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DETECTION OF GUANYL CYCLASE IN MAMMALIAN TISSUES

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SUMMARY

Guanyl cyclase was assayed by determining the rate of conversion of [α-^{32}P]GTP to cyclic 3',5'-[^{32}P]GMP. A two-step chromatographic procedure was developed for isolating the product. The enzyme was found in the soluble fraction after homogenizing tissues in 0.3 M sucrose. The reaction showed a pH optimum near 7.6 and was dependent on Mn^{2+}. The relative enzymatic activity for tissues of the rat was lung $\gg$ brain = spleen $>$ kidney = liver = heart.

INTRODUCTION

ASHMAN *et al.*[1] obtained evidence that guanosine 3',5'-monophosphate (cyclic 3',5'-GMP) occurs in the urine of rats along with the better-known cyclic nucleotide, adenosine 3',5'-monophosphate (cyclic 3',5'-AMP). The authors suggested that cyclic 3',5'-GMP might be another important mediator of hormone action in analogy to the recognized function of 3',5'-AMP[2]. PRICE *et al.*[3] further characterized this compound after large scale isolation of the radioactive nucleotide from rat urine and suggested that cyclic 3',5'-GMP was synthesized from GTP by a reaction analogous to that catalyzed by adenyl cyclase.

HARDMAN *et al.*[4] developed a method for measuring the concentration of cyclic 3',5'-GMP in urine and tissues and confirmed the occurrence of the nucleotide in urine. They found that urinary excretion of cyclic 3',5'-GMP by rats was reduced to 50% of normal by hypophysectomy, whereas excretion of cyclic 3',5'-AMP was unchanged. Excretion of cyclic 3',5'-GMP was restored toward normal by treatment with a mixture of adenohypophyseal hormones *plus* hydrocortisone or with large doses of thyroxine *plus* hydrocortisone. These observations raised the possibility that cyclic 3',5'-GMP was another "second messenger"[5] for hormone action.

In the current study we developed an assay for guanyl cyclase by measuring conversion of radioactive GTP to radioactive cyclic 3',5'-GMP. Application of the

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assay showed that the enzyme is distinct from adenyl cyclase and appears to be widely distributed in mammalian tissues. A portion of this work has appeared in abstract form[6].

EXPERIMENTAL PROCEDURE

Chemicals

Cyclic 3′,5′-GMP was purchased from Sigma Chemical Comp. GTP was obtained from P–L Laboratories, the other nucleotides from Calbiochem. [α-^{32}P]GTP was from International Chemical and Nuclear Corp. or from Schwarz BioResearch. The $(NH_4)_2SO_4$ was special enzyme grade from Mann Research Laboratories. Caffeine was obtained from Matheson, Coleman and Bell. Crystalline bovine serum albumin was a product of Armour Pharmaceutical Company. Dowex AG 50W-X8, 100–200 mesh in the H^+ form, was obtained from Bio-Rad Laboratories. ChromAR Sheet 1000 and 500 are products of Mallinckrodt Chemical Works. Avicel microcrystalline cellulose thin-layer plates were from Analtech.

Purification of tritiated cyclic 3′,5′-GMP

Thin-layer chromatography on Avicel plates was used for isolation and identification of cyclic 3′,5′-GMP. The two solvent systems used by PATAKI[7] for two-dimensional separation of nucleotides proved to be most useful for analysis of the guanine nucleotides. The R_F for cyclic 3′,5′-GMP was greater than that for GMP, GDP and GTP in System 1, *n*-propanol–conc. ammonia–water (6:3:1, by vol.) and less than the R_F for the latter guanosine phosphates in System 2, isopropanol–satd. $(NH_4)_2SO_4$–water (2:79:19, by vol.). The commercial preparation of cyclic 3′,5′-GMP showed one principal component and four small contaminants on two-dimensional chromatography. Part of this preparation (50 mg) was sent to Schwarz BioResearch for labeling with ^3H. The product was purified in 30-mC quantities, after removal of the solvent by lyophilization. It was applied to a 12 cm × 44 cm column of Dowex-50 (H^+) and eluted with 0.05 M HCl. Approx. 6-ml fractions were collected; cyclic 3′,5′-[^{3}H]GMP in the effluent was identified by chromatography on Avicel plates using System 2. Three tubes contained most of the tritiated cyclic 3′,5′-GMP, and these fractions were lyophilized together. The residue was dissolved in a small amount of dilute NH_4OH and lyophilized again. Final purification was effected by preparatory thin-layer chromatography on an Avicel plate using System 1. The cyclic 3′,5′-GMP area was scraped from the plate and the nucleotide was eluted from the cellulose with 50% ethanol. Specific activity was 870 mC/mmole. Chromatography on ChromAR Sheet 1000 with 99% ethanol–conc. NH_4OH (5:2, v/v) could be used instead of the Avicel thin-layer step for purification.

The identity of the tritiated cyclic 3′,5′-GMP was confirmed by hydrolysis with cyclic 3′,5′-nucleotide phosphodiesterase. The enzyme had been prepared in this laboratory from beef heart according to the procedure of BUTCHER AND SUTHERLAND[8]. 1 ml of this preparation catalyzed the conversion of 29.5 μmoles of 1 mM cyclic 3′,5′-AMP to AMP in 30 min at 30°. The reaction mixture contained 0.5 mmole of cyclic 3′,5′-GMP (containing 165 000 counts/min of cyclic 3′,5′-[^{3}H]GMP); 2.2 mM $MgCl_2$; 50 mM Tris–HCl (pH 8.0) and 20 μl of phosphodiesterase in a final volume of 82 μl. After incubating for 1 h at 30°, 1-μl aliquots were applied to two Avicel plates, and

each was overlaid with 0.02 μmole of carrier 5′-GMP. The plates were developed separately, one in System 1 and the other in System 2. The areas representing 5′-GMP were scraped from the plates and assayed for radioactivity. 65% of the applied radioactivity was recovered in the 5′-GMP area from the plate developed with System 2. There appeared to be 85% conversion to 5′-GMP in the test with System 1; however, resolution between cyclic 3′,5′-GMP and 5′-GMP was not as complete in the latter system.

Assay of guanyl cyclase

The assay is based upon detection of cyclic 3′,5′-[^{32}P]GMP formed from [α-^{32}P]-GTP. The latter product is separated from the labeled substrate by sequential chromatography on Dowex-50 and silicic acid medium (ChromAR Sheet). The reaction mixture contained in a final volume of 75 μl: 50 mM Tris–HCl buffer (pH 7.6); 20 mM caffeine; 10 mM or 1 mM $MnCl_2$; 0.2 μmole of cyclic 3′,5′-GMP; 0.4 mM [α-^{32}P]GTP (20–30 counts/min per pmole); 0.02% bovine serum albumin; and 25 μl of enzyme or tissue extract. Incubations were usually carried out for 10 min at 37° and were terminated by the addition of 10 μl of 40 mM GTP containing 78 pmoles of tritiated cyclic 3′,5′-GMP (approx. 30 000 counts/min). The mixture was boiled for 3 min, cooled in an ice bath, and 0.5 ml of 0.05 M HCl were added. After vigorous mixing on a Vortex mixer, the fractions were centrifuged for 10 min at 2500 rev./min. The supernatant fluid from each tube was applied to a 0.6 cm × 10 cm column of Dowex-50 (H^+) equilibrated with 0.05 M HCl. After the supernatant fluid had entered the resin bed, 5.0 ml of 0.05 M HCl were added. The column effluent was discarded. The column was eluted with a further 5.0-ml portion of 0.05 M HCl, and the eluate was collected in a 16 mm × 100 mm test tube. About 70–80% of the tritiated cyclic 3′,5′-GMP was recovered in this fraction. The fractions were evaporated overnight to dryness at 40° in the collection tubes with a current of dry air.

To each tube were added 75 μl of 50% ethanol (v/v), and the sides of the tube were washed by vigorous stirring on a Vortex mixer. The solution from each tube was removed with a capillary tube and applied 1 inch from the end, at 1-inch intervals to an 8-inch-long sheet of ChromAR Sheet 1000*. The sheets are thick enough to accept the entire volume of ethanol solution in a single application. Ascending chromatography was carried out with the solvent system: 99% ethanol–conc. NH_4OH (5:2, v/v). Adequate radiochemical purification was achieved by continuing development for 2 h which exceeded the time required for the solvent front to reach the end of the sheet. After development the sheets were dried. The areas corresponding to cyclic 3′,5′-GMP were visualized with ultraviolet light, marked with a pencil and cut from the sheet. The sections containing cyclic 3′,5′-GMP were placed in vials, 3 ml of water and 15 ml of scintillator solution[9] were added and the mixture was shaken vigorously in the capped vials to disperse the ChromAR. Tritium and ^{32}P were assayed with a

* It was anticipated that this chromatographic procedure alone without the Dowex-50 fractionation would suffice for an assay of guanyl cyclase measuring the rate of conversion of [α-^{32}P]GTP to cyclic 3′,5′-[^{32}P]GMP. However, a radioactive impurity in the commercial [α-^{32}P]-GTP moves with cyclic 3′,5′-GMP on the ChromAR Sheet. Attempts to purify the substrate by ion-exchange chromatography did not completely remove the contaminant. For this reason a one-step procedure, with application of incubation mixtures directly to the ChromAR Sheet, cannot be used routinely. On the other hand, when high concentrations of enzyme are available the reaction rate becomes great enough to make the high blank acceptable and a single chromatographic procedure with the silica medium can be used.

liquid scintillation spectrometer. Losses during the chromatographic procedures were determined from the amount of cyclic 3′,5′-[^{3}H]GMP recovered. One unit of enzyme catalyzed the formation of 1 pmole of cyclic 3′,5′-GMP per min at 37°. Protein was determined by a microprocedure[10] with biuret reagent for tissue extracts and by the method of LOWRY *et al.*[11] on the enzyme fraction from bovine lung.

Extraction of rat tissues

Sprague–Dawley rats from the N.I.H. colony were killed by decapitation, and the organs were removed, weighed and chilled in cold (4°) 0.3 M sucrose. Tissues were cut with scissors and homogenized in a Potter–Elvehjem-type homogenizer with 2 vol. of 0.3 M sucrose. A Kontes–Duall ground-glass homogenizer was used for heart, skeletal and intestinal muscle; a smooth glass tube with Teflon pestle was used for other tissues. The homogenates were centrifuged at 27 000 $-g$ for 20 min in a refrigerated centrifuge. The supernatant solution was assayed for enzyme.

Preparation of enzyme from bovine lung

Bovine lung was obtained packed in ice from a slaughter house. It was cut into pieces and washed with 0.9% NaCl. The large blood vessels and bronchi were discarded. Chunks of tissue were packed in solid CO_2 until frozen, then stored at −20° until used. The frozen tissue was wrapped in a towel, crushed with a hammer and then mixed with solid CO_2 and powdered in a Waring blender. The blender jar had been pre-cooled with solid CO_2. Solid CO_2 was allowed to sublime from the powdered mixture in the freezer (−20°). The frozen powdered tissue (69 g) was added quickly to 350 ml of 50 mM Tris–HCl buffer (pH 7.4) containing 0.9% NaCl and stirred for 30 min at 4°. The mixture was centrifuged, and the supernatant fluid was used as the crude extract. $(NH_4)_2SO_4$ was added to the crude extract to 20% saturation; the precipitate was removed by centrifugation. $(NH_4)_2SO_4$ was added to 40% saturation in the supernatant fluid causing precipitation of most of the enzyme. This fraction, obtained by centrifugation, was dissolved in 200 ml of water, the pH was adjusted to 7.4 and a second $(NH_4)_2SO_4$ fractionation was carried out. The enzyme-rich fraction again precipitated between 20 and 40% saturation. This fraction was dissolved in 100 ml of distilled water, and portions were quick-frozen in solid CO_2–acetone to be stored at −20°.

RESULTS

Silicic acid chromatography

Cyclic 3′,5′-GMP was separated efficiently from other guanine nucleotides and degradation products on ChromAR Sheet (Table I). The 500-μ-thick sheet was used in this experiment in order to obtain more sharply defined R_F values; sheets of 1000-μ thickness allow somewhat greater diffusion of compounds. This chromatographic system also affords separation of the adenine nucleotides (Table I). The mobilities of caffeine and theophylline are included in the table, since these compounds may be incorporated in assays to inhibit cyclic nucleotide phosphodiesterase. Caffeine, rather than theophylline, was used in the assay because it is completely retained on the Dowex-50 column, whereas a part of the theophylline elutes with cyclic 3′,5′-GMP. Theophylline interfered with solubilizing the dried cyclic 3′,5′-GMP samples.

TABLE I

SILICIC ACID CHROMATOGRAPHY

Chromatographic separations achieved on ChromAR Sheet 500 with the solvent system, ethanol–conc. NH_4OH (5:2, v/v). Compounds visualized with ultraviolet light or by dipping with reagents of RUNECKLES AND KROTKOV[12].

Compound	R_F
GTP	0.01
GDP	0.04
GMP	0.07
Cyclic 3′,5′-GMP	0.84
Guanosine	0.79
Guanine	0.80
ATP	0.05
ADP	0.16
AMP	0.42
Cyclic 3′,5′-AMP	0.88
Adenosine	0.86
Adenine	0.89
Ribose 5-phosphate	0
Pyrophosphate	0
Orthophosphate	0
Caffeine	0.90
Theophylline	0.88

Enzyme from bovine lung; fractionation with $(NH_4)_2SO_4$

Assays showed that most of the enzyme from bovine lung was precipitated with $(NH_4)_2SO_4$ between 20 and 40% saturation (Table II). The remainder of the enzyme appeared in the fraction precipitated with 20% $(NH_4)_2SO_4$ and in the supernatant fraction after precipitating with 40% $(NH_4)_2SO_4$. The enzyme obtained after the second $(NH_4)_2SO_4$ fractionation showed specific activity 4-fold greater than that of the crude extract.

Identification of product

The chromatographic systems with Dowex-50 and silicic acid provided evidence that the product of reaction was indeed cyclic 3′,5′-GMP. We have not found other nucleotides that chromatograph in these systems identically with cyclic 3′,5′-GMP. We made use of the cyclic 3′,5′-nucleotide phosphodiesterase to obtain further proof

TABLE II

FRACTIONATION OF ENZYME FROM BOVINE LUNG

	Total protein (mg)	*Total enzyme (units*)*	*Specific activity (units/mg)*	*Recovery (%)*
Crude extracts	3750	208 000	55	100
0–20% $(NH_4)_2SO_4$ precipitate	130	2 720	21	
20–40% $(NH_4)_2SO_4$ precipitate	1190	195 000	164	94
Second 20–40% $(NH_4)_2SO_4$ precipitate	608	134 000	220	64

* Standard assays with 0.4 mM GTP, 10.0 mM $MnCl_2$.

that the radioactive product of [α-^{32}P]GTP was cyclic 3′,5′-[^{32}P]GMP. This enzyme is relatively specific for cyclic 3′,5′-nucleotides and catalyzes hydrolysis to the corresponding 5′-mononucleotide. This preparation had been used earlier to prove the structure of the tritiated cyclic 3′,5′-GMP.

A standard incubation was carried out using the bovine lung preparation to generate a relatively large amount of product. Two mixtures were incubated for 10 min, using 10 mM $MnCl_2$ and 1 mM GTP. As usual cyclic 3′,5′-[^{3}H]GMP was added at the end of the reaction period. The reaction mixtures were carried through chromatography on Dowex-50 (H^+) and ChromAR Sheet. The cyclic 3′,5′-GMP regions were cut from the ChromAR Sheet, eluted with water and the eluate was dried with a stream of air. To the product of one reaction mixture were added 50 μl of a solution containing 8.3 μl of the cyclic 3′,5′-nucleotide phosphodiesterase preparation, 0.008% bovine serum albumin, 33 mM Tris–HCl buffer (pH 8.0) and 1.5 mM $MgCl_2$. The reaction product was carefully dissolved in this solution, and the mixture incubated for 3 h at 37°. At the end of this time the entire reaction mixture was applied to ChromAR Sheet, and at the same time the control mixture, incubated in Tris–HCl–$MgCl_2$ without cyclic 3′,5′-nucleotide phosphodiesterase, was dissolved in water and applied at another point at the origin. After chromatography with ethanol–NH_4OH, the control showed the usual cyclic 3′,5′-GMP region (Spot 1) while nothing was visible in the corresponding area of the enzyme-treated sample (Spot 2). A new compound, representing the product of hydrolysis catalyzed by phosphodiesterase, appeared near the origin (Spot 3) in the region where 5′-GMP migrates. These three areas were cut from the chromatogram and the radioactivity of each was determined. Spot 1, from the control reaction, contained 4979 counts/min of ^{3}H and 721 counts/min of ^{32}P, giving a ^{3}H:^{32}P ratio of 6.9. Spot 2 showed 970 counts/min of ^{3}H and 72 counts/min of ^{32}P, with a ^{3}H:^{32}P ratio of 13.4. Spot 3 contained 4781 counts/min of ^{3}H and 879 counts/min of ^{32}P, with a ^{3}H:^{32}P ratio of 5.4. It was concluded that the product had been converted to GMP

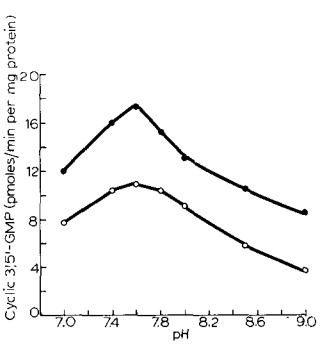

Fig. 1. Effect of pH on enzyme activity. The reaction mixture contained 0.2 mg of protein representing the enzyme fractionated from bovine lung, 50.0 mM Tris–HCl buffer, 20.0 mM caffeine, 0.2 mM GTP, 0.02% bovine serum albumin, 0.2 μmole cyclic 3′,5′-GMP and either 1.0 mM (○—○) or 10.0 mM (●—●) $MnCl_2$. 10-min incubations.

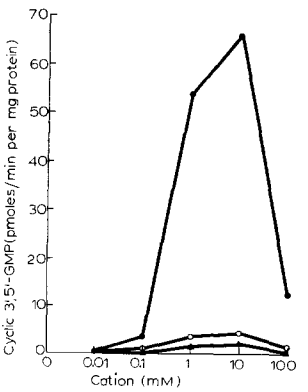

Fig. 2. Effect of cations on guanyl cyclase. Reaction conditions as in Fig. 1 except with 0.4 mM GTP (pH 7.6) and with $CaCl_2$ (▲—▲). $MgCl_2$ (○—○) or $MnCl_2$ (●—●) at the indicated concentrations.

through catalysis with cyclic 3′,5′-nucleotide phosphodiesterase without significant change in the original isotope ratio. It was known that the cyclic 3′,5′-nucleotide phosphodiesterase preparation contained a small amount of phosphomonoesterase activity, which would become evident during a 3-h reaction period. This would explain the ^{3}H in Spot 2 as probably representing guanosine, which migrates near cyclic 3′,5′-GMP and would be a product of GMP. The P_i removed from the GMP remains at the origin and would therefore reduce the ^{3}H:^{32}P ratio for the compound in Spot 3.

Kinetics and metal requirements

Tests for optimal conditions of assay with the enzyme from bovine lung are illustrated in Figs. 1–6. The pH optimum is near 7.6 (Fig. 1). The requirement for Mn^{2+} is illustrated by the results shown in Fig. 2. There is much less activity in the presence of Mg^{2+} and still less with Ca^{2+}. At the highest concentration of metal, 100 mM, there was a very low rate of apparent nonenzymatic formation of cyclic 3′,5′-GMP equivalent in radioactivity to less than twice the background of the method itself. This nonenzymatic reaction appeared to account for formation of 1, 0.3 and 2.6 pmoles of cyclic 3′,5′-GMP with Mn^{2+}, Mg^{2+} and Ca^{2+}, respectively, at 10 min with the highest concentration (100 mM) of metal used in the experiments represented in Fig. 2. It is likely that the greater enzyme activity obtained with the routine concentration of Mn^{2+}, 10 mM, rather than 1 mM, is due to precipitation of pyrophosphate, the other product of the reaction.

The effect of substrate concentration on the reaction at 1 or 10 mM Mn^{2+} is illustrated in Fig. 3. With 1 mM Mn^{2+}, the reaction rate reached a maximum at 0.4 mM GTP; at higher concentrations of substrate there was apparent inhibition. This inhibition was not apparent in assays at 10 mM Mn^{2+}. Reciprocal plots for these data are shown in Fig. 4; the curve for 1 mM Mn^{2+} was extrapolated from data with the

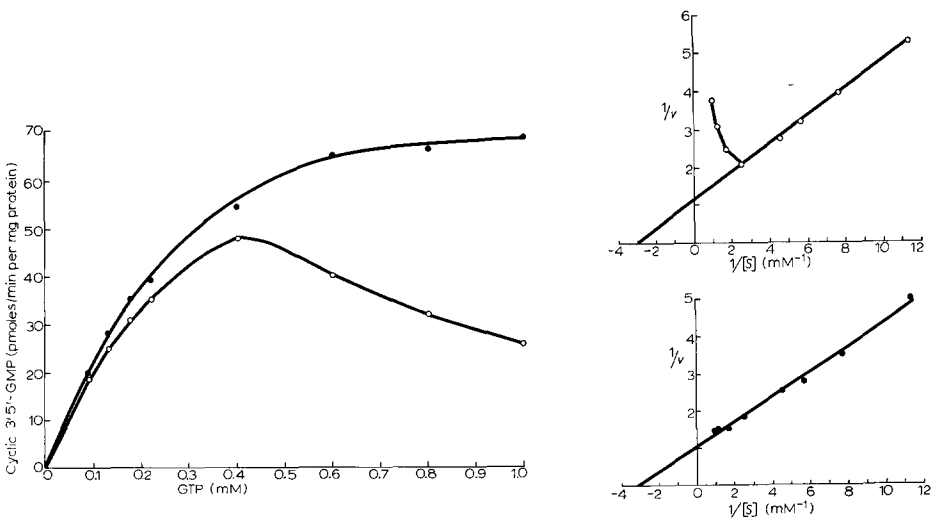

Fig. 3. Effect of substrate concentration on reaction rate. Reaction conditions as in Fig. 1, pH 7.6, with indicated concentrations of GTP at 1.0 mM (○—○) or 10.0 mM (●—●) $MnCl_2$.

Fig. 4. Plot of reciprocals of data shown in Fig. 3. ○—○, 1.0 mM $MnCl_2$; ●—●, 10.0 mM $MnCl_2$.

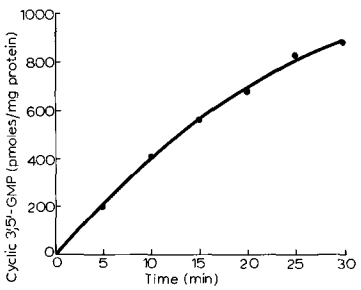

Fig. 5. Reaction rate as a function of time. Reaction conditions as in Fig. 1, pH 7.6, 1.0 mM $MnCl_2$ and 0.4 mM GTP.

Fig. 6. Reaction rate as a function of enzyme concentration. Reaction conditions as in Fig. 1, pH 7.6, 1.0 mM $MnCl_2$ and 0.4 mM GTP.

lower concentration of GTP where substrate inhibition was not apparent. The apparent K_m for GTP at 1 mM Mn^{2+} was calculated to be 0.32 mM, with a v_{max} of 87.0 pmoles/min. The apparent K_m at 10 mM Mn^{2+} was 0.30 mM with a v_{max} of 93.4 pmoles/min.

The reaction rate was linear for 10 min (Fig. 5) and 10 min incubations were used for assays of tissue extracts. Fig. 6 shows that with the beef-lung fraction, cyclic 3',5'-GMP formation was proportional to enzyme concentration.

TABLE III

OPTIMAL CONDITIONS FOR ASSAY OF GUANYL CYCLASE

In Expt. 1, each reaction contained 0.20 mg of enzyme fraction. In Expt. 2, each reaction contained 0.63 mg of enzyme fraction. GTP-regenerating system: 5 µg pyruvate kinase, 75 nmoles of disodium phosphoenolpyruvate and 3.75 µmoles of KCl per reaction mixture. NaF concentration was 10.0 mM when used. Other conditions cited in text.

	Activity as percent of control	
	10.0 mM $MnCl_2$, 1.0 mM GTP	*1.0 mM $MnCl_2$, 0.4 mM GTP*
Expt. 1. $(NH_4)_2SO_4$ fraction from bovine lung		
Control	100	100
Minus unlabeled cyclic 3',5'-GMP	53	62
Minus caffeine	104	115
Minus caffeine, *minus* cyclic 3',5'-GMP	3	4
NaF added	98	103
GTP-regenerating system added	101	100
Expt. 2. rat-kidney supernatant fraction		
Control		100
GTP-regenerating system added		105
NaF added		124
NaF *plus* regenerating system added		121
Minus caffeine		58
Minus caffeine, regenerating system added		58
Minus caffeine, NaF added		85
Minus caffeine, NaF and regenerating system added		88

Optimal conditions for assay of tissue extracts

Experiments were performed at the previously determined optimum pH, substrate and $MnCl_2$ concentrations. It was found that adding unlabeled cyclic 3',5'-GMP to the reaction mixture was more effective than adding 20 mM caffeine to reduce losses caused by cyclic 3',5'-nucleotide phosphodiesterase (Table III). This experiment also showed that, unlike the adenyl cyclase system[13], guanyl cyclase is not activated by F^-. The enzyme fraction from bovine lung apparently contained little GTPase activity, since inclusion of a GTP-regenerating system was without effect.

A similar study was carried out with the supernatant fraction from homogenates of rat kidney (Table III, Expt. 2). Greater enzyme activity was apparent when F^- was added. It was concluded that F^- probably inhibited nucleotidases capable of destroying GTP. On the other hand, there was little or no effect of adding a GTP-regenerating system. The effect of F^- varied from tissue to tissue, and we have not routinely added F^- to the system. In the assay of tissues such as kidney, where guanyl cyclase activity was low relative to cyclic 3',5'-nucleotide phosphodiesterase activity, both caffeine and cyclic 3',5'-GMP should be included.

Guanyl cyclase activity of rat tissues

Table IV shows the results for two assays of guanyl cyclase in tissues of the rat. Enzyme activity of the different tissues from a single animal was compared relative to

TABLE IV

DISTRIBUTION OF GUANYL CYCLASE IN THE TISSUES OF THE RAT

Rat tissues were prepared and assayed as described in the text. The reaction mixture contained 10.0 mM $MnCl_2$ and 1.0 mM GTP. Assays with brain enzyme contained 0.4 μmole of added cyclic 3',5'-GMP per sample. Whole blood was defibrinated with a stick. Intestinal mucosa cells were scraped from the luminal surface of the small intestine with a microscope slide. The remainder of this preparation was termed intestinal muscle. Each experiment was performed on the tissues from a single male rate of about 200 g except as noted.

Expt.		*Activity*	
		pmoles cyclic 3',5'-GMP per min per mg protein	*Relative to kidney*
1	Lung	89.5	16.0
	Brain	12.8	2.3
	Spleen	9.5	1.7
	Kidney	5.6	1.0
	Liver	5.5	1.0
	Heart	5.0	0.9
	Blood (whole)	0	0
2	Intestinal mucosa	13.6	3.0
	Intestinal muscle	5.4	1.2
	Kidney	4.5	1.0
	Thymus	3.2	0.7
	Skeletal muscle	2.9	0.6
	Testis	2.9	0.6
	Free fat cells*	2.3	0.5

* Free fat cells were prepared from epididymal fat pads of 15 rats according to RODBELL[14].

TABLE V

INTRACELLULAR DISTRIBUTION OF GUANYL CYCLASE IN LIVER AND LUNG OF RATS

Cell fractionation was performed according to the method of GONZÁLEZ-CADAVID AND CAMPBELL[15]. The liver from one rat and the pooled lungs from six rats were homogenized in 0.3 M sucrose using glass homogenizers with Teflon pestles. In this experiment the cell fractions were defined only by the relative centrifugal force used to sediment them ,*viz*; nuclear, 755 × *g* for 10 min; mitochondrial, 7710 × *g* for 10 min; microsomal and supernatant fractions, 104 000 × *g* for 60 min. Guanyl cyclase was assayed by the standard method, using 10.0 mM $MnCl_2$ and 1.0 mM GTP.

Cell fraction	*pmoles cyclic 3',5'-GMP per min per mg protein*	
	Lung	*Liver*
Nuclear	1.4	0.3
Mitochondrial	0	0.1
Heavy microsomal	0.9	0.4
Microsomal	0.8	0
Supernatant	68.9	7.5

the activity of kidney. The activity of brain is particularly difficult to measure because of the high concentration of cyclic 3',5'-nucleotide phosphodiesterase in this tissue. In this instance, it was necessary to double the amount of added cyclic 3',5'-GMP in the reaction mixture. The amount usually used, 0.2 μmole, was almost completely destroyed within 10 min and there was correspondingly a low yield of radioactive cyclic 3',5'-GMP.

Lung showed the highest concentration of guanyl cyclase. Brain and intestinal mucosa ranked next, but recovery of activity from the latter has been variable.

Intracellular distribution of guanyl cyclase

Guanyl cyclase was found entirely in the "soluble" fraction of tissue homogenates (Tables III, IV, and V). This is in contrast to the distribution of adenyl cyclase which is primarily located in cell membranes[16].

TABLE VI

EFFECT OF CERTAIN COMPOUNDS ON GUANYL CYCLASE

Addition	*Concn. (mM)*	*Activity (%)*
None		100*
$ZnSO_4$	0.12	4
Pyrophosphate	0.30	49
Orthophosphate	10.00	70
ATP	0.40	46
UTP	0.40	64
ITP	0.40	31
CTP	0.40	72
Cyclic 3',5'-AMP	1.00	97
Sodium pyruvate	10.00	98

* Standard assay as described in text using 25 μl of the beef-lung fraction containing 0.20 mg protein. The reaction mixture contained 1.0 mM $MnCl_2$ and 0.4 mM GTP.

Inhibitors

The effect of certain compounds upon the activity of guanyl cyclase is shown in Table VI. 0.12 mM $ZnSO_4$ caused marked inhibition as has been found with adenyl cyclase by RALL AND SUTHERLAND[17]. Both pyrophosphate and orthophosphate inhibit guanyl cyclase by precipitating Mn^{2+}. The more effective inhibition by pyrophosphate corresponds to the lower solubility of manganese pyrophosphate. Pyruvate caused no effect on guanyl cyclase whereas it causes activation of the adenyl cyclase obtained from *Brevibacterium liquifaciens*[18].

Cyclic 3′,5′-AMP was without effect, but the nucleoside triphosphates were inhibitory. The strong inhibition of ITP is of interest in view of the observations by MIECH AND PARKS[19] that inosine nucleotides may be substrates for several enzymes that react with GDP or GTP. On the other hand, it is possible that inhibition by the nucleoside triphosphates is attributable to competition with GTP for binding to Mn^{2+}. However, a similar degree of inhibition by nucleoside triphosphates was observed at 10 mM, a concentration of metal that appears to be in excess. The entire spectrum of substrate specificity for guanyl cyclase has not been determined but we found that [α-^{32}P]ATP is not converted to cyclic 3′,5′-[^{32}P]AMP by guanyl cyclase from bovine lung. The assay for this experiment was performed by a modification of the method of KRISHNA *et al.*[20].

DISCUSSION

The enzyme guanyl cyclase is clearly distinct from the adenyl cyclase system with regard to substrate specificity, intracellular distribution and metal activation. The difference in intracellular distribution is of particular importance. As has been mentioned earlier, all mammalian adenyl cyclases that have been studied so far have been membrane bound[16]. Such a localization is implicit in the concept of the second messenger function for cyclic 3′,5′-AMP, as developed by SUTHERLAND *et al.*[5]. In this concept a hormone need not enter the target cell in order to effect a response, since adenyl cyclase can be activated at the cell membrane; the resulting cyclic 3′,5′-AMP then transmits the signal to the intracellular effectors.

The physiological importance of cyclic 3′,5′-GMP remains to be established. MAGANIELLO *et al.*[21] found that 1 mM cyclic 3′,5′-GMP inhibited the lipolytic effect of exogenous cyclic 3′,5′-AMP in fat cells, when the Krebs–Ringer phosphate medium contained only Na^+ as cation. The inhibition appeared to be competitive. Under these conditions cyclic 3′,5′-GMP caused slight lipolysis, and this effect was potentiated by theophylline. In Krebs–Ringer phosphate buffer agents, such as epinephrine, which increase intracellular concentration of cyclic 3′,5′-AMP, lipolysis is also stimulated. Under these conditions cyclic 3′,5′-GMP also inhibits lipolysis and does so in the presence of epinephrine, glucagon or theophylline. This work suggests that in this system cyclic 3′,5′-GMP is an antogonist to the action of cyclic 3′,5′-AMP. In addition, it was also found that cyclic 3′,5′-GMP caused an increase in concentration of cyclic 3′,5′-AMP in the fat cell[22]. It is also possible that similar biochemical responses may be effected by several different cyclic nucleotides. LEVINE[23] found that cyclic 3′,5′-IMP and cyclic 3′,5′-7-deazaadenosine monophosphate were each equivalent to N^6-2′-*O*-dibutyryl cyclic 3′,5′-AMP in glycogenolytic activity in the isolated perfused rat liver.

We have not yet found a change in guanyl cyclase activity effected by addition

of hormones *in vitro*, and there is still no explanation for the findings of HARDMAN *et al.*[4] that hypophysectomy or hormone treatment influences urinary excretion of cyclic 3′,5′-GMP. These effects could be secondary or might be manifestations of direct influences of hormones on guanyl cyclase. Since guanyl cyclase appears to be a soluble enzyme, one might question whether cyclic 3′,5′-GMP functions like cyclic 3′,5′-AMP as an intracellular second messenger for polypeptide hormones, particularly those that do not enter cells. However, it is not possible to prove that our extraction method, though it appeared to be gentle, did not disrupt the enzyme from a membrane-bound locus. On the other hand, guanyl cyclase might represent an enzyme regulated by small molecules like amine or steroid hormones that may enter the cytoplasm of cells. Obviously, answers to these several questions require much further study.

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