*CYCLIC NUCLEOTIDE-DEPENDENT PROTEIN KINASES, IV. WIDESPREAD OCCURRENCE OF ADENOSINE 3′,5′-MONOPHOSPHATE-DEPENDENT PROTEIN KINASE IN VARIOUS TISSUES AND PHYLA OF THE ANIMAL KINGDOM**

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Abstract.—Adenosine 3′,5′-monophosphate-dependent protein kinase activity was found in about thirty sources including many mammalian tissues as well as species representative of eight different invertebrate phyla. The data support a unifying theory for the mechanism of action of adenosine 3′,5′-monophosphate, namely that its many and diverse effects are mediated through activation of tissue-specific protein kinases.

The great variety of actions that involve adenosine 3′,5′-monophosphate (cyclic AMP), including mediation of the effects of many hormones in vertebrates[1] as well as stimulation of enzyme synthesis in bacteria,[2] raises the question as to the mechanism by which this low molecular weight compound achieves its diversity of effects. Cyclic AMP-dependent protein kinase, which catalyzes the phosphorylation of casein, protamine and/or histone by ATP, was found initially in muscle,[3] and then in liver,[4] brain,[5] bacteria,[6] and adipose tissue.[7] A hypothesis was postulated[6] that all of the wide variety of effects elicited by cyclic AMP are mediated through stimulation of protein kinase. This hypothesis provides a unifying theory for the mechanism of action of cyclic AMP. There appears to be no evidence against this concept but there has been little data to support it. For this reason, we have examined a variety of vertebrate and invertebrate sources for the possible occurrence of cyclic AMP-dependent protein kinase activity.

Materials and Methods.—Fresh bovine organs were obtained from a local slaughterhouse and transported in ice to the laboratory. They were stored at −20° until the enzyme purification was started. Rabbit adipose tissue (epididymal) was purchased from Pel-Freez Biologicals. Rat organs were collected from 6 Sprague-Dawley rats (weighing about 260 gm each). Isolated adipose cells were prepared from the epididymal fat pads of rats by the method of Rodbell.[8] Worms, sponges, starfish, and jellyfish were obtained from the Marine Biological Laboratory, Woods Hole, Mass.; lobster, clam, squid, and carp, from a local fish market; protozoa (*Paramecium aurelia syngen* 4, stock 51s, grown in axenic medium, and then deciliated) were generously supplied by Dr. Henry M. Butzel, Jr., Union College. Cyclic AMP was purchased from Schwarz; inosine 3′,5′-monophosphate (cyclic IMP), guanosine 3′,5′-monophosphate (cyclic GMP), uridine 3′,5′-monophosphate (cyclic UMP), cytidine 3′,5′-monophosphate (cyclic CMP), and 2′-deoxythymidine 3′,5′-monophosphate (cyclic dTMP) from Boehringer Mannheim; histones, from Mann Research Labs.; casein, from Nutritional Biochemicals; ADP, AMP, adenosine, adenine, 2′-deoxyadenosine, FMN, GDP, GMP, guanosine, and diethylaminoethyl (DEAE) cellulose, from Sigma. γ^{32}P-ATP was prepared by the procedure of Post and Sen.[9]

Purification of protein kinase: Protein kinase from all sources except protozoa was

purified by a standardized procedure. Starting material was cut into small pieces and homogenized with 2 or 3 vol of neutral 4 mM EDTA solution for 2 min in a Waring Blendor. Protozoa were homogenized with glass beads in a Virtis homogenizer, as described for the enzyme from *Escherichia coli*.[6] The homogenates were centrifuged for 20 min at 27,000 $\times$ *g*, and the pH of the supernatant was adjusted to 4.8 with 1 *N* acetic acid. The precipitate formed was removed by centrifugation. The pH of the clear supernatant solution was adjusted to 6.9 with 1 *M* potassium phosphate buffer (pH 7.2), and solid ammonium sulfate (0.33 gm/ml) was slowly added to the enzyme solution. The protein kinase in the precipitate was collected by centrifugation, dissolved in a small volume (0.2 ml per gm wet weight of starting material) of 5 mM potassium phosphate buffer (pH 7.0) containing 2 mM EDTA, and dialyzed extensively against the same buffer. The enzyme was applied to a column of DEAE cellulose of appropriate size (56 gm dry cellulose/gm protein), and the column was washed with two bed volumes of 0.1 *M* phosphate buffer, pH 7.0, containing 2 mM EDTA. The protein kinase was eluted from the column with 0.3 *M* phosphate buffer, pH 7.0, containing 2 mM EDTA. The active fractions were pooled and dialyzed extensively against 5 mM phosphate buffer, pH 7.0, containing 2 mM EDTA. Purification of the enzyme from the various sources ranged from 10- to 50-fold for the ammonium sulfate step and from 12- to 250-fold for the DEAE-cellulose step.

Standard assay for protein kinase: The activity of the cyclic AMP-dependent protein kinase was assayed in an incubation volume of 0.2 ml containing: sodium glycerol phosphate buffer (pH 6.5), 10 μmoles; histone (mixture), 40 μg; γ^{32}P-ATP, 0.5 mμmole, containing 1.5 $\times$ 10^6 cpm; magnesium acetate, 2 μmole; NaF, 2 μmole; theophylline, 0.4 μmole; ethylene glycol bis-(β-aminoethylether)-N,N'-tetraacetic acid, 0.06 μmole; with or without 1 mμmole of cyclic AMP. Incubations were carried out for 5 min at 30° in a shaking water bath. The reaction was terminated and the protein-bound ^{32}P was determined as described for the enzyme from skeletal muscle,[3, 10] with minor modification.[11] Protein kinase activity is expressed as pmole ($\mu\mu$mole) of ^{32}P transferred from γ^{32}P-ATP to histone in the standard assay system.

Results.—Cyclic AMP-dependent protein kinase activity was found in every mammalian tissue examined, including 13 bovine tissues, 7 rat tissues, and 1 rabbit tissue (Table 1). Whole bovine blood was also processed and assayed for the protein kinase under conditions identical to those used for the other tissues; definite but only slight activity was detected. The possibility that protein kinase activity in various bovine tissues might have been due to contaminating blood was therefore excluded.

Cyclic AMP-dependent protein kinase activity was found in every animal species examined, which included representatives from nine different phyla (Table 2). Efforts to demonstrate cyclic AMP dependent protein kinase activity, under the present experimental conditions, in a series of plant tissues, including germinating barley seeds, green pepper, cabbage leaves, shell beans, and *Euglena gracilis* (strain Z) were unsuccessful.

A limited number of comparative studies of the properties of the cylic AMP-dependent protein kinase from various animal tissues and species was carried out in order to obtain information on the degree of tissue- and species-specificity of the enzyme. Three commercially available histone preparations ("mixture," "arginine-rich," and "slightly lysine-rich"), as well as casein, were tested for their capacity to serve as substrates for most of the protein kinase preparations listed in Tables 1 and 2. Concentrations of substrate found optimal for the enzyme from bovine brain were used. The data indicate that all four of these protein preparations can serve as phosphate acceptor for ATP. Interestingly, the his-

TABLE 1. *Cyclic AMP-dependent protein kinase in various mammalian tissues.*

	Protein Kinase Activity −Cyclic AMP	+Cyclic AMP
Experiment 1		
Bovine brain	0.43	6.24
pancreas	0.68	8.11
kidney	1.06	8.80
heart	0.54	5.54
lung	0.23	2.53
liver	1.22	12.85
thyroid	1.93	11.65
adrenal (whole)	1.31	11.74
muscle	1.18	12.43
testis	1.32	13.46
ovary	1.25	11.20
stomach	2.77	11.03
duodenum	1.49	10.40
Experiment 2		
Bovine brain	0.78	7.82
Rabbit adipose tissue	5.48	16.33
Rat adipose cells	0.55	6.38
interscapular brown fat pads	0.16	8.57
brain	1.29	7.94
liver	3.22	7.79
testis	2.62	5.17
heart	3.42	5.18
muscle	4.23	20.80

The protein kinase preparations used in Experiment 1 were from the DEAE cellulose step, whereas the preparations used in Experiment 2 were from the ammonium sulfate step. The data have been corrected for protein kinase activity which occurred in the absence of added histone, both in the absence and in the presence of cyclic AMP (5 μM).

tones (40 μg) were more effective than was casein (600 μg) as substrates for the mammalian enzyme, whereas the reverse was true for the protein kinase prepared from the nonmammalian species. Representative data from those experiments are presented in Table 3.

TABLE 2. *Cyclic AMP-dependent protein kinase activity in species from nine animal phyla.*

Phylum	Common name (genus)	Protein Kinase Activity −Cyclic AMP	+ Cyclic AMP
Protozoa	Paramecium (*Paramecium*)*	0.28	0.58
Porifera	Sponge (*Haliclona*)*	0.15	0.30
Coelenterata	Jellyfish (*Cyanea*)*	3.72	5.15
Nematoda	Roundworm (*Golfingia*)*	2.10	3.18
Annelida	Sandworm (*Nereis*)*	0.76	1.74
Mollusca (class Pelecypoda)	Clam (*Mya*)*	0.87	2.13
Mollusca (class Cephalopoda)	Squid (*Loligo*)†	3.45	6.01
Arthropoda	Lobster (*Homarus*)†	0.54	6.72
Echinodermata	Starfish (*Asterias*)*	0.29	0.74
Chordata	Fish (*Cyprinus*)†	0.55	1.15

Protein kinase preparations used were from the ammonium sulfate step, except for the protozoa preparation, which was from the pH 4.8 step. The data have been corrected for protein kinase activity which occurred in the absence of added histone, both in the absence and in the presence of cyclic AMP (5 μM).

* Whole animal was used as enzyme source.

† Muscle tissue was used as enzyme source.

TABLE 3. *Relative ability of histone (mixture) and casein to serve as substrate for various protein kinase preparations.*

Enzyme source	Protein Kinase Activity			
	Histone (40 μg)		Casein (600 μg)	
	−Cyclic AMP	+Cyclic AMP	−Cyclic AMP	+Cyclic AMP
Bovine brain	1.07	6.35	0.41	0.80
Rat brain	0.68	1.39	0.21	0.32
Rat adipose cells	0.59	4.45	0.23	1.47
Rat muscle	4.23	20.80	3.34	7.82
Lobster muscle	0.23	2.14	2.30	4.28
Roundworm	0.99	1.35	1.96	3.88
Fish	0.52	1.35	1.17	3.62

Assay conditions were as described in the text except for the variation in the kind and amount of protein substrate, as indicated. The data have been corrected for the values obtained in the absence of added protein substrate both in the absence and presence of cyclic AMP (5 μM). The enzyme preparations used were from the ammonium sulfate step.

The relative ability of some cyclic 3′,5′-nucleotides to stimulate the activity of various protein kinase preparations is shown in Table 4. For any given enzyme preparation examined, cyclic IMP, cyclic GMP, cyclic UMP, and cylic CMP, at higher concentrations, could stimulate protein kinase activity as, or almost as, effectively as could optimal concentrations of cyclic AMP. Cyclic dTMP (at high concentration) caused moderate increases in the activity of the enzyme from rat muscle and from lobster muscle but little or no increase in the activity

TABLE 4. *Comparison of the effects of cyclic 3′,5′-nucleotides on various protein kinase preparations.*

Cyclic nucleotide	Concentration (μM)	Protein Kinase Activity				
		Bovine brain	Rat brain	Rat adipose cells	Rat muscle	Lobster muscle
None	...	1.60	2.55	0.64	3.70	0.86
Cyclic AMP	0.5	10.18	7.58	5.17	20.78	4.55
	5	11.01	9.27	5.97	21.44	5.09
	50	11.60	9.12	5.82	20.01	6.40
	250	7.70	6.57	3.74	10.44	5.09
Cyclic IMP	0.5	9.17	8.20	3.05	11.58	3.89
	5	10.98	9.36	5.53	22.22	5.49
	50	12.83	9.69	6.44	21.48	6.16
	250	11.50	8.85	7.28	18.59	6.30
Cyclic GMP	0.5	2.43	2.36	1.35	5.43	4.45
	5	3.17	4.46	2.45	12.67	5.18
	50	7.13	8.30	6.16	20.53	6.23
	250	10.49	9.37	6.96	22.62	5.56
Cyclic UMP	0.5	1.53	2.90	1.12	4.87	2.17
	5	2.13	2.80	1.71	7.72	2.25
	50	5.58	6.22	4.55	17.34	4.64
	250	9.46	8.51	7.01	20.85	5.78
Cyclic CMP	0.5	1.86	2.36	0.60	3.95	1.63
	5	2.37	2.99	0.78	9.18	2.56
	50	4.27	5.49	1.50	16.35	5.30
	250	8.85	9.70	3.09	21.02	5.99
Cyclic dTMP	250	2.25	2.76	0.78	6.44	2.30

The protein kinase preparations used were from the ammonium sulfate step. Assay conditions were as described in the text except for the kind and amount of cyclic nucleotides present. The data have been corrected for zero time control values.

of the enzymes from bovine brain, rat brain, and rat adipose cells. At the lowest concentrations of the nucleotides tested, the effectiveness of the cyclic nucleotides in stimulating protein kinase activity followed the general pattern: cyclic AMP > cyclic IMP > cyclic GMP > cyclic UMP > cyclic CMP. However, there were exceptions to this pattern. For instance, protein kinase from lobster muscle was simulated by cyclic GMP as effectively as by cyclic AMP. The lobster enzyme, compared to the enzyme from other sources, was also stimulated quite effectively by cyclic UMP and cyclic CMP. Preliminary experiments have shown that one pmole of cyclic GMP and 10 pmoles of cylic UMP and cyclic CMP can be readily measured using the lobster protein kinase preparation; the development of assay methods for these compounds, based on use of the lobster muscle enzyme, is now in progress. Using protein kinase from bovine kidney, it is possible to measure cyclic AMP down to a level of 0.1 pmole.[12]

The effect of various adenine and guanine derivatives on the activity of some protein kinase preparations has been determined. In some instances the protein kinases from different tissues showed quite marked differences in their sensitivity to inhibition by these compounds. Thus, GDP inhibited the protein kinase activity of rat brain and muscle, and AMP inhibited that of rat and lobster muscle to a greater extent than either of them inhibited the enzyme from other sources (Table 5). A number of other compounds, which included adenine, adenosine, 2′-deoxyadenosine, ADP, guanosine, GMP, and FMN, gave varying degrees of inhibition of the protein kinases from various sources.

The results shown in Tables 3, 4, and 5 for the protein kinase preparations from bovine brain obtained after the ammonium sulfate step are similar to results obtained with the enzyme preparation from the DEAE cellulose step, or even with the highly purified (electrophoretically homogeneous) enzyme preparation from bovine brain.[11] Obviously, other pure protein kinase preparations need to be studied to determine whether any of the differences in properties of the enzyme from various tissues found in the present study are the result of our having used only partially purified enzyme preparations.

A comparison of the effects of divalent metal ions was carried out on protein kinase preparations from bovine brain, rat brain, and adipose cells, lobster muscle, carp muscle, and roundworm. The enzyme fom the six sources behaved in a similar way with regard to metal requirements. Co^{+2} (10 mM) was some-

TABLE 5. *Inhibition by AMP and GDP of the activity of various protein kinase preparations.*

Additive (50 μM)	Cyclic AMP (5 μM)	Protein Kinase Activity				
		Bovine brain	Rat brain	Rat adipose cells	Rat muscle	Lobster muscle
None	−	1.21	2.60	0.78	4.23	0.82
	+	9.28	10.23	6.00	20.80	4.96
AMP	−	0.82	0.87	0.59	0.30	0.04
	+	7.04	6.20	5.42	0.56	0.34
GDP	−	1.39	0.20	0.56	0.77	0.55
	+	7.74	2.53	5.79	3.87	4.02

The protein kinase preparations were from the ammonium sulfate step. Assay conditions were as described in the text except for the presence of inhibitors as indicated. The data have been corrected for zero time control values.

what more effective than either Mg^{+2} (10 mM) or Mn^{+2} (2.5 mM) in stimulating protein kinase activity either in the absence or presence of cyclic AMP. (The concentrations of Co^{+2}, Mg^{+2} and Mn^{+2} used were those found to be optimal for stimulation by cyclic AMP of the protein kinase from bovine brain.[11]) In the presence of Ca^{+2} (2.5 mM), or in the absence of any added metal, there was little or no protein kinase activity either in the absence or in the presence of cyclic AMP.

Discussion.—Cyclic AMP-dependent protein kinase activity was found in every animal tissue examined in the present study, which included 13 bovine tissues, 7 rat tissues, 1 rabbit tissue, fish muscle, and tissue from species representing 8 invertebrate phyla. In the case of a number of mammalian tissues subject to regulation by the endocrine system, there is compelling evidence[1] that cyclic AMP mediates the actions of the appropriate tissue-specific hormones. The present demonstration of the occurrence of cyclic AMP-dependent protein kinase activity in every animal tissue examined, including a number of mammalian tissues where regulation by the endocrine system is not known to occur, suggests that cyclic AMP may play a role in the regulation of all animal tissues, even those not controlled by specific hormones.

The inability to find cyclic AMP-dependent protein kinase activity in several plant tissues is interesting. Since the protein kinase from all tissues was prepared and assayed under the conditions found optimal[11] for the enzyme from bovine brain, the absence of enzyme activity in the plants studied is not at all conclusive. The cyclic AMP-dependent protein kinase in *E. coli*[6] could only be detected under assay conditions quite different from those used for the animal enzyme. The plant preparations were also assayed under the conditions found suitable for the bacterial enzyme, but no cyclic AMP-dependent protein kinase was detected.

If the hypothesis[6] that protein kinases mediate all the diverse effects of cyclic AMP should prove to be correct, then the tissue-specific and species-specific actions of cyclic AMP might be reflected in some specificity of the protein kinases and/or the protein substrates for these kinases. In the present investigation, evidence for some degree of tissue-specificity and species-specificity of the various protein kinases was provided by studies of the relative ability of various proteins to serve as substrates and of various cyclic 3′,5′-mononucleotides to serve as activators for the enzyme from different sources as well as by studies with a series of inhibitors.

The relative ability of histone and casein to serve as substrates (Table 3) may reflect an important difference between the protein kinase of mammals and that of lower species. Some degree of species-specificity of the protein kinases is further suggested by the sensitivity of the lobster enzyme to low concentrations of cyclic GMP, cyclic UMP and cyclic CMP, in contrast to mammalian preparations (Table 4). The experiments with inhibitors (Table 5) provide further evidence of species-specificity of the protein kinase. For instance, GDP inhibited the enzyme from rat muscle to a much greater extent than it did the enzyme from lobster muscle, and it inhibited the enzyme from rat brain to a greater extent than it did the enzyme from bovine brain. The experiments with inhibitors also provide evidence for tissue-specificity of the enzyme within a given

species. Thus, the protein kinase from rat muscle was much more sensitive to inhibition by AMP than that from rat brain or adipose cells. Moreover, GDP strongly inhibited the protein kinase activity of rat brain and muscle without significantly inhibiting that of rat adipose cells.

The apparently ubiquitous occurrence of cylic AMP-dependent protein kinase activity in the animal kingdom supports the hypothesis[6] that all the actions of cyclic AMP are mediated through such protein kinases. One particularly attractive feature of this hypothesis is that it provides a single reaction mechanism by which cyclic AMP can bring about its diverse effects, with the specificity of the action of cyclic AMP residing in the nature of the particular protein kinase and/or its substrates. The molecular mechanism by which the cyclic AMP-dependent protein kinase system achieves its highly specific effects in diverse systems will obviously require an enormous amount of further experimental work, involving the purification and characterization of the protein kinases and their natural substrates, using many tissues from many species.

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[1] Sutherland, E. W., G. A. Robison, and R. W. Butcher, *Circulation*, **37**, 279 (1968); Robison, G. A., R. W. Butcher, and E. W. Sutherland, *Ann. Rev. Biochem.*, **37**, 149 (1968).

[2] Perlman, R. L., and I. Pastan, *Biochem. Biophys. Res. Commun.*, **30**, 656 (1968); Perlman, R. L., and I. Pastan, *J. Biol. Chem.*, **243**, 5420 (1968).

[3] Walsh, D. A., J. P. Perkins, and E. G. Krebs, *J. Biol. Chem.*, **243**, 3763 (1968).

[4] Langan, T. A., *Science*, **162**, 579 (1968).

[5] Miyamoto, E., J. F. Kuo, and P. Greengard, *Science*, **165**, 63 (1969).

[6] Kuo, J. F., and P. Greengard, *J. Biol. Chem.*, **244**, 3417 (1969).

[7] Corbin, J. D., and E. G. Krebs, *Biochem. Biophys. Res. Commun.*, **36**, 328 (1969).

[8] Rodbell, M., *J. Biol. Chem.*, **239**, 375 (1964).

[9] Post, R. L., and A. K. Sen, in *Methods in Enzymology*, ed. R. W. Estabrook and M. E. Pullman (New York: Academic Press, 1967), vol. 10, p. 773.

[10] DeLange, R. J., R. G. Kemp, W. D. Riley, R. A. Cooper, and E. G. Krebs, *J. Biol. Chem.*, **243**, 2200 (1968).

[11] Miyamoto, E., J. F. Kuo, and P. Greengard, *J. Biol. Chem.*, **244**, 6395 (1969).

[12] Kuo, J. F., and P. Greengard, manuscript in preparation.