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Original Article

Molecular forms in human serum of enzymes synthesizing DNA precursors and DNA

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Abstract

Both thymidine kinase (TK) and DNA polymerase (DNAp) are present in measurable amounts in human serum. Even though the use of TK as a clinical marker is rapidly increasing there has been no attempt to characterize the serum TK in a wider extent, i.e.; with respect to Mw or other biochemical parameters. Therefore sera with high TK or DNAp activities derived from patients with cytomegalo-virus (CMV) infection, B_{12}-deficiency and leukaemia were fractionated by gel exclusion chromatography. The TK activity eluted as two peaks, one major TK activity with an apparent molecular weight (Mw) or 730 kD and one minor TK activity corresponding to a Mw of 58 kD. The amount of TK activity at 58 kD varied between 7 and 23% of total activity, depending on the serum fractionated. The DNAp activity in sera from patients with malignant disease and B_{12} deficiency eluted as a single peak corresponding to a Mw of 240 kD. A DNAp with a different Mw (>1000 kD) was recovered from 1 of 3 investigated immunosuppressed patients with CMV infection. A similar pattern of enzyme forms was observed when sera were separated by glycerol gradient centrifugation.

The effect of high salt and various reaction solution components on the enzymes were studied. The only condition found that affected the molecular forms of TK was the state of reduction. Incubation of sera with high concentrations of dithioerythritol (DTE) (400 mM) prior to separation transferred all serum TK to the 58 kD form, it also converted most of the serum DNAp from the 240 kD form to a smaller form (56 kD) without affecting the total recovery of enzymatic activity.

The reaction product from both TK forms was exclusively monophosphate and none of the TK forms could efficiently utilize cytidine triphosphate as phosphate donor. The substrate kinetics of the small serum TK fraction was identical with those of an enzyme with similar size purified from proliferating HeLa cells, indicating that both serum TK activities are forms of TK 1, the proliferation associated cellular isozyme.

Introduction

The incorporation of radiolabeled thymidine (dThd) into DNA is a commonly used method to measure cell proliferation. The radiolabeled DNA is the product of the catalytic action of several proliferation associated enzymes. Thymidine kinase (TK), ATP-: thymidine 5′phosphotransferase (EC 2.7.1.21), catalyses the conversion of dThd to thymidine monophosphate TMP, which is further

converted to thymidine-diphosphate and -triphosphate (TTP) by the action of the enzymes thymidine-monophosphate kinase and -diphosphate kinase respectively. The TTP is finally incorporated into DNA by DNA-polymerase (EC 2.7.7.7).

Two human TK genes designated TK 1 and TK 2 are known. TK 1 is located on chromosome 17 near the galactokinase locus, whereas TK 2 is located on chromosome 16 [1, 2]. TK 1, the cytosolar isozyme, is the main isozyme in proliferating cells (G1 to S), while TK 2 probably contributes to mitochondrial autonomy [3, 4]. A considerable variation in apparent molecular weight of proliferation associated TKs has been reported. The smallest functional gene for TK 1 has been estimated to 1421 basepairs, which corresponds to a protein with an estimated molecular weight (Mw) of 25 kD [5]. The Mw of a highly purified catalytically active TK 1 from HeLa cells has been estimated to be 96 kD. However, with SDS-PAGE a single band of 24 kD was obtained [6]. Studies of unstimulated and stimulated normal human lymphocytes revealed a proliferation associated TK with a Mw of 70–75 kD, that increased to 170–200 kD in the presence of adenosinetriphosphate (ATP). In addition several other distinct forms were recorded from leukemic lymphocytes [7, 8]. A large protein complex (Mw 500 kD) with the capacity to channel dThd directly into DNA has also been described [9]. The reason for the variability in Mws of TK is not clear, but the organization of DNA precursor synthesizing enzymes into functional polypeptide complexes has been suggested [9, 10, 11].

Three different DNA polymerases, α, β and τ have been clearly identified in human cells [12, 13]. α is considered to be responsible for DNA replication during cell division, while β and τ are involved in DNA repair and mitochondrial DNA synthesis, respectively. Several forms of DNA polymerase α with Mws ranging from 50 to 1000 kD have been described. This variability can partly by explained by the fact that DNAp-α is composed of several subunits and that it may be associated with additative auxilary proteins (for a review see 14).

DNA and DNA-precursor synthesizing enzymes are potential clinical markers for proliferative disorders, provided that they can be measured in serum or cell biopsies. The development of a TK assay utilizing ^{125}I-iododeoxyuridine (IUdR) as substrate, facilitated the measurement of serum Tk activity (S-TK) and made it possible to study its clinical relevance [15]. Significant increases in S-TK activity have mainly been found in three groups of diseases: 1) malignant diseases, especially blood malignancies, 2) metabolic disorders interfering with thymidylate synthetase, i.e. pernicious deficiency or therapy induced metabolic block, achieved with drugs such as metothrexate, 3) acute stages of certain viral diseases [15, 16]. S-TK has proved to be a useful clinical marker for several malignant and viral diseases, indicating disease activity, spread and prognosis [17–21].

In spite of the clinical usefulness, very little is known about the TK isozymes in human serum. Characterization of phosphate donor acceptance and migration in non-denaturating PAGE of TK in serum samples from patients with different diseases have been performed. In all instances except in connection with varicella, where a fraction of S-TK consisted of varicella zoster virus coded TK, the dominating S-TK isozyme had similar properties to those of human TK 1. However, the S-TK activity was found remarkably stable compared to TK 1 from cell preparations [15, 22].

Only a few studies of DNA polymerase in serum have been carried out. An unidentified DNA polymerase was detected in 4 out of 22 sera from children with acute lymphatic leukaemia [23]. Recently, DNA polymerase activity was recorded in sera from patients with leukaemia, B_{12}-deficiency and CMV infection. The activity detected was similar to α-polymerase with regard to sensitivity to salt and N-ethylmaleimide, and was inhibited by monoclonal antibodies that block α-polymerase [24].

The purpose of this study was to characterize the molecular forms of TK and DNA-polymerase that occur in serum during different types of disease, to identify the catalytically active forms present during assay conditions and to evaluate some of the enzymatic properties of the different forms.

Material and methods

Clinical samples

Sera from persons with different defined haematological malignancies were obtained from the department of Medicine, Akademic Hospital, Uppsala. Sera submitted for B_{12} analyses were obtained from the B_{12} laboratory at the department of Medical Chemistry, Uppsala. Sera from patients with CMV infection were selected from a recent study of kidney transplanted patients [18]. Most sera used in this study were selected for high levels of TK and/or DNA-polymerase activity.

Preparation of enzyme from HeLa cells

HeLa cells were grown in spinner culture utilizing 80% spinner medium [15], 10% tryptose phosphate broth and 10% newborn calf serum. Proliferating cells at a density 8–10 $\times$ 10^5 cells/ml were harvested, washed in PBS and extracted in digitonin buffer at pH 7,5 (80 mg/100 ml digitonin, 10 mM THCL, 250 mM Sucrose, 160 mM KCL, 50 mM ε-amino-n-caproic acid) as previously described [6]. Extracted cells were pelleted at 1000 g for 10 min at 4° C. The supernatant was centrifuged at 20000 g for 2 h, and the final supernatant was stored at −70° C. The kinetic studies were performed with enzyme further purified by gel exclusion chromatography on FPLC (see below).

Assay and determination of enzymatic properties of TK

Determinations of TK activities in sera and in the fractions from the separations, were basically performed according to Gronowitz *et al.* [15]. The assay is based on the use of ^{125}I-Iodo-deoxy-uridine-ribose (IUdR) as substrate. All TK activities are given as units per μl. One unit corresponds to a substrate turnover of 1.2×10^{-18} katal. The comparison of cytidine triphosphate and adenosine triphosphate as phosphate donors was done at equimolar concentrations (4.6 mM) and 1.1×10^{-7} M IUdR as substrate.

The kinetic studies were performed with 50–100 units of enzyme. Samples for product analysis were taken at 15 minutes intervals for 90 min. The reaction velocities were determined at seven different substrate concentrations ranging from 1.25×10^{-7} M to 2.0×10^{-6} M. Concentrations above 2×10^{-6} M were avoided due to the occurrence of substrate inhibition. To obtain Km and Vsat values, the experimental data were analysed by using a computer program based on the method of Hanes for the analysis of enzyme kinetics.

DNA polymerase (DNAp) assay

DNAp activity was determined by following the incorporation of ^{3}H-thymidine triphosphate (TTP) into deoxyribonuclease activated calf thymus DNA. One DNAp unit corresponds to the incorporation of 1.66×10^{-19} katal TTP into TCA precipitable DNA per min and μl of sample. For detailed assay procedure and final concentrations of components in the reaction solution see Neumüller *et al.* [24].

Assay of thymidylate kinase (TMPK)

The assay system utilize ^{125}I-IUdR monophosphate as substrate and has previously been described in detail by Karlström & Gronowitz [25].

Chromatography system for analysis of reaction products

Plastic sheets precoated with aluminium oxide, 60 F_{254} neutral type, were purchased from Merck. The system used for separation of nucleotides with different degrees of phosphorylation has recently been described [25]. The sheets were preequilibrated with 150 mM KH_2PO_4 and air-dried. Chromatographic analysis of different samples (1–3 μl) were performed by ascending chromatography with 50 mM KH_2PO_4. The ^{125}I spots were detected directly by autoradiography using Kodak XAR-5 X-ray sensitive film, while ^{3}H containing spots

were detected after pretreatment with En^{3}Hance spray (New England Nuclear)

Gel exclusion chromatography

FPLC technique was used to obtain rapid separation and high reproducibility. Sera and cell extracts were diluted twofold in buffer A (0.01 M Hepes, pH 7.6, containing 0.15 M NH_4Cl and 0.02% NaN_3) and thereafter sterile filtrated. 200 µl sample was fractioned on a Superose 12 (Pharmacia Fine Chemicals) column (1.0 × 30 cm). The column was eluted with buffer A and 200 µl fractions were collected. The protein content of the eluent was continuously monitored at 280 nm. The locations of α2-macro-globuline, IgG and albumin in the fractionated serum were determined by Mancini technique (Behring Partigen plates) and used as internal Mw – controls. In addition, the positions for different Mw were determined separately by the use of a set of protein markers (Pharmacia).

Determination of sedimentation behaviour in glycerol gradients

200 µl Serum samples diluted 1 to 4 in buffer A was layered on top of linear (10–40%) gradients of glycerol in buffer A. Each tube contained 4.8 ml. The tubes were centrifuged at 160 000 g for 16 h. The tubes were punctured and 13 drop fractions (approximately 150 µl) were collected. The positions of the different enzymes were determined as described above. The above mentioned serum proteins were used as internal Mw markers. The molecular weights of the enzymes were calculated according to Martin & Ames [26]. The following approximate values for molecular weights of serum proteins were used in the calculations; α2-macroglobulin 725 kD, IgG 150 kD and albumin 66 kD.

Results

Fractionation of serum TK and DNAp by gel exclusion chromatography

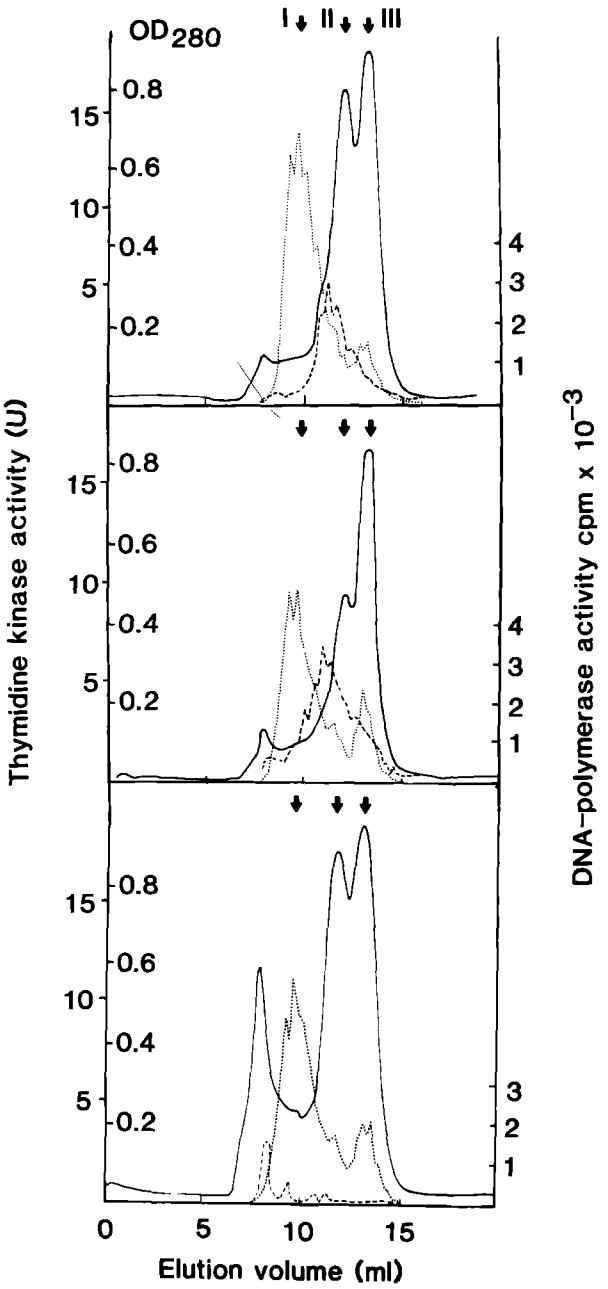

Fig. 1. Molecular forms of TK and DNA polymerase in human serum. A: Serum from a patient suffering from AML. B: Serum from a patient with B_{12}-deficiency. C: Serum from a kidney transplanted patient suffering from cytomegalovirus disease. The serum diluted 1 to 4 was sterile filtered, separated on a Superose 12 column and 200 µl samples were collected. A_{280} was continously monitored ———, TK ..... and DNA polymerase ------ activities were determined for each fraction. The locations of α2-macro-globulin (I, 725 kD), IgG (II, 150 kD) and albumin (III, 66 kD) were used as internal Mw – controls.

Sera with high TK or DNAp levels from patients with cytomegalo-virus (CMV) infection (n = 3), B_{12}-deficiency (n = 1), chronic myeloic leukaemia (CML) (n = 4), acute myeloic leukaemia (AML) (n = 1), acute lymphatic leukaemia (ALL) (n = 2) and Non-Hodgkin's lymphoma (NHL) (n = 1) were fractionated on a Superose 12 column. The distribution of TK and DNAp activity obtained is illustrated in Fig. 1, which also gives the protein distribution. The total recovery of TK activity over the column varied between 50 and 146%. The activity was always eluted in two distinctly separated peaks. The major TK activity eluted as a heterogeneous peak centered around 9.7 ml (730 kD) with one or sometimes two optima at 9.3 to 10.1 ml, corresponding to a range in molecular weight of 650–900 kD, according to internal standards. The minor TK activity eluted as a homogeneous peak around 13.3 ml, near the albumin protein peak, corresponding to a Mw of 58 kD (range 50–78 kD). The amount of 58 kD TK varied between 7 to 23% of the original activity, while 47 to 136% was recovered as the 730 kD form (Table 1). An extract of TK from proliferating HeLa cells, which was fractioned form comparison, contained exclusively the 58 kD TK.

In seven sera with a DNAp activity ranging between 9 and 67 U/μl serum, the total recovery of DNAp after chromatography varied between 76 to 147% (Table 1). Separation of four sera with rather low DNAp activity (1.3–3.4 U/μl) gave no recovery of activity. The DNAp activity in sera from patients with malignant disease and B_{12}-deficiency eluted as a single peak around 11.2 ml corresponding to a molecular weight of approximately 240 kD (range 220–290), distinctly separated from the two different TK forms. The studies of sera from patients with verified CMV infection gave less conclusive results. DNAp was only recovered from 1 out of 3 sera, selected for highest available DNAp activity. The activity eluted near the void at 8.2 ml (>1000 kD) with an additional, probably not significant, peak at 9.4 ml (Fig. 1, Table 1). Another serum from the same patient, sampled one week earlier, also contained a DNAp with a Mw >1000 kD (data not shown).

Table 1. Exclusion chromatography of serum from patients with various diseases. 200 μl serum diluted 1/2 was loaded on a Superose column and 200 μl fractions were collected. Thymidine kinase and DNA-polymerase activities were measured for each fraction.

Diagnosis	Thymidine kinase in serum					DNA-polymerase in serum		
	Activity in sample U/μl	[a]Total recovery %	Peak position 1 ml	Peak position II ml	[a]Recovery in peak II/I %	Activity in sample U/μl	[a]Total recovery %	Peak position ml
B12 deficency	96	111	9.7	12.8–13.3	14/97	64	124	11.2
NHL	38	108	9.7	13.3	14/94	11.5	76	11.2
CML	280	114	9.3, 9.7	13.2	13/101	3.4	nd[b]	–
CML	240	146	9.7	13.2	10/136	2.1	nd	–
CML	310	78	9.4, 9.7	13.2–13.6	7/71	nd	nd	–
CML	88	91	9.7	13.2	11/80	3.6	nd	–
ALL	252	114	9.3, 9.7	13.2	23/91	68.6	108	11.2
ALL	328	137	9.7	13.4	23/114	17	129	11.2–11.5
ALL	248	70	9.3, 9.7	13.2	14/56	63	147	11.2
AML	50	50	9.3, 9.9	13.2	11/39	49	88	11.1
CMV	126	55	9.5, 10.1	13.3	8/47	1.4	nd	–
CMV	120	110	9.5	13.3	22/88	8.6	88	8.2, (9.4)
CMV	60	100	9.5, 9.7	13.2	14/86	1.3	nd	–
Hela[c]	154	98	–	13.3	98.0			

[a] Percent of unfractioned sample.

[b] nd = not detected.

[c] Extract from proliferating cells prepared according to Sherley & Kelley[6].

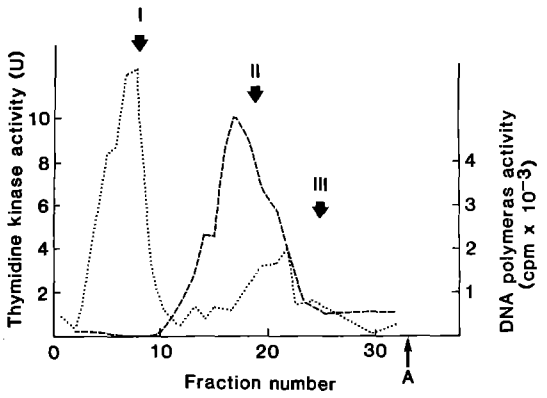

Fig. 2. Sedimentation rate of molecular forms of TK and DNA polymerase in human serum on glycerol gradients. 200 µl serum from a patient suffering from AML was diluted 1/5 and layered on top of 10 to 40% gradient of glycerol in buffer A. TK ..... and DNA polymerase ------ activities were determined for each fraction. The locations of α2-macro-globulin (I, 725 kD), IgG (II, 150 kD) and albumin (III, 66 kD) were used as internal Mw – controls. Arrow A indicates top of gradient.

Determination of sedimentation behaviour of serum TK and DNAp

To verify the distribution of serum enzyme forms found in gel exclusion chromatography, three sera; one from a patient with B_{12}-deficiency, one from a CML patient and one from the CMV patient with the deviating DNAp form were studied by density gradient centrifugation. Note that an identical buffer as in the exclusion chromatography separations was used.

The enzyme distribution obtained was determined and the results are exemplified in Fig. 2. The TK activity profiles were similar for the three sera investigated. The recovery of total TK activity varied from 48 to 60% of the frozen control and the activity was found in two distinct peaks. The major TK activity was found between 39.6 and 38.6 mm from meniscus, near the α-2 macroglobulin position. The minor activity eluted as a sometimes diffuse peak 16 to 18.6 mm from the meniscus, near the albumin position. Between 13 to 16% of the TK activity of the control was found around this position. The DNAp activity was found as a broad peak centered around 25 mm from meniscus both for the CML and the B_{12} serum. No DNAp activity was recovered from the CMV serum. Serum proteins were used as internal Mw markers as described in Materials and Methods. The estimated molecular weights were; Major TK 740–780 kD, Minor TK 80–100 kD and DNAp 270–290 kD.

Phosphate donor specificity and reaction products of the two TK peaks obtained by the gel filtration

Human TK 1 can be distinguished from TK 2 and from the herpesvirus TKs by a more narrow phosphate donor range (for a review see 4). Three sera from patients with B_{12}-deficiency, CML and CMV infection were separated on a Superose 12 column. The TK activity of each fraction was determined using either ATP or cytidine triphosphate (CTP) as phosphate donor. The rate of CTP to ATP mediated phosphorylation was calculated for each TK peak by comparing the areas below each graph. The CTP/ATP ratio varied from 4 to 6% for the major TK activity, which eluted around 9.7 ml. The corresponding range for the minor TK activity, which eluted at 13.3 ml was similar, 2–4%. TK 1 from HeLa cells gave 4%, while affinity purified mitochondrial TK 2 from human placenta [27], analysed for comparison, exhibited a CTP/ATP ratio of 67%.

Since a large protein complex with the capacity to channel dThd directly in to DNA has been described [9] it was of interest to examine the reaction products from the two peaks. Fractions of the two TK peaks obtained from the exclusion chromatography were pooled separately and incubated for 360 minutes in a complete reaction solution containing 10^{-7} M ^{125}I-IUdR at a specific activity of approximately 2000 Ci/mM. The reaction products were analysed by ascending thin layer chromatography as described in Material and Methods. The chromatograms showed prominent spots of ^{125}I-IUdR monophosphate as a reaction product for both TK activities. No traces of ^{125}I-IUdR di- or tri-phosphate were detected.

Factors affecting the molecular form of thymidine kinase

The finding of various proportions of two different molecular forms of TK with similar CTP/ATP ratio and reaction products challenged a study of factors affecting the molecular forms of TK. For this study we selected a serum from a CML patient with an extremely high TK activity (3500 U/μl). A good recovery of enzyme activity is necessary for this type of studies. Therefore we initially evaluated the stability of the serum TK activity during incubation with high salt or different combinations of reaction solution components (Table 2). The results showed that the enzyme was relatively stable in all tested combinations of components, except during incu-

Table 2. Stability of serum TK diluted 1 to 20 and incubated for 10 minutes at 37° C in indicated mixtures of reaction components and high salt

Components in incubation mixture	1	2	3	4	5	6	7	8	9	10
Buffer[a]	+	+	+	+	+	+	+	+	+	+
Substrate	0	+	0	0	+	+	+	0	0	0
ATP	0	0	+	0	+	+	0	+	+	+
Ditioerytriol	0	0	0	+	0	+	+	+	0	+
2 Mm NH_4CL	0	0	0	0	0	0	0	0	+	+
[b]Residual activity %	76	102	83	14	86	82	16	82	90	99

[a] Contains: 89 mM Hepes, 8 mM $MgCL_2$, 18 mM KCl, 2 mM NaF at pH 7.4.
[b] Percent of TK activity in original serum sample.

Table 3. The effect of preincubation of serum on the molecular appearance of TK and recovery in gel exclusion chromatography. Serum diluted 1/20 was incubated for 10 min at 37° C with indicated substances. The component concentrations are the same as in the reaction mixture if not otherwise is indicated. The analysis of molecular forms of serum TK were performed by exclusion chromatography on a Superose 12 column and 200 μl fractions were collected. TK activity was measured for each fraction

Serum	Preincubated with	[a]Recovery after pre-incubation %	[b]Total recovery after separation	Peak position I ml	Peak position II ml	[b]Recovery in peak II%
CML	–	–	78	9.5	13.0	9
CML	IUdR	100	75	9.5	12.9	7
CML	IUdR, 1 mM	[c]?	43	9.4, 9.7	13.2–13.6	7
CML	ATP	83	71	9.5	13.0	3
CML	IUdR, ATP	86	62	9.5	13.0	6
CML	IUdR, DTE	20	6	10.8	13.0	2
CML	ATP, DTE	82	58	10.6	13.2	12
CML	IUdR, ATP, DTE	82	43	10.4	13.0	9
CML	ATP, 1 M NH_4Cl	90	60	9.5	13.0	2
CML[d]	ATP	94	100	9.2, 9.8	13.0	5
CML[d]	ATP, 5 mM DTE	82	70	10.8	13.0	22
CML[d]	ATP, 25 mM DTE	97	70	10.9	13.0	17
CML[d]	ATP, 128 mM DTE	64	45	11.4	12.9	36
CML[d]	ATP, 400 mM DTE	102	73	–	12.8	73

[a] Percent of a control, with the indicated substance included, stored at 5° C.
[b] Percent of activity in original unfractioned serum.
[c] 1 mM IUdR prevents the measurment of the TK activity in the sample.
[d] Separations was performed with 1 mM ATP added to the separation buffer.

bation with dithioerythritol (DTE) in absence of ATP.

200 µl serum samples diluted 1/20 were then preincubated for 100 minutes at 37° C with the different compounds and thereafter separated by gelexclusion chromatography on a Superose 12 column. High concentration of salt (1 M), phosphate donor (ATP 3 mM) or substrate (IUdR 1 mM) had no effect on the apparent molecular weights of the enzymes (Table 3). The only factor found that affected the pattern of molecular forms to a significant extent was the reducing agent, dithioerythritol (DTE). After incubation with 5 mM DTE in presence of ATP the elution volume of the major TK activity increased from 9.7 ml to 11.0 ml, corresponding to an alteration in apparent Mw from 740 kD to 300 kD. In addition, the amount of original TK activity that was recovered as the 58 kD form increased from 3% to 12%. Incubation with standard assay conditions (5 mM DTE, 3.9 mM ATP and 1.1×10^{-7} M IUdR) resulted in recovery of a mixture consisting of 21% 58 kD TK and 79% 300 kD TK. The total recovery from the column was relatively low (43–58%) (Table 3).

To further investigate the effect of reducing agents on the molecular forms of TK, a series of experiments with sera incubated at different concentrations of DTE in the presence of 1 mM ATP was carried out. In these experiments, ATP was included also in the separation buffer to increase the recovery of enzyme activity. The results (Table 3, Fig. 3A, B) showed no or only marginal difference in enzyme profiles when the DTE concentration was increased from 5 to 25 mM. At 128 mM DTE, however, no enzyme activity was recovered as the 300 kD form, while 36% of the original activity was recovered as the 58 kD form. A new intermediate form contained 9% of the original activity (fig. 3C). After incubation with 400 mM DTE 73% of the original enzyme activity was recovered, all as the 58 kD form (fig. 3D).

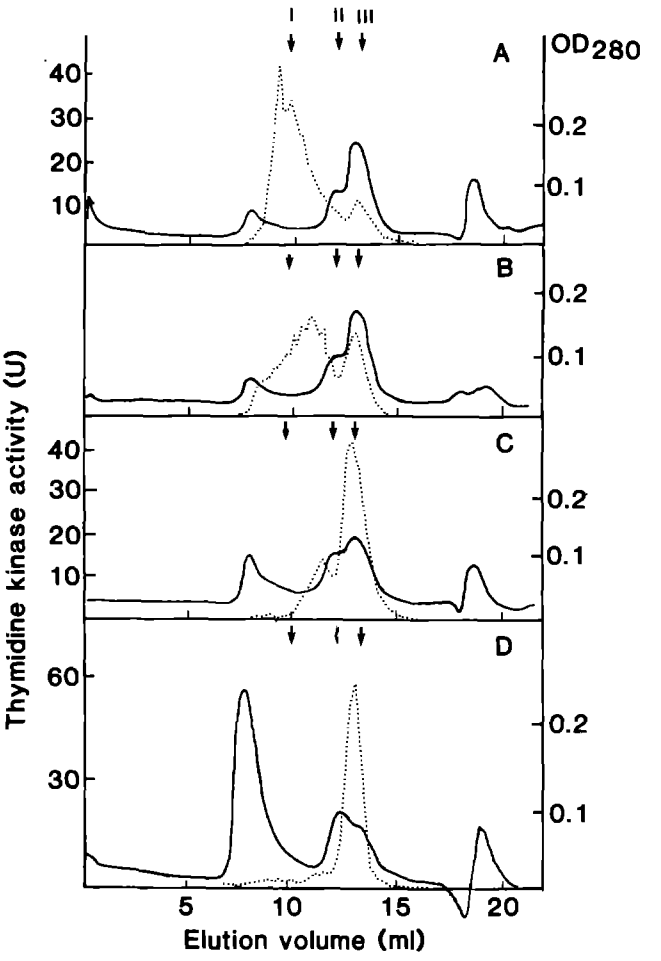

Fig. 3. The effects of a reducing agent (DTE) on the molecular forms of S-TK. Serum from a patient suffering from CML was diluted 1/20 in TK assay mixture containing 1 mM ATP but no substrate, and A: OmM DTE, B: 5 mM DTE, C: 120 mM DTE and D: 400 mM DTE. Two hundred µl samples were incubated for 10 min at 37° C, sterile filtered, separated on a Superose 12 column and 200 µl fractions were collected. A_{280} (———) was continously monitored, and TK activity (.....) was determined for each fraction. The locations of α2-macro-globulin (I, 725 kD), IgG (II, 150 kD) and albumin (III, 66 kD) were used as internal Mw – controls.

Effect of reducing agent (DTE) on the molecular forms of serum DNA polymerase

The drastic effect of DTE on the molecular forms of S-TK and the fact that DTE is included in the assay mixture for DNAp initiated a study of the effect of DTE on S-DNAp. The procedure described above for S-TK was used. The initial studies revealed that also serum DNAp required ATP for stabilization when DTE was present.

Diluted serum (1/5) was preincubated with dif-

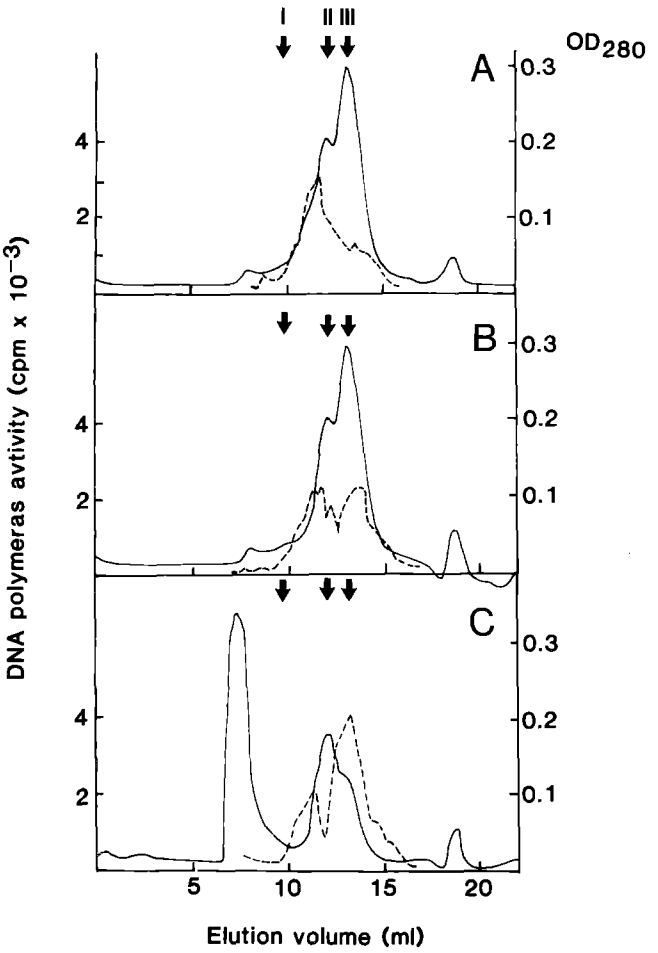

Fig. 4. The effects of a reducing agent (DTE) on the molecular forms of serum DNAp. Serum from a patient suffering from ALL was diluted 1/5 in DNA-polymerase buffer (containing 1 mM ATP but no template), and DTE was added to give final concentration of A: OmM, B: 10 mM, C: 400 mM DTE. Two hundred μl samples were incubated 10 min at 37° C, sterile filtered, separated on a Superose 12 column and 200 μl fractions were collected. A_{280} (———) was continuously monitored, and DNA polymerase (------) activity was determined for each fraction. The locations of α2-macro-globulin (I, 725 kD), IgG (II, 150 kD) and albumin (III, 66 kD) were used as internal Mw – controls.

ferent DTE concentrations and separated on a Superose 12 column. When 1 mM ATP was included in the incubation mixture and the elution buffer, more than 90% recovery of DNAp was obtained. The results (Fig. 4) showed that serum DNAp was converted from a large form (240 kD) to a smaller form (56 kD) with increasing DTE concentration. Separation of serum preincubated in buffer devoid to DTE gave a total recovery of 108% DNAp, 81% as the 240 kD form and 27% as a 'smear' with a Mw below 150 kD (Fig. 4A). Preincubation with 10 mM DTE, the concentration used in the DNAp assay, resulted in recovery of a mixture consisting of 42% large form and 52% of a distinct small form (total recovery 94%) (fig. 4B). At 400 mM DTE, a total recovery of 114% DNAp was obtained, consisting of 25% large form and 89% small form (Fig. 4C).

Evaluation of the possible presence of TMP-kinase activity in human serum

A collection of 10 sera containing high TK or DNAp activity was screened for TMPK activity. All sera were found to be negative. To investigate whether these sera, in spite of lack of activity in a direct assay, contained a TMP kinase activity which was hidden from detection by competitive nucleosides or product destroying enzymes, three sera were fractioned by gel exclusion chromatography and the fractions were assayed for TMPK activity. A small but significant peak (3 times background) was recovered at an elution volume of 14.0 ml (50 kD) from one of these sera (data not shown). To evaluate the possibility that TMPK could be bound to other proteins (e.g. TK or DNAp) in a complex form not detectable in our TMPK assay, the TMPK positive serum was incubated with 400 mM DTE, separated on gel exclusion chromatography and assayed for TMPK activity. The TK activity was determined as a control. No additional TMPK activity was found in this experiment although a good recovery of a 58 kD TK, 85% was obtained.

The relation between DTE concentration and reaction velocity in the TK assay

The finding of the conversion of large TK forms to smaller as a function of DTE concentration challenged a study of the relation between enzyme activity and DTE concentration in assay mixture.

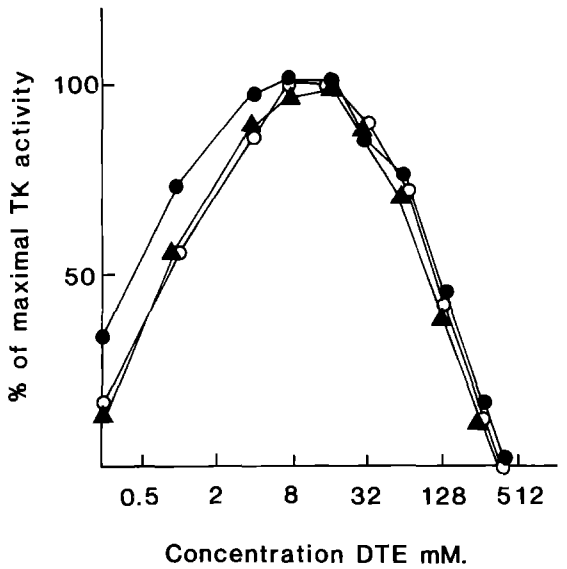

Fig. 5. The effects of the DTE concentration in TK assay mixture on reaction velocity. The 750 kD and the 58 kD TK forms were isolated from an AML serum by gel exclusion chromatography. The enzymatic activities of the two TK forms and of the unfractionated serum were determined in the standard assay with DTE concentrations ranging from 0.25 mM to 400 mM. The cpm values from a two hour assay were for each sample recalculated to percent of of the maximal activity obtained at optimal DTE concentration. Symbols: unfractioned serum (O–O–O); 750 kD form (▲–▲–▲); 58 kD form (●–●–●).

The enzymatic activity of the 750 kD form, the 58 kD forms and of the unfractioned serum were determined at DTE concentrations ranging from 0.25 mM to 400 mM. The result (Fig. 5) showed that the three preparations had a rather similar relation between DTE concentration and enzymatic activity. At DTE concentrations below 0.5 mM the three samples gave 12 to 34% of the maximal turnover, which was obtained at concentrations between 4 and 32 mM DTE in the assay mixture. All three preparations successively lost their enzymatic activity with increasing DTE concentration and were virtually inactive at 400 mM DTE (Fig. 5).

Comparison of substrate kinetics of 58 kD TK purified from human serum and from HeLa cells

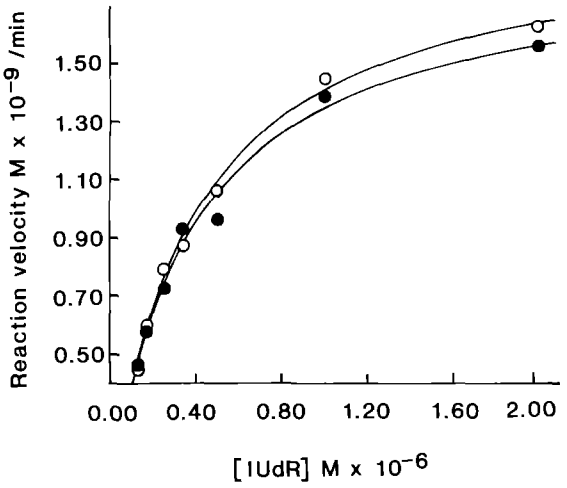

Fig. 6. Comparison of the substrate kinetics of the small S-TK form and TK 1 from HeLa cells. Menten plots showing the initial reaction velocities at different IUdR concentrations, (●–●) small S-TK form and (O–O) the enzyme with the same molecular size purified from HeLa cells.

Serum from a patient suffering from AML was preincubated with 400 mM DTE and fractioned by gel exclusion chromatography on FPLC. An extract from HeLa cells was separated on FPLC without preincubation. Both separations resulted in recovery of only the small TK form (58 kD). The reaction velocities of the purified TK preparations at different concentrations of IUdR were determined as described in Materials and Methods. Figure 6 shows initial reaction velocities plotted against substrate concentration for these two TK preparations. The enzyme from the AML patient had a Km value of 3.83×10^{-7} M compared to 3.88×10^{-7} M for the HeLa TK. When the reaction velocities were recalculated to the same amount of enzyme (100 U, Fig. 6) the preparations gave Vsat 1.86×10^{-9} and 1.96×10^{-9} mol/min respectively.

Discussion

Despite the clinical usefulness, very little is known about the TK isozymes in human serum, and the knowledge of DNAp in serum is even smaller. A

major problem concerning characterization of TK and DNAp in serum is the low activity present. As a consequence of this all investigations are restricted to sera selected from patients with diseases giving comparatively high TK or DNAp activity. Another consequence is that the sensitivity of the enzyme assays available is a limiting factor for this type of studies. The utilization of a ^{125}I-labeled substrate analogue gave an increase in sensitivity compared with a conventional TK assay utilizing ^{3}H- or ^{12}C-Thd, which was instrumental for this study. On the other hand the more conventional DNAp assay used, after serum fractionation, gave only significant detection of selected high activity sera. As a result of this, the present study contains comparatively more data on S-TK than on S-DNAp.

In the first part of this study we evaluated which enzyme forms that actually are present in the circulating blood. To obtain a gentle, reproducible and rapid separation according to molecular size we choose to utilize FPLC technique combined with gel exclusion chromatography. Initially a few fresh serum samples were separated before and after a freezing and thawing cycle. The enzyme profiles obtained were identical (data not shown). Therefore the results presented, mostly obtained by analysis of frozen samples, are likely to be representative for the actual situation in the blood. Two TK forms with distinct apparent Mws was detected in all investigated serum samples. The main form, contributing more than 80% of total enzyme activity recovered, exhibited a Mw of 730 kD while the minor form eluted as 58 kD. The results from the FPLC separations were verified by studies of the sedimentation behaviour in glycerol gradient centrifugation. The Mw estimated for the major TK form in this system was 740–780 kD, which is rather close to the Mw obtained by gel filtration (730 kD). The minor TK form gave an estimated Mw of 80–100 kD, which is significantly higher than the 58 kD obtained by gel filtration. The reason for this discrepancy is unknown, but one must consider the possibility that this TK form is slightly retarded in relation to albumin by the column material. The value calculated from the gradient centrifugations is in accordance with those reported for human cell TK 1 by others using similar methods. TK 1 isolated from human placenta was estimated to have a Mw of 90 kD [28], while affinity purified TK 1 from HeLa cells was reported to have a Mw of 96 kD [6]. When the elution volume of TK 1 from HeLa, prepared according to Sherley & Kelley [6], was determined in our FPLC system, the peak was found at the same position as the the small serum TK form. The mentioned HeLa TK peak fraction also had identical substrate kinetics as the small serum TK form. The ability of the two TK forms to utilize CTP as phosphate donor was investigated and the results showed that both forms were similar to TK 1, in regard to phosphate donor specificity. No significant TK 2 activity was isolated from any of the studied sera.

Despite the diverging results in the literature regarding molecular forms of TK isozymes in different specimens, 730 kD is an exceptionally high Mw for a TK. The reaction product of this TK form proved to be exclusively monophosphate, indicating that this was not the functional complex of DNA precursor synthesizing enzymes previously described [9]. This challenged a study of factors known to more or less specifically affect the molecular forms of protein complexes. High ionic strength (1 M NH_4Cl) did not disaggregate the large TK form, indicating that it was not an unspecific protein aggregate held together by ionic interactions. On the other hand, incubation with high concentrations of substrate or phosphate donor, which earlier has been shown to specifically affect the molecular forms of TKs did neither have any effect [7–9]. High concentrations of DTE were finally found to have the capacity to quantitatively convert the large TK form into the small one. From this it can be concluded that both TK forms in serum contained the same catalytical active peptide and that the large TK form consists of proteins linked by S-S bridges. Since DTE is an essential component in the TK assay mixture the dependence of the enzymatic activity on DTE was studied. A drastic decrease in activity was found when the DTE concentration in the assay mixture was decreased below 5 mM. The requirement of DTE for maximal activity and the ability of DTE to convert large TK forms into smaller represents an enigma

that probably only can be solved when the peptide composition of the 730 kD TK is elucidated. This is not easily achieved with current techniques, considering the small amounts of 730 kD protein that is present in selected human sera. Addition of HeLa TK to normal serum does, however, not result in generation of 730 kD TK (data not shown).

The enzymatic properties of the major TK form in human serum, the 730 kD form, can not be studied since it is not stable at the DTE concentrations required for reasonable enzymatic activity. In fact we do not know if the 730 kD TK form *per se* has any enzymatic activity, or if it is activated when the DTE in the assay mixture converts it into smaller forms. At our standard assay conditions, a mixture of 79% of a new 300 kD form, not present in the original serum sample and 21% of the 58 kD TK form was found. This 300 kD form apparently has approximately the same catalytical activity as the 58 kD form as similar recoveries of enzymatic activity were obtained in the dissociation experiments (Table 3), independent of the enzyme form recovered.

We were only able to detect a low TMPK activity in one of the sera investigated after separation on Superose 12, the serum with the highest TK activity. If this is due to a very low TMPK content in serum or to the assay method used has not yet been elucidated. However, pretreatment with DTE did not increase the TMPK activity showing that there are no disulfide bonds binding TMPK to any complex form not detectable in our assay.

The serum DNAp from patients with B_{12}-deficiencies or haematological malignancies was found to have a Mw in the range 240–270 kD both by gel exclusion chromatography and glycerol gradient centrifugation. One exceptionally large DNAp activity was recorded in a immunosuppressed patient suffering from CMV infection. We have recently shown that the characteristics of serum DNAp were similar to those of α-polymerase. The variation in molecular forms reported for mammalian α-polymerases is even wider than for TKs. In recent reports there seems to be a consensus on a Mw of 200–240 kD [29, 30]. The enzyme can be found in different polymeric forms and associated with several other proteins, see review [14]. If the $>10^6$ kD DNAp from the CMV serum represents another form of the α-polymerase or is a viral enzyme is an intriguing question not answered by this study, however, DNAp from several unfractioned CMV sera has earlier been shown to have similar properties as α-polymerase [24]. The 240 kD serum DNAp was found to be dissociated at high concentrations of reducing agent (DTE) into an enzymatically active 56 kD form (Fig. 4). Subunits of DNAp-α with Mws down to 40 kD have been reported previously, but are today regarded as partially degraded but enzymatically active enzyme molecules, for a review see [14]. At the DTE concentration used in our standard DNAp assay (10 mM) a 45/55% mixture of the two mentioned DNAp forms was recovered. The substrate turnover of these two DNAp forms was obviously similar, since the total recovery of DNAp always was around 100% (94–114%) regardless of the proportions of enzyme forms recovered.

The present study has probably raised more questions than it has answered. Both TK and DNAp occurs in other forms in serum than during catalytical conditions *in vitro*. This is an example of a classical problem in enzymology; the characteristics of an enzyme in an analytical system is not always identical to the properties of the catalytically active enzyme. The TK activity in serum that is measured by our TK assay is likely to originate from a protein consisting of a TK 1 linked by S-S bridges to other peptides. The greater stability of serum TK compared to TK 1 from cells earlier reported [15] is explained by the finding of this large (730 kD) TK protein in native serum. The nature and biological relevance of this form is still enigmatic. We are currently attempting to purify reasonable amounts of it to gain some information of peptide composition and enzymatic properties.

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References

1. Elsevier SM, Kucherlapati RS, Nichols EA, Creagan RP, Giles RE, Ruddle FH, Willecke K, McDougal JK: Assignment of the gene for galactokinase to human chromosome 17 and its regional localisation to band q21–22. Nature 251: 633, 1974
2. Willecke K, Reber T, Kucherlapati RS, Ruddle FH: Human mitochondrial thymidine kinase is coded for by a gene on chromosome 16 of the nucleus. Somatic Cell Genetics 3: 237, 1977
3. Bello LJ: Regulation of thymidine kinase in human cells. – Exp Cell Res 89: 263, 1974
4. Kit S: Viral-associated and induced enzymes. Pharmacol Ther 4: 501, 1979
5. Bradshaw HD Jr., Deininger PL: Human thymidinkinase gene: Molecular cloning and nucleotide sequence of a cDNA expressible in mammalian cells. Mol Cell Biol 4: 2316, 1984
6. Sherley JL, Kelly T: Human cytosolic thymidine kinase. Purification and physical characterization of the enzyme from HeLa cells. JBC: 263: 375, 1988
7. Munch-Petersen B: Differences in the kinetic properties of thymidine kinase isozymes in unstimulated and phytohaemagglutinine stimulated human lymphocytes. Mol Cell Biochem 64: 173, 1984
8. Munch-Petersen B, Tyrsted G: Thymidine kinase isozymes in human acute monocytic leukaemia. Mol Cell Biochem 66: 185, 1985
9. Wickremasinge RG, Yaxley JC, Hoffbrand AV: Gel filtration of a complex of DNA polymerase and DNA precursor-synthesizing enzymes from a human lymphoblastoid cell line. BBA 740: 243, 1983
10. Fridland A: DNA precursor in eukaryotic cells. Nat New Bio 243: 105, 1973
11. Taheri MR, Wickremasinghe RG, Hoffbrand V: Alternative metabolic fates of thymine nucleotides in human cells. Biochem J 194: 451, 1981
12. Kornberg A: DNA replication. San Francisco. W.H. Freeman and Company 1: 1, 1980
13. Weissbach A: Eukaryotic DNA poymaerases. Ann Rev Biochem 46: 25, 1977
14. Loeb LA, Liu PK, Fry M: DNA polymerase α: Enzymology, Function, Fidelity and Mutagenesis. Prog Nucleic Acid Res Mol Biol 33: 57, 1986
15. Gronowitz JS, Källander CFR, Diderholm H, Hagberg H, Petterson U: Application of an *in vitro* assay for serum thymidine kinase: results on viral disease and malignancies in humans. Int J Cancer 33: 5, 1984
16. Hagberg H, Gronowitz JS, Killander A, Källander CFR: Thymidine kinase activity in serum from patients with B12 deficiency. Scand J Haematol 32: 41, 1984
17. Gronowitz JS, Hagberg H, Källander CFR, Simonsson B: The use of serum deoxythymidine kinase as a prognostic marker, and in the monitoring of patients with non-Hodgkin's lymphoma. Brit J Cancer 47: 487, 1983
18. Gronowitz JS, Tötterman TH, Källander CFR, Hagström Å, Tufveson G: Serum Thymidine kinase, CMV specific antibodies, and total immunoglobulins in renal transplant patients during immunosuppression with cyclosporin A. Clinical Transpl 2: 26, 1988
19. Källander CFR, Simonsson B, Hagberg H, JS Gronowitz: Serum deoxythymidine kinase gives prognostic information in chronic lymphocytic leukaemia. Cancer 54: 2450, 1984
20. Hagberg H, Gronowitz JS, Killander A, Källander CFR, Simonsson B, Sundström C, Öberg G: Serum thymidine kinase in acute leukaemia. Brit J Cancer 49: 537, 1984
21. Simonsson B, Källander CFR, Brenning G, Killander A, Gronowitz JS: Evaluation of serum deoxythymidine kinase as a marker in multiple myeloma. Br J Haematol. 61: 215, 1985
22. Källander CFR, Gronowitz JS, Olding-Stenkvist E: Rapid diagnosis of varicella-zoster virus infection by detection of viral deoxythymidine kinase in serum and vesicle fluid. J Clin Microbiol 17: 280, 1983
23. Bondurant M, Aur J, Dahl GV, Tereba A: Possible prognostic indicator for high risk childhood acute lymphocytic leukaemia patients. Lancet jan 29: 234, 1983
24. Neumüller M, Källander CFR, Gronowitz JS: Detection and some characteristics of DNA polymerase activity in serum from patients with malignant, viral, or B12-deficiency disease. In press Enzyme 41: 1989.
25. Karlström AR, Gronowitz JS: An optimized thymidylate kinase assay; based on enzymatically synthesized 5(^{125}I) iododeoxyuridine monophosphate and its application to an immunological study of herpes simplex virus thymidine-thymidylate kinases. Anal Biochem 162: 500, 1987
26. Martin RG, Ames BN: A method for determining sedimentation behavior of enzymes: Application to protein mixtures. J Biol Chem 236: 1372, 1961
27. Karlström AR, Källander CFR, Abele G, Larsson A: Acyclic guanosine analogs as substrates for varicella-zoster virus thymidine kinase. Antimicrob Agents Chemother 29: 171, 1986
28. Eng Gan T, Brumley JL, Van Der Weyden MB: Human thymidine kinase: Purification and properties of the cytosolic enzyme of placenta. J Biol Chem 258: 7000, 1983
29. Lamothe P, Baril B, Chi A, Lee L, Baril E: Accessory proteins for DNA polymerase α activity with single-strand DNA templates. PNAS 78: 4723, 1981
30. Masaski S, Koiwai O, Yoshida S: 10 S DNA polymerase α of calf thymus shows a microheterogeneity in large polypeptide component. JBC 257: 7172, 1982

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