and Wolff, 1980). NECA in a concentration 10^{-7} M produced a larger response than $5x10^{-7}$ adenosine. This response was also reversible. PIA was the least effective of the three compounds tested. A final infusion of 10^{-5} M of adenosine showed that the sensitivity of the preparation had not substantially changed during the course of the experiment. Two other experiments produced similar results. As in our previous observations (Erlij, Silva and Reinach, 1978) chloride concentration in the resting and stimulated samples were not different.

This sequence of activity, NECA > adenosine > PIA, is characteristic of adenosine responses associated with activation of adenylate-cyclase (Ra receptor). While inhibition of adenylate cyclase is associated with the sequence PIA > adenosine > NECA; (Ri receptor; Londos, Cooper and Wolff, 1980). Another purine substituted analogue, 2 methyl-adenosine was tested in four perfused glands. Three of them were clearly stimulated to secrete by 10^{-5} M methyladenosine and all four responded markedly when 10^{-4} M was used; the increases in rate ranged between 2 and 8 times the resting rate ($\bar{x} \pm$ S.E. = 4.84 $\pm$ 1.47).

We also tested the ribose modified analogues 2' Deoxyadenosine and 2'3' isopropylidene-adenosine. Concentrations of 2' Deoxyadenosine ranging between 10^{-5} M and 10^{-4} M were without an effect in 8 glands. Of three experiments with 2'3' isopropylidene-adenosine, only one was stimulated to secrete with 10^{-4} M.

We tested two compounds that penetrate the cell membrane very slowly: adenosine 5'-0- thiomonophosphate and adenyl $(3'\ 5')_9$ - adenosine. Adenosine 5'-0-thiomonophosphate (10^{-5} M) produced about a three-fold stimulation of secretion in four preparations ($\bar{x} \pm$ S.E. = 3.49 $\pm$ 1.05). Adenyl $(3'5')_9$ adenosine, (10^{-5} M) was without effect in 3 preparations. The present experiments suggest a number of points concerning the adenosine effects. First, based on the sequence NECA > Adenosine > PIA it seems that a site analogous to the Ra site of other cell types is involved in the response (Londos, Cooper and Wolff, 1980). This site is usually associated with activation of adenylate cylase. In agreement with this pattern, we have found in a single experiment in which we measured cyclic AMP content in slices of rectal gland that 10^{-4} M adenosine increased the content of cyclic AMP from 5 to 12.9 pmoles/mg protein. Clearly this finding merits additional experiments. A second point, is that the P site, a nucleoside receptor site different from the Ra and Ri sites, and associated with a catalytic subunit of adenylate cyclase is not involved in the regulation of secretion in the rectal gland, since compounds that are effective activators of it, such as 2' deoxyadenosine and 2'3' isopropylidene-adenosine, had no effects or were poor stimulators of secretion.

Finally the effects of 5'-0-Thiomono phosphate, a compound that very likely does not penetrate the cell membrane, are in further agreement with the notion that the action of adenosine is the result of an interaction with a receptor on the outer surface of the cell membrane. This work was supported by Grants from the New York Heart Association and the NIH (AM 24064 and HLB 10384). We thank Dr. C. Londos for a generous gift of PIA and NECA and useful discussion of our results.

PROTEIN PHOSPHORYLATION IN THE DOGFISH RECTAL GLAND

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Cyclic AMP stimulates active Cl secretion in the rectal gland of Squalus acanthias (Stoff et al., J. Exp. Zool., 199: 443, 1977). The major mechanism whereby cyclic AMP is thought to accomplish its second messenger role is by activation of cyclic AMP-dependent protein kinases leading to phosphorylation of tissue-specific substrate proteins (Greengard, Science, 199: 146, 1978). This hypothesis has received support from the finding that the regulatory subunits of cyclic AMP-dependent protein kinases are the sole intracellular receptors for this

nucleotide, as determined by photoaffinity labeling in a variety of eukaryotic cells (Walter and Greengard, Handbook of Experimental Pharmacology, J.A. Nathanson and J.W. Kebabian, eds., Springer-Verlag, Berlin, in press, 1981). In view of the important role of cyclic AMP in the physiological response of the rectal gland it was of interest to examine the nature of the cyclic AMP-dependent protein kinases and their substrates in various subcellular fractions of this tissue. Because of the possible role of intracellular Ca^{2+} in the secretory response [as has been described in a number of other Cl-transporting epithelia, e.g., rabbit colon (Frizzell, J. Memb. Biol., 35: 175, 1977)] the presence of a recently discovered class of protein kinase regulated by the Ca^{2+}/calmodulin complex (Schulman and Greengard, Proc. Natl. Acad. Sci. U.S.A., 75: 5432, 1978) was also investigated.

Materials and Methods

Rectal glands were excised from adult dogfish and either briefly washed in dogfish Ringers at 4°C or perfused for 30 min with Ringers until their secretion rate decreased to the levels characteristic of unstimulated glands in the "basal" state, approximately 200 µl/h/gww. Similar results were obtained with both preparations. The glands were minced into small pieces with a pair of scissors and then homogenized in a Teflon-glass homogenizer in a solution containing 0.3 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 10 mM Tris·Cl, pH 7.4 (1:4 wt/vol). The resultant crude homogenate was filtered through cheesecloth, diluted 1:1 with the same buffer and centrifuged (International) at 1,200 x g for 8 min. The resulting pellet ("nuclear" fraction plus unbroken cells) was discarded and the supernatant, termed the "homogenate," centrifuged at 10,000 x g for 15 min (Sorvall SS34 rotor). The grey pellet was termed the "mitochondrial" fraction and was resuspended to the original homogenate volume in 10 mM Tris·Cl, pH 7.3. The supernatant was then centrifuged at 150,000 x g_{av} for 40 min (Beckman 50 Ti rotor) yielding a pale yellow pellet ("microsomal" fraction) and a clear supernatant ("cytosol" fraction). The microsomal pellet was resuspended in 1/5 the original homogenate volume of 10 mM Tris·Cl, pH 7.3. Although a systematic marker enzyme analysis was not performed in this study, the results of gel analysis suggested that each fraction contained a distinct set of proteins, as reflected both in the Coomassie blue staining patterns (e.g., see Fig. 4), and in the array of phosphorylated proteins (e.g., Figures 1 and 2). The microsomal fraction was found to be enriched in Na/K-ATPase (located on the basolateral membrane borders in this tissue, see Eveloff et al., J. Cell Biol., 83: 16, 1979) as incidated both by the enrichment of a 100,000 dalton molecular weight protein in this preparation (this protein being the major staining band in this fraction, See Figures 2 and 4) (cf. Hokin et al., J. Biol. Chem., 248: 2593, 1973) and by preliminary measurements of Na/K-ATPase activity in this preparation (kindly performed by Dr. B. Forbush, Department of Physiology, Yale University School of Medicine) indicating a sodium dodecyl sulfate (SDS)-activated specific activity of 326 µmol P_i/mg protein/hr, comparable to that observed by Skou and Esmann (Biochem. Biophys. Acta, 567: 436, 1979) and representing an approximately 5-fold enrichment over the homogenate value.

Samples of the four fractions (homogenate, cytosol, mitochondria, and microsomes) were taken for analysis of their cyclic AMP-binding proteins by photoaffinity labeling and for endogenous protein phosphorylating capacity using [γ^{-32}P]ATP. The photoaffinity labeling technique using 8-N_3-[^{32}P]cyclic AMP (ICN; 83 Ci/mmol) was that of Walter et al., (J. Biol. Chem., 252: 6494, 1977). The composition of the reaction mixture was 50 mM Tris·Cl (pH 7.3), 5 mM $MgCl_2$, 0.5 mM isobutylmethylxanthine (IBMX, a phosphodiesterase inhibitor), 5 mM β-mercaptoethanol, 1 µM 8-N_3-cyclic AMP (1-2 µCi), in the presence and absence of 10 µM cold cyclic AMP, plus 40 µl of the respective cell fractions in a final volume of 100 µl. This mixture was preincubated on ice for 30 min in a procelain multiwell plate prior to irradiation of the samples at a distance of 8 cm using a short-wave UV lamp (UVS mineralight). After 10 min the reaction was stopped by the additon of 50 µl "SDS-stop solution" (Alper, Palfrey,

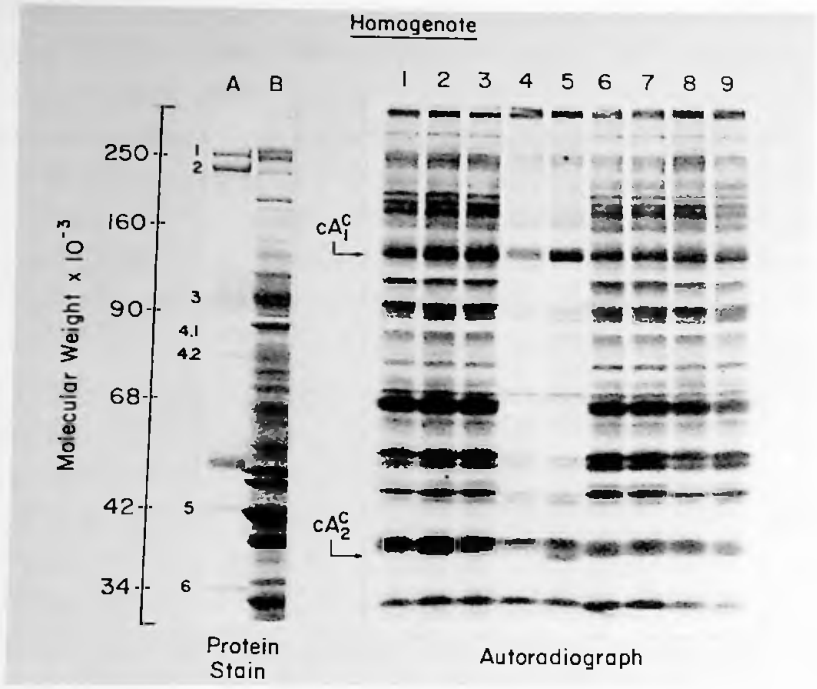

Figure 1.--Endogenous phosphorylation of rectal gland homogenate (250 μg) with [γ-^{32}P]ATP. Left: protein staining pattern of (B) homogenate, compared to (A) human erythrocyte membrane proteins (used as molecular weight markers; numbered as described by Steck, J. Cell Biol., 62: 1, 1974). Right: autoradiograph (exposure: 12 hr, Kodak XR-5 film). Lanes: (1) control; (2) 20 μM cyclic AMP; (3) 20 μM cyclic GMP; (4) 1 mM EGTA; (5) 1 mM EGTA + 20 μM cyclic AMP; (6) 1 mM EGTA + 1.1 mM $CaCl_2$; (7) same as 6 + 1 μg calmodulin; (8) control + 20 μg cyclic AMP-dependent protein kinase inhibitor. cA_1^c and cA_2^c refer to two (cytosolic) proteins whose state of phosphorylation is increased by cyclic AMP.

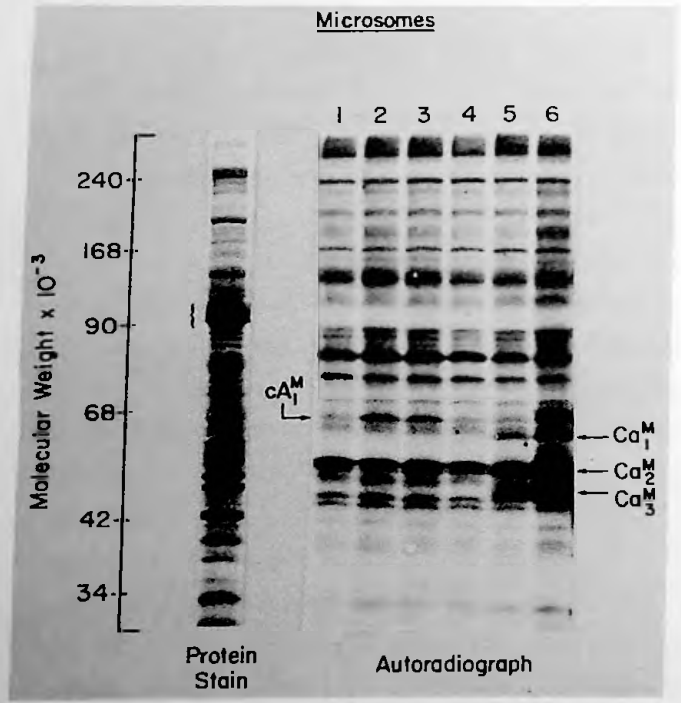

Figure 2.--Endogenous phosphorylation of rectal gland "microsomes" (85 μg) with [γ-^{32}P]ATP. Left: protein stain. Right: autoradiograph (exposure, 48 hr). Lanes: (1) control; (2) 20 μM cyclic AMP; (3) 20 μM cyclic GMP; (4) 1 mM EGTA; (5) 1 mM EGTA + 1.1 mM $CaCl_2$; (6) same as (5) + 1 μg calmodulin. Note the major protein in this preparation has a M_r of ~100,000 daltons and probably corresponds to the heavy subunit of the Na/K-ATPase (bracket on protein stain). In the autoradiograph the positions of the major cyclic AMP-stimulated band cA_1^M and Ca^{2+}/calmodulin-stimulated bands (Ca_1^M, Ca_2^M and Ca_3^M) are indicated.

De Reimer and Greengard, J. Biol. Chem., 255: 11029, 1980) and the samples boiled for 1 min, then electrophoresed on 10% SDS-polyacrylamide slab gels using the method of Laemmli (Nature, 227: 680, 1970). The gels were stained and destained, dried and exposed to X-ray film to produce an autoradiograph.

Endogenous protein phosphorylation was assayed by incubating cell fractions (40 μl) in a reaction mixture consisting of (final concentrations): 50 mM Tris·Cl (pH 7.3), 10 mM $MgCl_2$, 1 mM IBMX, with some samples containing one or more of the following: cyclic AMP (20 μM), EGTA (1 mM), $CaCl_2$ (1.1 mM), bovine brain calmodulin (10 μg/ml, purified according to Watterson et al., J. Biol. Chem., 251: 4501, 1976), bovine heart cyclic AMP-dependent protein kinase catalytic subunit (40 mM) (a gift of Dr. Angus C. Nairn), and rabbit skeletal muscle catalytic subunit inhibitor (200 μg/ml) (a gift of Dr. Werner Schiebler). The reaction mixture (final volume, 100 μl) was preincubated at 20°C for 1 min, and [γ-^{32}P]ATP was then added (20 μM, specific activity 1-2 x 10^4 cpm/pmol) and the reaction allowed to continue for 1 min before stopping the reaction as described above for photoaffinity labeling. The phosphorylated proteins were electrophoresed on 8% SDS-polyacrylamide gels as detailed above, then dried and autoradiographed. Estimation of the molecular weights of specific proteins on the gels was achieved by comparison with known molecular weight standards. On each gel separate lanes containing human erythrocyte membrane proteins were electrophoresed (e.g., Figure 1); the molecular weights of these proteins had been previously calibrated (H.C. Palfrey, unpublished results).

Identification of endogenous calmodulin in rectal gland extracts was performed as described by Alper et al., (op. cit.).

Results

The endogenous phosphorylation patterns of two of the four fractions prepared as described above are shown in Figures 1 and 2. The most striking feature of this reaction in the homogenate (Fig. 1) and cytosol (not shown) preparations was the large reduction in overall phosphorylation capacity on addition of EGTA, a Ca^{2+}-specific chelating agent (Fig. 1 compare lanes 1 and 4). This suggested that sufficient Ca^{2+} was present in the "control" (although no exogenous Ca^{2+} was added) to support the Ca^{2+}-dependent reactions and, in both fractions, re-addition of Ca^{2+} in excess of the EGTA present restored the original "control" pattern (compare lane 6 with lane 1 in Fig. 1). Addition of exogenous calmodulin, a Ca^{2+}-binding protein known to stimulate various protein kinases (Schulman and Greengard, op cit), failed to increase the effect of Ca^{2+} alone (lane 7 in Fig. 1). However, it was suspected that the Ca^{2+}-dependent protein kinase activity was supported by endogenous calmodulin already present in the extract. This hypothesis is probably correct as (a) shark rectal gland extracts contained a protein which comigrated with authentic bovine brain calmodulin on alkaline-urea polyacrylamide gels (Fig. 3) and (b) both homogenate and cytosol Ca^{2+}-dependent protein phosphorylation could be completely inhibited by trifluperazine (100 μM), an antipsychotic drug known to interfere with the activation of calmodulin-dependent enzymes by binding to calmodulin (see Cheung, Science, 207: 19, 1980, for review) (not shown). These results suggested the presence of a highly active calmodulin-dependent protein kinase(s) in rectal gland cytosol responsible for phosphorylating a large number of cytosolic proteins.

Addition of cyclic AMP (20 μM) to homogenate and cytosol samples, either in the presence or absence of EGTA, resulted in the phosphorylation of two major specific substrate proteins of M_r 135,000 and 37,000 daltons (cA_1^c and cA_2^c, Fig. 1). The phosphorylation of these proteins was also slightly stimulated by cyclic GMP at a concentration (20 μM) which would be expected to activate the cyclic AMP-dependent protein kinase. No phosphorylation specific to cyclic GMP addition could be detected when lower concentrations of cyclic GMP,

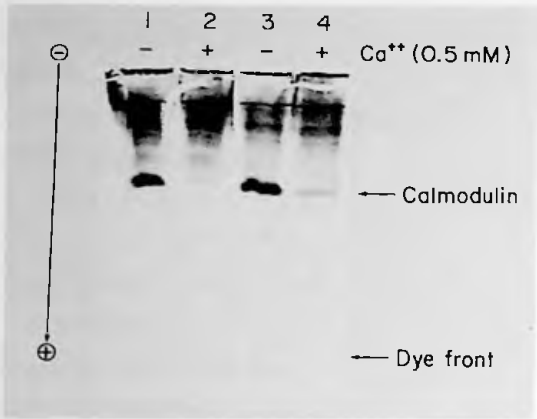

Figure 3.--Demonstration of calmodulin in rectal gland tissue. The highly acidic calmodulin is the fastest running protein in these alkaline-urea gels and can be easily recognized by its retardation due to binding to other cellular proteins upon addition of Ca^{2+}. Lanes 1 and 2 consist of rectal gland extract along, in the presence of EGTA (Lane 1), or excess $CaCl_2$ (Lane 2). Lanes 3 and 4 are identical to 1 and 2 except that 5 μg of pure bovine brain calmodulin has been added. Note that the added calmodulin runs in the same position as the intensely stained band in the rectal gland extract.

which would not activate cyclic GMP-dependent protein kinase, were used (data not shown). The phosphorylation of these proteins was presumably due to the activation of a cytosolic cyclic AMP-dependent protein kinase (cf. Fig. 4), and the substrates were restricted to the cytosol, not appearing in either particulate fraction. Visualization of cyclic AMP-stimulated protein phosphorylation was greatly enhanced by inclusion of EGTA, reducing the

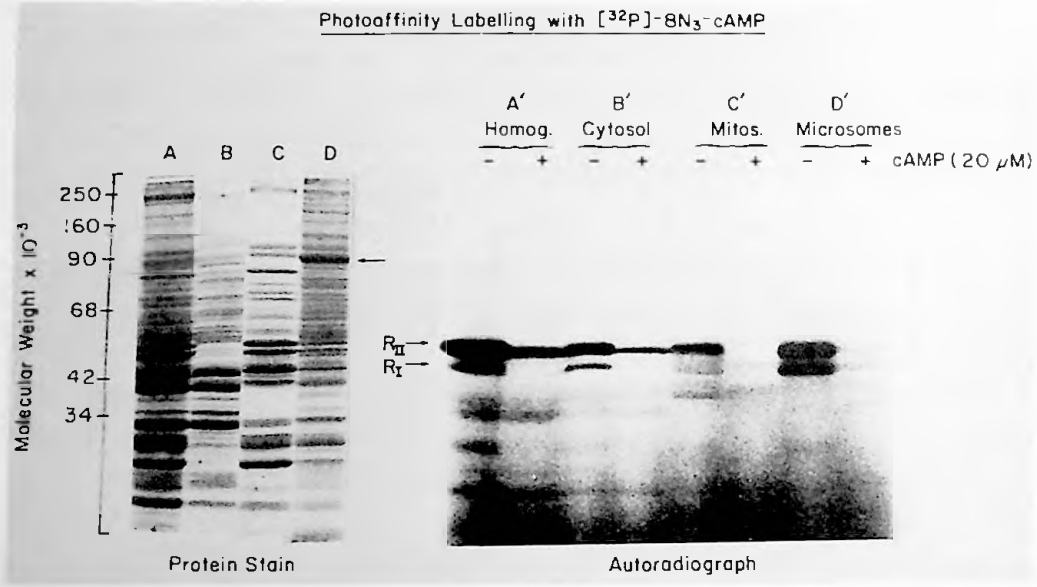

Figure 4.--Photoaffinity labeling of rectal gland fractions with 8-N_3-[^{32}P]-cyclic AMP. The protein concentrations were homogenate (250 μg), cytosol (140 μg), mitochondria (235 μg), microsomes (85 μg). Left: comparison of protein staining patterns of the four fractions (A) homogenate, (B) cytosol, (C) mitochondria, (D) microsomes. An arrow marks the putative Na/K-ATPase subunit. Right: autoradiograph (exposure, 24 hr). Alternate lanes contain cold 20 μM cyclic AMP to demonstrate competition with the 8-N_3-cyclic AMP in labeling the two subunits of the cyclic AMP-dependent protein kinase (R_I and R_{II}). The incomplete competition seen in lanes A'+ and B'+ is probably due to the presence of a highly active phosphodiesterase (evidently incompletely inhibited by 0.5 mM IBMX) which can hydrolyze some of the cold cyclic AMP included in the assay mixture, reducing its effective concentration. The faint lower molecular weight labeled bands seen primarily in A' were tentatively identified as proteolytic digestion products of R_I and R_{II}.

"background" due to Ca^{2+}-dependent protein kinase activity. Inclusion of purified cyclic AMP-dependent protein kinase inhibitor did not affect the basal pattern of phosphorylation in either homogenate (Fig. 1, lane 8) or cytosol

fractions (not shown), indicating that this phosphorylation was not mediated by the free catalytic subunit of this enzyme. Very little phosphorylation of proteins was detected in the "mitochondrial" fraction under any of the conditions tested (not shown). The very low level of Ca^{2+}/calmodulin-dependent protein phosphorylation in this fraction suggested that the contamination by other membranes was small. Of particular relevance to the physiology of the rectal gland were those results obtained with the "microsomal" fraction. This fraction presumably consists largely of basolateral plasma membranes and intracellular endoplasmic reticulum membranes. The presence of basolateral membranes was suggested by the enrichment in the putative 100,000 dalton Na/K-ATPase polypeptide, which was the major protein seen in stained gels from this fraction. This enzyme is highly localized in this membrane compartment in the rectal gland (Eveloff et al., op cit.) and preliminary measurements suggested a high activity of this enzyme in the "microsomal" preparation studied here (see above). Addition of cyclic AMP to this preparation led to a selective increase in ^{32}P incorporation into a 64,000 dalton protein which comigrated with a prominent Coomassie-blue stained band on the gel (Fig. 2). Previous work has suggested the presence on this membrane of a NaCl co-transport system crucial to the transepithelial movement of Cl in this tissue. Loop diuretics specific for cation-anion co-transport systems seem to act on the basolateral border in the rectal gland (Palfrey et al., Bull. MDIBL, 19: 58, 1979) and electrolyte measurements support the possibility that this system may be one of the targets for cyclic AMP activation of transport (Silva et al., Bull. MDIBL, 19: 70, 1979). Thus, the discovery of a specific substrate for a cyclic AMP-dependent protein kinase in a subcellular fraction enriched in basolateral membranes is of considerable interest. That this protein is a major substrate for this enzyme was confirmed by addition of the catalytic subunit of the purified cyclic AMP-dependent protein kinase (from heart) to the membrane preparation in the absence of added cyclic AMP. The 64,000 dalton protein was a major target for this enzyme (data not shown). In contrast to results obtained with the cytosolic fraction, addition of EGTA did not result in significant changes in protein phosphorylation in the microsomal fraction, probably indicating that endogenous calmodulin had been effectively removed during the separation of the membranes. Addition of Ca^{2+} in the presence of exogenous calmodulin led to the phosphorylation of three membrane substrates (Ca^{M}_{1}, Ca^{M}_{2}, Ca^{M}_{3}, Fig. 4) of M_r 60,000, 52,000 and 49,000 which were clearly distinct from the M_r 64,000 cyclic AMP-stimulated protein. The rectal gland can thus be added to other tissues known to have membrane associated Ca^{2+}/calmodulin-dependent protein kinases (Schulman and Greengard, op cit.; Alper et al., op cit.).

Photoaffinity labeling revealed the presence of cyclic AMP-dependent protein kinase regulatory subunits in each subcellular fraction, with relatively small amounts appearing in the mitochondrial fraction (Fig. 4). The estimated molecular weights of the two major 8-N_3-cyclic AMP-binding proteins found in the rectal gland, 46,000 and 52,000 daltons, agree well with the reported molecular weights of the two regulatory subunits (designated R_I and R_{II}) found in a variety of other tissues (Walter and Greengard, op cit.).

As with many other tissues, the two types of regulatory subunits are differentially distributed between the cytosol and particulate fractions of the rectal gland. Thus, R_{II} is the predominant form in the cytoplasm, while the microsomal fraction contains almost equivalent amounts of R_I and R_{II}.

In conclusion, these results provide a biochemical correlate of the physiological effects of cyclic AMP, and of the hormone which raises intracellular cyclic AMP levels (VIP, Stoff et al., Bull. MDIBL, 17: 66, 1977), in the dogfish rectal gland. Although the nature of the phosphorylated protein substrates described here is as yet unknown, it is encouraging that phosphorylation of a specific substrate in a membrane-enriched fraction could be increased by activation of an endogenous cyclic AMP-dependent protein kinase. Further experiments will be directed towards defining intact cell systems where protein phosphorylation can be studied in parallel with measurement of the physiological response of fluid secretion in the rectal gland.