the possibility that the appearance of TDP may only be transient, it seems to us most unlikely that the experiments they have carried out to investigate this possibility could throw any light on the situation. Thus, in these experiments, the phosphorylation of TdR by diluted enzyme preparations was examined in order to slow down the initial stages of the reaction. We do not understand why this would be expected to lead to any increased accumulation of TDP since dilution of the enzyme seems unlikely to slow down the reaction leading from TDP to TTP to a greater extent than that leading from TMP to TDP.

From our experiments it is clear that TMP and TTP are formed very rapidly from TdR and that TTP is formed very rapidly from TDP. In these circumstances it seems possible that the formation of TDP from TMP is the rate-limiting step in the overall reaction sequence, in which event TDP would only be expected to appear as a transient intermediate.

It is clear that the thymidine-kinase system is inhibited by the accumulation of reaction products, and hence the maintenance of a high rate of TTP formation is dependent on its efficient utilization. Thus Ives *et al.* (1962) have demonstrated negative-feedback control of thymidine kinase by TTP, and we have observed that the presence of high concentrations of TDP and TTP in the reaction mixture leads to inhibition of the phosphorylation of TMP. Further, the picture may be complicated by the influence of extraneous factors that might be present in the enzyme preparations. Thus, for example, the presence of phosphatases might distort the overall reaction by the dephosphorylation of TTP, as suggested by Bianchi *et al.* (1961), although in our experience the rate of disappearance of TTP was slow. In view of these difficulties, more detailed investigations of these reactions must await the purification of the enzymes concerned with the different stages of phosphorylation.

SUMMARY

1. The mechanism of formation of thymidine 5′-triphosphate from thymidine by soluble extracts of Landschutz ascites-tumour cells has been examined by an improved method for the complete separation of thymidine nucleotides on columns of ECTEOLA-cellulose.

2. Studies on the time-course of formation of thymidine 5′-triphosphate from [^{3}H]thymidine have invariably shown that thymidine 5′-monophosphate and thymidine 5′-diphosphate may be detected in the reaction mixture very soon after the start of the incubation. On no occasion was labelled thymidine 5′-triphosphate detected earlier than the diphosphate. In experiments with thymidine 5′-[^{32}P]-monophosphate, labelled diphosphate was again detected before labelled triphosphate.

3. When experiments were performed with thymidine 5′-diphosphate labelled in either the α- or β-phosphates with ^{32}P, thymidine 5′-triphosphate was formed very rapidly without the intermediate formation of thymidine 5′-monophosphate or any higher phosphates of thymidine than the triphosphate.

4. It is concluded that thymidine 5′-diphosphate is an intermediate in the formation of thymidine 5′-triphosphate from thymidine and thymidine 5′-monophosphate.

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