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Fractionation of Thymidine Phosphokinase, Thymidine 5′-Monophosphate Phosphokinase and Thymidine 5′-Diphosphate Phosphokinase in Extracts of Landschutz Ascites-Tumour Cells

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1. Extracts of Landschutz ascites-tumour cells have been fractionated by treatment with acid, alumina C_γ gel and Sephadex G-100 to yield purified preparations of thymidine phosphokinase, thymidine 5′-monophosphate phosphokinase and thymidine 5′-diphosphate phosphokinase. 2. These results clearly demonstrate the existence in Landschutz ascites tumour of three phosphokinases each of which catalyses one step in the reaction sequence: thymidine ⇌ thymidine 5′-monophosphate ⇌ thymidine 5′-diphosphate ⇌ thymidine 5′-triphosphate. Though these results do not preclude the participation of other enzymes in the formation of thymidine 5′-triphosphate from thymidine by Landschutz ascites-tumour cells, they provide strong support for the view that thymidine 5′-diphosphate is an intermediate in the formation of thymidine 5′-triphosphate from thymidine 5′-monophosphate by this system.

Previous investigations on cell-free extracts of ascites-tumour cells have indicated that the formation of TTP† from TdR probably proceeds by stepwise phosphorylation through TMP and TDP (Weissman, Smellie & Paul, 1960; Grav & Smellie, 1963), and may involve the participation of TdR kinase, TMP kinase and TDP kinase. The present investigation is concerned with attempts to separate and characterize these three phosphokinases in extracts of Landschutz ascites-tumour cells. A preliminary report of this work has appeared (Grav & Smellie, 1964).

EXPERIMENTAL

Biological material

The source of enzymes was an accumulated pool of freeze-dried extracts of Landschutz ascites-tumour cells prepared as described by Grav & Smellie (1963). This material was derived from about 400 tumour-bearing mice of the departmental colony, and the resulting carefully mixed stock of enzyme preparation was stored at − 50°.

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† Abbreviations: TdR, thymidine, TMP, TDP and TTP, thymidine 5′-mono-, di- and tri-phosphate respectively; TdR kinase, TMP kinase and TDP kinase denote respectively the enzyme systems responsible for the formation of TMP from TdR, TDP from TMP, and TTP from TDP.

Enzyme assays

TdR kinase and TMP kinase were assayed according to a modification of the procedure described by Grav & Smellie (1963) as follows:

Thymidine kinase. Reaction mixtures containing the following components in a total volume of 1·0 ml. were prepared: 100 μmoles of tris buffer, pH 8·0; 5 μmoles of ATP; 5 μmoles of $MgCl_2$; 0·1 μmole of 2-mercaptoethanol, 1·0 μc of [^{3}H]TdR of specific radioactivity 360 mc/m-mole; enzyme solution containing the quantities of protein given in the legends to the Figures. The mixtures were incubated in a shaking water bath for 15 min. at 37°. The reactions were stopped by heating for 2 min. at 100° and, after centrifuging to remove coagulated protein and dilution of the resulting supernatant fluid to 3·0 ml., a portion (2·0 ml.) was applied to a column (10 cm. × 1·5 cm. diam.) of ECTEOLA-cellulose for separation of TdR derivatives. The columns were eluted successively with 150 ml. of water and 100 ml. of 0·2 N-HCl, TdR being recovered in the water fraction and the thymidine nucleotides in the HCl fraction. Portions (0·1 ml.) from each fraction were plated on stainless-steel planchets and counted in a Nuclear–Chicago windowless gas-flow counter. The results were expressed either in terms of the percentage of total radioactivity recovered as (TMP + TDP + TTP) or as the number of mμmoles of TdR phosphorylated.

Thymidylate kinase. Two methods were employed for the measurement of TMP-kinase activity.

(*a*) Radioactivity assay. This method measured the rate of formation of (TDP + TTP) from [^{32}P]TMP and the reaction mixture contained the following components in a total volume of 1·0 ml.; 100 μmoles of tris buffer, pH 7·6; 5 μmoles

of $MgCl_2$; 5 μmoles of ATP; 20 mμmoles of [^{32}P]TMP (3×10^6 to 4×10^6 counts/min./μmole); enzyme in the quantities given in the legends to the Figures. The mixtures were incubated in a shaking water bath for 25 min. at 37° and were heated at 100° for 2 min. to terminate the reaction. Coagulated protein was removed by centrifuging, the supernatant fluid was diluted to 3·0 ml. and a portion (1·5 ml.) was reserved for analysis by paper chromatography. Of the remainder, 1·0 ml. was adsorbed on a column (10 cm. × 1·5 cm. diam.) of ECTEOLA-cellulose. The TdR nucleotides were eluted by applying successively 150 ml. of 0·015 N-HCl and 100 ml. of 0·2 N-HCl. TMP was recovered in the 0·015 N-HCl fraction and (TDP+TTP) were eluted in the 0·2 N-HCl fraction. Portions (0·1 ml.) from each fraction were plated on stainless-steel planchets and counted in a Nuclear–Chicago windowless gas-flow counter. The results were expressed as the number of mμmoles of TMP phosphorylated, and 1 unit of TMP-kinase activity was that amount catalysing the phosphorylation of 1 mμmole of TMP/25 min.

Additional information on the relative proportions of the reaction products due to TMP kinase and TDP kinase were obtained by paper chromatography in ammonium isobutyrate (Keir & Smellie, 1959). The positions of radioactive areas on chromatograms were ascertained by the use of the Nuclear–Chicago 2pi Actigraph with a windowless counting chamber. Estimates of the quantities of TMP, TDP and TTP were obtained either by cutting and weighing relevant peaks from the recorder chart or by cutting radioactive areas from the fully developed chromatogram, eluting the nucleotides by capillary flow in distilled water and counting portions of the eluate as described above. TDP-kinase activity was not measured in enzyme fractions in which TMP-kinase activity was absent.

In some enzyme experiments a relatively large amount of TMP was added as a stabilizing factor for TMP kinase during the kinase fractionation. Assays reflecting absolute values of TMP-kinase activity could therefore not be performed directly, owing to the dilution of [^{32}P]TMP resulting from the presence of the unlabelled nucleotide. On such occasions unlabelled TMP was removed from the enzyme fractions by treatment with Sephadex G-25. Sephadex G-25 (coarse grade) was pretreated with 0·01 M-phosphate buffer, pH 7·6, and, after equilibration, packed carefully into a column (10 cm. × 1 cm. diam.) (in the manner described below for Sephadex G-100). The column constants, V_0 and V_i (see below), were determined by the use of sheep haemoglobin and KCl respectively at a flow rate of 50 ml./hr. The sample from enzyme solutions containing TMP was layered on the bed surface and elution was performed with 0·01 M-phosphate buffer, pH 7·6. The protein fraction appearing at V_0 after the start of the elution was collected and employed for the assay of TMP kinase.

(*b*) Spectrophotometric assay. This method measured the rate of formation of TDP from TMP by coupling the reaction with the oxidation of $NADH_2$ (Kornberg & Pricer, 1951; Bello & Bessman, 1963). However, because of the non-specific nucleoside diphosphokinase present in the pyruvate-kinase preparation used, both ADP and TDP were converted into their respective triphosphates. Thus 2 moles of $NADH_2$ were oxidized for every mole of TMP phosphorylated. The complete reaction mixture contained the following components in a total volume of 2·29 ml.: 0·1 μmole of TMP; 10 μmoles of ATP; 10 μmoles of $MgCl_2$; 0·25 μmole of $NADH_2$; 0·5 μmole of phosphoenolpyruvate; 200 μmoles of tris buffer, pH 7·6; 20 μg. of lactate dehydrogenase; 20 μg. of pyruvate kinase; enzyme solution. The reaction was initiated by the addition of TMP and the rate of oxidation of $NADH_2$ was followed on a Beckman DB spectrophotometer at 340 mμ and at 30°. Because of endogenous $NADH_2$ oxidases and phosphatases in the crude ascites-tumour extracts, the assay was not used for other than highly purified TMP-kinase preparations.

Adenosine triphosphatase. Adenosine triphosphatase was measured by using the spectrophotometric procedure described above for TMP kinase in which TMP had been omitted from the reaction mixtures. Results were calculated in μmoles of ADP formed/25 min.

Protein estimations were performed by the method of Lowry, Rosebrough, Farr & Randall (1951).

Enzyme-fractionation techniques

All manipulations were carried out at 0–2°.

Dilute acid fractionation. In fractionation with dilute acetic acid, the method used was essentially that of Weissman *et al.* (1960), with minor modifications.

For enzyme fractionation, freeze-dried soluble extracts of Landschutz ascites-tumour cells were dissolved in 0·01 M-phosphate buffer, pH 7·6, to give a solution containing 6–8 mg. of protein/ml. To this solution was added 1·0 N-acetic acid drop by drop until the appropriate pH was reached (between 4·5 and 5·0); the resulting mixture was quickly transferred to centrifuge tubes and centrifuged at 2000 *g* for 7 min., and the supernatant fluid decanted and adjusted to pH 7·6 with 1·0 N-NaOH (AS fraction). The sediment, if required, was suspended in 0·5 vol. of 0·01 M-phosphate buffer, pH 7·6, and the pH similarly adjusted to 7·6. The enzyme preparations were preserved by freeze-drying and storage at −50°.

Alumina C_γ fractionation. A 4-year-old preparation of alumina C_γ gel (Willstätter & Kraut, 1923), stored in an aqueous suspension containing 11 mg. of $Al_2O_3,3H_2O$/ml., was used.

The fractionation procedure involved measurement of the kinase activity remaining in the supernatant fluid after the addition of increasing quantities of alumina C_γ gel. Enzyme solution was prepared by dissolving the freeze-dried AS fraction in 0·01 M-phosphate buffer, pH 7·6, to give a protein concentration of 4–8 mg./ml. In small-scale experiments, portions of the alumina C_γ suspension ranging from 0·5 to 10 ml. were added to 15 ml. centrifuge tubes, and the gel was packed tightly in the tubes by centrifugation, washed three times with 0·01 M-phosphate buffer, pH 7·6, and then allowed to equilibrate at 0° for 24 hr. in the same buffer. After centrifugation and decantation, a 1·0 ml. portion of enzyme solution was added to each tube and the gel was dispersed by gentle stirring over a 5 min. period. Thereafter, the mixtures were left at 0° for 30 min. to complete the adsorption. The gel was then sedimented by centrifugation at 1000 *g* for 15 min. and the supernatant fluid was decanted and assayed for the appropriate kinase activity. The initial enzyme activity was obtained by assaying the untreated enzyme solution and the results were calculated in terms of the percentage of enzyme activity adsorbed. The enzyme fractions were preserved by freeze-drying and storage at −50°.

Enzyme fractionation by gel-filtration. Pretreatment of Sephadex G-100 dextran gel (200–400 mesh) (A.B. Pharmacia, Uppsala, Sweden) was performed by adding the dry dextran powder to excess of 0·01 M-phosphate buffer, pH 7·6, and allowing the mixture to equilibrate and swell for 24 hr. While the process of swelling was in progress, it was convenient to remove fine particles by stirring the suspension and then allowing the gel to sediment. The supernatant fluid was removed by suction and washing was repeated until the supernatant fluid was clear.

The gel was packed into a column (98 cm. × 1 cm.), with the precautions given by Flodin (1962), and the gel was washed with 0·01 M-phosphate buffer, pH 7·6, until a homogeneous column bed had formed. The column parameters, column void volume (V_0) and column imbibed volume (V_1) (Flodin, 1962), were determined by measuring the volume of eluent required to move peak concentrations of, respectively, γ-globulin (mol.wt. 150000; Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) and KCl from the top to the bottom of the column. Samples were layered on the bed surface with a pipette after the removal of supernatant buffer and allowed to enter the bed, and the bed surface was then washed with a small volume of eluent. A larger volume of buffer was then added and the elution commenced. In enzyme-fractionation experiments, eluate fractions (2·0 ml.) were collected with a flow rate of 15–25 ml./hr., samples being removed from each fraction for the assay of TdR kinase, TMP kinase, TDP kinase and adenosine triphosphatase. The remainder of each enzyme fraction was preserved by freeze-drying and storage at −50°.

Materials

ATP and TdR were purchased from the Sigma Chemical Co., St Louis, Mo., U.S.A. [^{3}H]TdR of specific activity 360 mc/m-mole and TMP were obtained from Schwarz Bio-Research Inc., Mount Vernon, N.Y., U.S.A. TTP was obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A. [^{32}P]TMP was prepared as described previously (Tener, 1961; Grav & Smellie, 1963) from carrier-free inorganic [^{32}P] orthophosphate (The Radiochemical Centre, Amersham, Bucks.), 2-cyanoethanol and TdR. $NADH_2$, pyruvate kinase and lactate dehydrogenase were purchased from Biochimica Boehringer, Mannheim, Germany.

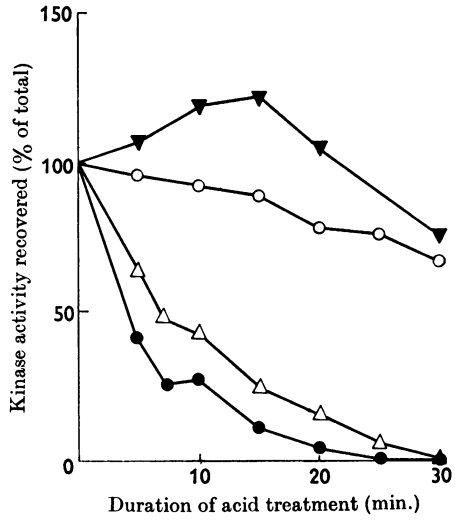

Fig. 1. Effect of treatment with dilute acetic acid on the activities of TdR kinase, TMP kinase and TDP kinase of cell-free extracts of Landschutz ascites-tumour cells. Two enzyme solutions were prepared by dissolving freeze-dried extracts of Landschutz ascites-tumour cells in 0·01 M-phosphate buffer, pH 7·6. Both solutions contained 8·5 mg. of protein/ml. To one solution was added 80 mμmoles of TMP/1·5 mg. of protein, and to both solutions 1·0 N-acetic acid was added until pH 4·5 was attained. Samples of the solution were either removed immediately, centrifuged and the pH of the supernatant fluid readjusted to pH 7·6, or allowed to stand at 0° for the times indicated on the diagram before being similarly neutralized. The details of the acid treatment were those given in the text. Enzyme activities were determined as indicated and the results were expressed as the percentage of total initial enzyme activity recovered in the neutralized supernatant fractions. For the solution containing added TMP, only the curve for TMP kinase is recorded, no change being observed in the recovery of TdR kinase and TDP kinase. △, TMP-kinase activity; ▼, TMP-kinase activity in the presence of TMP; ○, TdR-kinase activity; ●, TDP-kinase activity.

RESULTS

Preliminary experiments had demonstrated that TdR kinase, TMP kinase and TDP kinase were highly unstable in aqueous solution. Crude ascites-tumour extracts retained their kinase activities for a few hours only, and extracts aged for 24 hr. at 0° frequently lost all detectable TMP-kinase and TDP-kinase activities, while retaining 40% only of their TdR-kinase activity. Similarly, after dialysis of such extracts against three changes of 0·01 M-tris buffer, pH 7·8, in 0·1 mM-2-mercaptoethanol for 18 hr., only 30% of the TdR-kinase activity was recovered, and TMP-kinase and TDP-kinase activities were all but completely lost. However, all three kinases retained their activity when stored at −50° after freeze-drying. The differential stability of TdR kinase, TMP kinase and TDP kinase was further emphasized in experiments designed to study the response of the kinases to treatment with dilute acid.

In such experiments (Fig. 1) with stock enzyme preparation dissolved in phosphate buffer, pH 7·6, the percentage of the total TdR-kinase, TMP-kinase and TDP-kinase activities recovered in the fraction remaining soluble at pH 4·5 was determined at different time-intervals after precipitation with dilute acetic acid, the time-intervals being measured from the initial attainment of pH 4·5 to the readjustment of the acid-soluble fraction to pH 7·6. The results showed that the ability of such extracts to catalyse the formation of TTP from TdR was

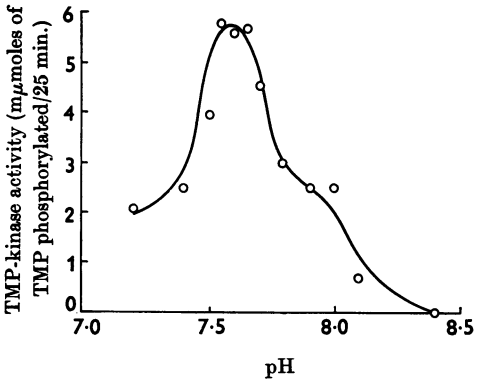

Fig. 2. Effect of pH on the activity of TMP kinase. The reaction mixtures contained the following components in a total volume of 1·0 ml.: 5 μmoles of ATP; 5 μmoles of $MgCl_2$; 20 mμmoles of [^{32}P]TMP of specific activity $3·5 \times 10^6$ counts/min./μmole; 1·0 mg. of protein; 100 μmoles of tris buffer of the pH indicated on the diagram. Incubation was for 25 min. in a shaking water bath at 37° and further treatment was as described in the text. The source of enzyme was the AS 4·7 fraction.

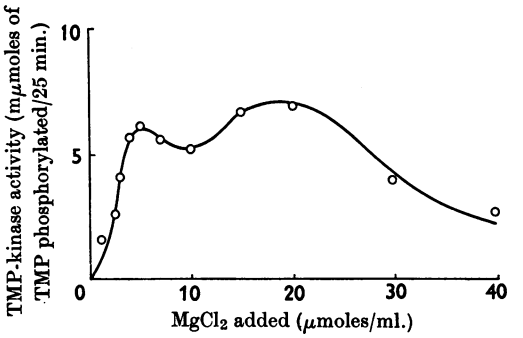

Fig. 3. Effect of Mg^{2+} concentration on the activity of TMP kinase. The reaction mixtures contained the following components in a total volume of 1·0 ml.: 5 μmoles of ATP; 100 μmoles of tris buffer, pH 7·6; 20 mμmoles of [^{32}P]TMP of specific activity $3·5 \times 10^6$ counts/min./μmole; 1·0 mg. of protein; $MgCl_2$ in the quantities indicated on the diagram. Incubation was for 25 min. in a shaking water bath at 37° and further treatment was as described in the text. The source of enzyme was the AS 4·7 fraction.

progressively lost over a 30 min. period of treatment with acid, the TMP-kinase and TDP-kinase activities being lost at a more rapid rate than was TdR-kinase activity. A preparation with TdR kinase alone may thus be obtained after a 30 min. acid treatment, when TMP-kinase and TDP-kinase activities are destroyed. The TdR-kinase preparation so obtained was purified 3–4-fold over the cell-free extracts and catalysed the phosphorylation of TdR to TMP with ATP as phosphoryl group donor. This reaction exhibited a relatively narrow pH optimum from 7·8 to 8·1 and showed an absolute requirement for Mg^{2+} (5 mM). Enhancement of TdR-kinase activity in the presence of 2-mercaptoethanol was only marginal, and addition of bovine serum albumin had no effect, in contrast with the observation by Okazaki & Kornberg (1964).

When an excess of TMP (80 mμmoles of TMP/1·5 mg. of protein) was added to the extract before the acid fractionation, 80–120% of the initial TMP kinase was recovered in the supernatant fraction (AS fraction), in a form purified 2–3-fold, after acidification to pH 4·7, whereas much of the TDP kinase was destroyed. Phosphorylation of TMP by this fraction (AS 4·7 fraction) had a sharp pH optimum of 7·6 (Fig. 2) and showed absolute requirements for ATP (5 mM) and for Mg^{2+} (5 or 20 mM) (Fig. 3). The double Mg^{2+} optimum may be due to different Mg^{2+} requirements of TMP kinase and TDP kinase. The reaction was inhibited by 2-mercaptoethanol but was unaffected by Na^+ and K^+.

Treatment of the AS 4·7 fraction at pH 7·6 with increasing concentrations of alumina C_γ gel led to adsorption of TMP kinase together with much of the protein (Fig. 4). Thus the addition of 5 ml. of alumina C_γ suspension to the enzyme solution (1·0 ml.) resulted in adsorption of all but a few per cent of the TMP-kinase activity, and further additions of gel did not markedly alter the proportion of adsorbed and unadsorbed enzyme. If excess of TMP (80 mμmoles of TMP/1·5 mg. of protein) was added to the AS 4·7 fraction before adsorption on the alumina gel, however, the rate of adsorption of the TMP kinase was markedly decreased, so that, though most of the protein was adsorbed along with TDP kinase and TdR kinase, a considerable proportion (40–80%) of the TMP kinase remained in solution. The fraction remaining in the supernatant fluid after the addition of 3·0 ml. of gel suspension/ml. of enzyme solution was purified 60–80-fold over the crude ascites-tumour extract and was designated the C_γ AS 4·7 fraction.

Further purification of the C_γ AS 4·7 fraction on a column of Sephadex G-100 by gradient elution with 0·01 M-phosphate buffer, pH 7·6, yielded TMP kinase purified 1000-fold over the crude ascites-tumour extract. This preparation (SC_γ AS 4·7 fraction) was devoid of TdR-kinase and TDP-kinase activities but was contaminated with adenosine triphosphatase. Because of this contamination, it seemed possible that the adenosine triphosphatase or some other phosphatase present in the preparation might be capable of hydrolysing TTP. If this were so, no TTP would be expected to appear among the reaction products in assays where

[^{32}P]TMP was used as substrate, since the activity of a possible TDP kinase would be masked by destruction of the TTP product, the TTP being reconverted into TDP as quickly as it was formed.

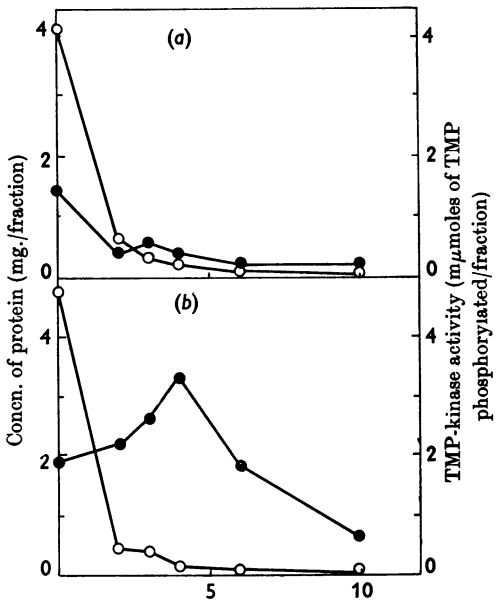

Fig. 4. Adsorption of TMP kinase on alumina C_γ gel in the presence and absence of added TMP: (*a*) TMP absent; (*b*) TMP added. The freeze-dried AS4·7 fraction was dissolved in 0·01 M-phosphate buffer, pH 7·6, and the resulting solution was divided into two parts, to one of which was added 80 mμmoles of TMP/1·5 mg. of protein. Portions (1·0 ml.) of these solutions were transferred to 15 ml. centrifuge tubes containing the volumes of alumina C_γ gel indicated on the diagram. Adsorption was carried out for 35 min. at 0°, after which the mixtures were centrifuged and the protein and TMP-kinase activity remaining in the supernatant fluid were measured by the radioactivity assay. The results were expressed in mμmoles of TMP phosphorylated/25 min./supernatant fraction. Protein concentrations were likewise expressed as mg./supernatant fraction. ○, Concn. of protein; ●, TMP-kinase activity.

No such TTP-phosphatase activity was detected in the TMP kinase obtained after elution from Sephadex G-100. The overall results of purification of TMP kinase based on a combination of these steps are shown in Table 1.

Fractionation of the AS4·7 fraction directly on a column of Sephadex G-100 by gradient elution with 0·01 M-phosphate buffer, pH 7·6, led to three peaks of kinase activity (Fig. 5). The first,

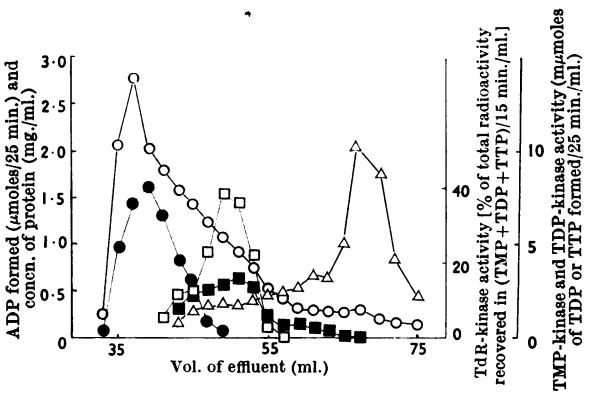

Fig. 5. Chromatography of the AS4·7 fraction on a column of Sephadex G-100 dextran gel. The eluent was 0·01 M-phosphate buffer, pH 7·6, and the details of the fractionation were those described in the text. Fractions (2·0 ml.) were collected at 2° and at a flow rate of 15 ml./hr. TdR-kinase activity was measured as described in the text with [^{3}H]TdR as substrate, and the results are recorded as the percentage of total radioactivity recovered in (TMP+TDP+TTP)/ml. of effluent after an incubation period of 15 min. TMP-kinase activity was measured by the radioactivity assay with [^{32}P]TMP as substrate. TDP-kinase activity was measured by the use of paper-chromatographic techniques. Adenosine-triphosphatase activity was determined by the spectrophotometric assay. The fractions containing TMP kinase uncontaminated with TDP-kinase activity (after 64–75 ml. of effluent had been collected) were combined and designated the SAS4·7 fraction. ○, Concn. of protein; □, adenosine-triphosphatase activity; ●, TdR-kinase activity; △, TMP-kinase activity; ■, TDP-kinase activity.

Table 1. *Summary of thymidylate-kinase purification*

TMP-kinase activity was measured by the radioactivity assay, with [^{32}P]TMP as substrate.

Enzyme fraction	Total activity (units)	Total protein (mg.)	Specific activity (units/mg.)	Purification
Crude extract	7800	9510	0·82	—
Redissolved freeze-dried extract	7000	5650	1·25	1·5
AS4·7 fraction	7950	2475	3·21	4
C_γAS4·7 fraction	7500	91	82·4	100
SC_γAS4·7 fraction	4150	5	950	1150

representing TdR kinase, appeared in the effluent superimposed on the main protein peak, and the second contained TDP kinase contaminated with adenosine triphosphatase and a small amount of TMP kinase accounted for by slight overlapping of the third peak of TMP kinase. This third peak contained TMP kinase, and the six fractions with the highest specific activity were combined and designated the SAS4·7 fraction. This preparation was purified 60-fold over the crude ascites-tumour extract and contained 40% of the TMP kinase applied to the column. Moreover, the TMP kinase so obtained was capable of phosphorylating TMP to TDP only, thus indicating the absence of TDP kinase; neither was there any evidence of TdR kinase or adenosine triphosphatase in this preparation. It did not contain phosphatases capable of degrading TTP, so that the failure to detect TTP among the reaction products was not due to simultaneous synthesis and degradation of the triphosphate.

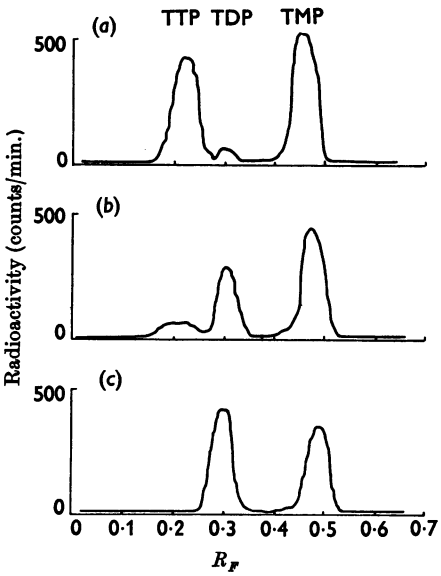

Fig. 6. Paper-chromatographic separation of reaction products obtained in the phosphorylation of [^{32}P]TMP by enzyme preparations at various stages of the purification of TMP kinase: (*a*) crude extract; (*b*) AS4·7 fraction; (*c*) SAS4·7 fraction. Enzyme assays with [^{32}P]TMP as substrate were carried out as described in the text, and the reaction products were subjected to descending paper chromatography in isobutyric acid–water–ammonia–EDTA (Keir & Smellie, 1959). Appropriate ^{32}P-containing lanes were cut from the chromatograms and scanned for radioactivity with the Nuclear-Chicago Actigraph windowless gas-flow detector. Marker compounds included in the chromatograms are shown.

The progressive removal of TDP kinase from TMP kinase during the fractionation is shown in Fig. 6, where chromatograms of reaction products run at different stages of the purification of TMP kinase show the gradual disappearance of biosynthetically formed TTP.

DISCUSSION

The enzyme-fractionation experiments described above provide strong evidence for the separate existence of three phosphokinases in soluble extracts of Landschutz ascites-tumour cells, each capable of catalysing one step in the phosphorylation of TdR to TTP. Moreover, these results strongly support the view that the formation of TTP from TdR proceeds by way of TMP and TDP, as suggested by Weissman *et al.* (1960) and Grav & Smellie (1963), so that the following sequence of reactions may be visualized:

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

Though the demonstration of the presence of TdR kinase, TMP kinase and TDP kinase makes the above mechanism likely, this evidence alone does not preclude the existence in the crude ascites-tumour extracts of a pyrophosphokinase capable of forming TTP directly from TMP, as suggested by Bianchi, Butler, Crathorn & Shooter (1961), or of other alternative pathways for the formation of TTP, but the purification in high yield of a TMP kinase that phosphorylates TMP to TDP only makes it probable that an enzyme of this type is a normal participant in the production of TTP from TMP in these extracts. This view is also consistent with the partial separation of a TMP kinase and a TDP kinase by the centrifugation of extracts of calf thymus in sucrose-density gradients (Bojarski, 1962).

Though the instability of TdR kinase and TMP kinase proved a major obstacle in their characterization, the evidence was consistent with the view of Weissman *et al.* (1960) that the kinases were not equally labile, and it was found that in order of decreasing stability was TdR kinase, TMP kinase and TDP kinase. The addition of excess of TMP to enzyme solutions protected TMP kinase, but was without effect on the TdR-kinase and TDP-kinase activities. Thus protection of TMP kinase by its substrate, originally predicted by Hiatt & Bojarski (1960), was confirmed.

The properties of the TdR kinase of Landschutz ascites-tumour cells generally occur with those ascribed to the Ehrlich ascites-carcinoma enzyme by Weissman *et al.* (1960), although TdR kinase in the present investigation did not respond to ammonium sulphate fractionation. However, the partially purified TdR kinase appeared to be different from

the TdR kinase of Novikoff hepatoma (Ives, Morse & Potter, 1963), since it was more labile and showed different requirements for ATP and Mg^{2+}. Though the specific activity of the TdR kinase purified from *Escherichia coli* (Okazaki & Kornberg, 1964) is several thousand times that of the Landschutz ascites tumour, the pH optima and Mg^{2+} requirements of the two enzymes are similar, as is the stabilization by 2-mercaptoethanol.

The TMP kinase had several properties that distinguish it from the non-specific nucleoside monophosphate kinase investigated by Strominger, Heppel & Maxwell (1959). The TMP kinase was much more labile, had a lower reaction velocity and was destroyed by acetone and ammonium sulphate fractionation. Similarly, the ascites-tumour TMP kinase differed from the *E. coli* TMP kinase described by Hurwitz (1959) in that acetone and ammonium sulphate fractionations resulted in serious loss of activity. The TMP kinase also appears to be distinct from the TMP kinase found in extracts of Novikoff hepatoma (Ives *et al.* 1963), which had different ATP and Mg^{2+} requirements. A deoxyribonucleoside monophosphokinase from *E. coli* infected with T2 bacteriophage has been purified (Bello & Bessman, 1963) and shown to catalyse the phosphorylation of TMP to TDP stoicheiometrically. This enzyme differed clearly from the ascites-tumour TMP kinase in that it had a broad pH optimum from 7·0 to 9·3, was much more stable in aqueous solution and had a much higher reaction velocity.

The TDP kinase was not studied in detail, but, insofar as it was found to be extremely unstable, it resembles the enzyme from regenerating rat liver (Breitman, 1963) and that from extracts of Ehrlich ascites-tumour cells (Weissman *et al.* 1960). That the crude extracts of Landschutz ascites-tumour cells contain a TDP kinase of relatively high reaction velocity has already been reported (Grav & Smellie, 1963).

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