Purification of Acetylcholine Receptors from *Torpedo californica* Electroplax by Affinity Chromatography†

J. Schmidt and M. A. Raftery*,‡

ABSTRACT: A variety of chromatographic resins were investigated with respect to acetylcholine receptor (AChR) purification. Resin selectivity toward AChR was found to depend on the nature and concentration of the incorporated ligand. A resin containing a quaternary ammonium function [NH-$(CH_2)_5CONH(CH_2)_3N^+(CH_3)_3$] at a concentration of 0.4×10^{-3} M was used for the large-scale purification of α-bungarotoxin-binding membrane components from the electric organ of *Torpedo californica*. The material, purified *ca.* 40-fold from electroplax membranes, binds one molecule of the neurotoxin per *ca.* 160,000 daltons of protein. Sodium dodecyl sulfate polyacrylamide gels show bands corresponding to 42,000 and 34,000 molecular weight and a minor component with an apparent molecular weight of 26,000. Complexity of the gel patterns may reflect the occurrence of several species of receptor in the tissue used.

Recent advances in the isolation of acetylcholine receptors (AChR)[1] are due to the introduction of polypeptide neurotoxins as specific and irreversible markers (Lee and Chang, 1966; Boquet *et al.*, 1966), and to the demonstration that neurotoxin-binding membrane components can be solubilized with detergents (Miledi *et al.*, 1971; Meunier *et al.*, 1971; Raftery *et al.*, 1971, 1972). In principle, this renders possible the utilization of conventional protein fractionation procedures for purposes of AChR purification. However, methods such as gel filtration, sedimentation velocity centrifugation, isoelectric focusing, and ion-exchange chromatography are of limited usefulness, because detergent-solubilized electroplax membrane proteins are very similar with respect to Stokes radius, sedimentation coefficient, isoelectric point, and chromatographic behavior on a variety of conventional chromatographic resins (Raftery *et al.*, 1971, 1972; Meunier *et al.*, 1972).

We have therefore investigated methods of affinity chromatography for AChR isolation. Chromatography on cobrotoxin–agarose, in a version that gives rise to purified toxin–receptor complex rather than free receptor, has been described elsewhere (Raftery, 1973). Since detailed structural and functional studies require isolation of neurotoxin-free receptor, we have more recently used resins which contain covalently bound quaternary ammonium functions. Such resins have been successfully used for the isolation of acetylcholinesterase from *Electrophorus electricus* electroplax (Kalderon *et al.*, 1970; Dudai *et al.*, 1972; Berman and Young, 1971; Rosenberry *et al.*, 1972) and bovine erythrocytes (Berman and Young, 1971). Employing this technique we have been able to achieve 15-fold purification of AChR from the electric organ of *Narcine entemedor* (Schmidt and Raftery, 1972).

In this paper we report experiments with several chromatographic resins and discuss requirements for resin selectivity toward AChR. We describe the use of an efficient affinity resin for the large-scale purification of AChR from *Torpedo californica* electroplax and discuss the significance of gel electrophoretic heterogeneity displayed by the purified material.

Experimental Section

Chromatographic Resins. DEAE-cellulose DE-52 was obtained from Whatman. QAE-Sephadex was from Pharmacia. Resin C (Table I) was prepared according to Berman and Young (1971). The synthesis of resin D followed the directions outlined by Cuatrecasas (1970): Sepharose 2B (Pharmacia) was activated with cyanogen bromide (Matheson Coleman & Bell) (65 mg/ml of settled resin) and reacted with spermine (Aldrich Chemical Co.) (140 mg/ml of settled resin); the resulting spermyl Sepharose was then succinylated. [^{14}C]Trimethyl(*p*-aminophenyl)ammonium chloride hydrochloride was synthesized and coupled to the carboxyl function as described by Berman and Young (1971). Ligand concentrations in resins C and D were determined by measuring incorporated radioactivity.

The ligand for the synthesis of resins E and F [*N*-(ε-aminohexanoyl)-3-aminopropyl]trimethylammonium bromide hydrobromide, was prepared as follows: 13.25 g of *N*-carbobenzoxy-ε-aminohexanoic acid, 5.75 g of *N*-hydroxysuccinimide, and 10.3 g of dicyclohexylcarbodiimide were dissolved in 300 ml of dioxane and left at room temperature for 3 hr after which the precipitate of dicyclohexylurea was filtered off. The solution was evaporated to an oily residue, triturated with ether, and dried to yield a semicrystalline product; yield 18 g of *N*-carbobenzoxy-ε-aminohexanoylsuccinimide ester (I). Compound I (14.5 g) and 3-dimethylaminopropylamine (4.1g) in 750 ml of dioxane were allowed to react overnight at room temperature. The product, *N*-carbobenzoxy-(ε-aminohexanoyl)-3-aminopropyldimethylamine (II) crystallized out of solution, was filtered and washed with dioxane and ether, and dried; yield 4 g of II. Quaternization was achieved by reaction of II, 3.5 g, with 1.9 g of methyl *p*-tosylate in 50 ml of chloroform under reflux for 3 hr. The crystalline product *N*-carbobenzoxy-(ε-aminohexanoyl-3-aminopropyl)trimethylammonium *p*-tosylate (III), was collected, washed with chloroform and ether, and dried; yield 3.3 g.

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[1] Abbreviations used are: AChR, acetylcholine receptor; α-Bgt, α-bungarotoxin; βDG, β-D-galactosidase from *E. coli*; HC,LC, rabbit IgG heavy and light chains; Pep, pepsin; Mb, myoglobin, Cbz, carbobenzoxy.

TABLE I: Nature and Concentration of Ligands in Chromatographic Resins Used.

Resin	Ligand	Concn (M)
A	$CH_2CH_2N^+H(CH_2CH_3)_2$	*Ca.* 3×10^{-1}
B	$CH_2CH_2N^+(CH_2CH_3)_2CH_2CHOHCH_3$	*Ca.* 10^{-1} [a]
C	$[NH(CH_2)_3NH(CH_2)_3NHCO(CH_2)_2CO]_2NHC_6H_4N^+(CH_3)_3$-*p*	11×10^{-3}
D	$NH(CH_2)_3NH(CH_2)_4NH(CH_2)_5NHCO(CH_2)_2CONHC_6H_4N^+(CH_3)_3$-*p*	8×10^{-3}
E	$NH(CH_2)_5CONH(CH_2)_3N^+(CH_3)_3$	2×10^{-3}
F	$NH(CH_2)_5CONH(CH_2)_3N^+(CH_3)_3$	0.4×10^{-3}

[a] Depends on degree of swelling of resin beads.

Removal of the carbobenzoxy-protecting group was effected by dissolving III, 3.0 g, in 50 ml of 50% HBr in acetic acid, leaving for 30 min at room temperature, and precipitation of the product, [*N*-(ϵ-aminohexanoyl)-3-aminopropyl]trimethylammonium bromide hydrobromide (IV) with ether. Compound IV was dried overnight in a desiccator containing NaOH pellets under high vacuum; yield 3.5 g. ^1H nuclear magnetic resonance (nmr) showed a ratio of methyl protons to methylene protons of 9:16 and ninhydrin analysis gave a value of 0.9 mol of amino groups/mol, using ϵ-aminohexanoic acid as a standard.

Resins E and F were prepared according to Cuatrecasas' procedure for the coupling of amines to agarose (1970): Sepharose 2B was activated with 50 and 25 mg, respectively, of CNBr per ml of settled resin; the washed resin was then allowed to react with the ligand (5.0 and 0.45 μmol, respectively, per ml of resin). After 3 days of stirring at 4° the resins were washed and stored in 0.02% sodium azide or used immediately. Upon acid hydrolysis (6 N HCl 100° 24 hr) 1-ml aliquots released 2.0 and 0.4 μmol of ϵ-aminohexanoic acid, respectively.

Electroplax Preparation. T. californica was purchased from Pacific Bio-Marine, Venice, Calif. A crude membrane fraction was obtained from freshly excised electric organs as described previously (Raftery *et al.*, 1972). Membranes were extracted by stirring with 1% Triton X-100 (v/v) in 10 mM sodium phosphate (pH 7.4) at 4° for 4 hr (approximately 3.5 g of original tissue/ml of extract), followed by centrifugation at 100,000*g* for 3 hr. In some experiments Triton X-100 was replaced by Emulphogene BC-720, an alkoxypoly(ethyleneoxy)-ethanol manufactured by General Aniline and Film Corp.; this detergent lacks aromatic residues thus permitting the determination of protein at 280 nm.

Assay Procedures. Toxin-binding activity was measured by a method utilizing DEAE-cellulose filter disks (Schmidt and Raftery, 1973). The specific acivity of the [^{125}I]α-bungarotoxin used ranged from 10^5 to 10^6 cpm per μg depending on the batch and the age of the preparation. To determine activity in column eluates, 0.1-ml aliquots were incubated with 25 μl each of a toxin solution of a suitable concentration, and 0.1-ml aliquots of the incubation mixtures were pipetted onto the filter disks. One unit of receptor activity is defined as that amount of receptor which binds 1 μg of [^{125}I]α-bungarotoxin (*i.e.*, 8 units = 1 nmol). Protein was determined by the method of Lowry *et al.* (1951) or by amino acid analysis. Acetylcholinesterase activity was determined by measuring the rate of acetylthiocholine hydrolysis with the method of Ellman *et al.* (1961).

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate gel electrophoresis of receptor preparations was performed according to Fairbanks *et al.* (1971). Densitometric traces were obtained using a Gilford spectrophotometer with a Model 2410 linear transport attachment.

Results

Evaluation of Resins. Elution profiles obtained by chromatography of crude membrane extract on a variety of ion-exchange and affinity resins are shown in Figure 1. Receptor purification achieved by means of DEAE-cellulose is poor. No significant improvement was seen upon replacement of the diethylaminoethyl group with the quaternary ammonium function of QAE-Sephadex (see Table I). Better results were obtained with resins C and D. The resin designed by Berman and Young for the purification of acetylcholinesterase (Berman and Young, 1971) seems to be fairly well suited for

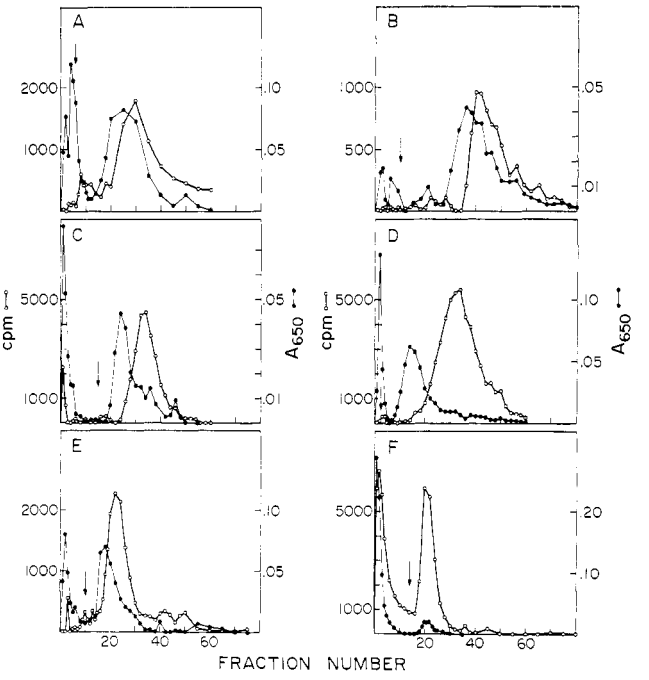

FIGURE 1: Chromatography of crude extract on various resins. Resins A–F (see Table I) were packed in Pasteur pipets (bed volume *ca.* 1.5–2 ml) and equilibrated with starting buffer (10 mM sodium phosphate (pH 7.4)–0.1% Emulphogene). Small aliquots of crude extract (2–6 units; 0.6–3.0 mg) were washed in with starting buffer. The arrow indicates the start of a linear salt gradient (total volume: 100 ml; final sodium chloride concentration: 1 M). Fractions of *ca.* 1.6 ml (*i.e.*, about a column volume each) were collected and assayed for protein and toxin-binding activity as described in the text.

TABLE II: Purification of Acetylcholine Receptor.

Fraction No.	Fraction	Protein (mg)	Act. (Units)	Sp Act. (Units/mg)	Purifcn -fold	Recov (%)
I	Membrane suspension	3700	5000	1.35		100
II	Triton X-100 extract	1400	3550	2.53	1.65	71
IIIa[a]	Purified AChR	30	1600	53	39.5	32
IIIb[b]		*Ca.* 60	2750	*Ca.* 46	*Ca.* 34	55

[a] Fractions 405–420 of Figure 2; protein determined by amino acid analysis. [b] Entire activity peak; protein value estimated from value of IIIa and Lowry profile.

acetylcholine receptor isolation as well; resin D appears to be even more promising. It is noteworthy that both sorbents display ion-exchange properties, as evidenced by the large quantity of inactive protein eluted with low concentrations of sodium chloride. This residual ion-exchange activity which presumably arises from an excess of charged sites in the resin, has to be eliminated for specificity of retention to be achieved. One way to accomplish this is further reduction of ligand concentration. As demonstrated in Figure 1 ligand "dilution" from 2×10^{-3} to 4×10^{-4} M, *i.e.*, by less than an order of magnitude, converts the weak ion exchanger E into F, a resin of high selectivity. Although this modification is accompanied by a marked reduction of receptor binding capacity (indicated by the large amount of activity eluted with the bulk of the protein) resin F was used to purify α-bungarotoxin-binding material on a large scale.

Affinity Chromatography. Electroplax (850 g) was processed and extracted as described in the Experimental Section. Results of a preparative-scale affinity chromatography are shown in Figure 2. A clean separation of receptor activity from the bulk of the inactive protein as well as from acetylcholinesterase is achieved by this method. The two-step purification procedure (involving detergent extraction of a crude membrane suspension and affinity chromatography) is summarized in Table II.

The purified product has a specific activity of about 50, *i.e.*,

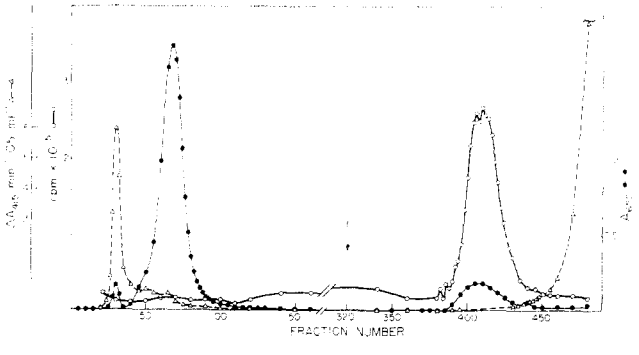

FIGURE 2: Large-scale purification of toxin-binding material. The Triton X-100 extract (fraction II) was applied to a column of resin F (5 × 80 cm), equilibrated in 10 mM sodium phosphate (pH 7.4)–0.1% Triton X-100. After sample application the resin was washed with approximately five column volumes of the same buffer before a linear gradient (4 l. of starting buffer and 4 l. of 10 mM sodium phosphate (pH 7.4)–0.5 M NaCl–0.1% Triton X-100) was started. Fractions of about 25 ml were collected and assayed for toxin binding, esterase activity and protein content. Specific activity was approximately constant between fractions 405 and 420; these fractions were pooled and designated "fraction IIIa."

there are about 160,000 daltons of protein for every bound toxin molecule. Electrophoretic analysis indicates that the purified material contains more than a single polypeptide component; two major components of apparent molecular weight 42,000 and 35,000 are observed, with an additional, faster moving band of approximate mol wt 26,000 (see Figures 3 and 4).

Discussion

Affinity Chromatography. The investigation reported here indicates that resins containing covalently linked cholinergic ligands bind not only acetylcholinesterase, but also acetylcholine receptor, although with lower affinity. Selectivity toward these macromolecules is lost when a critical ligand concentration is exceeded, *i.e.*, when the resin begins to act as a nonspecific ion exchanger. This effect was first described by Kalderon *et al.* (1970) who noticed loss of acetylcholinesterase affinity resin specificity upon raising the ligand concentration from 0.15×10^{-3} to 1.6×10^{-3} M. Their values are close to those of the effective resin F (0.4×10^{-3} M) and the nonspecific resin E (2.0×10^{-3} M), respectively, of the work reported here. The critical ligand concentration is approximately 10^{-3} M in both cases which corresponds to a nearest-neighbor distance of about 100 Å, assuming ligands distributed evenly throughout the resin and positioned at the intersections of a cubic lattice. It is conceivable that, at concentrations below 10^{-3} M, ligands are spaced sufficiently far apart to prevent nonspecific proteins from interacting with more than one charged group at a time; only molecules possessing choline recognition sites rather than nonspecific negatively charged functions would then be retained on the resin.

This interpretation also explains why resins C and D are less efficient than resin F, although their quaternary ammonium functions are attached to the resin matrix by much longer flexible "arms" and therefore should be particularly well suited to interact with large macromolecules. It is their high ligand concentration that gives rise to the adsorption of nonspecific proteins. Isolation of AChR using such resins could conceivably be effected by eluting with low concentrations of specific ligands. Alternatively, it appears that high selectivity of an affinity resin can only be achieved by reducing the concentration of resin-bound inhibitor, at the cost of low receptor capacity. However, this is not a severe drawback as resin synthesis and chromatographic procedures can be scaled up fairly easily.

Receptor Purification. The results reported here are remarkable with respect to the quantity as well as the purity of the toxin binding material isolated. Purification of AChR from electroplax membranes is about 40-fold (corresponding to an

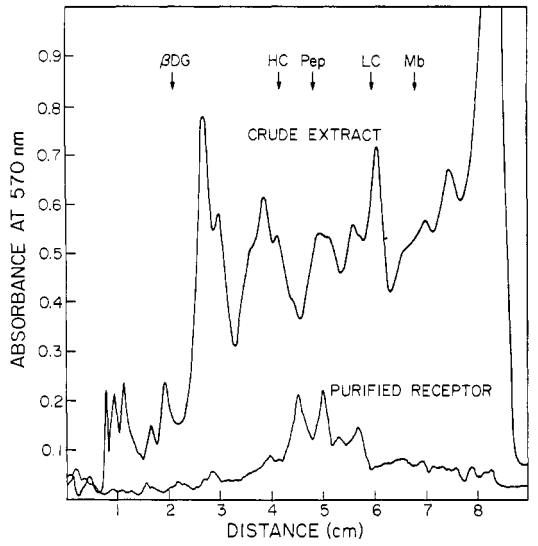

FIGURE 3: Gel electrophoresis of fractions II and IIIa (see Table II). About 320 μg of crude extract and *ca.* 7 μg of purified receptor, both fractions representing approximately 0.2 g of original electroplax were electrophoresed and scanned as described in Methods. Arrows indicate positions of protein markers used for calibration.

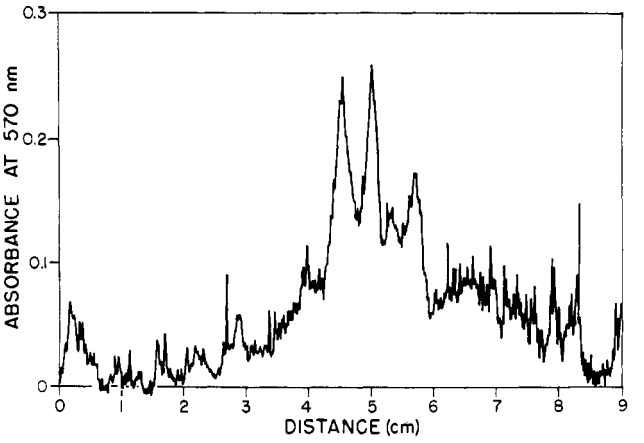

FIGURE 4: Gel electrophoresis of fraction IIIa. Approximately 7 μg of purified receptor was electrophoresed as described in legend to Figure 3 and scanned at high sensitivity.

approximate 300-fold purification from tissue homogenate) and the amount obtained is sufficient for a variety of structural studies.

Although affinity chromatography appears superior to other purification techniques, it does not give rise to a unique molecular species. Analysis of the purified material by sodium dodecyl sulfate gel electrophoresis (Figures 3 and 4) indicates that it is not composed of a single polypeptide chain. This may be due to contamination with inert proteins, as some residual ion-exchange activity of the affinity resin cannot be ruled out; clearly the leading edge of the receptor peak (Figure 2) is of lower specific activity than the center part. Alternatively, the apparent heterogeneity may reflect receptor multiplicity. Multiple ligand binding sites in electric tissue of *Torpedo marmorata* have been described by Eldefrawi *et al.* (1971) and two toxin binding components in *T. californica* have recently been distinguished by Raftery *et al.* (1972). Further purification, *i.e.*, increasing specific activity (units per milligram) would then be impossible; one could only hope to fractionate the product obtained by affinity chromatography into various toxin-binding constituents. This might be achieved by techniques that have permitted resolution of toxin-binding activity into several peaks (Raftery *et al.*, 1972). Preliminary studies utilizing isoelectric focusing and gel electrophoresis without sodium dodecyl sulfate have yielded promising results in this regard (M. Martinez, J. Schmidt, and M. A. Raftery, unpublished data).

Finally, multiple bands in polyacrylamide gels may reflect a complex subunit structure of the receptor; however, this would require simple stoichiometric relationships between individual components. It is conceivable that the two slower moving bands are related in such a fashion. Similar electrophoresis patterns have also been observed for acetylcholine receptors isolated from *Narcine entemedor* electroplax (Schmidt and Raftery, 1972) and for AChR–α-Bgt complexes isolated from *T. californica* (Raftery, 1973).

Although it is likely that isolation of a single homogeneous receptor has not been accomplished, statements concerning the possible subunit molecular weight are possible. A comparison of the gel electrophoresis pattern of purified receptor with that of crude membrane extract clearly indicates that out of a variety of proteins over a wide spectrum of molecular weights only a few species with subunit molecular weights in the neighborhood of 40,000 daltons are selected (Figure 3). Even when large sample sizes are electrophoresed, only traces of material are observed in the 80,000 molecular weight region. This is in disagreement with the subunit molecular weight of the AChR of *T. marmorata* as proposed by Miledi *et al.* (1971) and is also lower than that reported by Meunier *et al.* (1972). Species differences are unlikely to account for the discrepancy, as the subunit molecular weight of the acetylcholine receptors from *N. entemedor*, another torpedinid, also appear to fall in the range of 30,000–45,000 (Schmidt and Raftery, 1972). Differences in the relative concentrations of each polypeptide component may be accounted for by the particular chromatographic fractions utilized for gel electrophoresis.

Acknowledgments

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Comparison of Catalytic Units of Muscle and Liver Adenosine 3′,5′-Monophosphate Dependent Protein Kinases†

Hirohei Yamamura, Kaoru Nishiyama,*‡ Reiko Shimomura,§ and Yasutomi Nishizuka

ABSTRACT: Rabbit skeletal muscle adenosine 3′,5′-monophosphate (cAMP) dependent protein kinase is resolved into two fractions by ammonium sulfate fractionation followed by DEAE-cellulose and hydroxylapatite column chromatography. These fractions are activated by cAMP in a similar manner resulting in the release of common active protein kinase. The muscle active kinase shows essentially identical kinetic and catalytic properties with rat liver active protein kinase which is prepared as described previously (Kumon, A., Nishiyama, K., Yamamura, H., and Nishizuka, Y. (1972), *J. Biol. Chem. 247*, 3726). Both muscle and liver protein kinases phosphorylate the same specific seryl and threonyl residues of histone and protamine, and are capable of phosphorylating muscle glycogen phosphorylase *b* kinase and glycogen synthetase resulting in the activation and inactivation of the respective enzymes. The active kinases and regulatory proteins obtained from these tissues are crosswise reactive, and either one of these active kinases is inhibited by regulatory protein from the homologous as well as from the heterologous tissue. Upon isoelectrofocusing electrophoresis either muscle or liver active kinase is resolved further into two components of isoelectric points of pH 7.4 and 8.2. Nevertheless, each component is catalytically active and indistinguishable from the other in kinetic and catalytic properties. The two components show apparently the same molecular weights as estimated by gel filtration procedure. The exact nature of this microheterogeneity has remained unexplored.

A preceding report from this laboratory (Kumon *et al.*, 1972) has described that in rat liver multiple cAMP[1]-dependent protein kinases may be distinguished which differ from each other in their associated R proteins, and that cAMP activates all kinases by binding to R proteins in an allosteric manner and then releases apparently common active protein kinase. A preliminary survey has shown that at least two cAMP-dependent protein kinases may be obtained from most tissues and organs including rabbit skeletal muscle, rat brain, and bovine adrenal gland (Yamamura *et al.*, 1971a). Reimann *et al.* (1971a) have also described that two cAMP-dependent protein kinases obtained from rabbit skeletal muscle yield one type of active protein kinase upon sucrose density gradient centrifugation in the presence of the cyclic nucleotide. Comparative studies have revealed that active protein kinases obtained from various mammalian tissues including rabbit skeletal muscle and rat liver show closely similar kinetic and catalytic properties. The active kinases exhibit rather broad and identical substrate specificities, and incorporate the terminal phosphate of ATP into several regulatory enzymes and functional proteins. A plausible evidence may imply, at least in part, a molecular basis of pleiotropic action of cAMP in controlling several biochemical reactions in each tissue. The present paper is a full account for the preliminary report published earlier (Yamamura *et al.*, 1971b) and will describe the experiments of such comparison and cross-reactions of rabbit skeletal muscle and rat liver cAMP-dependent protein kinases as representatives.

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‡ Graduate Fellow of California Foundation of Biochemical Research.
§ On leave from Tokiwa College, Kobe, Japan.

[1] Abbreviations used are: cAMP, adenosine 3′,5′-monophosphate: R protein, regulatory protein.

Experimental Section

Materials and Chemicals. Wistar albino rats (200–250 g) and domestic rabbits (3–3.5 kg) maintaining *ad libitum* on