Biochemical Pharmacology, Vol. 24, pp. 791–795. Pergamon Press, 1975. Printed in Great Britain.

INHIBITION OF RAT HEPATIC GUANYLATE KINASE BY 6-THIOGUANOSINE-5′-PHOSPHATE AND 6-SELENOGUANOSINE-5′-PHOSPHATE*

KAILASH C. AGARWAL and ROBERT E. PARKS, JR

Division of Biological and Medical Sciences, Brown University, Providence, R.I. 02912, U.S.A.

(*Received* 30 *July* 1974; *accepted* 20 *September* 1974)

Abstract—Guanylate kinase has been partially purified from rat liver and subjected to isoelectric focusing. Three peaks of enzymic activity at isoelectric points of 4·7, 4·9 and 5·1 were obtained. The greatest amount of activity occurred at pH 4·9 with an over-all purification of about 250-fold. GMP, dGMP and 8-azaGMP act as substrates and no essential differences were detected in the K_m and V_{max} values of the three isoelectric focusing peaks. Initial velocity studies with the principal activity peak (pI 4·9), with either GMP or ATP as the changing–fixed substrate, confirmed earlier results with guanylate kinase preparations from hog brain and human erythrocytes and were consistent with either a "random" or "ordered" reaction mechanism. 6-Thioguanosine-5′-phosphate (6-thioGMP) and 6-selenoguanosine-5′-phosphate (6-SeGMP) are good competitive inhibitors of the main activity peak (pI 4·9) with inhibition constants (K_i) of $6{\cdot}8 \times 10^{-5}$ M and $9{\cdot}5 \times 10^{-5}$ M for 6-thioGMP and 6-SeGMP respectively. No substrate activity was detected for 6-thioGMP or 6-SeGMP with any of the three isoelectric focusing enzyme peaks in assays capable of detecting reaction velocities greater than 1 per cent of that seen with GMP as the substrate.

Earlier reports from this laboratory have shown that guanylate kinases (ATP:GMP phosphotransferase, EC 2.7.4.8) from hog brain, human erythrocytes and Sarcoma 180 cells are competitively inhibited by the analog nucleotides, 6-thioguanosine-5′-phosphate (6-thioGMP) [1–4] and 6-selenoguanosine-5′-phosphate (6-SeGMP) [5]. When these analog nucleotides were examined at high concentrations of guanylate kinases, they proved to be alternative substrates with V_{max} values of 0·1 per cent or less than those observed with GMP. A recent finding of interest is that variants of guanylate kinase have been identified in human erythrocytes (four) and Sarcoma 180 cells (two) [4]. However, no marked differences were seen in the enzymatic behaviour and physical properties of these guanylate kinase variants.

A guanylate kinase from rat liver was purified about 250-fold and some of its properties were described by Buccino and Roth [6]. In many of its characteristics, this preparation resembles the guanylate kinases isolated from other tissues [1–3]. The molecular weight, estimated by gel filtration, is about 20,500, and the enzyme is highly specific for GMP or dGMP and ATP or dATP. However, in contrast to other guanylate kinase preparations, the liver enzyme was reported to be weakly inhibited by 6-thioGMP, which also serves as a substrate with a V_{max} about 12 per cent of that measured with GMP. In view of this surprising apparent difference between the rat hepatic enzyme and other guanylate kinase preparations, it was decided to examine the electrophoretic composition and enzymatic behavior of rat hepatic guanylate kinase in greater detail.

* This investigation was supported by Grant IC-62 from the American Cancer Society and Grant CA 07340 from the National Cancer Institute, National Institutes of Health.

MATERIALS AND METHODS

The sources of the various nucleotides, NADH, phosphoenol pyruvate (PEP), pyruvate kinase and lactate dehydrogenase were described elsewhere [4]. Bovine plasma albumin was purchased from Armour Pharmaceutical Co. Ampholine solutions (40%) were obtained from LKB products. All other chemicals used were of the highest purity commercially available.

6-ThioGMP was prepared enzymatically according to Miech *et al.* [2] except that the ratio of 6-thioguanine to 5-phospho-α-D-ribosylpyrophosphate (PRPP) in the reaction mixture was 1:1. 6-SeGMP was prepared chemically from 6-selenoguanosine [5]. The lyophilized samples of 6-SeGMP and 6-thioGMP were stored under nitrogen in a desiccator containing Silica gel at −20° until required for study.

Perfused frozen rat livers were obtained from Pel-Freez Biologicals.

Enzyme assay

The methods for enzymatic assay, protein determination and the definition of specific activity are as described earlier [4].

One μM unit of guanylate kinase is defined as the amount of the enzyme that will catalyze the phosphorylation of 1 μmole GMP/min at 30°. The phosphorylation of 1 μmole GMP in the presence of ATP ultimately results in the oxidation of 2 μmoles NADH, because both ADP and GDP serve as substrate for pyruvate kinase [3].

Partial purification of guanylate kinase from rat livers

Twenty perfused, frozen rat livers (about 150 g), obtained from Pel-Freez, were used for purification of the enzyme. The livers were added to 3 vol. cold 0·1 M Tris–acetate buffer, pH 7·5, and homogenized in a

Waring blender for 1 min. The homogenate was centrifuged at 12,000 *g* for 1 hr and the supernatant fraction (Fraction I) was collected, which had about 60 units of guanylate kinase activity with a specific activity of 0·008 unit/mg of protein.

Ammonium sulfate fractionation

Solid ammonium sulfate was added slowly to Fraction I until 33 per cent saturation was reached. After about 12 hr, the precipitated proteins were removed by centrifugation at 15,000 *g* for 20 min, and discarded. The supernatant fluid was brought to 65 per cent saturation by adding solid ammonium sulfate and was stirred overnight. The precipitated proteins were collected by centrifugation at 15,000 *g* for 40 min and dissolved in small volume of 0·05 M potassium phosphate buffer, pH 7·5. The solution (Fraction II) was dialyzed against 6 liters of the 5 mM phosphate buffer, pH 7·5, and the traces of precipitated proteins were removed by centrifugation.

Calcium phosphate gel adsorption

Calcium phosphate gel [7] was added to Fraction II in a protein to gel ratio of 20: 1. After thorough mixing for 30 min, the supernatant fluid was collected by centrifugation at 5000 *g* for 10 min. The pellet was washed two times with the 5·0 mM phosphate buffer (pH 7·5). The washings were added to the supernatant fluid, and the proteins were precipitated by adding solid ammonium sulfate to 65 per cent saturation. The precipitated proteins were sedimented by centrifugation at 15,000 *g* for 40 min, and dissolved in a small volume of 0·1 M Tris–acetate buffer, pH 7·5. In the resulting solution (Fraction III), the total activity of guanylate kinase recovered was about 50 units with a specific activity of 0·15 unit/mg of protein. Fraction III was used in isoelectric focusing procedures to separate the guanylate kinase variants.

Isoelectric focusing

Isoelectric focusing was carried out in an LKB electrofocusing column (110 ml) in a sucrose gradient with 2% Ampholine of pH 4–6 for 50–60 hr at 500 volts by methods similar to those described elsewhere [8].

RESULTS

Isoelectric resolution of guanylate kinase

In the isoelectric focusing experiment described in Fig. 1, an aliquot of partially purified guanylate kinase (Fraction III) was subjected to isoelectric focusing. The guanylate kinase activity resolved into one major peak at pH 4·9 with two smaller shoulders of activity at pH 4·7 and 5·1. In order to achieve better separation of these enzymatic activity peaks, the contents of the tubes 40 through 70 were pooled and again subjected to isoelectric focusing after the establishment of a new sucrose gradient. As shown in Fig. 1b, the refocusing procedure completely separated the three peaks of enzymatic activity at isoelectric points of about 4·7, 4·9 and 5·1 with the greatest amount of activity occurring at pH 4·9. The specific activity in the peak tube of the pI 4·9 enzymic variant is about 2 units/mg of protein, which represents approximately 250-fold purification from the supernatant fraction (Fraction I).

Two or three tubes containing the greatest enzymatic activity in each of the peaks of Fig. 1b were pooled and employed for substrate specificity and kinetic studies, after dialysis against 0·1 M Tris–acetate buffer, pH 7·5. These separated isoelectric fractions of guanylate kinase were stable for at least 4 months at −15° in the presence of 1% bovine plasma albumin.

Substrate specificity and kinetic parameters

Substrate specificity studies with guanylate kinases isolated from various tissues [1–4] indicate that the nucleoside monophosphate binding sites are highly specific for the guanine moiety. Similar observations have now been made with the three rat liver guanylate kinase isoelectric peaks. In the presence of about 0·005 unit of the enzymic activity, no substrate activity for IMP, CMP, dCMP, UMP, dUMP, dAMP, 6-thioIMP, 6-thioGMP and 6-SeGMP (at 0·2 mM concentrations) could be demonstrated with any of the three liver guanylate kinase peaks. AMP had weak substrate activity (less than 1 per cent with two of the peaks, pI values 4·7 and 5·1). This may have been due to trace contamination by ATP:AMP phosphotransferase.

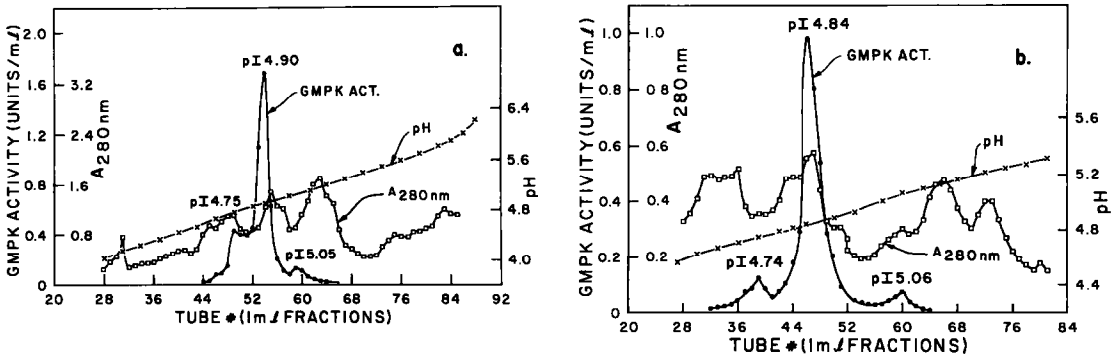

Fig. 1. Electrofocusing profile of partially purified guanylate kinase. (a) About 10 units of guanylate kinase (Fraction III in Materials and Methods) was electrofocused in an electrofocusing column (110 ml) in a sucrose gradient with 2% Ampholine (pH 4–6). After electrofocusing for 50 hr at 500 volts, 1-ml fractions were collected. Enzymatic activity, pH and absorbancy at 280 nm were determined in each fraction. (b) The contents of the tubes 40–70 (pH 4·4 to 5·4) were pooled and again subjected to electrofocusing for 60 hr at 500 volts after the establishment of a new sucrose gradient. Enzymatic activity, pH and absorbancy at 280 nm were again measured in each 1-ml fraction collected.

Table 1. Kinetic parameters of guanylate kinase variants*

Enzyme variants	pI	GMP $K_m \pm$ S. E. ($\times 10^{-5}$ M)	GMP $V_{max} \pm$ S.E. ($-A_{340}$ min^{-1})	dGMP $K_m \pm$ S. E. ($\times 10^{-5}$ M)	dGMP $V_{max} \pm$ S. E. ($-A_{340}$ min^{-1})	8-azaGMP $K_m \pm$ S. E. ($\times 10^{-5}$ M)	8-azaGMP $V_{max} \pm$ S. E. ($-A_{340}$ min^{-1})
I	4·7	1·65 ± 0·21	0·055 ± 0·003	3·32 ± 0·13	0·031 ± 0·001	5·53 ± 0·83	0·011 ± 0·004
II	4·9	0·98 ± 0·29	0·058 ± 0·004	2·82 ± 0·34	0·035 ± 0·002	7·04 ± 0·80	0·014 ± 0·005
III	5·1	1·13 ± 0·18	0·047 ± 0·002	2·17 ± 0·43	0·027 ± 0·002	4·69 ± 0·50	0·011 ± 0·003

* In each kinetic measurement, 1 ml of the reaction mixture contained: 0·0032 unit of the enzyme; 100 μmoles Tris-acetate, pH 7·5; 100 μmoles KCl; 10 μmoles $MgCl_2$; 1·5 μmoles phosphoenol pyruvate; 1·25 μmolar units pyruvate kinase; 2·25 μmolar units lactate dehydrogenase; 0·15 μmole NADH; 4 μmoles ATP; and the substrate of varying concentrations (GMP, dGMP and 8-azaGMP) was added and the oxidation of NADH was followed with time by change in absorbancy at 340 nm. The K_m and V_{max} values with standard errors were obtained by using Cleland's computer program [9]. The enzymic fractions used for the kinetic experiments were aliquots of pooled and dialyzed samples of the peaks in Fig. 1b.

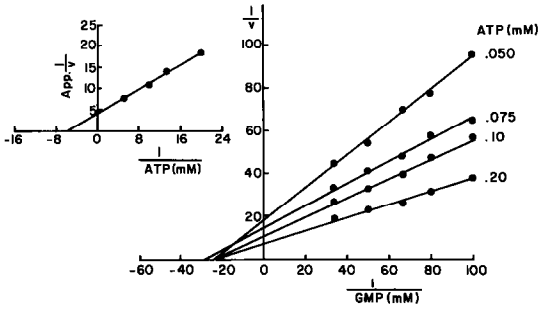

Fig. 2. Initial velocity analysis of the guanylate kinase (pI 4·9) variant with GMP as the variable substrate and ATP as the changing–fixed substrate. One ml of the reaction mixture contained 0·0084 unit of the enzyme; the other constituents were similar to those described in Table 1. The substrates ATP and GMP were added at the indicated concentrations. The inset is a replot of the reciprocal of apparent V_{max} vs the reciprocal of ATP. From the replot, the K_m value for ATP was found to be $1·78 \times 10^{-4}$ M.

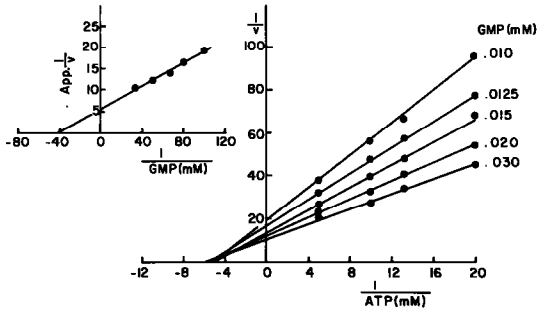

Fig. 3. Initial velocity analysis of the guanylate kinase (pI 4·9) variant with ATP as the variable substrate and GMP as the changing–fixed substrate. One ml of the reaction mixture contained 0·0084 unit of the enzyme; all the other constituents were similar to those described in Table 1. The substrates ATP and GMP were added at the indicated concentrations. The inset is a replot of the reciprocal of apparent V_{max} values vs the reciprocal of GMP concentrations. From the replot, the K_m value for GMP was found to be $2·50 \times 10^{-5}$ M.

Table 1 compares the apparent K_m and V_{max} values with each of the guanylate kinase peaks for GMP, dGMP and 8-azaGMP. It is seen that all three enzymic peaks are very similar in their kinetic behavior.

Studies with guanylate kinase pI 4·9 isoelectric peak

Initial velocity studies. Initial velocity studies gave patterns of crossing lines using either GMP or ATP as the changing–fixed substrate as shown in Figs. 2 and 3. This is consistent with previous studies with ATP:GMP phosphotransferase isolated from human erythrocytes and hog brain [1, 3], which indicate that no product is released from the enzyme surface between the addition of GMP and ATP. Therefore, this observation rules out a "ping-pong" reaction mechanism, and is consistent with either a "random" or "ordered" reaction sequence. Product inhibition studies will be necessary to distinguish these two possibilities (unfortunately product inhibition studies cannot be performed satisfactorily with the assay used in these studies).

Effect of temperature on K_m and V_{max}. In order to determine the effect of temperature on the K_m and V_{max} values, assays were performed at temperature varying from 24 to 41°. The K_m and V_{max} values were obtained from double reciprocal plots at each temperature. When the reciprocal of the absolute temperature (1/T) was plotted against log V_{max} (Arrhenius plot) or the negative logarithm of the Michaelis constant (pK_m), linear graphs were obtained as seen in Fig. 4. From the

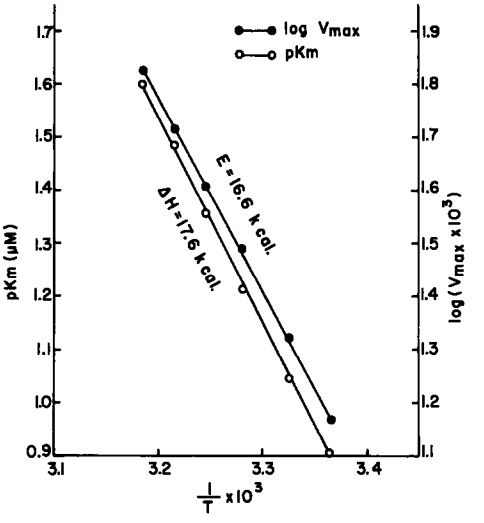

Fig. 4. Effect of temperature on K_m and V_{max} of guanylate kinase (pI 4·9) variant. The reaction constituents were similar to those described in Table 1. The reaction mixture containing GMP was incubated at the desired temperature for about 5 min. After temperature equilibration, the reaction was started with the addition of 0·0036 unit of the enzyme. K_m and V_{max} values were obtained from the Lineweaver–Burk plot at each temperature.

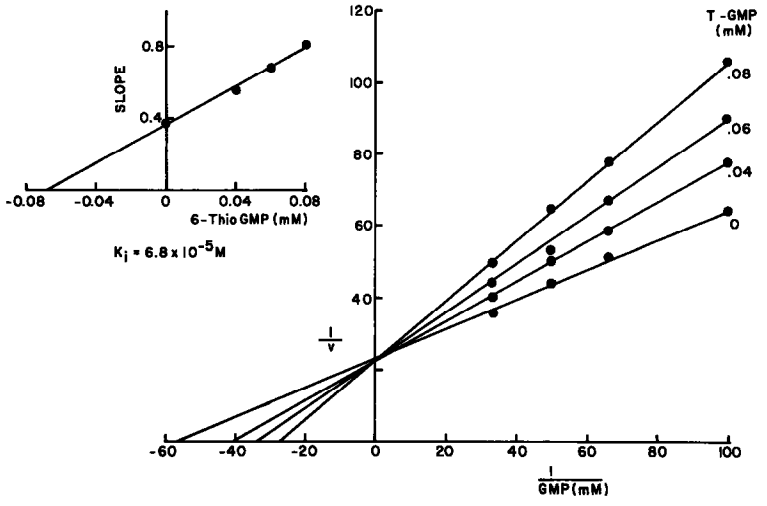

Fig. 5. Competitive inhibition of the guanylate kinase (pI 4·9) variant by 6-thioGMP with GMP as the variable substrate. Reaction conditions were identical with those described in Table 1. The substrate, GMP, and the inhibitor, 6-thioGMP, were added in the indicated concentrations. The reciprocal of the initial velocities is plotted against the reciprocal of the concentrations of GMP at three different concentrations of 6-thioGMP. The inset is a plot of the values of the slopes against the concentrations of 6-thioGMP. The abscissa intercept yields a K_i value of $6{\cdot}8 \times 10^{-5}$ M for 6-thioGMP.

Arrhenius plot (log V_{max} vs 1/T), an activation energy E of 16·6 kcal was obtained. From the plot of pK_m vs 1/T, one can obtain apparent ΔH for the reaction which is actually a very complex function as discussed by Dixon and Webb [10]. The "ΔH" obtained was 17·6 kcal.

Molecular weight estimation. The "molecular weight" of the pI 4·9 guanylate kinase was estimated by the gel filtration method of Andrews [11] with the use of a Sephadex G-100 column and the marker proteins, creatine kinase, horse radish peroxidase, chymotrypsinogen and cytochrome c by methods similar to those described previously [4]. A molecular weight of about 20,000 was estimated, which is in good agreement with the value reported elsewhere [6]. It should be noted that gel filtration offers an estimate of the Stoke's radius which is related to the molecular weight for symmetrical molecules. An estimate of the sedimentation constant will be needed to determine more precisely the molecular weight.

Inhibition by 6-thioGMP and 6-SeGMP. In view of the failure of Buccino and Roth [6] to observe strong inhibition by 6-thioGMP of the guanylate kinase preparation isolated from rat liver, we have performed inhibition studies of the pI 4·9 isoelectric peak with both 6-thioGMP and 6-SeGMP. As may be seen in Figs. 5 and 6, both analog nucleotides gave classical patterns for competitive inhibition, with inhibition constants (6-thioGMP, $K_i = 6{\cdot}8 \times 10^{-5}$ M; and 6-SeGMP, $K_i = 9{\cdot}5 \times 10^{-5}$ M) that compare favorably with the values determined with guanylate kinases from other tissues [2–5]. In addition, no measureable substrate activity was detected with either 6-thioGMP or 6-SeGMP at the concentrations of enzyme employed. Unfortunately, insufficient amounts of the pI 4·7 and 5·1 guanylate kinase peaks were obtained for detailed inhibition

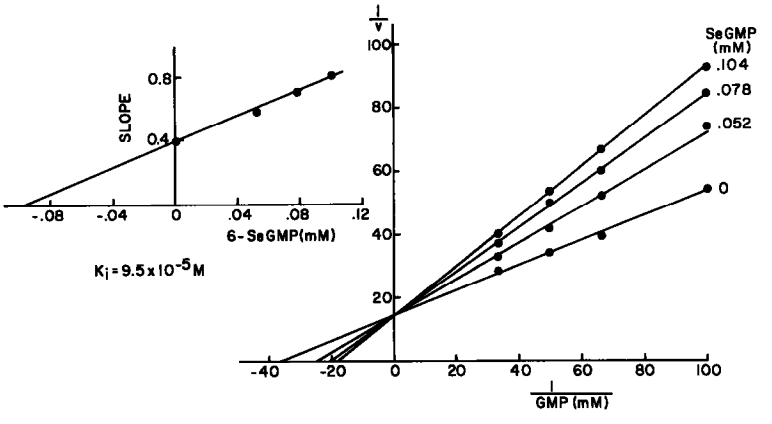

Fig. 6. Competitive inhibition of the guanylate kinase (pI 4·9) variant by 6-SeGMP with GMP as the variable substrate. Reaction conditions were identical with those described in Table 1. Substrate, GMP, and the inhibitor, 6-SeGMP, were added at the indicated concentrations. The reciprocal of the initial velocities is plotted against the reciprocal of the concentrations of GMP at three different concentrations of 6-SeGMP. The inset is a plot of the values of the slopes against the concentrations of 6-SeGMP. The abscissa intercept yields a K_i value of $9{\cdot}5 \times 10^{-5}$ M for 6-SeGMP.

studies. However, neither 6-thioGMP or 6-SeGMP displayed substrate activity with either enzymic peak. If these analog nucleotides serve as weak alternative substrate for the rat hepatic guanylate kinase, as they do with other guanylate kinases, the reaction velocities must be less than 1 per cent of those observed with GMP.

DISCUSSION

The results of these studies demonstrate that, as with guanylate kinases from human erythrocytes and Sarcoma 180 cells, the enzyme is heterogeneous in rat liver with three activity peaks with isoelectric points about pI 4·7, 4·9 and 5·1. The greatest amount of activity is found in the pI 4·9 variant. These activity peaks differ very little from one another in their substrate specificity and their kinetic parameters with the substrates GMP, dGMP and 8-azaGMP. Unfortunately, the quantities of the three guanylate kinase peaks separated by isoelectric focusing were too small to permit further characterization to determine whether they represent true isozymes or are due to some other phenomenon such as non-specific binding with other proteins or nucleotides, sulfhydryl group modification, etc.

In contrast to the results of Buccino and Roth [6], who observed only weak inhibition by 6-thioGMP, and report that 6-thioGMP serves as a substrate with about 12 per cent of the reaction velocity measured with GMP, we have found that 6-thioGMP and 6-SeGMP are both potent inhibitors, competitive with GMP, and no substrate activity was observed with either analog nucleotide. It must be noted that the relatively weak inhibition by various analog-nucleotides including 6-thioGMP reported by these workers undoubtedly resulted from inclusion in their assay reaction mixture of GMP-8-^{14}C at a concentration of 0·4 mM, which is about 40-fold greater than the K_m values presented above (Table 1). Unfortunately, insufficient amounts of the purified activity peaks were available to enable examination for low substrate activity at high concentrations of enzyme. No substrate activity was detected for either 6-thioGMP or 6-SeGMP with the pI 4·7 and 5·1 variants which were not available in sufficient amounts to permit detailed inhibition studies. On the basis of these findings, we have not observed significant differences between the guanylate kinase isoelectric variants isolated from rat liver and those reported earlier by this laboratory [1–4]. We do not have a clear-cut explanation for the discrepancy between our results and those reported by Buccino and Roth [6]. However, their sample of 6-thioGMP was prepared chemically whereas ours was synthesized enzymatically by a method described earlier [2]. Because of the notorious instability of 6-thioGMP, our preparations were isolated by DEAE chromatography in the presence of dithiothreitol and were lyophilized and stored under nitrogen at −20°. These preparations were dissolved immediately prior to kinetic analysis. A possibly significant observation was made in the course of these studies of a factor that can greatly affect the degree of inhibition of guanylate kinase by 6-thioGMP or 6-SeGMP. If, in the performance of inhibition studies, the 6-thioGMP or 6-SeGMP was preincubated with the pyruvate kinase-lactate dehydrogenase indicator system and the enzymatic reaction was started by the addition of the guanylate kinase preparation, the inhibitory effect was abolished. However, if the enzymatic reaction was started by the addition of the substrate, GMP, and the 6-thioGMP or 6-SeGMP was added a few seconds earlier, satisfactory inhibition patterns were observed as seen in Figs. 5 and 6. In examining this phenomenon further, it was learned that if 6-thioGMP or 6-SeGMP is incubated with NADH or NAD, loss of the inhibitory activity occurs, presumably by a nonenzymatic chemical reaction. The explanation of this observation is being sought. However, since the studies of Buccino and Roth [6] did not involve the use of coupled enzymatic assays and NADH, this cannot explain their failure to observe strong inhibition by 6-thioGMP. The reaction velocity of 12 per cent with 6-thioGMP reported by Buccino and Roth [6] suggests that their 6-thioGMP preparation was contaminated by significant quantities of GMP.

REFERENCES

1. R. P. Miech and R. E. Parks, Jr., *J. biol. Chem.* **240,** 351 (1965).
2. R. P. Miech, R. York and R. E. Parks, Jr., *Molec. Pharmac.* **5,** 30 (1969).
3. R. P. Agarwal, E. M. Scholar, K. C. Agarwal and R. E. Parks, Jr., *Biochem. Pharmac.* **20,** 1341 (1971).
4. K. C. Agarwal and R. E. Parks, Jr., *Molec. Pharmac.* **8,** 128 (1972).
5. A. F. Ross, K. C. Agarwal, S.-H. Chu and R. E. Parks, Jr., *Biochem. Pharmac.* **22,** 141 (1973).
6. R. J. Buccino, Jr. and J. S. Roth, *Archs. Biochem. Biophys.* **132,** 49 (1969).
7. K. K. Tsuboi and P. B. Hudson, *J. biol. Chem.* **224,** 879 (1957).
8. H. Haglund, *Sci. Tools* **14,** 17 (1967).
9. W. W. Cleland, *Adv. Enzymol.* **29,** 1 (1967).
10. M. Dixon and E. C. Webb, *Enzymes.* 2nd Edn., p. 156. Academic Press, New York (1964).
11. P. Andrews, *Biochem. J.* **96,** 595 (1965).