[CANCER RESEARCH 36, 1740-1743, May 1976]

Regulation of the Adenylate Cyclase System in Transplantable Hepatomas[1]

Wayne E. Criss[2] and Harold P. Morris

Department of Obstetrics-Gynecology and Biochemistry, University of Florida Medical School, Gainesville, Florida 32610 [W. E. C.], and Department of Biochemistry, Howard University Medical School, Washington, D. C. 20059 [H. P. M.]

SUMMARY

Adenylate cyclase systems were examined in purified membrane preparations from normal rat liver and several Morris hepatomas with differing growth rates. All tumor membrane preparations had lower relative specific activities than did liver preparations. Liver adenylate cyclase was stimulated by fluoride, glucagon and guanyl-5'-yl imidodiphosphate [Gpp(NH)p]. Membranes from two slow-growing hepatomas (hepatomas 20 and 21) contained adenylate cyclase activities which are also stimulated by each of these three modulators. Membrane adenylate cyclases from several fast-growing hepatomas (hepatomas 3924A, 7777, 5123tc, and $9618A_2$) were marginally stimulated by glucagon but were readily stimulated by fluoride and Gpp(NH)p. Examination of the highly specific binding of ^{125}I-glucagon to the various membrane preparations revealed much less binding in all the tumor membranes than in liver membranes.

More detailed kinetic examination of membranes prepared from liver, slow-growing hepatoma 21 (which had reasonable binding to and stimulation by glucagon), and fast-growing hepatoma 3924A (which had marginal binding to and stimulation by glucagon) revealed major differences in rates of cyclic adenosine 3':5'-monophosphate production in the absence and presence of glucagon, Gpp(NH)p, and glucagon plus Gpp(NH)p and in the combined alteration of magnesium:adenosine 5'-triphosphate ratio and temperatures. The different kinetic characteristics in the hepatoma adenylate cyclase systems may be due to different structural characteristics of the tumor membranes or may be due to altered hormonal receptors, catalytic units, or receptor-catalytic unit interrelationships within the tumor membrane.

INTRODUCTION

Adenylate cylcase systems in mammalian cells are activated by biogenic amines, polypeptide hormones, and prostaglandins. These systems are therefore important components in the regulatory controls of cellular functioning. It is likely that these systems are composed of membrane receptors that are specific for certain hormones, a catalytic or enzyme unit that is capable of coverting ATP or cAMP,[3] and some form of transducing elements that allows the activation of the catalytic unit when the receptor interacts with a modulator (12, 13, 18, 21, 22, 24).

Liver cell membranes have been reported to have an adenylate cyclase system that responds to glucagon, secretin, GTP, fluoride, prostaglandins, epinephrine, and calcium. The type, extent, and time dependency of responses vary with the quantity of modulators and conditions of incubation (16, 24, 25). Therefore, extremely careful assay conditions must be observed if meaningful data on liver adenylate cyclase systems are to be obtained.

Several laboratories have examined the adenylate cyclase system in preneoplastic and neoplastic tissues (1-6, 14, 15, 19). The reports are somewhat conflicting, possibly because of the variations in membrane purity, tissue sources, assay methods, etc. In the general assay of hepatic adenylate cyclase, one must consider the interrelationships of the following modulators: fluoride, GTP or Gpp(NH)p which is not hydrolyzed, Ca^{2+}, and hormones. Interesting is the fact that hormonal activation occurs with a distinct time lag, while Gpp(NH)p desensitizes the hormone site and abolishes the time lag (20-25). Therefore the normal liver adenylate cyclase system is composed of hormone receptor, coupling processes, and nucleotide-regulatory sites, and a catalytic unit. In this study we will compare liver and liver tumors: (*a*) characteristics of activation by glucagon; (*b*) activation and properties of Gpp(NH)p; (*c*) the effects of the substrate on hormonal and Gpp(NH)p regulations; and (*d*) specific binding of glucagon and relationship to activity.

MATERIALS AND METHODS

Preparation of Membranes. A modification of Neville's method (16, 20, 25) of preparing plasma membranes from liver tissue was also used for the hepatoma preparations. Liver and tumor tissue were removed, cleaned of necrotic elements, and homogenized in 1 mM $NaHCO_3$ plus 1 mM dithiothreitol. The homogenate was filtered through cheesecloth, diluted 1:100 in the homogenizing media, stirred for 3 min, and centrifuged at 1500 × *g* for 30 min. The pellets were resuspended and layered on top of a 42.3% sucrose solution in cellulose nitrate tubes. The tubes were centrifuged in a SW25 rotor in a Spinco refrigerated centrifuge at 25,000 rpm for 150 min. Only floated membrane pellets at the top of the cellulose nitrate tubes were removed with a spoon spatula, resuspended, washed in the bicarbonate buffer, and stored in liquid nitrogen. The yield was approximately 40 to 60 mg of membrane protein per 100 g of

[1] This research was supported in part by USPHS Grants CA-11818, CA-10729, and CA-10906.

[2] Recipient of NIH Research Career Development Award CA-70187.

[3] The abbreviations used are: cAMP, cyclic adenosine 3':5'-monophosphate; Gpp(NH)p, guanyl-5'-yl imidodiphosphate.

Received October 2, 1975; accepted January 7, 1976.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/36/5/1740/2396869/cr0360051740.pdf by guest on 07 August 2024

tissue. The resulting preparation allowed recovery of 60 to 80% of the original adenylate cyclase activity and resulted in a 7- to 10-fold purification of the enzyme.

Enzymatic Assay. Adenylate cyclase activity (except where indicated) was routinely determined at 37° in a 100-μl total volume containing 25 mM Tris-HCl (pH 7.6), 1 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM ATP, 1 mM EDTA, 0.01 mM cAMP, 5 mM creatinine phosphate, 3 units of creatinine phosphokinase (Sigma-rabbit muscle; 155 units/mg), and 3 to 5 (10^6) cpm of [α-^{32}P]ATP (radioactivity, 30 to 50 cpm/pm nucleotide). [α-^{32}P]ATP was purchased from ICN Chemical Radioisotopes Division, Irvine, Calif., at ~100 Ci/mmole. It was less than 2% [^{32}P]AMP, less than 0.1% [^{32}P]ADP, less than 2% all other impurities, and greater than 96% [^{32}P]ATP. The reaction was initiated with 10 to 20 μl (20 to 40 μg of protein) of membrane preparation. The assay was terminated with 1 ml of 2% sodium dodecyl sulfate plus 1 mM ATP. Time-course studies were performed with 10× assay reagents. Protein was performed as described (17).

Separation of Nucleotides. [^{3}H]cAMP was added to the reaction mixture. The labeled nucleotides were separated on columns of Dowex AG-50W-X4 and alumina (20–25). Purified cAMP was counted in Aquasol in a Beckman scintillation counter, and the data were calculated by computer analysis.

Binding of ^{125}I-Glucagon. Each binding assay contained 200 μg of membrane protein in a final volume of 1500 μl. ^{125}I-Glucagon was prepared by a chloramine-T method to give a specific activity of 1.2 cpm/pmole (16, 23). Two assay conditions were used for each determinant. Assay condition 1 contained 2 × 10^4 cpm of ^{125}I-glucagon, 1.5 mg of bacitracin, and 1% bovine serum albumin in 20 mM Tris-HCl buffer, pH 7.5. Assay Condition 2 contained 10^{-5} M unlabeled glucagon in addition to the components of Assay Condition 1. All were incubated for 10 min at 30°, filtered on oxoid membranes, and washed with two 1-ml portions of the bovine serum albumin-Tris buffer. Each membrane fraction was counted in a γ counter. Assay Condition 2 was substrated from Assay Condition 1 to determine the net cpm bound. Each tissue was assayed in duplicate in each of the 3 separate experiments.

Other Reagents. Modulators, resins, chemicals, etc., were purchased as previously described (16, 20–25).

RESULTS

Regulation of Adenylate Cyclase Activity in Various Purified Membrane Preparations. Rat liver membrane-bound adenylate cyclase was stimulated by fluoride, glucagon, and Gpp(NH)p (Table 1). Membranes from 2 slow-growing hepatomas (hepatomas 20 and 21) had adenylate cyclases that were also stimulated by these 3 modulators. While membrane-bound adenylate cyclases prepared from several fast-growing hepatomas (hepatomas 3924A, 7777, 5123tc, and 961A_2) were only marginally stimulated by glucagon, they were readily stimulated by fluoride and Gpp(NH)p. Epinephrine was ineffective in all liver and hepatoma preparations examined.

Glucagon Binding to Liver and Tumor Membranes. Measurement of the highly specific binding of glucagon to liver membranes under non-equilibrium-low-protein concentration conditions showed 0.7 pmole ^{125}I-glucagon bound per mg membrane protein (Table 2). Under identical conditions and within the same experiment, less than 0.002 and 0.15 pmole of ^{125}I-glucagon were bound per mg of membrane protein to the slow-growing hepatomas 20 and 21, respectively. All of the fast-growing hepatomas had 0.002 and 0.04 pmole of ^{125}I-glucagon bound per mg of membrane protein. This experiment was repeated 3 times with variations in ^{125}I-glucagon preparations and incubation times. With only 1 exception (hepatoma 20), there is a general relationship of binding and activation.

Activation Characteristics at Low Temperature. Examination of liver adenylate cyclase activity in the absence of modulators at 10° showed that the rate of production of cAMP began dropping off around 7 to 9 min (Chart 1). In the presence of Gpp(NH)p, the liver enzyme showed an increased rate only after a 4- to 6-min lag and no rate dropoff. Glucagon caused an immediate increase in enzymatic activity, but the dropoff in rate was observed at 8 to 9 min, while glucagon plus Gpp(NH)p showed an immediate increase in cAMP production which was linear for over 20 min.

Two hepatoma adenylate cyclase systems were examined at 10°. The enzymes from the slow-growing tumor 21 (which has reasonable glucagon binding and activation) and the rapid-growing tumor 3924A (which has poor glucagon binding and activation) had kinetic characteristics that differed from those of the liver system and of each other. The basal enzymatic activity of hepatoma 21 showed a large rate increase at 5 to 7 min (Chart 2). In the presence of Gpp(NH)p, the rate of production of cAMP was initially higher than basal and increased even further at 4 to 5 min. Glucagon caused an immediate and continuously elevated rate. The glucagon plus Gpp(NH)p rate was approximately additive.

The basal rate of adenylate cyclase from hepatoma 3924A showed a spontaneous rate increase at 7 to 9 min (Chart 3). Gpp(NH)p caused a rate increase after a 4- to 5-min lag. While incubation with glucagon resulted in an immediate and continuous rate increase, incubation with glucagon plus Gpp(NH)p gave an immediate and continuous increase in the rate of production of cAMP.

Activation Constants. Titration of adenylate cyclase activity in membranes from liver, hepatoma 21, and hepatoma 3924A with 10^{-5} to 10^{-11} M concentrations of glucagon yielded different curves (Chart 4). However, 5 × 10^{-8} M glucagon caused stimulation to one-half maximum with all 3 enzymatic systems. 10^{-7} M Gpp(NH)p stimulated one-half maximal activities of enzymes from liver, hepatoma 21, and hepatoma 3924A (data not presented).

Temperature Influence on Enzymatic Activity. Basal liver adenylate cyclase activities were similar when measured at 30° or 37° (data not presented). Addition of glucagon also gave similar activities at 30° *versus* 37°. However, fluoride or Gpp(NH)p produced larger activities at 37° *versus* 30°. With both hepatoma 21 and hepatoma 3924A, only the fluoride-activated activities were greater at 37° *versus* 30°.

Influence of Mg:ATP Ratio. Comparison of adenylate cyclase activities at various Mg:ATP ratios was observed (data not presented). Fluoride did not activate any of the 3 enzymes at a Mg:ATP ratio of 1. The activation of the liver enzyme by Gpp(NH)p at 30° was similar at Mg:ATP ratios of

Downloaded from http://aacrjournals.org/cancerres/article-pdf/36/5/1740/2396869/cr0360051740.pdf by guest on 07 August 2024

Table 1

Stimulation of adenylate cyclase in purified liver and hepatoma membranes

These experiments were performed with 2 to 5 preparations of each tissue and were averages ± S.E. of 3 to 10 experiments.

	nmoles cAMP formed/10 min/mg							
		Slow-growing hepatomas		Intermediate-growing hepatoma	Fast-growing hepatomas			
	Liver	20	21	9633F	3924A	7777	5123tc	$9618A_2$
Basal	0.65 ± 0.06	0.06 ± 0.01	0.08 ± 0.01	0.27 ± 0.02	0.11 ± 0.01	0.19 ± 0.01	0.49 ± 0.02	0.40 ± 0.03
+ Fluoride	3.52 ± 0.30	0.44 ± 0.02	0.98 ± 0.06	1.97 ± 0.09	2.80 ± 0.16	1.99 ± 0.12	4.88 ± 0.25	3.01 ± 0.19
+ Epinephrine	0.69 ± 0.05	0.06 ± 0.01	0.11 ± 0.01	0.30 ± 0.02	0.08 ± 0.01	0.24 ± 0.01	0.65 ± 0.04	0.40 ± 0.02
+ Glucagon	3.08 ± 0.31	0.21 ± 0.02	0.35 ± 0.02	0.36 ± 0.02	0.20 ± 0.02	0.26 ± 0.02	1.26 ± 0.10	0.41 ± 0.02
+ Gpp(NH)p	3.27 ± 0.33	0.38 ± 0.02	0.84 ± 0.05	0.56 ± 0.03	2.31 ± 0.09	0.69 ± 0.03	2.33 ± 0.12	1.33 ± 0.09
+ Glucagon + Gp	5.28 ± 0.41	0.61 ± 0.03	1.82 ± 0.06	0.59 ± 0.03	3.29 ± 0.13	1.53 ± 0.08	9.24 ± 0.46	1.32 ± 0.11

Table 2

Stimulation of adenylate cyclase and glucagon binding

	Ratio of specific activities		
	Glucagon:fluoride	Glucagon:Gpp (NH)p	^{125}I-Glucagon binding (pmole/mg)
Liver	0.85	0.91	0.720
Slow-growing tumors			
20	0.48	0.55	<0.002
21	0.35	0.41	0.146
Intermediate-growing tumors			
9633F	0.18	0.64	0.063
Fast-growing tumors			
3924A	0.071	0.087	0.014
7777	0.131	0.377	0.021
5123tc	0.258	0.541	0.038
$9618A_2$	0.136	0.308	<0.002

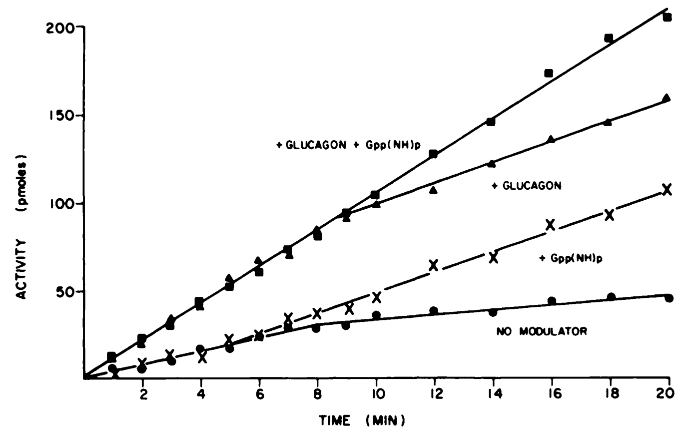

Chart 1. Time curve of liver adenylate cyclase activity at 10°. ●, no activities added; ×, 10^{-5} M, Gpp(NH)p; ▲, 10^{-6} M glucagon; ■, 10^{-5} M Gpp(NH)p plus 10^{-6} M glucagon.

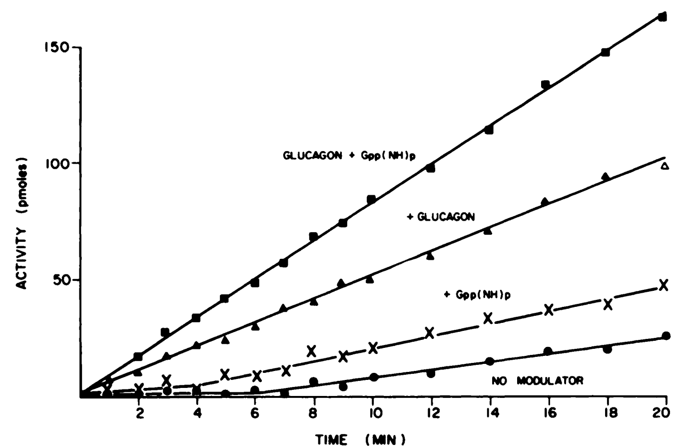

Chart 2. Time curve of hepatoma 21 adenylate cyclase activity at 10°. ●, no activators added; ×, 10^{-5} M Gpp(NH)p; ▲, 10^{-6} M glucagon; ■, 10^{-5} M Gpp(NH)p plus 10^{-6} M glucagon.

8 and 1, but it was significantly greater at 37° with a Mg:ATP ratio of 8 *versus* 1. This was not observed with either tumor enzyme. Other stimulated activities were not affected by Mg:ATP ratios from 8 to 1.

DISCUSSION

These studies compare the regulatory responses of several normal and neoplastic liver adenylate cyclase systems. It is apparent that several differences exist. The basal and glucagon-stimulated activities were always lower in the hepatoma membranes. With 1 exception, there was a general relationship between the binding and activation of glucagon to the various purified membrane preparations.

Membranes from 1 slow-growing and 1 fast-growing tumor were kinetically examined in more detail and compared with adult liver membranes. The time course of reaction in the presence and absence of activators at 10° was different for each system. Differences were also observed when the various modulators were compared at different temperatures and ratios of Mg:ATP.

It would appear from these comparisons that the hepatoma adenylate cyclase systems are not identical to the adult liver adenylate cyclase systems. While the tumors seem to have lost their responsiveness to glucagon, they retain their responsiveness to Gpp(NH)p and fluoride. Since many studies (see reviews, Refs. 7 to 11) have revealed modified metabolite and hormonal responses in these hepatomas, and because the hormonally responsive adenylate cyclase system is postulated to be one of the major modes of hormonal action in mammalian cells, it is logical to examine this hormonal system in these tumors in an attempt to understand the observed modifications in hormonal con-

Downloaded from http://aacrjournals.org/cancerres/article-pdf/36/5/1740/2396869/cr0360051740.pdf by guest on 07 August 2024

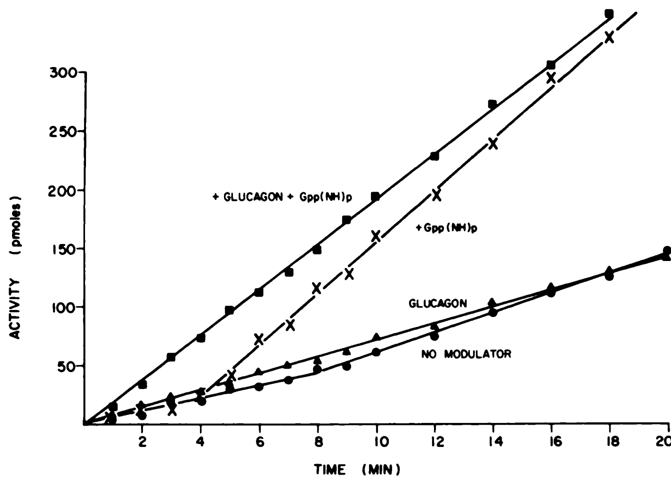

Chart 3. Time curve of hepatoma 3924A adenylate cyclase activity at 10°. ●, no activators added; ×, 10^{-5} M Gpp(NH)p; ▲, 10^{-6} M glucagon; ■, 10^{-5} M Gpp(NH)p plus 10^{-6} M glucagon.

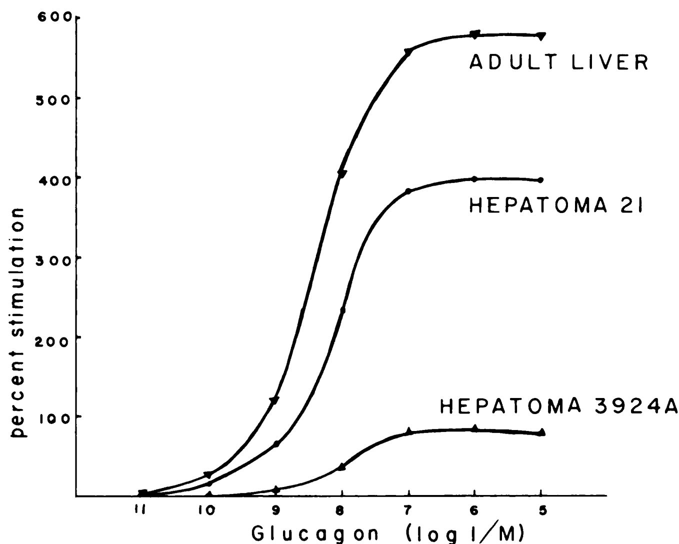

Chart 4. Activation of adenylate cyclases by glucagon. ▼, adult rat liver; ▲, hepatoma 3924A; ●, hepatoma 21.

trols in neoplasia. The current study indicates that the following may be modified in tumors: (*a*) decreased specific hormone binding; (*b*) variations in the catalytic components; and (*c*) modified responses to modulators. It is not known whether these characteristics reflect changes in membrane structure and composition or whether they reflect changes in the individual components of the adenylate cyclase system. However, it does allow us to begin to explore in detail a possible major loss of regulatory controls in solid tumors.

ACKNOWLEDGMENTS

We wish to express our sincere appreciation for the support and guidance of Dr. Martin Rodbell, Dr. Michael Lin, and Dr. Constantine Londos during the course of these investigations. We gratefully acknowledge the technical assistance of Willis Parker, Charity Jackson, and Louise Lawson.

REFERENCES

1. Allen, D. O., Munshower, J., Morris, H. P., and Weber, G. Regulation of Adenyl Cyclase in Hepatomas of Different Growth Rates. Cancer Res., *31:* 557-560, 1971.
2. Boyd, H., Louis, C. J., and Martin, T. J. Activity and Hormone Responsiveness of Adenyl Cyclase during Induction of Tumors in Rat Liver with 3'-Methyl-4-dimethylaminoazobenzene. Cancer Res., *34:* 1720-1725, 1974.
3. Brown, H. D., Chattopadhyay, S. K., Morris, H. P., and Pennington, S. N. Adenyl Cyclase Activity in Morris Hepatomas 7777, 7794A, and 9618A. Cancer Res., *30:* 123-126, 1970.
4. Bruch, J. S., Sutliff, L. S., and Sharma, R. K. Metabolic Regulation and Adenyl Cyclase Activity of Adrenocortical Carcinoma Cultured Cells. Cancer Res., *34:* 1495-1502, 1974.
5. Chayoth, R., Epstein, S. M., and Field, J. B. Glucagon and Prostaglandin E_1 Stimulation of cAMP Levels and Adenylate Cyclase and Activity in Benign Hyperplastic Nodules and Malignant Hepatomas of Ethionine-treated Rats. Cancer Res., *33:* 1970-1974, 1973.
6. Christoffersen, T., and Øye, I. Alterations in Hormone Responsiveness of Hepatic Adenylate Cyclase during Ontogenesis and Oncogenesis. Acta Endocrinol., *77* (Suppl. 191): 67-71, 1974.
7. Criss, W. E. Hormonal Control of Genetic Expression in Tumors. Am. J. Obstet. Gynecol., *116:* 753-759, 1973.
8. Criss, W. E. Metabolite and Hormonal Control of Energy Metabolism in Experimental Hepatomas. *In:* K. W. McKerns (ed.), Hormones and Cancer, Chap. 6, pp. 169-202. New York: Academic Press, Inc., 1974.
9. Criss, W. E. The Second Messenger System in Neoplasia. Oncology, *30:* 43-80, 1974.
10. Criss, W. E. Enzymes as Chemotherapeutic Targets in Cancer. Pharmacol. Res. Commun., in press.
11. Criss, W. E., and Pradhan, T. K. Regulation of Tumor Metabolism by the Adenylate and Guanylate Energy Charges. *In:* W. E. Criss, T. Ono, and J. Sabine (eds.), Modified Cellular and Molecular Controls in Neoplasia. New York: Raven Press, in press.
12. DeRubertis, F., Zenser, T. V., and Curnow, R. T. Inhibition of Glucagon-mediated Increases in Hepatic Cyclic Adenosine 3':5'-Monophosphate by Prostaglandin E_1 and E_2. Endocrinology, *95:* 93-100, 1974.
13. Harwood, J. P., Löw, H., and Rodbell, M. Stimulatory and Inhibitory Effects of Guanyl Nucleotides on Fat Cell Adenylate Cyclase. J. Biol. Chem., *248:* 6239-6245, 1973.
14. Hickie, R. A., Jan, S. H., and Datta, A. Comparative Adenylate Cyclase Activities in Homogenate and Plasma Membrane Fractions of Morris Hepatoma 5123tc(h). Cancer Res., *35:* 596-600, 1975.
15. Klein, I., Levey, G. S., Bricker, L. A., and Morris, H. P. Glucagon and Epinephrine Activation of Adenylate Cyclase and Glucagon Binding in Morris Hepatomas. Endocrinology, *94:* 279-283, 1974.
16. Lin, M., Salomon, Y., Rendell, M., and Rodbell, M. Hepatic Adenylate Cyclase System. II. Substrate Binding and Utilization and the Effects of Magnesium Ion and pH. J. Biol. Chem., *250:* 4246-4252, 1975.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem., *193:* 265-275, 1951.
18. Oka, H., Kaneko, T., Yamashita, K., Suzuki, S., and Oda, T. The Glucagon and Fluoride Sensitive Adenylate Cyclase in Plasma Membranes of Rat Liver. Endocrinol. Japan, *20:* 263-270, 1973.
19. Prasad, K. N., Gilmer, K. N., Sahu, S. K., and Becker, G. Effect of Neurotransmitters, Guanosine Triphosphate, and Divalent Ions on the Regulation of Adenylate Cyclase Activity in Malignant and Adenosine Cyclic 3':5'-Monophosphate-induced "Dfferentiated" Neuroblastoma Cells. Cancer Res., *35:* 77-81, 1975.
20. Rendell, M., Salomon, Y., Lin, M., Rodbell, M., and Berman, M. Hepatic Adenylate Cyclase System. II. A Mathematical Model for the Steady State Kinetics of Catalysis and Nucleotide Regulation. J. Biol. Chem., *250:* 4253-4260, 1975.
21. Robison, G. A., Butcher, R. W., and Sutherland, E. W. Adenyl Cyclase as an Adrenergic Receptor. Ann. N. Y. Acad. Sci., *139:* 703-723, 1967.
22. Robison, G. A., Butcher, R. W., and Sutherland, E. W. *In:* J. F. Danielli, J. F. Moran, and D. J. Triggle (eds.), Fundamental Concepts in Drug-Receptor Interaction, pp. 59-110. New York: Academic Press, Inc., 1968.
23. Rodbell, M., Krans, H. M. J., Pohl, S., and Birnbaumer, L. Glucagon Sensitive Adenyl Cyclase System in Plasma Membranes of Rat Liver. III. Binding of Glucagon-Method of Assay and Sensitivity. J. Biol. Chem., *246:* 1861-1871, 1971.
24. Rodbell, M., Lin, M. C., Salomon, Y., Londos, C., Harwood, J. P., Martin, B. R., Rendell, M., and Berman, M. Role of Adenine and Guanine Nucleotides in the Activity and Response of Adenylate Cyclase Systems to Hormones: Evidence for Multisite Transition States. Acta Endocrinol. Suppl., *191:* 11-37, 1974.
25. Salomon, Y., Lin, M. C., Londos, C., Rendell, M., and Rodbell, M. Hepatic Adenylate Cyclase System. I. Evidence for Transition States and Structural Requirements for Guanine Nucleotide Activation. J. Biol. Chem., *250:* 4239-4245, 1975.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/36/5/1740/2396869/cr0360051740.pdf by guest on 07 August 2024