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IDENTIFICATION AND ISOLATION ON A LARGE SCALE OF GUANYLATE KINASE FROM HUMAN ERYTHROCYTES

EFFECTS OF MONOPHOSPHATE NUCLEOTIDES OF PURINE ANALOGS*

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Abstract—Guanylate kinase has been identified and partially purified from human erythrocytes. A simple method has been developed, using calcium phosphate gel and DEAE-cellulose, for the purification of guanylate kinase and several other erythrocytic enzymes such as purine nucleoside phosphorylase, nucleoside diphosphokinase, adenylate kinase, lactic dehydrogenase, pyruvate kinase and phosphoglucose isomerase. The method is adaptable for both small and large scale purification and has been applied successfully to as much as 40 to 50 l. of human blood. Studies with the partially purified erythrocytic guanylate kinase showed that GMP, dGMP, 8-azaGMP and IMP serve as substrates while UMP, CMP and 6-thioIMP do not. 6-ThioGMP was not a substrate but was a competitive inhibitor with GMP with a K_i of $7{\cdot}5 \times 10^{-5}$ M. Preliminary findings suggest the occurrence of several isozymes of this enzyme. Other kinetic parameters of the enzyme are described.

NUMEROUS investigations have been performed on the behavior of many chemicals with enzymes from a variety of sources, plant, microorganism and animal in the hope of learning fundamental information about drug action. Since the understanding of drug action in man is of primary interest to many pharmacologists, it is of obvious importance that studies of this sort be performed on enzymes of human origin. In the past few years, this laboratory has been engaged in investigation of a number of enzymes involved in purine metabolism that interact with nucleotides and nucleosides of carcinostatic purine analogs. It has been found that the human erythrocyte provides a rich and readily available source for several enzymes of great interest to us, such as purine nucleoside phosphorylase and nucleoside diphosphokinase.[1–3] A procedure has been developed by this laboratory which is an adaptation of methods published previously, employing adsorption on calcium phosphate gel[1] and DEAE cellulose (phosphate)[4] to remove the proteins of interest from the overwhelming amount of hemoglobin in hemolyzates. This general procedure has been found adaptable to amounts of blood ranging from a few milliliters to as much as 50 l.

Recently, in addition to a number of other enzymes of interest in purine metabolism, we have identified the enzyme ATP:GMP phosphotransferase (EC 2.7.4.8) (guanylate kinase) in human erythrocytes. This report documents the methods employed in the

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general procedure for the large scale purification of erythrocytic enzymes and describes the substrate specificity, some of the kinetic properties, and the effects of various analog nucleotides on guanylate kinase. The analog nucleotide 6-thioguanosine 5′-phosphate (6-thioGMP), which was earlier shown to inhibit guanylate kinase from hog brain and Sarcoma 180 cells[5] also inhibits this enzyme from the human erythrocyte.

MATERIALS AND METHODS

The sodium salts of AMP, ADP, ATP, GMP, GDP, dGMP, UMP, CMP, IMP and inosine were purchased from P-L Biochemicals. The sodium salts of pyruvic acid, NADP, NADH, phosphoenolpyruvic and 6-phosphogluconic acids were purchased from Sigma Chemical Co. Also purchased from Sigma were glucose 6-phosphate, glucose 6-phosphate dehydrogenase from yeast, crystalline pyruvate kinase, lactic dehydrogenase and adenylate kinase from rabbit muscle. The nucleotides, 8-azaGMP* and 6-thioIMP, were obtained from the Cancer Chemotherapy National Service Center. The concentration of 8-azaGMP was determined by its molar absorbance at pH 7·4 ($\epsilon = 10{\cdot}5 \times 10^3$ at 257 mμ).[6,7] DEAE-cellulose (Cellex-D), DTT, and the lithium salt of dTDP were purchased from Calbiochem. Sephadex was a product of Pharmacia. Xanthine oxidase was obtained from Worthington Biochemicals. Whatman chromatography cellulose powder (coarse fibers Grade CF 11) was purchased from H. Reeve Angel. All other chemicals used were of the highest purity. Outdated human blood was obtained from Miriam Hospital, Providence, R.I., and from the Red Cross of Boston. In all experiments this blood was used unless otherwise mentioned.

6-ThioGMP was prepared enzymatically according to Miech *et al.*[5] Calcium phosphate gel was prepared according to the method of Tsuboi and Hudson.[1] The relative affinity of the gel for various enzymes was determined by varying the gel to protein ratio as in Fig. 1. In further purification experiments a 0·05 gel to protein ratio† was selected because at this concentration a clear separation was obtained between NDPK, PNP, LDH and other enzymes. Crystalline PNP was prepared according to the method of Agarwal and Parks.[8]

Enzyme assays. PNP was assayed by the coupled xanthine oxidase method of Kalckar.[9] The assay conditions and definition of enzyme units are described by Kim *et al.*[2] NDPK was assayed by the coupled pyruvate kinase–lactic dehydrogenase method as described by Mourad and Parks[3] except that the substrate was 0·4 mM dTDP instead of dGDP.

Adenylate kinase activity was measured spectrophotometrically by a coupled

* Abbreviations used: adenylate kinase, ATP:AMP phosphotransferase (EC 2.7.4.3); 8-aza GMP, 5′-monophosphate of 8-azaguanosine; catalase, H_2O_2:H_2O_2 oxidoreductase (EC 1.11.1.6); DTT, dithiothreitol (Cleland's Reagent); G 6-PDH, glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: NDAP oxidoreductase) (EC 1.1.1.49); guanylate kinase, ATP:GMP phosphotransferase (EC 2.7.4.8); LDH, lactic dehydrogenase (L-lactate: NAD oxidoreductase) (EC 1.1.1.27); NDPK, nucleoside diphosphokinase (ATP: nucleoside diphosphate phosphotransferase) (EC 2.7.4.6); PEP, phosphoenol pyruvate; PK, pyruvate kinase (ATP: pyruvate phosphotransferase) (EC 2.7.1.40); 6-phosphogluconic acid dehydrogenase (6-phospho-D-gluconate: NAD (P) oxidoreductase) (EC 1.1.1.43); phosphoglucose isomerase, D-glucose 6-phosphate ketol-isomerase (EC 5.3.1.9); PNP, purine nucleoside phosphorylase (purine nucleoside: orthophosphate ribosyl transferase) (EC 2.4.2.1); PRPP, 5-phosphoribosyl pyrophosphate; 6-thioGMP, 6-thioguanosine-5′-monophosphate; 6-thioIMP, 5′-monophosphate of 6-mercaptopurine riboside.

† One mg dry calcium phosphate gel: 20 mg protein (as calculated by absorbance at 280 mμ).

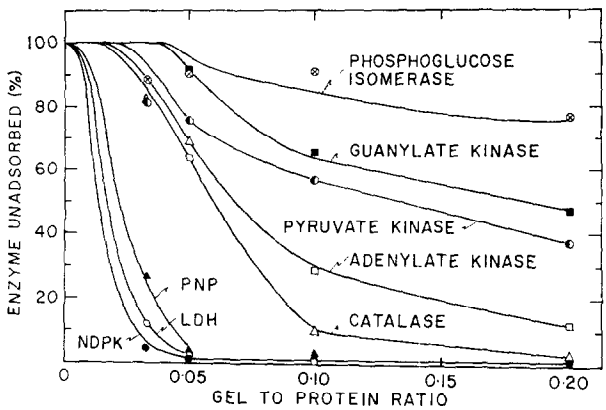

FIG. 1. Relative enzyme affinities for calcium phosphate gel. The gel and hemolyzate were thoroughly mixed for 30 min and centrifuged. The gel was washed once by suspending in distilled water. The wash was combined with the supernatant and residual enzyme activity was determined.

pyruvate kinase–lactic dehydrogenase system identical to that used for NDPK except that the substrate was 0·1 mM AMP.

Guanylate kinase activity was measured spectrophotometrically, as with the NDPK assay using 0·1 mM GMP, by determining the rate of formation of ADP and GDP in the presence of nonrate-limiting concentrations of phosphoenolpyruvate, NADH, Mg^{2+}, pyruvate kinase and lactic dehydrogenase.[10] In most experiments the reactions were started by the addition of enzyme. Appropriate blanks were included to detect background activity resulting from the presence of contaminants such as phosphatases. With the purified guanylate kinase preparations background activity was negligible. The phosphorylation of 1 μmole of GMP in the presence of ATP ultimately results in the oxidation of 2 μmoles of NADH, because both ADP and GDP serve as substrates for pyruvate kinase. To test whether this is true for diphosphates other than ADP and GDP, the stoichiometry of the reaction was examined. Since dGDP and 8-azaGDP are very poor substrates for pyruvate kinase as seen in Fig. 2, it is assumed in the initial velocity studies that 1 μmole of either dGMP or 8-azaGMP resulted in the oxidation of only 1 μmole of NADH.

Catalase was assayed by a slight modification of the perborate method of Feinstein.[11] Lactic dehydrogenase was assayed spectrophotometrically by a modification of the method of Kornberg.[12] Pyruvate kinase was assayed spectrophotometrically in a coupled system by the method of Bucher and Pfleiderer,[13] phosphoglucose isomerase was assayed by the spectrophotometric method described by Noltmann,[14] glucose 6-phosphate dehydrogenase was assayed spectrophotometrically by the method of Kornberg and Horecker,[15] phosphogluconic acid dehydrogenase activity was measured by the method of Pontremoli and Grazi.[16]

Protein determination. Protein concentrations were determined by the method of Warburg and Christian[17] in which the absorbance at 280 mμ was multiplied by a factor of 1·1 to obtain protein concentration in milligrams per milliliter.

Preparation of calcium phosphate gel-cellulose for columns. Calcium phosphate gel-cellulose columns were employed as described earlier.[18] Coarse fibered cellulose powder (120 g) was suspended in 1 l. of 0·005 M KCl and allowed to swell for at least 24 hr.

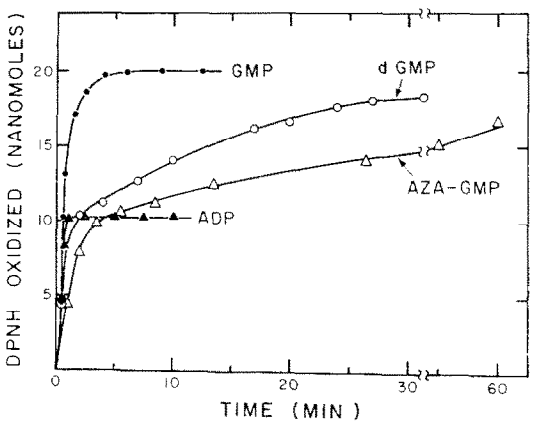

FIG. 2. Stoichiometry of the reaction of nucleoside diphosphates with pyruvate kinase. To 1-m reaction mixture containing an excess of guanylate kinase, 100 μmoles Tris acetate, pH 7·5; 100 μmoles KCl; 24 μmoles $MgCl_2$; 2 μmoles ATP; 3 μmoles PEP; 1·25 μM units of pyruvate kinase; 2·25 μM units of lactic dehydrogenase; 0·3 μmoles of NADH; and 10 nmoles of either GMP, dGMP or 8-azaGMP were added and the oxidation of NADH was followed with time by change in absorbancy at 340 mμ. As a standard, ADP (10 nmoles) replaced the mononucleotide.

Calcium phosphate gel (5 g) was added to the cellulose suspension and mixed thoroughly. This suspension was used for packing the column. When attempts were made to employ higher gel:cellulose ratios, e.g. 1:10, or finer grades of cellulose powder, poor flow rates resulted.

Preparation of DEAE-cellulose for columns. About 100 g of DEAE-cellulose was washed free of fines and converted to the phosphate form by suspension in 1 M potassium phosphate buffer, pH 7·5. After thorough washing with water this was suspended in 0·005 M potassium phosphate buffer, pH 7·5, for column chromatography.

These column procedures were appropriate for the preparation of columns with dimensions of about 5 × 40 cm. The amounts employed were successfully adjusted for columns varying in dimensions from 2 × 20 cm to about 14 × 150 cm.

RESULTS

A. *General procedure for purifying erythrocytic enzymes*

A survey was made of enzyme levels in freshly drawn and outdated human erythrocytes and compared with known literature values (Table 1). The table illustrates that the enzyme activities reported here agree reasonably well with those in the literature. Enzymes such as NDPK, LDH, guanylate kinase and adenylate kinase seem to be relatively stable in erythrocytes stored under usual blood-bank conditions. On the other hand, PNP, pyruvate kinase, 6-phosphogluconic acid dehydrogenase and glucose 6-phosphate dehydrogenase activities in stored blood were 2-to 3-fold lower than those of freshly drawn blood. The purification procedure described below is a general one that has been found adaptable for amounts of whole blood as small as 5 ml or as large as 40–50 l. A simple modification of the relative amounts of materials and reagents following the relative proportions described below have proved satisfactory over this wide range of starting material. All purification procedures were

TABLE 1. COMPARISON OF ENZYME LEVELS IN FRESH AND OUTDATED ERYTHROCYTES*

Enzyme	Fresh blood (Units†/ml packed cells)	Outdated blood (Units/ml packed cells)	Literature value with references‡
PNP	13·5	6·5	14 (1); 8 (2)
NDPK	76·0	74·0	30 (3)
LDH	28·0	21·5	21 (19)
Pyruvate kinase	3·1	1·0	2·6 (19)
Phosphoglucose isomerase		3·1	2·5 (19, 20); 10·5 (21)
Guanylate kinase	0·35	0·40	
Adenylate kinase	1·3	1·5	
Glucose 6-phosphate dehydrogenase	1·4	0·33	1·2 (22)
6-Phosphobluconic acid dehydrogenase	1·1	0·70	1·02 (22)
Catalase§	1090	800	

* Fresh blood was drawn from volunteers just prior to the experiment. Outdated blood (stored about 8 weeks in ACD solution) was obtained from the blood bank.

† An enzyme unit is defined as that amount which converts 1 μmole of substrate per minute.

‡ References are indicated in parentheses.

§ Activity expressed as milliequivalents of perborate reduced in 5 min/ml as described in Materials and Methods section.

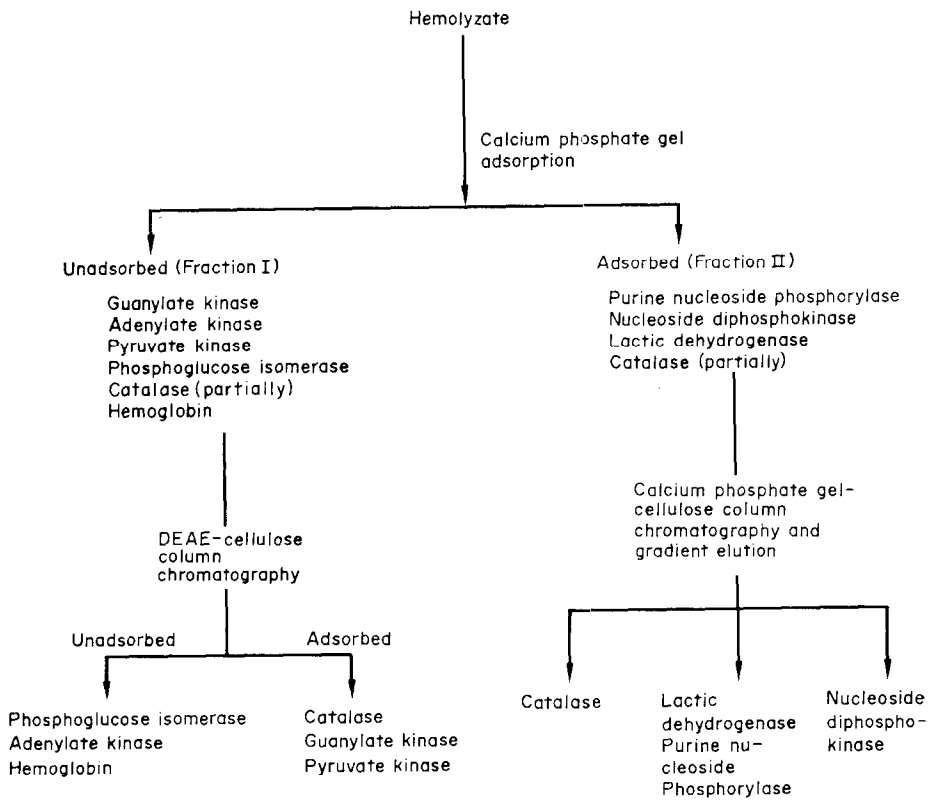

FIG. 3. Scheme for enzyme purification.

carried out at 4°. A schematic diagram of the purification procedure is presented in Fig. 3.

Table 2 presents the results of a typical small-scale purification which employs the following procedures and demonstrates that both good purification and good recoveries can be obtained.

Preparation of hemolyzate. Erythrocytes were collected by centrifugation at 2000 *g* for 10 min. The cells were washed three to four times with an equal volume of cold 0·9% sodium chloride solution. The washed cells were then suspended in 1 vol. of distilled water and frozen until ready for use. Just before using, the mixture was thawed and diluted with 4 vol. of distilled water (overall dilution was 10-fold) and erythrocytic stroma was removed by centrifugation. The stroma may be collected for study if desired.

TABLE 2. PURIFICATION OF ERYTHROCYTIC ENZYMES*

	Total activity (Units)	Specific activity (Units/mg protein)	Recovery (%)	Purification (Fold)
PNP				
Hemolyzate	13·8	0·013	100	1
Fraction II†	12·1	0·23	88	18
Calcium phosphate gel column	11·6	1·46	84	112
NDPK				
Hemolyzate	148·0	0·11	100	1
Fraction II	148·5	2·83	100	26
Calcium phosphate gel column	102·2	9·00	69	82
LDH				
Hemolyzate	32·0	0·024	100	1
Fraction II	32·4	0·62	100	26
Calcium phosphate gel column	23·2	2·90	72	121
Catalase‡				
Hemolyzate	1440	1·7	100·0	1
Fraction I	380	0·4	25·0	
Fraction II	428	8·1	30·0	5
DEAE cellulose column from Fraction I	380	37·6	25·0	22
Calcium phosphate gel column from Fraction II	380	5·4	6·5	3
Pyruvate kinase				
Hemolyzate	2·4	0·002	100	1
Fraction I	2·2	0·002	92	1
DEAE cellulose column	0·59	0·054	25	28
Guanylate kinase				
Hemolyzate	0·80	0·001	100	1
Fraction I	0·77	0·001	96	1
DEAE cellulose column	0·93	0·086	116	86
Adenylate kinase				
Hemolyzate	2·4	0·002	100	1
Fraction I	1·5	0·002	64	Nil
DEAE cellulose column	1·7	0·002	71	Nil

* Table describes the purification of enzymes from 2 ml of packed human erythrocytes.

† See text and Fig. 3 for description of each fraction. The figures given here are after dialysis of these fractions.

‡ Since catalase is only partially absorbed on calcium phosphate gel, the purifications for both the DEAE cellulose and gel cellulose columns are given.

Calcium phosphate gel adsorption. Calcium phosphate gel was added to the hemolyzate at a gel to protein ratio of 0·05 (see Materials and Methods). After thorough mixing for 30 min the gel was collected by centrifugation (5000 *g* for 10 min) and washed with water by repeated centrifugation and resuspension at least three times. The supernatant and washings were pooled and saved (Fraction I) for DEAE cellulose (phosphate) adsorption. Enzymes were eluted from the gel with 20% saturated ammonium sulfate solution (Fraction II). Calcium phosphate gel adsorption proved to be a very useful tool in our purification scheme. It allowed the separation of the enzymes into two major groups as seen in Fig. 3. PNP, NDPK, LDH, and catalase (partially) which were adsorbed to the calcium phosphate gel were obtained in good purity as most of the hemoglobin is not adsorbed. It should be noted that the gel to protein ratio could be modified so as to adsorb other enzymes which would not adsorb at the ratio used here as seen in Fig. 1. For example, as reported by Tsuboi and Hudson,[1] pyrophosphatase and tripeptidase were adsorbed on calcium phosphate gel. This will still leave unadsorbed a large percentage of the hemoglobin which is a major problem in the purification of erythrocytic enzymes. The enzymes can be easily eluted from the gel with ammonium sulfate or phosphate solutions.

Calcium phosphate gel: cellulose chromatography. To Fraction II, solid ammonium sulfate was added to a concentration of 75 per cent saturation. After standing overnight the precipitate was collected by centrifugation at 10,000 *g* for 60 min and dissolved in 0·05 Tris-acetate, pH 8, containing 1·0 mM DTT (in the large preparations it has been possible to replace the DTT with 10 mM mercaptoethanol). The solution, after being dialyzed overnight against the same buffer, was placed on a gel-cellulose column (relative dimensions 1 × 10) equilibrated with the above buffer. Elution was carried out with a linear gradient of potassium phosphate (0 to 0·3 M in 0·05 M Tris-acetate

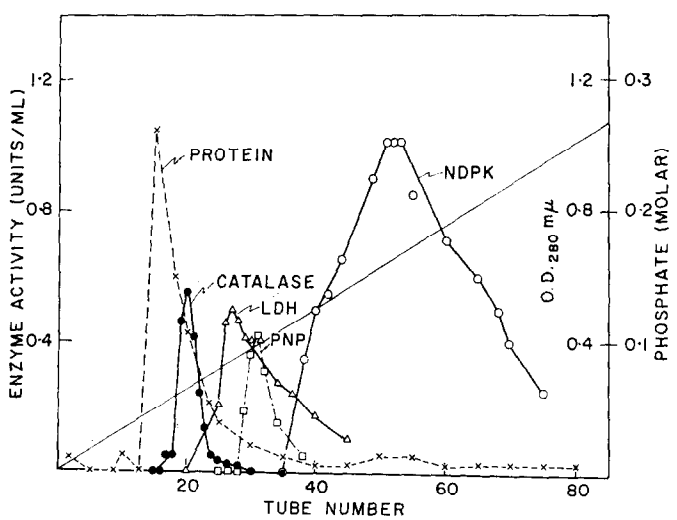

FIG. 4. Calcium phosphate gel cellulose column chromatography. Fraction II, after being dialyzed, was placed on a gel cellulose column (2 × 20 cm) equilibrated with 0·05 M Tris-acetate, pH 8, containing 1 mM DTT. Enzymes were eluted with a linear potassium phosphate gradient (0 to 0·3 M in 0·05 M Tris-acetate buffer, pH 8, containing 1 mM DTT, 1 l. total volume). Ten-ml fractions were collected.

buffer, pH 8, containing 1·0 mM DTT). This step resulted in the separation of several enzymes as shown in Fig. 4. Relatively good purification of all these enzymes was obtained with the exception of catalase which elutes with the bulk of the protein. By varying the gradient concentrations, better purification may be obtained for any one of these enzymes. In fact, we have been able to separate NDPK and PNP on a large scale using this method.

DEAE-cellulose column chromatography. Fraction I (material not adsorbed on calcium phosphate gel) was applied to a DEAE cellulose phosphate column (relative dimensions 1 × 10) equilibrated with 0·005 M potassium phosphate buffer, pH 7·5. Adenylate kinase, phosphoglucose isomerase and the hemoglobin were not retained on DEAE-cellulose and were recovered in the wash. The remaining enzymes were eluted with a linear gradient of potassium phosphate (0·005 to 0·5 M) as shown in Fig. 5. As is seen in the figure, catalase, guanylate kinase and pyruvate kinase are eluted very close to each other. Since gradient elution does not result in good separation, the much quicker method of batch-wise elution with potassium phosphate buffer may be used instead. This step demonstrated for the first time that adenylate kinase and guanylate kinase are different enzymes in human erythrocytes.

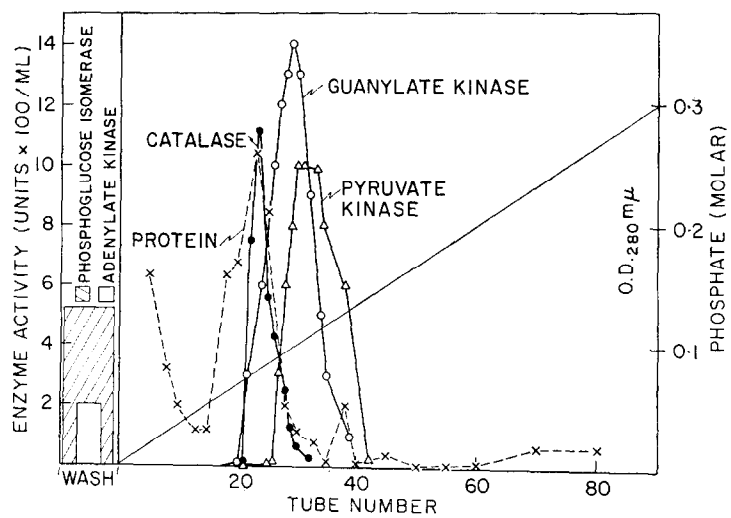

FIG. 5. DEAE-cellulose column chromatography. Fraction I was placed on a 2 × 20 cm DEAE-cellulose column (phosphate form) equilibrated with 0·005 M potassium phosphate, pH 7·5. The column was washed with the same buffer until most of the red protein was removed (about 60 ml). The remaining enzymes were eluted with a linear gradient of potassium phosphate, pH 7·5, (0·005 M to 0·5 M, 500 ml total volume). Five-ml fractions were collected.

Results of large-scale purification. The New England Enzyme Center at Tufts University Medical School, Boston, Mass., carried out a large-scale purification of PNP, NDPK and guanylate kinase by the above procedures. In this preparation, 14 l. of washed human erythrocytes was used. The hemolyzate was freed of stroma by centrifugation and carried through the calcium phosphate gel adsorption, calcium phosphate-cellulose and DEAE-cellulose column chromatography steps described above.

The yields of separated enzymic activities obtained were: PNP, 33,000 units, sp. act. 1·2; NDPK, 177,000 units, sp. act. 7–10; guanylate kinase, 2000 units, sp. act. 0·14.

The separated fractions were brought to 70 per cent saturation with ammonium sulfate and stored at 4° until subjected to further purification. It should be noted that since this laboratory has crystallized human erythrocytic PNP at a specific activity of 96 units per milligram of protein,[8] the amount of PNP partially purified in this preparation represents about 340 mg of pure enzyme. The highest activity of human erythrocytic NDPK obtained in this laboratory is about 1000 units per mg of protein.* This represents about 177 mg of NDPK of highest purity. The success of the large scale-up of this procedure may make available amounts of these enzymes sufficient that chemical and other studies of the protein structure will become practical.

B. *Studies with guanylate kinase*

Further purification of guanylate kinase. Because of the importance of guanylate kinase in nucleotide metabolism it was of interest to purify further and characterize this enzyme. As described above, we received from the Enzyme Center approximately 2000 units of guanylate kinase (sp. act. 0·14) purified about 140-fold from hemolyzates of human erythrocytes. Approximately one-half of this material was employed for further purification and study. It was dialyzed overnight against several changes of 0·005 M potassium phosphate buffer, pH 7·5. The dialyzed solution was then added to a DEAE-cellulose phosphate column (2 × 20 cm). The enzyme was eluted with a linear gradient varying from 0·005 M to 0·5 M potassium phosphate, pH 7·5, in a total volume of 2 l. Ten-ml fractions were collected and those containing the enzyme were pooled and the enzyme was precipitated by the addition of solid ammonium sulfate to achieve 65 per cent saturation. The recovery of enzyme in these final two steps was approximately 30 per cent, with a final specific activity of 2·2 units/mg and an overall purification from the hemolyzate of about 2000-fold. The further studies described below employed this enzyme preparation.

This preparation of enzyme was stable to storage in saturated ammonium sulfate at −20°. Under these conditions, only 35 per cent of the enzyme activity was lost over a period of 18 months. When heat stability studies were performed in 0·1 Tris-acetate buffer, pH 7·5, 90 per cent of the activity was lost in 10 min at 50°, and 27 per cent of the activity at 40°, which suggests that the enzyme is unusually heat labile. When an aliquot of purified enzyme was incubated with 2×10^{-5} M *p*-chloromercuribenzoate for 30 min, 70 per cent of the activity was lost. However, when dithiothreitol was added in a concentration of 1×10^{-3} M, the activity was completely restored. This finding indicates the presence of an essential sulfhydryl group in erythrocytic guanylate kinase.

Substrate specificity and kinetic parameters. Table 3 compares the V_{max} and the K_m values of erythrocytic guanylate kinase with several substrates. These studies indicate that the nucleoside monophosphate binding site in guanylate kinase is highly specific for the guanine moiety. GMP, dGMP and 8-azaGMP were found to be good substrates. The erythrocytic enzyme catalyzes a very slow reaction with IMP; the V_{max} is 0·2 per cent of that with GMP. AMP also showed a small amount of activity (less than 0·2 per cent) with guanylate kinase. In the presence of a 50-fold increased amount of guanylate kinase, UMP, CMP and 6-thioIMP were neither substrates nor inhibitors. 6-ThioGMP was not a substrate at the concentrations of enzyme studied but was active as an inhibitor with a K_i of $7{\cdot}5 \times 10^{-5}$ M (Fig. 6). This value is of the same order

* R. P. AGARWAL and R. E. PARKS, JR., unpublished observations.

TABLE 3. KINETIC PARAMETERS OF GUANYLATE KINASE*

Nucleoside monophosphate	Apparent K_m	Apparent V_{max}
GMP	$7{\cdot}7 \times 10^{-5}$	0·100
dGMP	$7{\cdot}4 \times 10^{-5}$	0·043
8-azaGMP	$9{\cdot}09 \times 10^{-5}$	0·016
IMP†	$1{\cdot}3 \times 10^{-3}$	0·0002
AMP		0·0015
UMP‡		No detectable
CMP		activity
6-thioIMP		

* The reaction mixture contained in 1 ml: 0·004 μmolar units of guanylate kinase; 100 μmoles of Tris acetate, pH 7·5; 100 μmoles KCl; 24 μmoles $MgCl_2$; 3 μmoles PEP; 8 μmoles ATP; 1·25 μmolar units of pyruvate kinase; 2·25 μmolar units of lactate dehydrogenase; 0·3 μmoles of NADH; and the substrate at varying concentrations (GMP, dGMP and 8-azaGMP) was added and the oxidation of NADH was followed with time by change in absorbency at 340 mμ. The K_m and V_{max} were determined from double reciprocal plots.

† With IMP as substrate the reaction mixture, in addition to the above components, contained 2·5 μmolar units of adenylate kinase to detect any contamination of the IMP with AMP. The reaction was started with the addition of 0·2 μmolar units of guanylate kinase. It should be noted that IDP is a good substrate for pyruvate kinase, and therefore in the assay 2 moles of NADH are oxidized per mole of IMP that reacts.

‡ The concentrations of UMP, CMP, and 6-thioIMP were 1·0 mM, 1·0 mM, and 0·1 mM respectively. All other conditions were the same as with GMP except that 0·2 μmolar units of guanylate kinase were used.

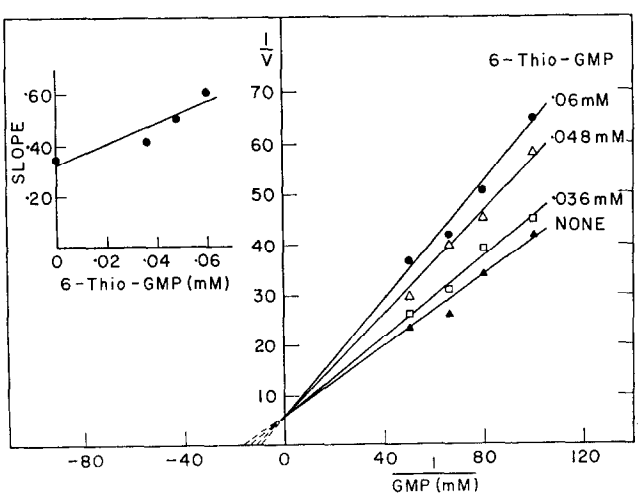

FIG. 6. Competitive inhibition of erythrocytic guanylate kinase by 6-thioGMP with GMP as the variable substrate. The reciprocal of the initial velocities is plotted against the reciprocal of the concentrations of GMP at different concentrations of 6-thioGMP. Reaction conditions were identical with those described in the Materials and Methods section, and the concentration of ATP was 4 mM. The inset is a plot of the slopes against the concentration of 6-thioGMP. From this the K_i value was calculated to be $7{\cdot}5 \times 10^{-5}$ M.

of magnitude as the K_i values found with guanylate kinase from hog brain and Sarcoma 180 ascites cells.[5] Although the Lineweaver–Burk plot indicates that the inhibition by 6-thioGMP is competitive with GMP, the replot gives a suggestion of nonlinearity. The cause of this apparent nonlinearity is currently under study and will be discussed in a subsequent publication. When 0·03 unit of erythrocytic guanylate

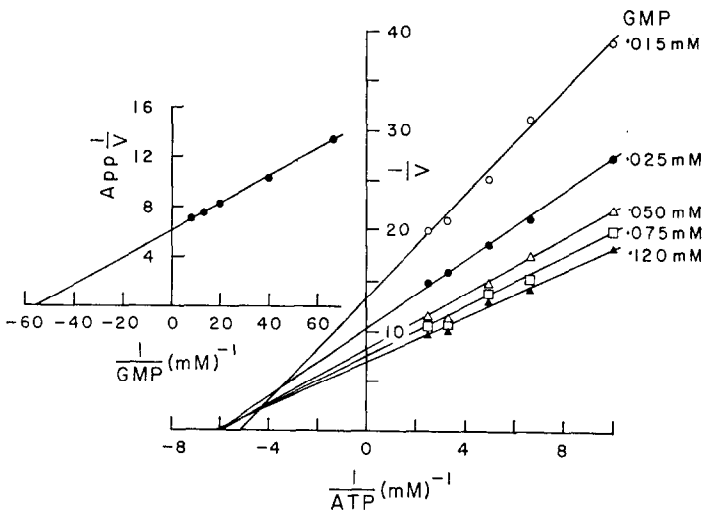

FIG. 7. Initial velocity analysis of guanylate kinase. Reciprocal plot of the initial velocities versus reciprocal of concentrations of ATP at different concentrations of GMP. Reaction conditions and components were identical with those described in the Materials and Methods section. The inset is a replot of the reciprocal of apparent V_{max} versus the reciprocal of GMP concentration. From the replot, the K_m value for GMP was found to be $1{\cdot}8 \times 10^{-5}$ M.

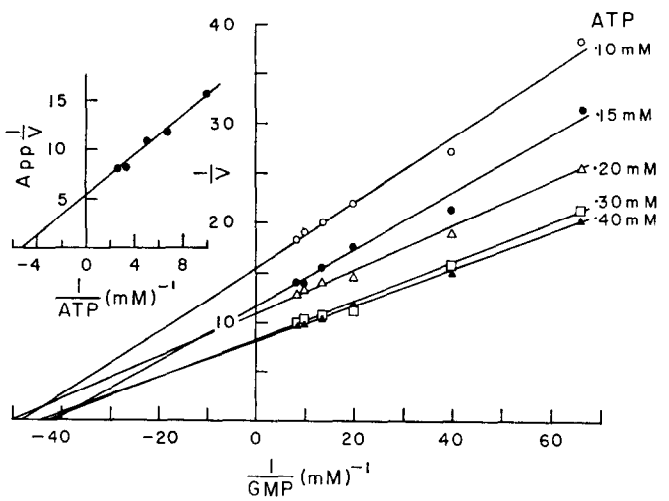

FIG. 8. Initial velocity analysis of guanylate kinase. Reciprocal plot of the initial velocity versus reciprocal of concentrations of GMP at different concentrations of ATP. Reaction conditions and components were described in the Materials and Methods section. The inset is a replot of the reciprocal of apparent V_{max} versus the reciprocal of ATP concentration. From the replot, the K_m value for ATP was found to be $1{\cdot}9 \times 10^{-4}$ M.

kinase (sp. act. 2·2) was incubated with 0·22 mM 6-thioGMP at room temperature and assayed with excess GMP every 10 min for a 90-min period, no inhibition was seen. This is in accord with findings reported with brain guanylate kinase[5] and indicates that the erythrocytic enzyme is not subject to progressive or irreversible inhibition by 6-thioGMP.

Initial velocity studies. Initial velocity studies using either GMP or ATP as the changing fixed substrate gave patterns of crossing lines as shown in Figs. 7 and 8. The results indicate that no product is released from the enzyme surface between the addition of ATP and GMP.[23] In agreement with the results obtained with hog brain guanylate kinase,[10] these findings rule out a "ping–pong" reaction mechanism but are consistent with either a "random" or "ordered" mechanism. It will be necessary to perform product inhibition studies to distinguish between these two possibilities.[23] Replots of the vertical intercepts of Figs. 7 and 8 (insets) give the Michaelis constant of one substrate at saturating concentrations of the second substrate. The Michaelis constants of GMP and ATP, as determined from the replots, are $1{\cdot}8 \times 10^{-5}$ M and $1{\cdot}9 \times 10^{-4}$ M respectively. The K_m's with the hog brain enzyme[10] are $0{\cdot}6 \times 10^{-5}$ M and $1{\cdot}2 \times 10^{-4}$ M.

Identification of isozymes. Preliminary studies by one of us (K.C.A.) employing agarose gel electrophoresis and an overlay technique for identification of the enzymic activity have given clear evidence for the occurrence of at least three isozymes of guanylate kinase in human blood. Attempts are now being made to separate these isozymes so that their characteristics may be studied in detail.

DISCUSSION

To our knowledge guanylate kinase has not been identified previously in non-nucleated mammalian erythrocytes. The guanine nucleotides, guanosine 5′-mono, di- and triphosphates have been identified in human erythrocytes, although as in most tissues their concentrations are significantly below the levels of the adenine-containing nucleotides.[24] It is significant that with the identification of guanylate kinase in human erythrocytes the complete enzymatic pathway for the conversion of guanine to GTP is demonstrated:

1. $\text{Guanine} + \text{PRPP} \xrightarrow[\text{pyrophosphorylase}]{\text{Inosinate-guanylate}} \text{GMP} + \text{PPi}$[25]
2. $\text{GMP} + \text{ATP} \underset{}{\overset{\text{Guanylate kinase}}{\rightleftharpoons}} \text{GDP} + \text{ADP}$
3. $\text{GDP} + \text{ATP} \overset{\text{NDPK}}{\rightleftharpoons} \text{GTP} + \text{ADP}$[3]

It is intriguing that to date no specific function has been assigned to GTP in the metabolism of the mature human erythrocyte, although in other tissues a number of key metabolic reactions require GTP rather than ATP. A question to be answered by further studies is whether this enzymatic pathway serves a useful purpose in the mature erythrocyte or whether it represents a vestige that served one or more vital functions in earlier stages of erythrocytic development but that is functionless in the mature cell.

In an earlier joint communication from this laboratory and that of Sartorelli[26] an hypothesis was offered to explain the action of 6-thioguanine based in part upon the very low substrate activity and competitive inhibition by 6-thioGMP of guanylate

kinase from Sarcoma 180 cells. Thus it was proposed that 6-thioGMP, being readily formed but poorly metabolized, accumulates in tumor tissues to levels in the range of 0·1 mM or greater. This makes possible feedback inhibitions of inosinic dehydrogenase and phosphoribosyl amidotransferase. It is significant to note that guanylate kinase from human erythrocytes is also competitively inhibited by 6-thioGMP with an inhibition constant in the same order of magnitude (about 5–7·5 $\times$ 10^{-5} M) as those found with guanylate kinases from hog brain and Sarcoma 180 cells.[5] Since this enzyme is present in mature erythrocytes, it is reasonable to assume that the enzyme also exists in earlier stages of erythrocytic development, i.e. erythroblasts, normoblasts, etc. Therefore, if the hypothesis proposed to explain the action of 6-thioguanine in Sarcoma 180 cells is valid, it seems likely that a similar explanation may also apply to the bone marrow toxicity seen in human patients as well as in animals treated with 6-thioguanine and related compounds.[27]

The human erythrocytic enzyme catalyzes a slow reaction between IMP and ATP (V_{max} is about 0·2 per cent of that with GMP when tested under the same conditions). The high K_m value (1·3 $\times$ 10^{-3} M) makes IMP an unlikely physiological substrate for this enzyme, since the normal concentrations of IMP in most tissues examined are far below this level. Concentrations of ITP of about 1 $\times$ 10^{-5} M have been reported in human erythrocytes[28] and we assume that the IMP levels must be at least ten times below this value. However, there is always the possibility that this reaction may be allosterically regulated so that the reactivity with IMP might be much greater in the cell under certain physiological conditions. Also it should be noted that genetic studies of certain families have been reported[29] in which high levels of ITP were identified in erythrocytes. This was ascribed to a deficiency in erythrocytic inosine triphosphatase. It would be of interest to examine the substrate activity of IMP with the erythrocytic guanylate kinase from these individuals. One might expect that the conversion of IMP to IDP would occur more readily than in the normal subject and that the kinetic parameters of an erythrocytic guanylate kinase isozyme would be such that IMP is a superior substrate.

A number of methods have been devised for the separation of enzymic proteins from the large amount of hemoglobin in hemolyzates. For example, Caffrey *et al.*[30] and Adams *et al.*[31] used ammonium sulfate to precipitate hemoglobin in the isolation of adenosine triphosphatase and tripeptidase. Ethanol–chloroform mixtures have been used to denature the hemoglobin in purification of carbonic anyhdrase from human and horse erythrocytes[32] as well as catalase from blood.[33] Rickli *et al.*[34] separated human erythrocytic carbonic anhydrase from hemoglobin on a Sephadex G-75 column, and Armstrong *et al.*[35] used DEAE-Sephadex columns at pH 8·7 to remove hemoglobin from hemolyzates in the purification of carbonic anhydrase. The above methods, although useful for the isolation of specific enzymes, have various disadvantages which make them unlikely candidates for inclusion in a general procedure. Several of these methods employ conditions that are likely to damage more sensitive proteins. This laboratory carried out preliminary experiments testing most of the procedures mentioned above and found that for our purposes the two procedures described here, calcium phosphate gel and DEAE-cellulose adsorptions, are most generally useful. The method, in addition to being of demonstrated use in the isolation of a number of enzymes, employs conditions that are relatively mild and avoids such factors as extremes of pH or temperature, the use of heavy metals or organic solvents,

etc., which may be damaging to sensitive proteins. It has been our experience that the use of calcium phosphate gel has the added advantage that it is relatively inexpensive and therefore may be discarded after a single use, whereas the cellulose ion exchangers for the sake of economy must be recovered and regenerated for repeated use.

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