Inhibition of Thymidine-5′-monophosphokinase from L5178Y Mouse Leukemia by Diethystilbestrol and 17-Ethynyl-β-estradiol

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SUMMARY

Diethylstilbestrol and 17-ethynyl-β-estradiol irreversibly inhibited thymidine-5′-monophosphokinase (EC 2.7.4.9) which had been purified from L5178Y mouse leukemia cells. Preparations of TMP-kinase from mouse spleen, calf thymus, and *Escherichia coli* were not inhibited by these compounds. A high concentration of thymidylate protected TMP-kinase from inactivation by diethylstilbestrol but could not reactivate the inhibited enzyme. The inhibition of TMP-kinase by the synthetic estrogens was prevented by several sulfhydryl compounds of which dithiothreitol was most effective. The addition of dithiothreitol to the inhibited enzyme did not restore activity. Diethylstilbestrol-^{14}C did not specifically associate with the enzyme protein in a partially purified TMP-kinase and the addition of the protective agents, dithiothreitol and thymidylate, did not alter the degree of binding. Treatment of the inhibition kinetics by the method of Ackermann and Potter showed that diethylstilbestrol did not titrate enzyme activity.

Diethylstilbestrol caused no inhibition of thymidylate metabolism in L5178Y cells incubated *in vitro* nor of the development of ascitic tumor in the mouse.

INTRODUCTION

Reports of steroid modification of enzymic reactions (1–9) include several effects on nucleic acid and nucleotide metabolism (10–12). In studies on deoxynucleotide metabolism in extracts of L5178Y mouse leukemia cells we have found that certain synthetic estrogens inhibited phosphorylation of thymidylic acid. Diethylstilbestrol was a strong inhibitor, and 17-α-ethynyl-β-estradiol, although less active, was also effective. However, several other estrogens were not inhibitors. The inhibitory effects were found only on cell-free mouse leukemia extracts. TMP-kinase prepared from several other mammalian tissues was not inhibited.

METHODS

Purification of TMP-kinase from L5178Y cells. L5178Y cells were grown intraperitoneally in B6D2F1 male mice and harvested as previously described (13). The following procedures were carried out at 2–4°. Packed cells (10 ml) were suspended in 7 volumes of buffer A, which contained Tris-HCl, 0.1 M (pH 7.8); sucrose, 0.25 M; 2-mercaptoethanol, 0.002 M; and thymidylic acid, 10^{-4} M. The cell suspension was sonicated with a MSE Ultrasonic Disintegrater, No. 3000, set at maximum output, for 3 periods of 30 sec each. The mixture was cooled with an ice-ethanol bath (—15°) for 1 min between each period. Particulate material was removed by centrifugation at 33,500 *g* for 60 min and

the supernatant fluid was stored at −80°.

The supernatant fraction was adjusted to pH 4.5 with 1 N acetic acid, centrifuged immediately at 10,000 *g* for 10 min, and the supernatant fluid was adjusted quickly to pH 7.0 with 1 N sodium hydroxide. This was called the 4.5 AAS fraction.

Negative absorption on alumina C_γ[1] was employed in the next purification step. A preparation of alumina C_γ was washed with buffer A, then allowed to equilibrate overnight with buffer A. For each milligram of protein in the 4.5 AAS fraction, 15 mg of the washed alumina C_γ was added and the suspension was stirred for 30 min. The alumina C_γ was removed by centrifugation and the supernatant was applied to a hydroxylapatite column.

A column of hydroxylapatite, Biorad HT, 4 cm × 3 cm, was washed with 100 ml of sodium phosphate buffer, 0.01 M (pH 7.0), then charged with 100 ml of the enzyme fraction. The column was eluted with a linear gradient of potassium phosphate buffer, pH 7.0, which contained 2-mercaptoethanol, 0.002 M, and thymidylic acid, 5×10^{-4} M. The mixing bottle contained 100 ml of 0.5 M buffer, and the reservoir contained 100 ml of 0.005 M buffer. Fractions which contained maximum TMP-kinase activity appeared at about 0.08 M phosphate buffer and were stored at −80°. The enzyme was stable for several months when prepared by this method. The overall purification was 100- to 140-fold with 10–15% recovery of initial units of activity (Table 1).

Assay of TMP-kinase activity. Standard assay conditions were adopted so that optimal TMP-kinase activity was obtained: ATP, 0.01 M; $MgSO_4$, 0.01 M; Tris-HCl, pH 7.8, 0.1 M; phosphoenolpyruvate, 0.005 M; pyruvate kinase, 10 μg; and TMP at 3×10^{-4} M was required for maximum velocity. TMP-^{3}H (1.8 C/mmole) was added in an amount sufficient to yield approximately 500,000 dpm per assay. Usually the volume of enzyme used per assay was 0.025 ml and the total volume of the reaction was 0.10 ml. The estrogens were added in methanol solution so that the final concentration of methanol in the reaction was 1%; at this concentration methanol had no effect on the rate of the reaction. The rate of formation of TDP + TTP was linear for 1 hr at 37°. The reaction was stopped by heating at 100° for 2 min.

A slight modification of the method of Weissman *et al.* (14) was used for the nucleotide analysis. The reaction mixture was diluted to 10 ml with water, and the nucleotides were adsorbed on a column of ECTEOLA-cellulose, 0.6 cm × 3 cm, which had been washed and equilibrated with distilled water. Thymidine was eluted with water, and TMP, TDP, and TTP were eluted sequentially with 50 ml of 0.01 N HCl, 25 ml of 0.02 N HCl, and 25 ml of 0.05 N HCl, respectively. Radioactivity in each fraction was determined by counting a 1.0-ml aliquot in 10 ml of Bray's liquid scintillation mixture (15). A Nuclear-Chicago scintillation spectrometer, model 720, was employed, and the appropriate quenching corrections were used to calculate disintegrations per minute. TMP-kinase activity was usually expressed as the sum of the micromoles of TDP and TTP formed in the reaction per hour per milligram protein. Other nucleoside phosphokinase activities in L5178Y sonicates were measured under the same incubation conditions, except that the appropriate tritium-labeled nucleoside or nucleotide substrate was substituted for TMP.

Catalase activity was measured spectrophotometrically (16), and hemoglobin concentration was determined by its absorption at 415 mμ. Protein was measured by the method of Lowry *et al.* (17).

Diethylstilbestrol was obtained from California Biochemical Corporation and from Mann Research Laboratories, Inc. Diethystilbestrol-^{14}C (28 mC/mmole) was purchased from Nuclear-Chicago, Des Plaines, Illinois; TMP-^{3}H (1.80 C/mmole) was the product of Schwarz BioResearch, Inc. ECTEOLA-cellulose and 17-ethynylestradiol were obtained from Sigma Chemical Co. Before use, the ECTEOLA-cel-

[1] The alumina C_γ was provided by Dr. T. W. Rall. This preparation of C had been aged for several years and was stored under distilled water at room temperature.

lulose, 100 *g*, was washed with 1 N sodium hydroxide, 2 liters, then 1 N hydrochloric acid, 2 liters, then water to pH 5.5.

RESULTS

Purification of Thymidine Monophosphokinase from L5178Y Mouse Leukemia Cells

Initial experiments showed that thymidine kinase, TMP-kinase, and TDP-kinase were very active in extracts of L5178Y mouse ascites cells, but that these activities were lost during storage unless sucrose, 2-mercaptoethanol, and the respective substrate were present. When frozen at —80° under the latter conditions, the enzyme preparations were stable for several months. The results of a partial purification of TMP-kinase are summarized in Table 1.

TABLE 1

Partial purification of TMP-kinase from L5178Y mouse leukemia

Values have been calculated for 1.0 ml of packed L cells. One unit equals 1 μmole of TDP formed per hour and specific activity equals units per milligram of protein.

Fraction	Protein (mg)	Units	Specific activity	Purification
Sonicate supernatant	112	7.62	0.068	—
pH 4.5 AAS	5.3	7.12	1.35	20
Alumina C_γ supernatant	2.2	5.5	2.52	37
HA fraction	0.13	1.05	8.0	117

This procedure was adapted from a scheme described by Grav and Smellie (18), who purified thymidine monophosphokinase from Landschutz ascites tumor cells. A 20-fold purification was achieved in the initial acetic acid step. However, subsequent fractionation with alumina C_γ and Sephadex gel filtration, as described by Grav and Smellie, did not give the impressive purifications reported for the Landschutz ascitic cell preparations. Fractionation on hydroxylapatite (HA) yielded the highest purification of TMP-kinase. This preparation had a specific activity somewhat higher than that reported by Grav and Smellie (18) for purified TMP-kinase. The HA fraction of TMP-kinase had no thymidine kinase and very little TDP-kinase activity, as indicated by the predominant formation of TDP rather than TTP in the reaction. Gel filtration of the hydroxylapatite-purified enzyme on Sephadex G-100 resulted in the loss of 50–60% of TMP-kinase activity with no significant increase in specific activity. By gel filtration it was estimated that the molecular weight of TMP-kinase was approximately 65,000 based on comparison with the elution volumes of catalase (Boehringer, mol. wt. 250,000) and hemoglobin (endogenous mouse, mol. wt. 64,000).

The purified TMP-kinase had optimal activity at pH 7.8 and an optimal ATP: Mg ratio of 1:1. The enzyme was about 50% as active when Mg^{2+} was replaced with Mn^{2+}. The reaction rate was linear for the first 60 minutes and the Michaelis constant, K_m (TMP) was about 1×10^{-4} M.

Inhibition of TMP-Kinase by Diethylstilbestrol and 17-Ethynyl-β-estradiol

TMP-kinase which was purified from L5178Y mouse leukemia cells was inhibited by the addition of diethylstilbestrol or 17-ethynyl-β-estradiol and there was no significant change in sensitivity of TMP-kinase to inhibition at different stages in the purification. The inhibition was dependent upon the concentration of the synthetic estrogens as shown in Fig. 1. Estradiol-17β was slightly, but not consistently, inhibitory, whereas cortisol, cortisone, hydrocortisone, prednisolone, 17-ethynyl-β-estradiol-3-methyl ether, triamcinolone, 17-nortestosterone, estriol, and estradiol-17α were without inhibitory activity.

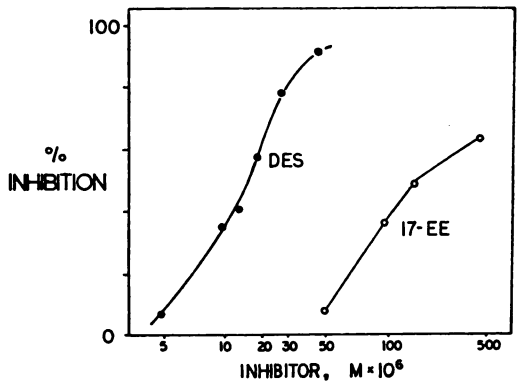

FIG. 1. *Inhibition of TMP-kinase by diethylstilbestrol and 17-ethynylestradiol*

Each reaction mixture contained TMP, 10^{-5} M, and TMP-kinase, 4.5 AAS fraction, 72 μg protein, and the standard components described in Methods in a total volume of 0.1 ml. The mixture was incubated for 15 min at 37°. The control, with no estrogen added, generated 52 mμmoles of TDP/hr/mg protein.

The inhibitory action of diethylstilbestrol and 17-ethynylestradiol was specific for the TMP-kinase isolated from L5178Y mouse leukemia cells (Table 2). TMP-kinase was partially purified from several other tissues, including mouse spleen, mouse thymus, calf thymus, three different human leukemias, and from *E. coli*. These activities were not inhibited by diethylstilbestrol at a concentration of 10^{-4} M.

The time course of the diethylstilbestrol and 17-ethynylestradiol inhibition is shown in Fig. 2. A period of up to 15 min was required before the low concentration (10^{-5} M) of diethylstilbestrol exerted its maximum inhibitory effect. When a higher concentration of diethylstilbestrol was added (10^{-4} M), the time required for complete inhibition was less. Diethylstilbestrol, present at 10^{-5} M, caused the rate of phosphorylation of TMP to decrease rapidly during the first 15 min, then the inhibited reaction rate appeared to remain constant.

The inhibition of TMP-kinase by 17-ethynyl-β-estradiol, at 10^{-4} M, shown in Fig. 2, indicated that the time required for maximum inhibition was about twice that needed for diethylstilbestrol. The concentration of 17-ethynyl-β-estradiol which

TABLE 2

Effect of estrogens on TMP-kinase obtained from different sources

TMP-kinase was prepared from *E. coli* B by a method previously described (23) and the eluate from alumina C_γ was used for assay. Calf thymus was homogenized in 4 volumes of 0.05 M Tris-HCl buffer and an enzyme fraction was obtained by precipitation with ammonium sulfate between 35 and 50% saturation. An alumina C_γ fraction of L5178Y mouse leukemia cells was employed. The standard assay mixture was used with 3×10^{-4} M TMP.

Human granulocytic leukemia cells from three individuals with no chemotherapeutic history were obtained from Dr. Rune Stjernholm. TMP-kinase in sonicates of the human leukemic cells was not inhibited by either diethylstilbestrol or β-estradiol. Homogenates of spleen and thymus from mice bearing the L5178Y ascites tumor also contained TMP-kinase which was not inhibited by these estrogens.

Enzyme source	Addition	TMP-kinase activity TDP and TTP (mμmoles/hr/mg protein)	Percent inhibition
E. coli	None	157	—
	Diethylstilbestrol, 10^{-4} M	162	0
	β-Estradiol, 10^{-4} M	147	6
Calf thymus	None	19.6	—
	Diethylstilbestrol, 10^{-4} M	21.8	0
	β-Estradiol, 10^{-4} M	23.6	0
L5178Y	None	87.8	—
	Diethylstilbestrol, 10^{-4} M	1.8	98
	β-Estradiol, 10^{-4} M	87.0	0

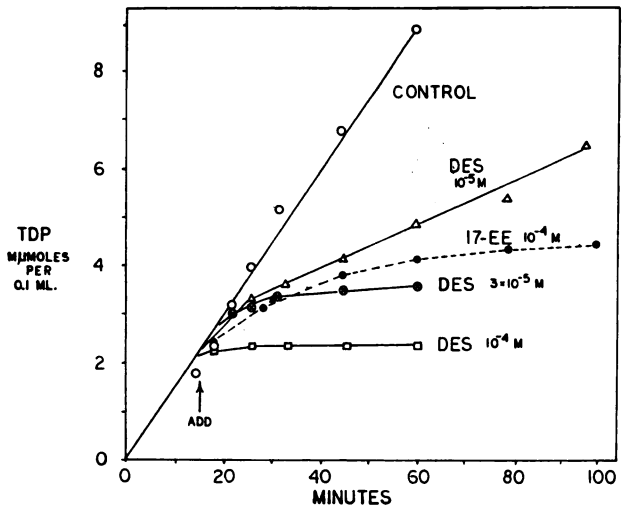

FIG. 2. *Time course for inhibition of TMP-kinase by diethylstilbestrol and 17-ethynylestradiol*

The reaction mixture contained TMP, 3×10^{-4} M, and TMP-kinase, alumina C_γ fraction, 98 μg protein, and the standard components in a final volume of 0.7 ml. The estrogen was added 16 min after the reaction was started. At the times indicated a 0.1-ml aliquot was removed from the reaction mixture for analysis.

caused 50% inhibition was about 10-fold greater than that required for diethylstilbestrol.

Since the initial inactivating reaction of diethylstilbestrol with TMP-kinase required approximately 15 min, this time was chosen in the following experiments for preincubation of the enzyme with diethylstilbestrol and all other reaction components except radioactive TMP. Following the preincubation, the rate of reaction was measured by addition of radioactive TMP, continuing the incubation for 30 min, and chromatographically analyzing the formation of TDP. The rate of TDP formation was markedly reduced in the presence of diethylstilbestrol. The inhibition was not a linear function of the reciprocal TMP concentration as shown in Fig. 3. These data indicate that the inhibition kinetics are complex and that diethylstilbestrol was not competitive with TMP in the reaction. These data were also graphically represented by the method of Dixon (19) as shown in Fig. 4. It is clear that when the substrate concentration was high (TMP = 6×10^{-3} M, a concentration of TMP 20 times the amount needed to saturate the enzyme), there was no inhibition of TMP-kinase activity. At substrate concentrations of TMP (6×10^{-4} M) which gave maximum velocity of the reaction, the inhibition of TMP-kinase was a nonlinear function of diethylstilbestrol concentration.

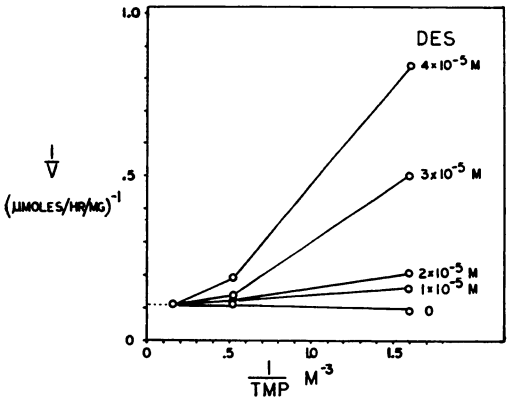

FIG. 3. *Double reciprocal plot of the kinetics of inhibition of TMP-kinase by diethylstilbestrol*

TMP-kinase, HA fraction, 20 μg protein, was incubated for 15 min in a standard reaction mixture with the indicated concentrations of unlabeled TMP and diethylstilbestrol; then TMP-^{3}H, 0.3 μC, 0.05 mμmole, was added to each tube and the incubation was continued for 30 min and the amount of TDP-^{3}H formed was measured.

The incubation of TMP-kinase with diethylstilbestrol (2×10^{-5} M) and a low concentration of TMP, 10^{-4} M, for 15 min, followed by an assay for enzymic activity with a high concentration of TMP, 2×10^{-3} M, which ordinarily protected the enzyme from diethylstilbestrol inactivation, showed that TMP-kinase was irreversibly inactivated during the preincubation with diethylstilbestrol.

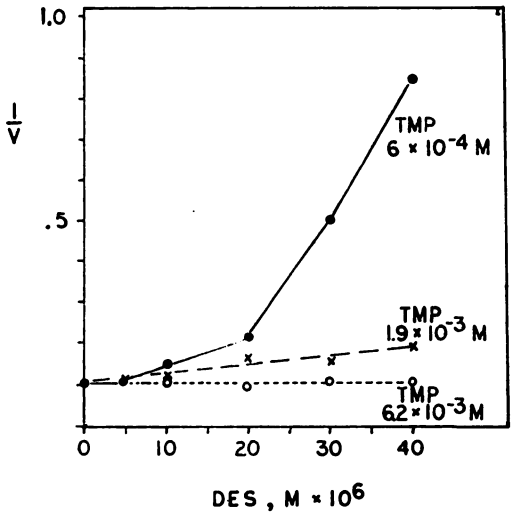

FIG. 4. *Kinetics of inhibition of TMP-kinase by diethylstilbestrol plotted by the Dixon method* (19)

The data were obtained in the experiment described in Fig. 3.

The relationship of diethylstilbestrol inhibition and TMP-kinase concentration is shown in Fig. 5. The plot of velocity versus enzyme concentration showed that diethylstilbestrol did not act as a "titration" inhibitor throughout the concentration range of enzyme protein employed [Ackermann-Potter plot; (20)]. These data cannot be rigorously interpreted because of the absence of proof of a homogeneous enzyme, but the similarity of the inhibitor-enzyme relationship to that observed by Yielding and Tompkins (21) for diethylstilbestrol inhibition of glutamic dehydrogenase is apparent.

Other enzymes that phosphorylate thymidine in the L5178Y cell extracts were not inhibited by the synthetic estrogens. Thymidine kinase was fully active in the presence of diethylstilbestrol at 10^{-4} M. TDP-kinase, which catalyzes the phosphorylation of TDP to form TTP, was not inhibited by diethylstilbestrol, as shown in Fig. 6. In this experiment TDP was generated from TMP in a reaction mixture with a hydroxylapatite purified enzyme preparation for 30 min, then diethylstilbestrol, 10^{-4} M, was added. Further synthe-

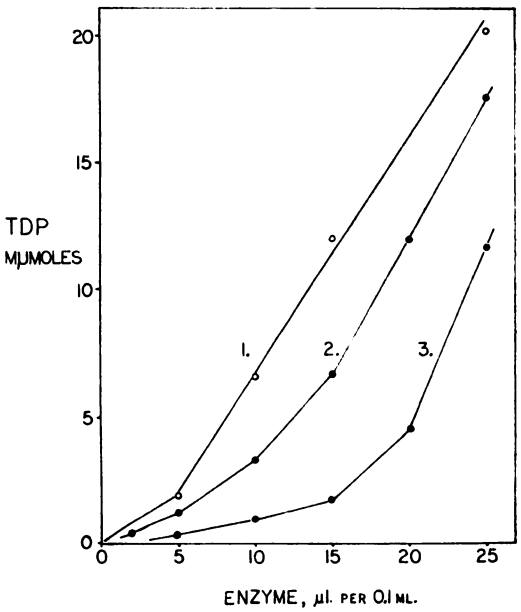

FIG. 5. *Effect of enzyme concentration on inhibition of TMP-kinase by diethylstilbestrol*

An HA fraction, which contained 1.2 mg protein per ml, was incubated for 20 minutes in an assay mixture with unlabeled TMP at 1×10^{-4} M and diethylstilbestrol at the concentrations shown. The tubes were chilled to 0° and TMP-^{3}H was added to 6×10^{-4} M; the assay incubation proceeded at 37° for 30 min. Curve *1*, control; *2*, diethylstilbestrol, 1×10^{-5} M; *3*, diethylstilbestrol, 2×10^{-5} M.

sis of TDP was inhibited, whereas the rate of synthesis of TTP from TDP was not appreciably affected. To accentuate the specificity of the inhibition of TMP-kinase, an alumina C_γ enzyme preparation that contained both TMP-kinase and a highly active TDP-kinase was preincubated with diethylstilbestrol, 10^{-4} M, for 10 min. The diethylstilbestrol-treated enzyme was added to the reaction mixture at 41 min. Within

4 min all the TDP was converted to TTP. In a separate experiment it was shown that no TMP-kinase activity remained in the alumina C_γ enzyme preparation after the 10-min preincubation with diethylstilbestrol.

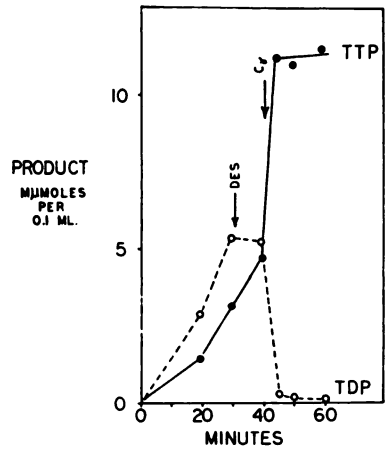

FIG. 6. *Selective inhibition of the formation of thymidine diphosphate from thymidylic acid by diethylstilbestrol*

A standard incubation contained TMP, 3×10^{-4} M, and TMP-kinase, HA fraction, 52 μg protein, in 0.5 ml; at 20 min and at 30 min a 0.1-ml aliquot was removed for analysis. At 31 min, diethylstilbestrol was added to a final concentration of 10^{-4} M. An aliquot was removed at 40 min, and at 41 min a second preparation of TMP-kinase, alumina C_γ fraction, 65 μg protein in 60 μl, was added. The alumina C_γ fraction had been incubated with diethylstilbestrol, 10^{-4} M, for 10 min at 37° immediately prior to its addition. Three aliquots were removed at 45, 50, and 60 min, respectively, which were analyzed for radioactive TDP and TTP.

Experiments in which the appropriate radioactive substrates were employed with extracts of L5178Y cells demonstrated no diethylstilbestrol inhibition of uridine kinase, cytidine kinase, cytidine monophosphokinase, deoxycytidine monophosphokinase, and deoxyadenosine monophosphokinase.

Prevention of Diethylstilbestrol Inhibition of TMP-Kinase by Sulfhydryl Compounds

Dithiothreitol, at a concentration of 10^{-3} M in the reaction mixture, prevented the inhibitory action of diethylstilbestrol and 17-ethynylestradiol on TMP-kinase. If dithiothreitol was added 2 min after the addition of diethylstilbestrol, there was still virtually complete protection as shown in Fig. 7. However, if 9 min elapsed before the addition of dithiothreitol, then TMP-kinase activity was irreversibly inhibited. Therefore, the presence of the sulfhydryl compound during the exposure of the enzyme to diethylstilbestrol was necessary to

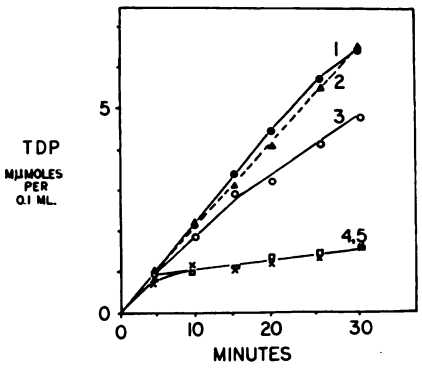

FIG. 7. *Time dependence of the protective effect of dithiothreitol on TMP-kinase inhibition by diethylstilbestrol*

A standard reaction mixture which contained TMP, 5×10^{-4} M, and TMP-kinase, alumina C_γ fraction, 140 μg, in a total volume of 0.6 ml was added to five small test tubes. Diethylstilbestrol in a final concentration of 3×10^{-5} M was added 1 min after the 37° incubation was begun in tubes numbered *2*, *3*, *4*, and *5*. Dithiothreitol was added to a final concentration of 10^{-3} M in tubes *2*, *3*, and *4* at 0, 3, and 10 min, respectively. Reaction mixture No. 1 served as a control. Aliquots of 0.1 ml were removed from each tube and analyzed at indicated times.

prevent inhibition. This was also demonstrated by prior treatment of TMP-kinase with 10^{-3} M dithiothreitol, followed by Sephadex G-25 gel filtration of the mixture to remove the sulfhydryl compound. This procedure yielded an enzyme preparation which was not resistant to diethylstilbestrol inhibition (Table 3).

Glutathione, cysteine, and mercaptoethanol also prevented diethylstilbestrol inhibition, but the required concentration of these compounds was about 100–1000

TABLE 3

Effect of pretreatment of TMP-kinase with diethylstilbestrol and dithiothreitol followed by Sephadex gel filtration

One milliliter of TMP-kinase, HA fraction, was incubated at 37° for 10 min with water, diethylstilbestrol at 3×10^{-5} M, or dithiothreitol at 10^{-3} M, as indicated in experiments B, C, and D; then these agents were removed by gel filtration on identical columns of Sephadex G-25, 1×20 cm, previously equilibrated with buffer A. The protein peak was assayed for TMP-kinase activity under the conditions described in Methods. The concentration of TMP in the assay was 5×10^{-4} M.

Pretreatment conditions	Assay conditions	TMP-kinase activity (μmoles/hr/mg)
A. No pretreatment	Methanol, 1%	5.56
	DES, 2×10^{-5} M	1.84
	DES, 2×10^{-5} M and DDT, 10^{-3} M	5.70
B. H_2O	Methanol, 1%	4.72
	DES, 2×10^{-5} M	1.78
	DES, 2×10^{-5} M and DTT, 10^{-3} M	4.82
C. DES, 3×10^{-5} M	Methanol, 1%	0.84
	DES, 2×10^{-5} M	0.86
	DES, 2×10^{-5} M and DTT, 10^{-3} M	0.58
D. DTT, 10^{-3} M	Methanol, 1%	4.20
	DES, 2×10^{-5} M	1.34
	DES, 2×10^{-5} M and DTT, 10^{-3} M	4.46

times higher than that found for dithiothreitol.

TMP-kinase was found to be irreversibly inhibited by 10^{-4} M concentration of *p*-mercuribenzoate, iodoacetamide, and *N*-ethylmaleimide. To test the possibility that diethylstilbestrol reacted with sulfhydryl groups, diethylstilbestrol-^{14}C was incubated with dithiothreitol and glutathione under several conditions, and the reaction mixture was examined by paper, thin layer, and gas chromatography. There was no evidence for the conversion of diethylstilbestrol-^{14}C to a separable radioactive product.

The enzyme glyceraldehyde phosphate dehydrogenase[2] has an exacting requirement for sulfhydryl compounds and, therefore, was examined for diethylstilbestrol inhibition under conditions where the concentration of added dithiothreitol was rate limiting. Diethylstilbestrol did not diminish enzyme activity and, therefore, did not react with the enzyme or dithiothreitol.

Attempts to Demonstrate Binding of Diethylstilbestrol-^{14}C to a Purified Preparation of TMP-Kinase

The irreversible inhibition of TMP-kinase by diethylstilbestrol suggested that there might be strong binding of the synthetic estrogen to the enzyme. This was tested by incubating the purified enzyme with an inhibitory concentration of diethylstilbestrol-^{14}C and measuring the distribution of radioactivity in the protein fractions obtained from Sephadex-gel filtration of the treated enzyme. Radioactivity was found to be distributed throughout the high molecular weight region as shown in Fig. 8. This fraction contained 3–4% of the total diethylstilbestrol-^{14}C added to the enzyme reaction. Figure 8 shows that the small amount of radioactivity in the protein region did not specifically associate with TMP-kinase activity. Furthermore, it was found in other experiments that the association of diethylstilbestrol-^{14}C with the protein of a purified TMP-kinase preparation was not altered by the presence of

[2] Glyceraldehyde-3-phosphate dehydrogenase (Boehringer, rabbit muscle) was assayed by the method of S. Velick as described in *Methods in Enzymology*, Vol. 1, p. 401, with the exception that dithiothreitol replaced cysteine in the reaction mixture.

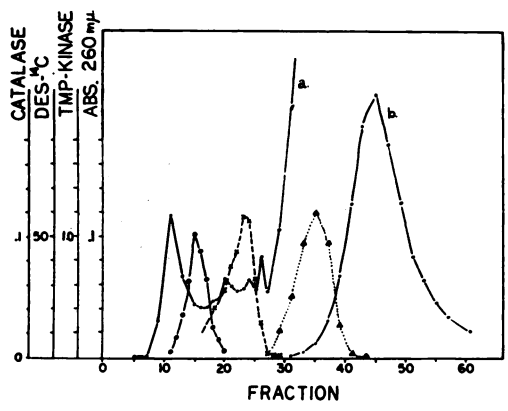

FIG. 8. *Sephadex G-200 gel filtration of a TMP-kinase preparation incubated with diethylstilbestrol-¹⁴C*

TMP-kinase, 5.0 ml of a 4.5 AAS fraction, was incubated for 5 min at 37° with diethylstilbestrol-¹⁴C, 0.15 μmole, 990,000 dpm, then cooled to 0°. Catalase, 200 μg, was added and the mixture was subjected to gel filtration on a Sephadex G-200 column 1.5 × 70 cm, previously equilibrated with buffer A. The column was eluted with buffer A at a rate of about 6 ml per hour, and each fraction contained 3.3 ml. The void volume of the column was 30 ml. Catalase (△ 240 mμ/min/10 μl, ○—○) appeared at 50 ml, TMP-kinase (%TDP/hr/25 μl, ×---×) was eluted with 79 ml, and low molecular weight substances absorbing at 260 mμ (△· · ·△) appeared after 115 ml. Diethylstilbestrol-¹⁴C distribution was plotted as dpm/ml in curve *a* and dpm × 10^{-3}/ml in curve *b*.

dithiothreitol at 10^{-3} M or by the presence or absence of the components of the enzyme assay mixture. When the TMP-kinase fraction from the experiment illustrated in Fig. 8 was rechromatographed on Sephadex G-200, under the same conditions, about 65% of the original radioactivity remained associated with the reisolated protein component.

Effect of Diethylstilbestrol on L5178Y Mouse Leukemia Cells

Mice were innoculated intraperitoneally with L5178Y ascites cells; after 4 days, 1 mg of diethylstilbestrol or 17-ethynylestradiol was injected intraperitoneally morning and evening for 3 days. There was no significant effect on the growth of the ascites cells in the mice under these conditions. Also, there was no difference between TMP-kinase in extracts which were prepared from the control and treated ascites cells, when the specific activity and sensitivity to diethylstilbestrol inhibition were compared.

In order to determine the distribution of diethylstilbestrol in mice bearing L5178Y ascites cells, animals were given intraperitoneally a single dose of diethylstilbestrol-¹⁴C, 1.5 mg, 1.50 × 10^6 dpm, dissolved in sesame oil. The animals were sacrificed 24 hr later. About 27% of the dose was excreted in the urine, and about 0.1% was found associated with the washed ascites cells. The cells were disrupted by alternate freezing and thawing, and upon centrifugation at 33,500 *g*, 85% of the radioactivity sedimented with the particulate fraction. The ascites cell supernatant fraction contained about 0.01% of the initial dose or about 0.15 μg of diethylstilbestrol per 0.5 ml of packed cells. In the cell-free system 20–50 times this concentration was required for inhibition of TMP-kinase. Although this is only an approximation of the intracellular concentration of diethylstilbestrol, 24 hr after a single dose, it appears that diethylstilbestrol did not accumulate to an inhibitory level in these ascites cells.

DISCUSSION

The mechanism of diethylstilbestrol inhibition of the TMP-kinase of mouse L5178Y leukemia cells has not been established. The evidence we have presented shows that the inhibition is irreversible, that the substrate, TMP, when present in high concentration protects the enzyme from the inhibitory effect of diethylstilbestrol, and that the sulfhydryl compound, dithiothreitol, can prevent diethylstilbestrol inhibition, but cannot reverse it. The association of diethylstilbestrol-¹⁴C with TMP-kinase protein appeared to be nonspecific and was not influenced by the presence of dithiothreitol and TMP. All these studies are limited by the purity of the enzyme preparation and until a crystalline or homogeneous preparation is available, interpretation must be tentative.

The kinetic studies of diethylstilbestrol

inhibition showed that the synthetic estrogen did not act as a titration inhibitor of TMP-kinase (Fig. 5). In the Dixon plot (Fig. 4) there was evidence that the inhibition might require a cooperative effect of several molecules of diethylstilbestrol on the enzyme, since at substrate concentration of TMP, the inhibition of the enzyme increased nonlinearly as the concentration of diethylstilbestrol was increased. The ability of high concentrations of TMP to protect against these effects might indicate that the substrate stabilized the tertiary structure of the enzyme against a diethylstilbestrol-induced conformational change.

The demonstration by Yielding and Tompkins (22) that diethylstilbestrol induced the disaggregation of glutamic dehydrogenase into subunits led us to examine TMP-kinase interaction with diethylstilbestrol by sucrose density gradient centrifugation and Sephadex-100 gel filtration. The low apparent molecular weight of TMP-kinase and the lack of homogeneity of the preparation limits these studies, but preliminary experiments have not indicated that diethylstilbestrol causes particle disaggregation of TMP-kinase.

The selective inhibitory activity of diethylstilbestrol and 17-ethynylestradiol upon TMP-kinase of L5178Y mouse leukemia cell extracts is remarkable. Thymus and spleen from mice with the ascitic leukemia were examined and although TMP-kinase activity was high in extracts of these tissues, the enzyme activity was not inhibited by diethylstilbestrol. A purified preparation of calf thymus TMP-kinase was also studied and found resistant to diethylstilbestrol. It was also demonstrated in studies on the purified thymus preparation mixed with the leukemia enzyme that a substance was not present in the thymus enzyme which was protective against the inhibitory action of diethylstilbestrol on the leukemia cell enzyme.

Despite the sensitivity of the leukemia cell TMP-kinase to inhibition by diethylstilbestrol, no inhibitory effect of the synthetic estrogen was found at the cellular level either *in vivo* or *in vitro*. This may be due to a failure of sufficient intracellular penetration of the drug as indicated by distribution studies with diethylstilbestrol-^{14}C in the tumor-bearing mouse. These observations do not encourage the belief that the synthetic estrogens may be exploited in L5178Y mouse leukemia therapy or that estrogens play a role in the physiological regulation of leukemia TMP-kinase. However, analogs of diethylstilbestrol which may possess different pharmacologic properties will be investigated further.

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