Concentrations of Cyclic AMP-dependent Protein Kinase Subunits in Various Tissues*

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The concentrations of the regulatory (R) and catalytic (C) subunits of adenosine 3':5'-monophosphate (cyclic AMP)-dependent protein kinase(s) were measured in extracts of skeletal muscle, heart, liver, kidney, and brain. These concentrations were also estimated for the particulate fraction from brain, the only tissue in which a major part of the total activity was not readily extracted in a soluble form. Values for R were determined by measuring the amount of cyclic[3H] AMP bound to protein in these tissue fractions under specified conditions; it was assumed that 1 mol of cyclic AMP binds to 1 mol of R. Values for C were determined from measurements of the specific protein kinase activity of the fractions utilizing the turnover number of pure C in the calculations. Turnover numbers for C were found to be identical for this subunit obtained in the pure form from rabbit skeletal muscle, rabbit liver, and beef heart. The methods used for measuring C were evaluated by kinetic studies and through the use of the specific heat-stable protein inhibitor of cyclic AMP-dependent protein kinase(s). R and C were found to exist in a 1:1 molar ratio in all of the tissue fractions that were studied. The absolute concentrations of R and C ranged from 0.23 μmol/kg wet weight for liver to 0.78 μmol/kg wet weight for brain. For brain this value was based on the amount of each subunit in the particulate as well as the soluble fraction. For other tissues the values were based solely on the subunit content of the latter fraction. It was noted that the molar concentrations of R are close to those of cyclic AMP under basal conditions in the various tissues.

Cyclic AMP-dependent1 protein kinases (EC 2.7.1.37; ATP:protein phosphotransferase) from several tissues are known to contain two types of subunits: catalytic subunits (C), which catalyze the transfer of the y-phosphate of ATP to certain proteins, and regulatory subunits (R), which, in the absence of cyclic AMP, inhibit the activity of the catalytic subunits (see Ref. 1). Cyclic AMP activates these enzymes by causing dissociation of the inactive holoenzyme (R,C) to yield free C subunits and a cyclic AMP regulatory subunit complex (R-Cyclic AMP) according to the following equation established for the enzyme purified from skeletal muscle (2) or heart (3, 4):

\[ R_C + 2 \text{ cyclic AMP} \rightarrow R \cdot \text{cyclic AMP}_2 + 2C \]  

(1)

At least two types of the cyclic AMP-dependent protein kinase exist in various mammalian tissues. These have been referred to as type I and type II according to their order of elution from DEAE-cellulose with increasing salt concentration (5). It is not known, however, whether "type I" and "type II" enzymes from different tissues are necessarily identical. Both types of protein kinase have been purified to near homogeneity from rabbit skeletal muscle and were referred to as Isozyme I and Isozyme II (6). In this instance it was found that each form contained identical C subunits but differed in the properties of R. In general, work from several laboratories has suggested that variability in the properties of the different cyclic AMP-dependent protein kinases resides in differences in the regulatory properties of R in various mammalian tissues. These have been studied include: (a) the binding of MgATP to type I protein kinase and its effect on recombination of the subunits (2, 4, 10–12); (b) autophosphorylation of the regulatory subunit of type II protein kinase and the effect of this reaction on cyclic AMP binding and dissociation of the enzyme (3, 4); (c) dissociation and activation of the protein kinases by basic protein substrates (13, 14); and (d) the presence of an inhibitory protein other than R that is capable of binding to C (15). In addition to the above, it has been stressed that it is necessary to take total enzyme concentration into account when attempting to estimate the degree of kinase activation by a given concentration of cyclic AMP (12, 16). Finally, it has been shown (10), and would be anticipated from Equation 1, that the relative concentrations of R and C subunits affect the responses to cyclic AMP in vivo.

The present study was undertaken in an attempt to gain

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1 The abbreviations used are: cyclic AMP, adenosine 3':5'-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether) N,N',N''-tetraacetate acid, Mes, 2(N-morpholino)ethanesulfonic acid. The regulatory subunit of cyclic AMP-dependent protein kinases are referred to as R. The catalytic subunit are referred to as C.
further knowledge as to the concentration of the two types of cyclic AMP-dependent protein kinase subunits in different animal tissues. It was thought that such information would be useful with respect to our understanding of the activation of the enzyme by cyclic AMP, as discussed above, and would also provide a basis for considering possible alternative functions of the regulatory subunit. In addition, knowledge concerning the relative concentrations of the catalytic and regulatory subunits would have a bearing on work related to their biosynthesis or degradation or both.

MATERIALS AND METHODS

Preparation of Tissue Fractions—Rabbits, 2.5 to 3 kg, (New Zealand Whites) were anesthetized deeply with a overdose of sodium pentobarbital and bled from the jugular veins. Tissues were quickly removed and immediately chilled in ice. All subsequent steps were carried out at 4°C. Skeletal and heart muscle were passed through the coarse disc of a meat grinder but the preliminary mincing of the other glandular tissues was done with scissors. The ground of minced tissues were then placed in either 2/3 or 5 volumes per kg wet weight of 6 mM EGTA (pH 7.0) containing 15 mM mercaptoethanol and homogenized for 45 s at high speed in a Waring Blender; these homogenates will be identified as 3°C homogenate. The homogenate was centrifuged at 10,000 × g for 25 min and the supernatants decanted through glass wool. The sediment from centrifugation of the brain homogenates was extracted two times with 2 volumes of homogenization buffer and the resulting supernatants were combined. The final pellet was resuspended in 2/3 volumes of homogenization buffer. For the various types of assays (see below), extracts were diluted in 25 mM Mes buffer, pH 6.9, containing 0.5 mg/ml of bovine serum albumin. Assays were carried out the same day the extracts were prepared, usually within 2 to 3 h after death of the animals.

Assay for Cyclic AMP-dependent Protein Kinase Activity—Protein kinase activity was measured at pH 6.9 in reaction mixtures with a final volume of 0.1 ml containing 2 μmol of Mes, 0.2 μmol of EGTA, 0.75 μmol of magnesium acetate, 0.01 μmol of γ-32P-ATP (100 cpm/μmol), 100 μg of histone f2b, 100 μmol of cyclic AMP, and 20 μl of diluted enzyme (see above for method of dilution) to start the reaction. Incubations were carried out for 2 min at 30°C. Reactions were terminated and the amount of 32P incorporated into the protein substrate determined as described (Method B) by Reimann et al. (17). Inasmuch as the holoenzyme forms of the cyclic AMP-dependent protein phosphotransferase were known to dissociate completely at the cyclic AMP concentration used in the assay (1), it was assumed that the activity expressed in these determinations was that of the free catalytic subunit. One unit of activity is taken as that amount of enzyme catalyzing the transfer of 1 pmol of phosphate to histone f2b per min.

Assays for Regulatory Subunit Concentration—The concentration of the regulatory subunits of cyclic AMP-dependent protein kinase(s) was determined by measuring the cyclic AMP binding capacity in tissue fractions using the method of Gilman (18). Binding reactions were carried out at pH 4.0 in a 0.1 ml reaction mixture containing 5 μmol of sodium acetate buffer, 10 μg of bovine serum albumin, cyclic[6H] AMP (28 Ci/mmol), and aliquots of the extracts. After incubation for at least 60 min, the reaction mixtures were diluted with 2 ml of cold 20 mM potassium phosphate buffer (pH 6.2) and poured through cellulose ester filters. The filters were washed twice with 5 ml of cold diluting buffer, dissolved in 2 ml of ethyloxyethanol, and counted in a toluene-based scintillator.

Assays for Cyclic AMP Phosphodiesterase and Histone Phosphatase Activities—Assays for cyclic AMP phosphodiesterase and histone phosphatase were carried out according to Beavo et al. (19) and England et al. (20), respectively, except for the following changes. The reaction mixtures had the composition of those used for deter-

RESULTS

Purification and Properties of Cyclic AMP-dependent Protein Kinase Catalytic Subunit(s) from Different Tissues

In order to calculate molar concentrations of C in crude tissue fractions from enzyme activity measurements, it was necessary to know the specific activity and molecular weight of the subunit for each tissue to be examined. To isolate and characterize C from all tissues was not feasible, but it was possible to do this for three representative tissues. The tissues selected for this purpose included rabbit skeletal muscle, rabbit liver, and beef heart. In this connection, it should be noted that prior to this study it was already known that C prepared from skeletal muscle or from liver interacts with R from the opposite tissue (28). In addition, it was shown (9) that C derived from each of two different skeletal muscle cyclic AMP-dependent protein kinase isozymes appeared to be identical in substrate specificity and physicochemical properties.

Preparation of Pure Cyclic AMP-dependent Protein Kinase Catalytic Subunit from Rabbit Liver and Beef Heart—The catalytic subunit (C) of cyclic AMP-dependent protein kinase(s) was isolated from rabbit liver and beef heart by adapting the skeletal muscle procedure (Method B) of Beavo et al. (21) to these tissues. The following modifications were required: (a) the amount of DEAE-cellulose was increased to 1 liter of settled resin per kg wet weight of muscle and to 2 liters for the same amount of liver; (b) the CM50 batch absorption steps were carried out twice at pH 6.6, twice at pH 6.1, and once at pH 6.5 before cyclic AMP was added; and (c) the active fractions from the CM50 cellulose column were pooled and diluted with distilled H2O containing 0.1 mg/ml of bovine serum albumin to a final conductivity of 2.5 mhos. The catalytic subunit was then absorbed on a 1 to 2 ml CM50 column. After extensive washing of the column, the subunit was eluted using a high salt buffer. This last step prevented inactivation of C from liver which otherwise occurred during dialysis.

Treatment of Skeletal Muscle Extract with Antibody to R type I and to R type II—Antibody to purified bovine skeletal muscle R type I C was made in rabbits and antibody to bovine heart muscle R type II C was made in goats.(10) In each case, the antibody was precipitated at 45% saturation with ammonium sulfate, dissolved in 100 mM NaCl, 1 mM EDTA, 10 mM Mes buffer, pH 6.5, and dialyzed to remove excess salt. Varying amounts of antibody were incubated with 20 μl of bovine skeletal muscle extract for 60 min at 23°C and the mixture was centrifuged at 1000 × g for 15 min. The supernatants were assayed for cyclic AMP binding capacity.

Miscellaneous—[γ-32P]-ATP was prepared using a modification (22) of the method described by Glynn and Chappell (23). 32P-labeled histone f2b was prepared according to Meisler et al. (24) using purified catalytic subunit of rabbit skeletal muscle protein kinase as the phosphotransferase; the protein was free of γ-32P-ATP. The specific activity of the labeled histone was calculated from the specific activity of γ-32P-ATP. Protein was determined by a biuret method (25) using bovine serum albumin as standard. Sodium dodecyl sulfate-gel electrophoresis was carried out as described by Weber and Osborne (26) using the Tris/glycine system (pH 8.2). The standards used for estimating molecular weights included glycogen phosphorylase (94,000), catalase (60,000), ovalbumin (49,000), lactate dehydrogenase (36,000), and lysisome (14,300). Homogenous cyclic AMP-dependent protein kinase catalytic subunit from rabbit skeletal muscle and preparation of the holoenzyme (type II C) from this same source were kindly provided by Dr. J. A. Beavo of the University of California. Cyclic [3H]AMP was purchased from Schwarz/Mann; ATP and cyclic AMP were obtained from Sigma; cellulose ester filters (HAWP) were from the Millipore Corp. Other chemicals used were of analytical grade. Crystalline skeletal muscle phosphofructokinase was generously provided by Dr. Robert G. Kemp of Marquette University. Histone f2b, isolated from calf thymus according to the method of Johns (27), and the purified heat-stable inhibitor of cyclic AMP-dependent protein kinases were kindly provided by Dr. Donal A. Walsh of the University of California.

Preparation of Pure Cyclic AMP-dependent Protein Kinase Catalytic Subunit(s) from Different Tissues

In order to calculate molar concentrations of C in crude tissue fractions from enzyme activity measurements, it was necessary to know the specific activity and molecular weight of the subunit for each tissue to be examined. To isolate and characterize C from all tissues was not feasible, but it was possible to do this for three representative tissues. The tissue selected for this purpose included rabbit skeletal muscle, rabbit liver, and beef heart. In this connection, it should be noted that prior to this study it was already known that C prepared from skeletal muscle or from liver interacts with R from the opposite tissue (28). In addition, it was shown (9) that C derived from each of two different skeletal muscle cyclic AMP-dependent protein kinase isozymes appeared to be identical in substrate specificity and physicochemical properties.

2 Details concerning the production and characterization of antibodies to the two types of R subunit will be published elsewhere (P. J. Bechetel, J. A. Beavo, and E. G. Krebs, manuscript in preparation). It was confirmed (see below) that antibody to Subunit R type I does not cross-react with Subunit R type II, nor does antibody to Subunit R type II cross-react with the type I subunit as evidenced by precipitation reaction or inhibition of both of cyclic AMP binding.
The purification procedure designed for the isolation of C from rabbit skeletal muscle (21) was found to be applicable to rabbit liver or beef heart after slight modification, and essentially homogenous preparations were obtained from each of the latter tissues. For skeletal muscle this required a 3,600-fold enrichment, for liver an 11,000-fold enrichment, and for heart a 2,800-fold enrichment i.e. calculating on the basis of the specific activity starting with crude extracts. The sodium dodecyl sulfate-gel electrophoresis patterns of the purified preparations are shown in Fig. 1. No detectable difference in the molecular weight was found for the subunits from the three sources; values ranged from 39,700 to 40,500. The specific activity was also the same for each catalytic subunit, all determinations falling in the range of 10.1 to 10.5 pmol of phosphate incorporated into histone f2b per min mg of protein.

Extrapolating from these data it appeared reasonable to assume that C from other tissues would probably have similar properties, and that, as a first approximation a molecular weight of 40,000 and a specific activity of 10.3 could be assumed in all cases; these values are used in subsequent calculations. It was recognized, however, that the possibility exists that one tissue or another could contain a catalytic subunit with different properties.

Development of Methodology for Determining the Concentration of Catalytic Subunit in Tissue Fractions by Activity Measurements

Dilution of Fractions—In order to carry out meaningful enzyme assays using crude tissue fractions it was essential that the assays be performed at high dilution. For example, with rabbit skeletal muscle extract specific activities did not become constant until at least a 50-fold dilution had been carried out (Fig. 2). Similar behaviour was exhibited by fractions from tissues other than skeletal muscle, but in all cases it was possible to dilute out apparent inhibitory factors in the crude fractions. With proper dilution of the fraction, reaction rates were linear and were proportional to the amount of enzyme used. The choice of purified histone f2b as a substrate, rather than mixed histones, was essential in order to achieve workable reaction rates at these high enzyme dilutions.

Competing Reactions—Possible interference with the protein kinase assay due to competing reactions was examined systematically under conditions chosen to resemble as closely as possible those of the phosphoryl transfer reaction itself. Phosphoprotein phosphatase activity was estimated using 32P-labeled histone f2b as a substrate at a concentration equal to that maximally obtained in the protein kinase reaction. Under these conditions less than 1% of the phosphate was released at the high dilution of tissue fractions ordinarily employed, although as much as 50% of the substrate was hydrolyzed at low dilutions with some of the fractions. Cyclic AMP phosphodiesterase assays showed that negligible amounts (2 to 5%) of this component were degraded under assay conditions. The phosphorylation of endogenous protein, determined by omitting histone f2b, did not contribute significantly to the reaction product at the dilutions used.

Recovery of Added Enzyme—The validity of the enzyme assay was further evaluated by adding homogenous catalytic subunit to diluted extract in order to see whether or not it could be recovered quantitatively. In these experiments either the amount of extract or the concentration of added catalytic subunit was varied. The added activity was recovered completely when amounts of extract corresponding to the linear part of the dilution curve were used or when increasing concentrations of catalytic subunit were added. The latter type of experiment is illustrated in Fig. 3. Identical results were obtained, whether catalytic subunit prepared from rabbit skeletal muscle, liver, or beef heart were used. Recovery measurements as described were carried out routinely for all the tissue examined.

Fig. 1. Sodium dodecyl sulfate-gel electrophoresis of purified catalytic subunits. Proteins were heated at 80° for 60 min in 2% sodium dodecyl sulfate containing 5% 2-mercaptoethanol and examined by electrophoresis using 7.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Gels were stained with Coomassie blue for 12 h and destained by diffusion in 40% methanol, 7% acetic acid. L = C from rabbit liver, 2 μg; H = C from beef heart, 3 μg; and M = C from rabbit skeletal muscle, 3.5 μg.

Fig. 2. Effect of dilution on the apparent specific activity of cyclic AMP-dependent protein kinase(s) in crude skeletal muscle extract. An extract of skeletal muscle prepared from a 3.5 to 1 homogenate was diluted as shown and 20-μl samples were assayed for protein kinase activity in the presence of cyclic AMP. At the highest dilution tested, the amount of 32P incorporated into protein due to the enzymic reaction was approximately twice the background value determined using heat-inactivated extract.

Fig. 3. Recovery of protein kinase activity on addition of catalytic subunit to tissue fraction. Increasing amounts of pure rabbit skeletal muscle C were assayed for activity in the presence (O—O) or absence ( — —) of a fixed amount of crude tissue fraction. Clockwise beginning with upper left panel: skeletal muscle extract prepared from a 3:1 homogenate and used at a 1:100 dilution; heart, kidney, brain, and liver extracts prepared from 6:1 homogenates and used at a 1:50 dilution; and particulate fraction from the brain homogenate resuspended in 2.5 volumes of buffer per kg wet weight of tissue and used at a 1:50 dilution. The protein concentrations of the undiluted extracts were 15.8 mg/ml for skeletal muscle, 5.7 mg/ml for heart, 10.1 mg/ml for kidney, 5.1 mg/ml for brain supernatant, 22.0 mg/ml for liver, and 13.6 mg/ml for the brain particulate fraction.
Specificity – It was found that at least 95% of the protein kinase activity being measured in each of the tissue fractions could be inhibited by the heat-stable protein inhibitor of cyclic AMP-dependent protein kinases (Fig. 4). This finding supported the concept that the possible contributions of other protein kinases was insignificant in these assays, since the heat-stable protein inhibitor has been shown to be specific for the cyclic AMP-dependent enzyme (30). The results obtained here differ slightly from those reported by Walsh and Ashby (30) who found a small but significant amount of protein phosphorylation using several tissue extracts that was not inhibited by the inhibitor. A probable explanation for this difference is that these workers carried out their assays at much lower dilutions of tissue extracts and appreciable phosphorylation of endogenous proteins probably occurred under their conditions. It is not unlikely that such phosphorylation reactions would be catalyzed by protein kinases other than the cyclic AMP-dependent enzyme. As noted earlier, the extent of endogenous protein phosphorylation was negligible in the present experiments.

Development of Methodology for Determining Concentration of Regulatory Subunits in Tissue Fractions

General Conditions for Assay – It was determined that the saturating concentration of cyclic AMP was 1 mM when brain extracts were used and 0.5 mM for other tissue extracts and for the particulate fraction from brain. In each instance the amount of cyclic AMP that was bound was proportional to the amount of the fraction used. No increase or loss in the amount of bound cyclic AMP was observed when samples were incubated longer than 40 min indicating that the reaction had reached equilibrium in this period of time and that there was no apparent destruction of R under these conditions. Experiments were carried out in which known amounts of cyclic AMP binding capacity in the form of pure skeletal muscle cyclic AMP-dependent protein kinase (holoenzyme) was added to tissue fractions and quantitative recovery was achieved in all cases. Finally, the Gilman procedure used in these studies was compared with two other methods of measuring cyclic AMP binding to proteins, the gel filtration method of Hummel and Dreyer (31) and the equilibrium dialysis method. Essentially identical results were obtained when each of these methods was applied to purified skeletal muscle cyclic AMP-dependent protein kinase.

Specificity – It was difficult to establish with certainty that the only proteins in tissue extracts that would bind cyclic AMP with high affinity and be retained on Millipore filters at pH 4.0 were the regulatory subunits of cyclic AMP-dependent protein kinases. An unequivocal answer to this question was not achieved for all of the tissues studied, but appreciable evidence was obtained that this was so, at least with reference to binding proteins that might be present in significant amounts. As a first approach it was shown that the known cyclic AMP binding protein, phosphofructokinase (32), was not retained by the filters under the assay conditions. This was determined in tests carried out using pure rabbit muscle phosphofructokinase. It was also shown that the cyclic AMP binding protein from Escherichia coli (33) is not retained by the Millipore filters.

Another approach to gain information as to the specificity of the binding assay for the regulatory subunit(s) was the use of specific antibody to the two known types of R. Antisera for this purpose were available in very limited amounts and it was necessary to restrict the studies that were carried out to skeletal muscle. Moreover, the experiment was carried out using bovine muscle rather than rabbit muscle. As can be seen in the experiment of Fig. 5, incubation of muscle extract with increasing amounts of antibody to R type I followed by centrifugation, removed approximately 55% of the total cyclic AMP binding capacity, while titration with antibody to R type II removed about 35% of the binding capacity. Three different muscle extracts were then treated using a combination of the two antibody preparations, 50 μl of each, and it was found that 85 ± 3.1% of the cyclic AMP binding capacity was precipitable. It is probable that even more of the total binding "activity" could have been removed had more antibody been employed, since it is evident from Fig. 5 that plateaus had not been reached at the 50-μl point.

Detection of Regulatory Subunit Already Existing as Cyclic AMP Complex in Tissue Fractions – In order to detect cyclic AMP binding proteins from bovine skeletal muscle extract with antibodies to R subunit (type I) and R subunit (type II). Bovine skeletal muscle extract was prepared by exactly the same method described for preparing this fraction from rabbit muscle and 20 μl aliquots were treated with antibody to R type I, O-O, or with antibody to R type II, • • •, as described under "Materials and Methods." After centrifugation, aliquots of the supernatant were assayed for cyclic AMP binding as described.
were made on tissue fractions as described in the text. The values are AMP binding capacities, and protein concentration determinations.

Specific protein kinase activities and cyclic AMP binding capacities of subunit recombination by mass action (see Equation 1). Additional catalytic subunit was provided since it would favor recombination of protein kinase subunits (2, 4, 10–12, 34), at least with one of the isozyme forms of the enzyme. Additional catalytic subunit was provided since it would favor subunit recombination by mass action (see Equation 1). Two different results were obtained depending on the tissue examined. When skeletal muscle extracts were used, little or no increase in binding capacity was obtained regardless of whether or not the extracts were preincubated in the absence or presence of the various components. Similar results were obtained with extracts from liver and brain and with the particulate fractions from the latter tissue. In contrast, when heart or kidney extracts were used, up to a 2-fold increase in binding capacity was observed. The extent of the increase was variable from extract to extract and usually smaller in kidney than in heart. The binding capacity also increased in the

Table I
Specific protein kinase activities and cyclic AMP binding capacities of various rabbit tissue fractions

| Tissue       | N | Specific activity (units/mg) x 10^3 | Specific binding capacity (µmol/mg) x 10^6 |
|--------------|---|-----------------------------------|-------------------------------------------|
| Skeletal muscle | 6 | 2.91 ± 0.17                       | 5.45 ± 0.25                              |
| Heart        | 6 | 3.60 ± 0.25                       | 7.20 ± 0.25                              |
| Kidney       | 6 | 2.36 ± 0.07                       | 4.20 ± 0.25                              |
| Liver        | 8 | 0.95 ± 0.10                       | 1.96 ± 0.08                              |
| Brain (extract) | 6 | 5.90 ± 0.19                       | 10.19 ± 0.10                             |
| Brain (particulate) | 4 | 3.58 ± 0.40                       | 8.50 ± 0.30                              |

Concentrations of Protein Kinase Subunits

Concentrations of Catalytic and Regulatory Subunits in Various Tissues

A preliminary examination of the distribution of cyclic AMP-dependent protein kinase activity showed that in the tissues selected for study; namely, rabbit skeletal muscle, heart, kidney, liver, and brain, nearly all of the enzyme appeared to reside in the soluble fraction, obtained from homogenates except for brain. For that tissue approximately 60% of the enzyme appeared to be soluble and 40% was sedimented at 10,000 x g. Other workers have previously noted the high concentration of insoluble enzyme in brain (35). It was arbitrarily decided, therefore, to limit the study of subunit concentration to the soluble fraction except for brain in which the particulate as well as the soluble fraction were examined. The decision to limit the investigation to the soluble fraction of tissue homogenates was not meant to infer that the cyclic AMP-dependent protein kinase present in other fractions might not be important functionally, but only that its contribution to the total cellular concentration of enzyme subunits would be very small.

Protein kinase activities in the presence of cyclic AMP (catalytic subunit activities) and cyclic AMP binding capacities were determined for various tissue fractions. The results of a series of measurements are shown in Tables I and II. It will be noted that the specific catalytic subunit activity varied considerably from fraction to fraction, being highest in the brain supernatant and lowest in liver extract (Table I). The total activity per wet weight of tissue (Table II) showed less variation. The total activity for whole brain i.e. the sum of the soluble and particulate fractions, was appreciably higher than that for the other tissues (see above). The specific cyclic AMP binding capacities showed a similar variability from fraction to fraction (Table I), but again, binding capacities based on the wet weight of tissues (Table II) were more nearly constant. Molar concentrations of catalytic subunit in the different tis-

Table II
Catalytic and regulatory subunit concentrations in rabbit tissues based on measurements of enzyme activity and cyclic AMP binding capacity

| Tissue            | N | Activity (units/kg w.wt.) | Concentration (µmol/kg w.wt.) | Concentration (µmol/kg w.wt.) | R/C |
|-------------------|---|---------------------------|-------------------------------|-------------------------------|-----|
| Skeletal muscle   | 6 | 134 ± 7                   | 0.32 ± 0.01                   | 0.30 ± 0.02                   | 0.94|
| Heart             | 6 | 162 ± 6                   | 0.38 ± 0.02                   | 0.31 ± 0.02                   | 0.82|
| Kidney            | 6 | 144 ± 4                   | 0.34 ± 0.01                   | 0.31 ± 0.02                   | 0.77|
| Liver             | 8 | 120 ± 7                   | 0.28 ± 0.02                   | 0.25 ± 0.01                   | 0.88|
| Brain (extract)   | 6 | 180 ± 12                  | 0.42 ± 0.07                   | 0.40 ± 0.01                   | 0.95|
| Brain (particulate) | 4 | 145 ± 15                  | 0.36 ± 0.01                   | 0.36 ± 0.01                   | 1.00|

For skeletal muscle, heart, kidney, and liver, the molar concentrations are arbitrarily based on the amount of subunits in the soluble fraction (see text).
 Concentrations of Protein Kinase Subunits

sues, calculated from the enzyme activity data, and molar concentration of regulatory subunit based on the binding capacity measurements, were found to be nearly identical. The R/C ratio was close to unity for all tissues (Table II).

The protein kinase activities recorded in Tables I and II were all performed in the presence of cyclic AMP but measurements on the various fractions were also carried out in the absence of the cyclic nucleotide. The ratio, activity (−cyclic AMP)/activity (+cyclic AMP), was on the average 0.1 for skeletal muscle, 0.15 for heart, 0.1 for kidney, 0.2 for brain extract as well as for the particulate fraction from this tissue, and 0.3 for liver. These results alone would require that the ratio, R/C, be as high as 0.7 to 0.9, assuming that Equation 1 accurately depicts the mechanism of activation of the cyclic AMP-dependent protein kinase.

### DISCUSSION

This study describes methods that can be used to provide estimates of the molar concentrations of catalytic and regulatory subunits of cyclic AMP dependent protein kinase(s) in crude tissue fractions. For skeletal muscle, heart, kidney, and liver, measurements carried out on the cytosol fraction alone give values that reflect the total tissue concentrations, since only a small proportion of either subunit is present in the particulate fraction. For brain, however, an appreciable amount of each subunit is present in the particulate as well as in the soluble fraction, and it is necessary to take both fractions into account in estimating the subunit content of the cell.

The methods used for measuring concentrations of the subunits depended upon (a) determinations of protein kinase activity using histone H2b as the substrate in the presence of sufficient cyclic AMP to completely dissociate the holoenzyme (see Equation 1) and (b) measurements of cyclic AMP binding capacity using the Millipore method. A necessary requirement for the validity of the catalytic subunit measurements was that the turnover number of the enzyme and that its molecular weight be identical for different tissues. This was established for three of the tissues examined and assumed to hold for the others. The reliability of the regulatory subunit determinations, as well as that of the catalytic subunit measurements, required that the methods used be specific for these proteins. Lending credence to the idea that the Millipore assay was specific for R, was the finding that essentially all of the assayable cyclic AMP binding protein present in muscle extract could be precipitated by antibody to pure type I and type II regulatory subunits. Similar experiments were not carried out, however, for the other tissue studied. Evidence that the only cyclic AMP binding protein detected in the Millipore assay was R was also derived from the results themselves. The molar ratio of R/C was found to be approximately 1 (Table II). Corroborating evidence for the presence of equimolar concentrations of C and R in crude tissue extracts can be taken from work with antibodies directed against the R subunit of bovine heart muscle protein kinase (type II) (36). In this case the ratio between catalytic activity versus either cyclic AMP binding capacity or immunoreactive R subunit remained constant throughout purification of cyclic AMP-dependent protein kinase from bovine heart muscle suggesting a similar ratio of C and R in extracts and purified preparations of holoenzyme.

This latter work was also the first to document the immunological similarity of type II R values from a number of tissues and the lack of cross-reactivity of type I and type II R values even from the same species and tissue. Since the minimal ratios of R/C, as obtained from ratios of activity (−cyclic AMP)/activity (+cyclic AMP), were in the range of 0.7 to 0.9, it is clear that the tissue fractions could not have contained appreciable amounts of some other binding protein that was retained by the Millipore filter. The specificity of the catalytic subunit determination was supported by showing that essentially all of the detectable activity in the different tissue fractions could be inhibited by the heat-stable protein inhibitor of cyclic AMP-dependent protein kinases.

The molar concentrations of regulatory subunit determined for the various tissues were surprisingly close to the reported basal levels of cyclic AMP i.e. to levels present under non-stimulated conditions in these tissues. Thus, in micromoles/kg wet weight, the basal level for cyclic AMP in skeletal muscle has been reported as 0.25 (37), whereas the regulatory subunit concentration determined here was 0.30 μmol/kg wet weight. Basal cyclic AMP levels of 0.36 μmol/kg wet weight have been reported for heart (37), 0.46 for liver (37), 0.67 for kidney (38), and 0.92 for brain (39). The range of regulatory subunit concentrations for these latter tissues was 0.75 to 0.9. The physiological implications of a situation in which the concentration of ligand and enzyme (or in this case regulatory protein) are the same order of magnitude have been discussed earlier (12).

It would appear that the relative rates of synthesis and degradation of the two type of subunits present in the cyclic AMP-dependent protein kinase are linked in such a manner that equimolar amounts are maintained within the cell. This finding makes it seem improbable that the regulatory subunit is involved in multiple regulatory events i.e. that it functions to regulate enzymes other than the protein kinase catalytic subunit.

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