Protein Kinases of the Chick Oviduct: A Study of the Cytoplasmic and Nuclear Enzymes†

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ABSTRACT: Subcellular fractionation of oviduct tissue from estrogen-treated chicks indicated that the bulk of the protein kinase activity of this tissue is located in the cytoplasmic and nuclear fractions. DEAE-cellulose chromatography of cytosol revealed a major peak of cAMP stimulatable activity eluting at 0.2 M KCl. This peak was further characterized and found to exhibit properties consistent with cytoplasmic cAMP dependent protein kinases isolated from other tissues: it had a K_m for ATP of 2×10^{-5} M, preferred basic proteins such as histones, as substrate, and had a M of 165 000. Addition of 10^{-6} M cAMP caused the holoenzyme to dissociate into a cAMP binding regulatory subunit and a protein kinase catalytic subunit. Extraction of purified oviduct nuclei with 0.3 M KCl released greater than 80% of the kinase activity in this fraction. Upon elution from phospho-cellulose, the nuclear extract was resolved into two equal peaks of kinase activity (designated I and II). Peak I had a sedimentation coefficient of 3S and a K_m for ATP of 13 μM, while peak II had a sedimentation coefficient of 6S and a K_m for ATP of 9 μM. Both enzymes preferred α-casein as a substrate over phosvitin or whole histone, although they exhibited different salt-activity profiles. The cytoplasmic and nuclear enzymes were well separated on phospho-cellulose and this resin was used to quantitate the amount of cAMP dependent histone kinase activity in the nucleus and the amount of casein kinase activity in the cytosol. Protein kinase activity in nuclei from estrogen-stimulated chicks was found to be 40% greater than hormone-withdrawn animals. This increase in activity was not due to translocation of the cytoplasmic protein kinase in response to hormone, but to an increase in nuclear (casein) kinase activity. During the course of this work, we observed small but significant amounts of cAMP binding activity very tightly bound to the nuclear fraction. Solubilization of the binding activity by sonication in high salt allowed comparison studies to be performed which indicated that the nuclear binding protein is identical with the cytoplasmic cAMP binding regulatory subunit. The possible role of the nuclear binding activity is discussed.

Previous work from several laboratories has suggested that most mammalian cytoplasmic protein kinases are stimulated by cAMP[1] and preferentially phosphorylate basic proteins, such as histones, in vitro (Walsh et al., 1968; Chen and Walsh, 1971; Majumder and Turkington, 1972). Krebs and co-workers (1972) have shown that cAMP activates these protein kinases by binding to a regulatory subunit (R), thereby causing the dissociation of an inactive holoenzyme (RC) into regulatory and catalytic (C) subunits: RC + cAMP $\leftrightharpoons$ R·cAMP + C. In contrast to the cytoplasmic enzymes, protein kinases from nuclear fractions have generally been found to be unresponsive to cAMP and to prefer acidic proteins, such as casein and phosvitin, as in vitro substrates (Takeda et al., 1971; Ruddon and Anderson, 1972; Kamiyama and Dastugue, 1971; Desjardins et al., 1972, 1975a,b). Given the nature of the nuclear protein kinases, the report several years ago by Langan (1971) must be considered paradoxical. This author found that glucagon or dibutyryl cAMP induced in rat liver the phosphorylation of a specific seryl residue in histone F_1 in vivo. An explanation for this finding has recently been afforded by the work of Palmer et al. (1974) and Castagna et al. (1975), who showed that glucagon or dibutyryl cAMP elicits a redistribution of the protein kinase catalytic subunit from the cytoplasmic to nuclear fraction in rat liver. This novel mechanism, which would allow cytoplasmic protein kinases to influence nuclear events such as transcription, has also been reported to occur in calf ovary in response to chorionic gonadotrophins (Jungmann et al., 1974a,b).

In view of these findings, and in light of recent work indicating that steroid hormones can induce the phosphorylation of nuclear proteins (Ahmed, 1971; Ahmed and Ishida, 1971; Ahmed and Wilson, 1975; Allfrey et al., 1973; Liew et al., 1973), we decided to investigate the possible role of protein kinases in the estrogen-induced growth and differentiation of the chick oviduct. Studies in this laboratory in the last several years have underscored the advantages of the oviduct system in investigating steroid hormone action (O'Malley et al., 1969; O'Malley and Means, 1974; Means et al., 1975). In the present work, we have carried out studies in the oviduct designed to: (1) establish the subcellular distribution of cAMP responsive and unresponsive protein kinases; (2) determine if protein kinases from different cell fractions are distinct; (3) ascertain if the kinase activity of any cell fraction is affected by steroid hormone treatment; and (4) if affected, determine if the change is due to an alteration in enzyme activity per se or a translocation of enzyme from one subcellular fraction to another.

Materials and Methods

Radioactive Materials and Counting. [^{32}P]cAMP (3.6 Ci/mmol), [^{3}H]cAMP (22 Ci/mmol), and [γ^{32}P]ATP

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[1] The abbreviations used are as follows: cAMP, cyclic adenosine 3′,5′-monophosphate; Des, diethylstilbestrol; MIX, 1-methyl-3-isobutylxanthine; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

(1-10 Ci/mmol) were purchased from New England Nuclear. Radioactive materials collected on nitro-cellulose filters (Millipore, HAWP, 002400; 045 μm) were measured in 5 ml of Liquifluor (Amersham-Searle)-toluene after drying the filters in a vacuum oven. Scintillation counting was performed in a Beckman LS-233 scintillation counter with counting efficiencies of 30 and >95% for ^3H and ^{32}P, respectively. Aqueous samples (0.5 ml total volume) were counted in 5 ml of Aquasol (New England Nuclear).

Animals. White Leghorn female chicks were used in all experiments. Unless otherwise stated animals received daily doses of 2 mg of Des for a period of 2-3 weeks prior to sacrifice.

Assay for Protein Kinase. Cytoplasmic protein kinase activity was assayed in a 0.075 ml reaction volume containing 30 mM sodium phosphate buffer, pH 7.0, 10 mM $MgCl_2$, 3 mg/ml of calf thymus whole histone (Sigma), 0.3 mM [^{32}P]ATP (1-2 μCi per assay), and when added, 1×10^{-6} M cAMP. The reaction was started by the addition of enzyme and allowed to proceed for 6 min at 30 °C before stopping with 1.5 ml of 5% trichloracetic acid containing 1.5% sodium pyrophosphate. The reaction products were collected on nitro-cellulose filters and washed with two 2-ml portions of the stopping reagent. The filters were dried and counted. One unit of activity is defined as that amount of activity incorporating 1 pmol of ^{32}P from [^{32}P]ATP in 6 min at 30 °C.

Nuclear protein kinase activity was assayed exactly as described for cytoplasmic activity except that 2.5 mg/ml of α-casein (Sigma) was used as substrate and assays were carried out in the absence of cAMP unless otherwise stated.

cAMP Binding Assay. The cAMP binding assay was performed according to Kumon et al., (1972). The reaction was carried out in a final reaction volume of 0.4 ml containing 50 mM sodium acetate, pH 4.5, 0.5 mM MIX, 25 mM $MgCl_2$, and 1×10^{-7} M [^3H]cAMP. Binding was allowed to proceed for 20 min at room temperature. Time studies indicated that saturation was complete within 10 min. Following saturation, the reaction was stopped by dilution with 2 ml of cold 10 mM Tris-Cl, 40 mM $MgCl_2$, pH 7.5, and passed over nitro-cellulose filters. After washing, the filters were dried and counted as described above. For the assay of bound [^3H]cAMP in column fractions, aliquots were taken and mixed with 2 ml of cold 10 mM Tris-Cl, 40 mM $MgCl_2$, pH 7.5, before filtering over Millipore filters and counting.

cAMP binding to nuclei was carried out in a final reaction volume of 0.1 ml containing 250 mM sucrose, 10 mM $MgCl_2$, 10-20 μg of nuclear DNA, 50 mM Tris-Cl, pH 7.5, and 10^{-7} M [^3H]cAMP. After 15 min at room temperature, the reaction was stopped with 2 ml of cold 10 mM Tris-Cl, 40 mM $MgCl_2$, pH 7.5, and filtered over nitro-cellulose filters. The filters were washed and treated as described above.

Analytical Methods. Protein was determined by the method of Lowry et al. (1951). Protein in column fractions was monitored by the absorbance at 280 nm. Salt concentrations in column effluents were determined by conductance. DNA was measured using the diphenylamine procedure (Burton, 1962).

Determination of s Values. The sedimentation coefficients of oviduct protein kinases were determined by sucrose gradient centrifugation according to Martin and Ames (1961) on linear sucrose gradients (5-20%) at 4 °C in the SW 50.1 rotor. Centrifugation was at 48 000 rpm for 17 h.

Determination of Stokes Radii (R_s). A column (1.6 × 30 cm) of Sephadex G-200 (Pharmacia) was employed. The purified enzyme or standard proteins were applied to the column in a total volume of 0.5 ml and chromatographed at 4 °C with 10 mM Tris-Cl, 1 mM EDTA, pH 7.5 as the eluent. The flow rate was 10 ml per hour and 2-ml fractions were collected. The column was calibrated with the following standards: chymotrypsinogen (R_s = 22.4 Å), ovalbumin (R_s = 27.0 Å), bovine serum albumin (R_s = 35 Å), aldolase (R_s = 45 Å). Blue dextran and [^3H]cAMP were used to obtain the void and internal volumes, respectively. Values of K_{av} (Laurent and Killander, 1964) ($K_{av} = (V_e - V_0)/(V_i - V_0)$ where V_e is the elution position of the protein, V_i is the volume of gel, and V_0 is the void volume) were determined for the standards. These values were then plotted vs. their corresponding R_s to yield the standard curve from which the R_s of the holoenzyme and its subunits were determined.

Cell Fractionation of Oviduct Tissue. Homogenization and all subsequent steps were carried out at 0-4 °C. Oviducts (2.2 g) from two 3 week Des-stimulated chicks were homogenized in 5 volumes (per g of wet tissue) of 250 mM sucrose, 50 mM Tris-Cl, 0.5 mM $MgCl_2$, pH 7.5. Homogenization was carried out in two stages. Tissue was first dispersed in a Polytron homogenizer for 10 s at a setting of 5. Homogenization was then continued in a glass-Teflon homogenizer using five up and down strokes. The homogenate was poured through cheesecloth and centrifuged at 1000*g* for 5 min. The supernatant fraction was saved and the pellet rehomogenized in 10 ml of the starting buffer and centrifuged as above. The pellet, representing the crude nuclei, was saved and the two supernatants were combined and centrifuged at 10 000*g* for 20 min. This spin yielded the postmitochondrial supernatant and the mitochondrial-lysosome fraction. The supernatant fluid was centrifuged at 100 000*g* for 1 h to yield the microsomal fraction and the high-speed supernatant (17 ml). The three pellet fractions were suspended in starting buffer (nuclei, 7 ml; mitochondria-lysosomes, 4 ml; microsomes, 1 ml) before assaying for protein kinase activity and protein. The nuclei and high-speed supernatant each contained about 40% of the total protein while the mitochrondria-lysosome and microsome fractions contained 12 and 6%, respectively.

Preparation of Oviduct Cytosol. Oviducts weighing between 1 and 2 g were excised from chicks which had been given daily doses of Des for a period of 2-3 weeks. The tissue was cleaned of adhering fat and mesentary, rinsed in 0.9% NaCl, weighed, and homogenized in 5 volumes (per g of wet tissue) of cold 10 mM Tris-HCl, 1 mM EDTA, 0.5 mM MIX, pH. 7.5. Homogenization was performed as described above for cell fractionation studies. The homogenate was centrifuged at 10 000 rpm in the JA 20 rotor for 10 min in a Beckman J-21 centrifuge. The resulting supernatant fraction was centrifuged at 40 000 rpm in a Beckman SW 50.1 rotor for 1 h. This procedure yielded the high-speed supernatant, or cytosol.

Partial Purification of Cytoplasmic Protein Kinase. The cytosol was treated dropwise (with stirring) with saturated ammonium sulfate (buffered to pH 7.4 with Tris-Cl) to 55% saturation. Precipitation was allowed to proceed 10 min before centrifuging at 10 000 rpm for 10 min in a Beckman J-21 centrifuge. The precipitate, containing the bulk of the enzyme activity, was taken up in 5 ml of 10 mM Tris-Cl, pH 7.5, and dialyzed 1:1000 against the same buff-

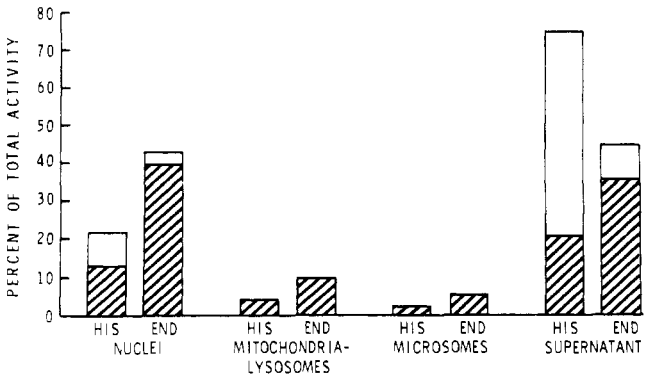

FIGURE 1: Subcellular distribution of oviduct protein kinase activity. Subcellular fractions were prepared as described in Methods. Fractions were assayed in the presence and absence of 10^{-6} M cAMP using both histone (HIS) and endogenous proteins (END) as substrates. For the percent total activity in the presence of cAMP the activity of each fraction in the presence of cAMP against the indicated substrate was divided by the total activity of all fractions (+cAMP) against the given substrate (total area). For the percent total activity in the absence of cAMP, the activity of each fraction in the absence of cAMP against the indicated substrate was divided by the total activity of all fractions (+cAMP) against the given substrate (hatched area).

er for at least 4 h. The dialyzed enzyme was applied to a column (2.5 × 3 cm) of DE-52 (Reeve Angel) previously equilibrated with 10 mM Tris-Cl, pH 7.5. After washing with 2–3 column volumes of buffer, a 100-ml linear gradient from 0 to 0.3 M KCl was used to develop the column. The flow rate was 60 ml per hour and 2-ml fractions were collected and assayed for protein kinase activity in the presence and absence of 10^{-6} M cAMP, cAMP binding activity, and KCl concentration.

Isolation of Nuclei. Purified chick oviduct nuclei were prepared according to Conn et al. (1976). Oviducts were washed with 0.14 M NaCl and thoroughly cleaned of adhering mesentery and blood. The tissue was minced into fine pieces and homogenized at room temperature at 15 ml per g of original tissue in 0.5 M hexylene glycol [2 methyl-2,4-pentanediol (Eastman)], 1 × 10^{-6} M $CaCl_2$, 1 mM Pipes (Calbiochem), pH 7.5. Homogenization was performed in a glass-Teflon homogenizer using 10–12 strokes. The homogenate was passed through eight layers of cheesecloth and one layer of organza before being centrifuged 7 min at 1200*g* in the JA-20 rotor of a Beckman J-21 centrifuge. The crude nuclear pellet was suspended by vortexing in 15 ml (per gram of original tissue) of the hexylene glycol buffer and the suspension centrifuged as above. This procedure was repeated once again and the final pellet was homogenized in 5 ml (per gram of original tissue) of 2.1 M sucrose, 0.1 mM $MgCl_2$, 10 mM Tris-Cl, pH 8.5. Centrifugation of this suspension at 25 000*g* for 1 h pelleted the purified nuclei. The yield of DNA in this procedure is generally around 40%. Nuclei were judged to be free of cytoplasmic contamination by enzymatic assays for cytoplasmic enzymes and by phase contrast and electron microscopy (Conn et al., 1976).

Partial Purification of Oviduct Nuclear Protein Kinases. Nuclei were homogenized in ice-cold 50 mM Tris-Cl, 0.3 M NaCl, pH 7.5 (1 ml per gram of original tissue), and then incubated for 30 min at 0 °C. Following incubation, the sample was centrifuged at 10 000*g* for 10 min. The resulting supernatant fraction, containing the majority of the nuclear kinase activity, was applied to phospho-cellulose P-11 (Reeve Angel).

Phospho-cellulose Chromatography. The resin was pretreated according to the recommendation of the manufacturer and stored in 50 mM Tris-Cl, 0.3 M NaCl, pH 7.5. For the routine purification of the nuclear protein kinases from 2–5 g of starting tissue, a 2.5 × 2 cm column was used. After applying the enzyme sample, the column was washed with 15 ml of starting buffer and then developed with a 100-ml linear gradient from 0.3 to 0.8 M NaCl in 50 mM Tris-Cl, pH 7.5. Three-milliliter fractions were collected and 20-μl aliquots assayed for protein kinase activity.

Preparation of Nuclear and Cytoplasmic cAMP Binding Proteins. For the preparation of the nuclear cAMP binding protein, nuclei were first taken up in 1 ml per gram of original tissue (200–400 μg of DNA/ml) in cold 10 mM Tris-Cl, pH 7.5. In some cases the nuclei were labeled with 10^{-7} M [^{3}H]cAMP at this point. Solid ammonium sulfate was then added to a final concentration of 0.3 M. The suspension was sonicated at 4 °C in a Branson sonifier fitted with a microtip at a setting of 3.0 in four 15-s periods with 15-s intervals for cooling. The nuclei were then diluted with two volumes of 10 mM Tris-Cl, pH 7.5, and centrifuged at 10 000*g* for 10 min. The supernatant fluid was made 2.4 M in ammonium sulfate, stirred for 10 min, and centrifuged at 10 000*g* for 10 min. The pellet was taken up in 10 mM Tris-Cl, pH 7.5.

To prepare the cytoplasmic cAMP binding protein, oviduct cytosol was prepared as described above. In some cases cytosol was labeled with 10^{-7} M [^{3}H]cAMP at 0 °C for 2 h. Saturated $(NH_4)_2SO_4$ (brought to pH 7.4 with Tris) was then added dropwise with stirring to 50% saturation. Precipitation was allowed to proceed 20 min in the cold followed by centrifugation at 10 000*g* for 10 min. The pellet containing the cAMP binding protein was taken up in 10 mM Tris-Cl, pH 7.5.

Results

Subcellular Distribution of Chick Oviduct Protein Kinase Activity. Oviduct tissue was homogenized in buffer containing 250 mM sucrose and subcellular fractions prepared by differential centrifugation. The various fractions were assayed for protein kinase activity using whole histone (at 3.0 mg/ml) and endogenous proteins as substrates. Figure 1 indicates that the high-speed supernatant, or cytosol, contained the majority of the cAMP-stimulatable protein kinase activity. The cAMP-stimulatable activity present in the crude nuclear fraction was probably due to cytoplasmic contamination since most of this activity was lost upon purification of the nuclei through 2.1 M sucrose. However, a considerable amount of endogenous protein kinase activity remained associated with the purified nuclei.

Protein Kinase Activity of Oviduct Cytosol. Two lines of evidence indicate that the protein kinase activity of chick oviduct cytosol is cAMP dependent. First, as shown above, protein kinase activity was stimulated threefold by the addition of 10^{-6} M cAMP (Figure 1). Second, the concentration of cAMP required for half-stimulation of enzyme activity was similar to the concentration required for half-saturation of cAMP binding (not shown). From these latter results, a dissociation constant of 30 nM was calculated for cAMP binding. This value is in close agreement with that reported for protein kinases from other tissues (Walsh et al., 1971; Garren et al., 1971; Tao and Hackett, 1973).

In order to characterize the cytoplasmic protein kinase, it was desirable to partially purify it from oviduct cytosol. A 50-fold purification was achieved using the procedure de-

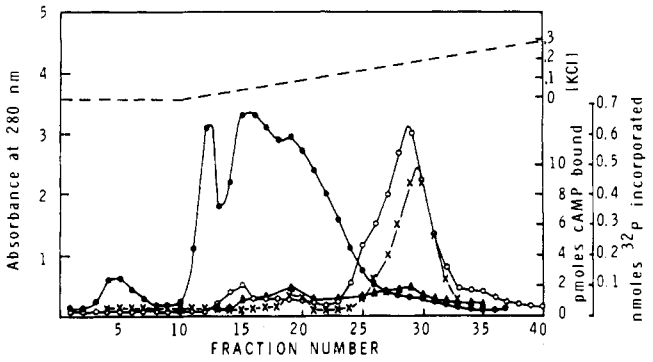

FIGURE 2: Purification of chick oviduct protein kinase on DE-52. Two oviducts (3 g) were homogenized and cytosol was prepared as described. The cytosol was treated with 100% $(NH_4)_2SO_4$ to 55% saturation and centrifuged. The precipitate was dissolved in 5 ml of 10 mM Tris-Cl, 1 mM EDTA, 0.5 mM MIX, pH 7.5. After dialyzing against this same buffer, the sample was applied to a 2.5 × 3 cm column of DE-52. The column was developed with a linear salt gradient from 0 to 0.3 M KCl. Fractions (2 ml) were collected and assayed for absorbance at 280 nM (●); cAMP binding (X); and protein kinase activity in the presence (○) and absence (▲) of 10^{-6} M cAMP.

Table I: Substrate Specificity of Oviduct Cytoplasmic Protein Kinase.[a]

Substrate	Units	% of Whole Histone
None		0
Whole histone	140	100
F_1 (I)	290	210
F_{2a} (IIb_1, IV)	74	53
F_{2b} (IIb_2)	660	470
F_3 (III)	175	125
Protamine sulfate	44	31
Ovalbumin	8	6
Bovine serum albumin	6	4
α-Casein	21	15
Cytosol		4

[a] Enzyme purified through DE-52 was used in the standard assay in the presence of 10^{-6} M cAMP with 0.25 mg of protein substrate. For "cytosol" the activity of cytosol was determined in the standard assay in the absence of substrate and compared with the activity of cytosol determined in the presence of 0.25 mg of whole histone.

scribed in the Methods section. Figure 2 shows the profile obtained when the enzyme was chromatographed on DEAE-cellulose with a linear gradient from 0 to 0.3 M KCl. The enzyme eluted at a salt concentration of 0.2 M, considerably behind the majority of oviduct proteins. Fractions 25–33 were pooled and used as the enzyme source in subsequent studies.

Although the activity profile of Figure 2 shows one major form of enzyme activity at 0.2 M NaCl, a second smaller peak of activity, also stimulated by cAMP, occasionally appeared at concentrations of NaCl below 0.1 M. The major peak probably corresponds to a type II protein kinase as recently described by Corbin et al. (1975) while the minor peak probably corresponds to a type I protein kinase. Since this early eluting peak of activity was always present in considerably smaller quantities than the peak eluting at 0.2 M NaCl, it has not been characterized further.

K_m Determination for Cytoplasmic Enzyme. The partially purified cytoplasmic enzyme followed Michaelis–

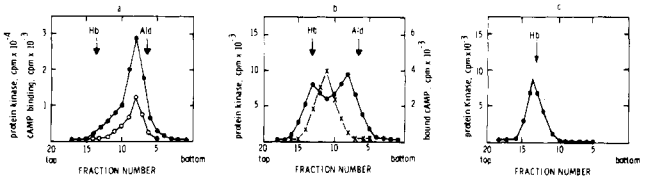

FIGURE 3: Sucrose gradient centrifugation. (a) Purified enzyme (200 μl) in 50 mM Tris-Cl, 0.1 M NaCl, pH 7.5, was layered over a 5–20% sucrose gradient in 50 mM Tris-Cl, 0.1 M NaCl, pH 7.5. Centrifugation was for 17 h at 48 000 rpm in the SW 50.1 rotor. Fractions (0.25 ml) were collected and 25-μl aliquots assayed for protein kinase activity in the presence of 10^{-6} M cAMP (●). Aliquots (0.1 ml) were used for the assay of cAMP binding activity (○). (b) Same as (a) except that the enzyme was labeled with 10^{-8} M [^{3}H]cAMP prior to centrifugation. Samples were assayed for bound [^{3}H]cAMP (X). (c) Same as (a) except that the enzyme was treated with 10^{-6} M cAMP prior to centrifugation in 10^{-6} M cAMP. Arrows indicate migration of hemoglobin (Hb) and aldolase (Ald) standards.

Menten kinetics for ATP. The K_m (1.8×10^{-5} M) agrees favorably with the K_m for cytoplasmic kinases derived from other tissues (Erlichman et al., 1973; Kuo and Greengard, 1970; Langan, 1968).

Substrate Specificity. Table I lists the proteins tested as substrates for the protein kinase of oviduct cytosol. Of the various histone fractions, F_{2b} was found to be an excellent substrate, incorporating ^{32}P at a rate five times greater than whole histone. Other than the histone fractions, none of the other proteins tested was found to be a good substrate under the conditions employed.

Determination of Sedimentation Coefficient. Since the cytoplasmic enzyme was found to be stimulated by cAMP, it was likely to possess regulatory and catalytic subunits, as has been shown for other cAMP dependent protein kinases. This was found to be the case. As shown in Figure 3a, when purified enzyme was centrifuged in a linear 5–20% sucrose gradient, the protein kinase activity (assayed in the presence of 10^{-6} M cAMP after centrifugation) sedimented as a 7.2S component. It can also be seen that cAMP binding activity co-sedimented with the enzyme. Moreover, the protein kinase activity was dependent on cAMP since deletion of cAMP from the assay resulted in a threefold loss in activity. These data suggested that the 7.2S species was composed of both catalytic and cAMP binding regulatory subunits. When [^{3}H]cAMP was added to the enzyme at a final concentration of 10^{-8} M before applying the enzyme to the gradient, the RC complex was still evident, but a lighter sedimenting form of kinase activity now appeared at 4.3S (Figure 3b). This form of the enzyme was not stimulated by cAMP and presumably represented free catalytic subunit which dissociated from the RC complex upon addition of cAMP. The regulatory subunit, labeled with [^{3}H]cAMP, sedimented as a single 6.2S component. If the enzyme were labeled with saturating amounts (10^{-6} M) of cAMP before centrifugation, and the gradient run in 10^{-6} M cAMP, all activity then sedimented as a 4.3S component, indicating complete dissociation of the RC complex (Figure 3c).

Determination of Stokes Radii. The dissociation of holoenzyme into regulatory and catalytic subunits by cAMP could also be demonstrated by gel filtration. Chromatography of purified unlabeled protein kinase on G-200 yielded a single peak of cAMP regulated activity which corresponded to a Stokes radius of 53 Å. When the enzyme was labeled with 10^{-7} M [^{3}H]cAMP before applying it to the column, a new peak of kinase activity, not stimulated by 10^{-6} M cAMP, was observed at an elution position corresponding to

Table II: Comparison of Nuclear Protein Kinase Activity in Oviduct Nuclei from Stimulated and Withdrawn Chicks.[a]

Experiment	Sample Assayed	Protein Kinase (pmol of ^{32}P Incorp)
I	Nuclei (WD)	14
	Nuclei (S)	19
II	Nuclei (WD)	13.4
	Nuclei (S)	19
	0.3 M extract (WD)	11
	0.3 M extract (S)	15.6

[a] Oviduct nuclei were prepared from chicks stimulated with estrogen for 3 weeks (S) and chicks stimulated 2 weeks followed by 1 week of no hormone (WD). Nuclei were suspended in 50 mM Tris-Cl, 0.3 M NaCl, pH 7.5, to an A_{260} = 10. Both nuclear samples had an A_{260}/A_{280} = 1.6. Fifteen microliters of nuclei were assayed for 1 min in the standard protein kinase assay. In experiment II, portions of the nuclei were centrifuged and 15 μl of the supernatants (0.3 M extract) was assayed in the presence of α-casein (2.5 mg/ml). Values reported are the average of duplicate assays.

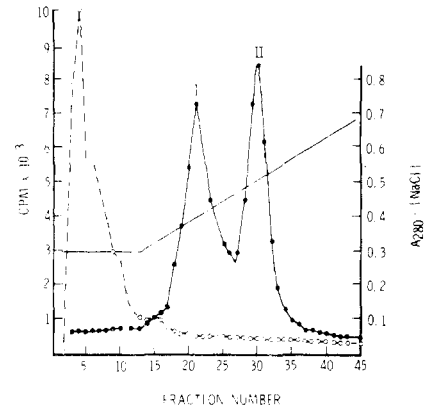

FIGURE 4: Phospho-cellulose chromatography of 0.3 M NaCl nuclear extract. Nuclei from 3 g of oviduct were extracted with 50 mM Tris-Cl, 0.3 M NaCl, pH 7.5, as described in the text. The extract was applied to a 2.5 × 2 cm column of phospho-cellulose equilibrated with the extraction buffer. The column was washed with 15 ml of this buffer and then eluted with a linear gradient from 0.3 to 0.8 M NaCl in 50 mM Tris-Cl, pH 7.5. Three-milliliter fractions were collected and 25-μl aliquots were assayed for casein kinase activity (●) and A_{280} (○).

a Stokes radius of 25 Å. This peak of activity represented free catalytic subunit since addition of 10^{-6} M cAMP to the kinase assay did not stimulate the enzyme. The regulatory subunit, containing bound [^{3}H]cAMP, eluted between the holoenzyme and free catalytic subunit at a Stokes radius of 47 Å. By combining the Stokes radii with the sedimentation coefficients determined above, the M's of the holoenzyme, catalytic component, and regulatory component were determined to be 165 000, 125 000, and 46 000, respectively.

Protein Kinase Activity of Oviduct Nuclei. Since appreciable amounts of the total protein kinase activity of oviduct tissue were found in the nuclear fraction (Figure 1) and since several reports have implicated nuclear kinase activity in steroid hormone action, we were interested in determining the nature of the nuclear activity. Specifically, we wanted to know if this activity was related to or derived from the cytoplasmic protein kinase and if the activity was influenced by estrogen.

Unlike oviduct cytosol, which catalyzes incorporation of ^{32}P into exogenous histone for well over 30 min (data not shown), the incorporation of ^{32}P into endogenous or exogenous protein by highly purified oviduct nuclei reaches a plateau within 2 min. This effect was found to be due to a very active nuclear ATPase which degrades greater than 95% of the added ATP within 5 min. Rickwood et al. (1973) in their studies on the phosphorylation of mouse liver nuclei also reported the presence of appreciable nuclear ATPase activity. The presence of this ATP hydrolyzing activity severely hampered phosphorylation studies with intact nuclei. In the studies reported below with isolated nuclei, phosphorylation was allowed to proceed for 1 min only, during which time ^{32}P incorporation is linear.

Increase in Nuclear Protein Kinase Activity during Hormonal Stimulation. Ahmed and co-workers have recently presented evidence indicating that dihydrotestosterone increases protein kinase activity in rat ventral prostrate nuclei (Ahmed and Ishida, 1971; Ahmed, 1971; Ahmed and Wilson, 1975). To determine if a similar situation exists in the oviduct system, oviduct nuclei from estrogen stimulated and 7-day withdrawn chicks were assayed for protein kinase activity. Table II indicates that oviduct nuclei from estrogen-primed chicks incorporate approximately 40% more ^{32}P than nuclei from withdrawn chicks. Table II also shows that extracts of nuclei from stimulated chicks have greater protein kinase activity in the presence of excess casein than extracts of nuclei from withdrawn chicks. This suggests that the increase in protein kinase activity is due to an increase in enzyme activity per se, and not to an increase in endogenous acceptors.

Partial Purification of the Nuclear Enzymes. Since previous workers have shown that a substantial purification of nuclear protein kinases can be achieved using phospho-cellulose (Takeda et al., 1971; Desjardins et al., 1972), this resin was chosen for the oviduct enzyme. Kinase activity is loosely bound to oviduct nuclei since extraction with 0.3 M NaCl released the protein kinase activity in good yield (>80%). Following extraction, the sample was applied to a 2.5 × 2 cm column of phospho-cellulose and eluted with a linear salt gradient from 0.3 to 0.8 M NaCl (Figure 4). When the fractions were assayed for protein kinase activity using casein as a substrate, two peaks of activity (I and II) were detected, eluting at sodium chloride concentrations of 0.4 and 0.5 M, respectively. Neither of the enzymes were stimulated by 10^{-6} M cAMP when assayed in the presence of casein. Both peaks of activity eluted well behind the majority of the material absorbing at A_{280}, affording an overall purification of 100- and 120- fold for peaks I and II, respectively. Recoveries of both enzymes were generally around 70%. Following phospho-cellulose chromatography, both enzymes incorporated ^{32}P into casein linearly for over 30 min. This was due to the fact that the ATPase activity, which was partially released with the 0.3 M extract, was not retarded by the phospho-cellulose column under the conditions employed.

Determination of K_m for the Nuclear Enzymes. Enzymes I and II both gave linear Lineweaver–Burk plots when assayed in the presence of varying ATP concentrations. The computed K_m's for ATP were 13 μM for peak I and 9 μM for peak II. We interpret these results to indicate that two enzymes have either very similar or identical ATP binding sites.

Substrate Specificity and Salt Dependence. As has been found with nuclear protein kinases from other sources (Desjardins et al., 1972; Gibson et al., 1974; Grummt,

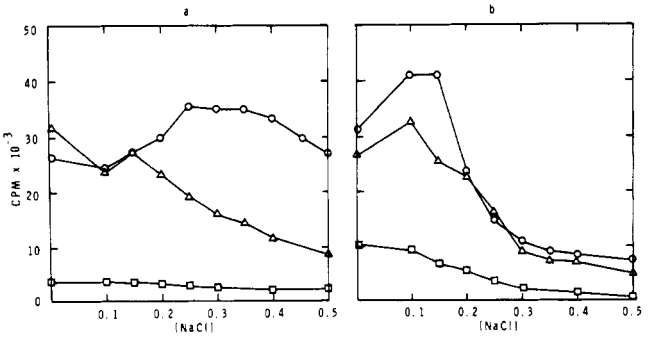

FIGURE 5: Substrate specificity and effect of salt on nuclear protein kinases I and II. Kinase I (panel a) or II (panel b) was incubated in the standard assay conditions with either 2.5 mg per ml of α-casein (○), phosvitin (△) or calf thymus whole histone (□) in the presence of various concentrations of NaCl.

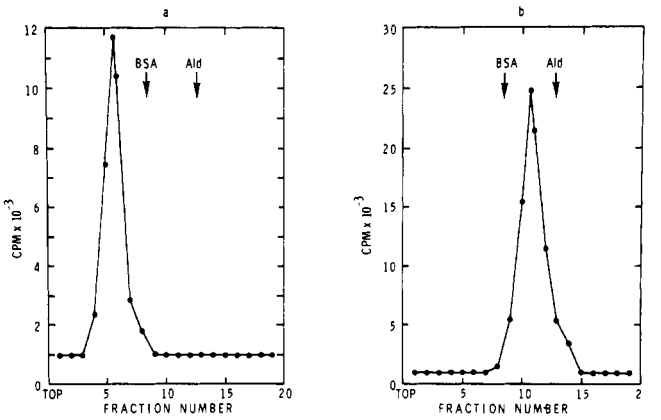

FIGURE 6: Sucrose gradient centrifugation of protein kinases I and II. Peak I (panel a) and II (panel b) (200 μl) from phosphocellulose were applied to 5–20% linear sucrose gradients prepared in 50 mM Tris-Cl, 0.3 M NaCl, pH 7.5. Centrifugation was for 16 h at 45 000 rpm in the SW 50.1 rotor. Fractions (0.25 ml) were collected and 25 μl aliquots were assayed for protein kinase activity using α-casein as substrate. Arrows indicate migration of bovine serum albumin (BSA) and aldolase (Ald) standards.

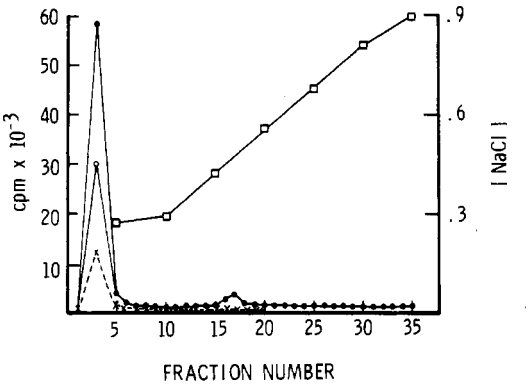

FIGURE 7: Phospho-cellulose chromatography of oviduct cytosol. Cytosol was made 0.3 M in NaCl and applied to a phospho-cellulose column (2.5 × 2 cm) equilibrated in 50 mM Tris-Cl, pH 7.5. The column was washed and eluted exactly as described in Figure 4. Fractions were assayed for histone kinase activity in the presence (●) and absence (○) of cAMP and for cAMP binding activity (X - - - X). An identical sample was labeled with 10^{-6} M cAMP before chromatography and chromatographed in an identical manner in the presence of 10^{-6} M cAMP. In this case, cAMP independent protein kinase activity eluted in the void volume exactly as found with the untreated cytosol (data not shown).

1974), the activity of the oviduct enzymes was markedly affected by salt. As shown in Figure 5a, enzyme I exhibited a NaCl optimum around 0.25 M for the phosphorylation of casein while the phosphorylation of both phosvitin and histone decreased with increasing concentrations of NaCl. Enzyme II gave a salt optimum near 0.10 M using either casein or phosvitin (Figure 5b) as a substrate. The effect of NaCl on enzyme I, therefore, may be on the conformation of the protein substrates used, whereas the salt effect observed with enzyme II is probably due to an effect on enzyme activity per se. It is worth mentioning that neither enzyme was affected by 10^{-6} M cAMP, regardless of the substrate used (data not shown).

In addition to the three protein substrates tested above, the purified progesterone receptor of oviduct was also assayed for its ability to serve as a substrate. Neither enzyme could significantly catalyze ^{32}P incorporation into the receptor, although, due to the limited quantities of material, the concentration of receptor in the assay was tenfold less than that employed for the other substrates tested (0.25 mg/ml). Nevertheless, this concentration of receptor should be sufficient to detect phosphorylation if it is a physiological substrate since casein at this concentration is phosphorylated approximately 60% as well as at 2.5 mg/ml. The possibility that the receptor already exists in a phosphorylated state was ruled out by amino acid analyses of homogeneous receptor preparations. These studies indicate that the receptor contains no phosphoserine or phosphothreonine residues.[2]

Stability. After storing for a period of 1 month at −20 °C in 50 mM Tris-Cl, 0.5 M NaCl, pH 7.5, both enzymes retained approximately 80% of their original activity.

Sucrose Gradient Centrifugation. Centrifugation of kinase I in 5–20% sucrose gradient gave a peak of casein kinase activity at a position slightly lighter than that of bovine serum albumin, corresponding to 3S (Figure 6a). Centrifugation of protein kinase II in an identical gradient yielded a single peak of activity sedimenting between bovine serum albumin and aldolase at 6S (Figure 6b). The sedimentation position of neither nuclear enzyme was affected by the addition of 10^{-6} M cAMP to the gradient.

Comparison of Nuclear and Cytoplasmic Protein Kinases. Although purified nuclei exhibited little or no cAMP dependent phosphorylation, the possibility existed that one of the nuclear protein kinases could be a catalytic subunit derived from the cytosol. To investigate this possibility, we divided an oviduct cytosol into two fractions. One fraction was made 10^{-6} M in cAMP and both fractions were incubated for 2 h at 0 °C. Following incubation, the samples were chromatographed on phospho-cellulose. The fraction treated with cAMP was chromatographed in the presence of 10^{-6} M cAMP. The cytosol protein kinase activity in both samples eluted in the void volume (Figure 7), indicating that unlike the two nuclear enzymes, neither holoenzyme nor catalytic subunit of cytosol was retained by phospho-cellulose under these conditions. These data plus the sedimentation data (Figures 3 and 6) strongly suggest that the nuclear and cytoplasmic protein kinases are not related.

cAMP Binding to Oviduct Nuclei. When highly purified oviduct nuclei were tested for cAMP binding activity, a significant amount of specific, saturable binding was observed. As shown in Figure 8, oviduct nuclei bound cAMP with K_d of 2×10^{-8} M. This value is similar to the K_d of 3×10^{-8}

[2] Kuhn, R. W., Schrader, W. T., and O'Malley, B. W., submitted for publication.

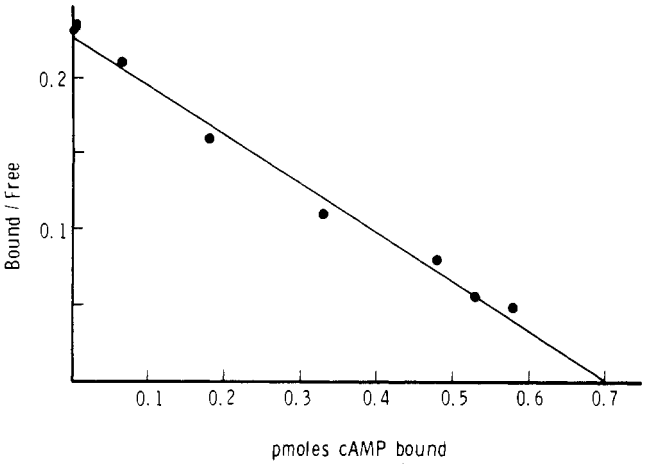

FIGURE 8: Scatchard plot of [^{3}H]cAMP binding to purified oviduct nuclei. Preparation of nuclei and cAMP binding were carried out as described in Methods. Purified nuclei (25 μg of DNA) were assayed for cAMP binding in the presence of varying concentrations of [^{3}H]cAMP. A 1000-fold excess of unlabeled cAMP was used to determine nonspecific binding.

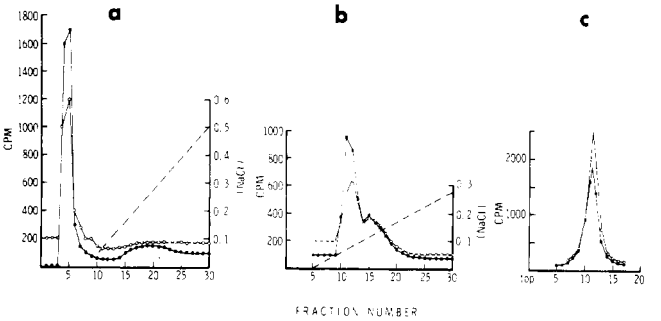

FIGURE 9: Comparison of nuclear and cytoplasmic cAMP binding proteins on phospho-cellulose, DEAE, and sucrose gradients. Nuclear and cytoplasmic cAMP binding proteins were prepared from 2 g of tissue as described in Methods and labeled with 10^{-7} M [^{3}H]cAMP and [^{32}P]cAMP, respectively. Each sample was then passed over G-75 in 50 mM Tris-Cl, pH 7.5, to remove $(NH_4)_2SO_4$ and free cAMP. The void volume fractions containing the bound cAMP were mixed to give equal amounts of ^{3}H and ^{32}P. The double-labeled sample (4 ml) was then chromatographed on a 2.5 × 2 cm column of phospho-cellulose (a). A linear gradient from 0 to 0.8 M NaCl in 50 mM Tris-Cl, pH 7.5, was applied. The drop-through fractions (8 ml) containing the bound cAMP were pooled. Seven milliliters was applied directly to a DEAE column (2.5 × 2 cm) and eluted with a 0 to 0.3 M NaCl gradient in 50 mM Tris-Cl, pH 7.5 (b). The pooled fractions (0.25 ml) from phospho-cellulose were applied to 5–20% sucrose gradients (c) and centrifuged as described in Figure 6. (○) ^{3}H cpm; (●) ^{32}P cpm.

Table III: Solubilization of cAMP Binding Component from Chick Oviduct Nuclei.[a]

Fraction	cAMP Bound (pmol)	% Recovery
Nuclei (1 ml/g of tissue)	22	100
0.3 M NaCl wash	2	9
0.3 M $(NH_4)_2SO_4$ sonicate	16	73
Final pellet	2	9

[a] Purified nuclei from 5 g of oviduct were suspended in 10 mM Tris-Cl, pH 7.5, and treatment with 1/9 volume of 3 M NaCl. After 30 min at 0 °C, the nuclei were collected by centrifugation and taken up in 5 ml of 0.3 M $(NH_4)_2SO_4$. The nuclei were then sonicated and diluted as described in Methods. The sonicate was centrifuged and the pellet suspended in 5 ml of 10 mM Tris-Cl, pH 7.5. One-hundred-microliter samples of all fractions were assayed for cAMP binding. The values given correspond to that amount of activity resulting from 1 g of tissue. Nonspecific binding, determined in the presence of a 1000-fold excess of unlabeled cAMP, has been subtracted.

M found for cAMP binding to the cytoplasmic regulatory subunit. The nuclear binding represents approximately 1–2% of the total binding activity found in the cytosol and corresponds to about 20 000 sites per nucleus. The binding activity was tightly associated with the chromatin since extraction with salt concentrations up to 0.3 M failed to release significant activity. However, substantial extraction of activity could be effected using the procedure described by Roeder and Rutter (1970) for RNA polymerase. As shown in Table III, 70% of the binding activity could be solubilized by sonication in 0.3 M ammonium sulfate.

Comparison of Cytoplasmic and Nuclear cAMP Binding Proteins. Since the nuclear cAMP binding activity could have arisen as a result of contamination with the cytoplasmic protein kinase holoenzyme or regulatory subunit, it was of interest to determine if the nuclear and cytoplasmic cAMP binding proteins could be distinguished. To test this possibility, nuclei were labeled with [^{3}H]cAMP while cytosol was labeled with [^{32}P]cAMP. The respective binding proteins were then prepared as described in Methods and portions of the preparations containing equivalent ^{3}H and ^{32}P counts per minute were mixed. The double-labeled sample was then applied to phospho-cellulose. As shown in Figure 9a, neither protein was retained by phospho-cellulose. The active fractions were pooled and applied to a DEAE column or to a 5–20% sucrose gradient. Figures 9b and 9c show that the nuclear and cytoplasmic proteins are indistinguishable by these methods.

Is the Nuclear cAMP Binding Protein Attached to a Protein Kinase? The finding of a cAMP binding protein in nuclei indistinguishable from the cytoplasmic cAMP binding regulatory subunit raised the possibility that the nuclear binding protein may be attached to a protein kinase catalytic subunit. To test this possibility, the sedimentation rates of the nuclear cAMP binding protein prepared in the absence and presence of [^{3}H]cAMP were compared. The sample prepared in the absence of cAMP was assayed *after* sucrose gradient centrifugation by the cAMP binding assay. If the nuclear cAMP binding protein is attached to a protein kinase catalytic subunit in the nucleus, then labeling with cAMP prior to sedimentation should dissociate the complex, resulting in a lowering of the sedimentation coefficient. Sucrose gradient centrifugation of the labeled and unlabeled nuclear extracts yielded bound [^{3}H]cAMP and cAMP binding activity, respectively, in the 6S region, suggesting that the cAMP binding protein is not associated with a protein kinase. However, control studies carried out with the cytoplasmic holoenzyme indicated that the sonication in high salt carried out to extract the binding protein dissociates the cytoplasmic holoenzyme into regulatory and catalytic subunits. Hence, it appears that more gentle methods of extraction are required before it can be firmly established whether the cAMP binding protein is ever associated with a kinase in the nucleus in vivo.

Discussion

The purpose of the present investigation was to gain information on the protein kinases of chick oviduct and to explore their possible role in the action of hormones on this

tissue. Subcellular fractionation of oviduct homogenates (Figure 1) revealed that, as had been found for other tissues, the bulk of the cAMP-stimulatable protein kinase activity is located in the cytosol, while appreciable amounts of kinase activity not stimulated by cAMP are localized in the nuclear fraction.

Chromatography of chick oviduct cytosol on DEAE-cellulose yielded one major peak of protein kinase activity eluting near 0.2 M KCl. This elution position suggests that the enzyme may be a type II cAMP-dependent protein kinase, as recently defined by Corbin et al. (1975). According to Corbin et al. (1975), this type of protein kinase is characterized by a slow rate of salt- or substrate-induced dissociation. Our results with the oviduct enzyme are consistent with this description since we have been unsuccessful in dissociating the protein kinase with histone (unpublished observations).

The substrate specificity of oviduct cytoplasmic protein kinase is similar to that reported for protein kinases from a variety of sources (Langan, 1968; Shepherd et al. 1971; Corbin et al., 1972; Farago et al., 1973) and is consistent with the hypothesis that the response of a particular cell to protein kinase activation is largely dictated by the nature of the protein substrates which are present in that cell (Langan, 1973). Although the basis for the specificity of cAMP-dependent protein kinases is not known, recent results suggest that an arginine residue N-terminal to the serine residue which undergoes phosphorylation may be a major determinant (Bylund and Krebs 1975; Kemp et al., 1975). In the case of the oviduct, it is especially difficult to speculate on possible cytoplasmic protein substrates since it is not known what factors may increase oviduct cAMP levels or what enzymes might be activated in response to cAMP-mediated phosphorylation. Previous results from our laboratory have shown (Kissel et al., 1970; Rosenfeld and O'-Malley, 1970; Rosenfeld et al., 1970) that estrogen and progesterone treatment do not change adenylate cyclase and cAMP levels during the initial 24 h following administration, suggesting that these hormones do not act by directly activating cAMP dependent protein kinase. On the other hand, our finding that oviduct nuclei contain appreciable amounts of protein kinase activity raised the possibility that this activity may be derived from the cytosol and may function in the phosphorylation of nuclear proteins. Alternatively, it was possible that the nuclear kinase activity of withdrawn chicks is due to a separate nuclear kinase, but that the greater nuclear activity observed in stimulated chicks is due to translocation of the cytoplasmic enzyme into the nuclear fraction, as has been suggested to occur in the case of polypeptide hormones (Palmer et al., 1974; Castagna et al., 1975; Jungmann et al., 1974a,b).

Both of these possibilities now appear to be eliminated since we have shown that oviduct nuclei from estrogen-stimulated animals contain two protein kinases, neither of which is related to the cytoplasmic enzyme. Thus, we have shown that neither of the two nuclear enzymes display substrate specificity or sedimentation behavior similar to that of the cytoplasmic enzyme. Also, the nuclear enzymes are not stimulated by cAMP nor do they bind cAMP (data not shown). Final proof that the nuclear and cytoplasmic enzymes are not related comes from chromatography on phospho-cellulose. When the cytoplasmic enzyme was preincubated in the presence or absence of 10^{-6} M cAMP and applied to phospho-cellulose in 0.3 M NaCl, essentially all of this histone-specific protein kinase activity passed through the column unretarded (Figure 7). The nuclear activity, by contrast, was retained by the column under these conditions and eluted as two peaks of casein kinase activity at 0.4 and 0.5 M NaCl (Figure 4). Our results also show that the increase in oviduct nuclear protein kinase activity observed in response to estrogen (Table II) is not due to translocation of cytoplasmic protein kinase into nuclei since phospho-cellulose chromatography of 0.3 M NaCl nuclear extracts from fully stimulated chick oviduct or hen oviduct (unpublished observations) failed to reveal significant cytoplasmic protein kinase activity. It is interesting to note in this respect that Keely et al. (1975) have recently presented evidence suggesting that the observations of protein kinase translocation from the cytoplasmic to particulate fractions may be due to artifactual binding of the protein kinase catalytic subunit brought about by homogenization in low salt containing buffers.

The differential elution of the cytoplasmic and nuclear protein kinases from phospho-cellulose proved to be useful in quantitating the amount of each enzyme in the nuclear and cytoplasmic fractions. Hence, when cytosol eluted from phospho-cellulose was assayed for nuclear kinase activity using casein as a substrate, less than 2% of the total kinase activity extractable from nuclei was present in the region where the nuclear enzymes elute (0.4–0.5 M NaCl). Conversely, when the 0.3 M NaCl extract of nuclei was chromatographed on phospho-cellulose and assayed for cytoplasmic kinase activity using histone in the presence of cAMP, less than 1% of the total kinase activity extractable from cytoplasm was observed in the region where the cytoplasmic enzyme elutes (void volume). When nuclear and cytoplasmic enzymes were mixed and applied to phospho-cellulose, they quantitatively retained their activity and separate elution positions (data not shown), indicating that each enzyme can be assayed in the presence of the other. From these results, we conclude that most, if not all, of the cAMP dependent histone kinase activity is derived from the cytosol, whereas most, if not all, of the casein kinase activity unresponsive to cAMP is derived from the nucleus (however, see below). This conclusion is similar to that reported by Desjardins et al. (1975a) in a recent study on rat liver nuclear protein kinases. These investigators found marginal quantities of cAMP dependent protein kinase in purified liver nuclei and concluded that this enzyme behaves as if it is of cytoplasmic origin.

The presence of light and heavy forms of cAMP-independent protein kinases in nuclei has also been reported by Takeda et al. (1971) and Desjardins et al. (1972) in rat liver, and Kemp et al. (1975) in human lymphocytes. In agreement with these groups we found that the two forms extractable from oviduct nuclei have similar K_m's for ATP (10–20 μM), the heavier form having a slightly lower K_m in our work and the work of Takeda et al. (1971) and Kemp et al. (1975). As in the work of Takeda et al. (1971) and Desjardins et al. (1972), we observed significant differences in the specificity of the two forms for exogenous substrates. In the former study this difference was quite dramatic since the two enzymes exhibited a 13-fold difference in the phosphoserine/phosphothreonine ratios when casein was used as an exogenous substrate.

The substrate specificty of oviduct nuclear kinases I and II for exogeneous proteins indicates that these enzymes prefer acidic proteins as substrates and suggest that their in vivo substrates are the nonhistone nuclear proteins. Preliminary experiments in which intact nuclei were phosphorylat-

ed bear out this conclusion. When oviduct nuclei were phosphorylated with [^{32}P]ATP and Mg^{2+}, we found that greater than 90% of the protein bound radioactivity was associated with the nonhistone proteins, in agreement with the substrate specificity of the isolated enzymes. It is interesting to note that following phosphorylation of nuclei, approximately one-half of the ^{32}P incorporated into protein was extractable with 0.3 M NaCl, indicating that a significant portion of the phosphorylated proteins is loosely bound to the nucleus. A possible role for loosely bound phosphoproteins in regulating gene transcription has recently been suggested by the work of Kostraba et al. (1975).

Our finding of a cAMP binding activity in isolated nuclei must be viewed with caution since the activity represents only 1–2% of that found in the cytosol and, therefore, could be due to contamination. However, the fact that the cAMP binding protein is strongly attached to chromatin argues against contamination and suggests that the protein could have a functional role in the nucleus. It is of interest that Rikans and Ruddon have reported the presence of two cAMP binding protins in nuclei from rat liver (Rikans and Ruddon, 1973). These binding proteins slightly inhibited one of the rat liver nuclear protein kinases, and it was suggested that the nuclear kinases may be regulated by cAMP binding proteins. The possibility that the oviduct nuclear protein kinases might be regulated by the nuclear cAMP binding protein seems remote since the nuclear and cytoplasmic cAMP binding proteins appear identical (Figure 9) whereas the protein kinases derived from nuclei and cytosol are quite different. In the absence of cAMP, the regulatory subunit binds tightly to the cytoplasmic kinase, but we have no evidence to suggest that it binds and regulates the unrelated nuclear enzymes. Studies carried out by Desjardins et al. (1972) indicate that in rat liver at least, the nuclear protein kinases are not inhibited by the heat-stable, cAMP-dependent protein kinase inhibitor, suggesting that these enzymes are not regulated by the same mechanisms as the cytoplasmic enzyme. We suggest that, if the cAMP binding protein of oviduct nuclei is attached to a protein kinase, it is probably the cytoplasmic catalytic subunit. Unfortunately we have been unable to find conditions gentle enough to allow extraction of the cAMP binding protein while maintaining the holoenzyme intact. Moreover, the detection of cAMP dependent protein kinase activity has been frustrated by the quantity of cAMP independent kinase activity, which is present in appreciable amounts even after extraction of nuclei with 0.3 M NaCl. Indeed, it is possible to calculate that, if the cAMP binding protein is attached to a protein kinase with activity similar to the cytoplasmic enzyme, then it is not present in sufficient quantity to allow detection of cAMP dependent histone kinase activity over the cAMP independent endogenous activity. We are therefore unable to draw any firm conclusions concerning the state of the cAMP binding protein in the intact nucleus.

Very recently, Johnson et al. (1975) reported that purified calf thymus nuclei contain significant amounts of cAMP dependent protein kinase and cAMP binding activity. The cAMP binding activity was found associated with a protein fraction indistinguishable from the cytoplasmic cAMP binding regulatory subunit. As in the present work, these authors were unable to quantitate the amount of binding protein which is unattached to a catalytic subunit. They suggest, however, that, since the cAMP dependent protein kinase activity is relatively small, some of the binding activity may not be associated with the protein kinase.

If the cAMP binding protein that we and others (Rikans and Ruddon, 1973; Johnson et al., 1975) observe in nuclei exists as an uncomplexed subunit, it might function in a capacity different from regulation of a kinase. For example, it might participate in genetic regulation, similar to the cAMP binding protein in bacteria (Pastan and Perlman, 1970; Perlman and Pastan, 1971). Nevertheless, in light of the present evidence, this possibility can only be considered speculative.

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Isolation of Brain Endopeptidases: Influence of Size and Sequence of Substrates Structurally Related to Bradykinin†

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ABSTRACT: Two thiol-activated endopeptidases with pH optima near pH 7.5 were isolated from the supernatant fraction of rabbit brain homogenates by DEAE-cellulose chromatography, gel filtration and isoelectrofocusing. Peptide bond hydrolysis was measured quantitatively by ion-exchange chromatography with an amino acid analyzer. Brain kininase A hydrolyzes the Phe^5-Ser^6 peptide bond in bradykinin (Bk), Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9. It is isoelectric near pH 5.2 and has a molecular weight of approximately 71 000. The enzyme also hydrolyzes the Phe–Ser peptide bond in Lys-Bk, Met-Lys-Bk, des-Arg^1-Bk, Lys^9-Bk, Pro-Gly-Phe-Ser-Pro-Phe-Arg, and Gly-Pro-Phe-Ser-Pro-Phe-Arg, but does not hydrolyze (0.1%) this bond in des-Phe^8-Arg^9-Bk. Brain kininase B hydrolyzes the Pro^7–Phe^8 peptide bond in Bk. It is isoelectric at pH 4.9 and has a molecular weight of approximately 68 000. Brain kininase B also hydrolyzes the Pro–Phe bond in Lys-Bk, Met-Lys-Bk, Lys^9-Bk, Ser-Pro-Phe-Arg, and Phe-Ser-Pro-Phe-Arg. Pretreatment of denatured kininogen with brain kininase A or B did not reduce the amount of trypsin-releasable Bk from this precursor protein, indicating that the Bk sequence, when part of a large protein, is not a substrate for either enzyme. However, kininase A and B hydrolyze the octadecapeptide Gly-Leu-Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Ser-Val-Gln-Val. The data show that a large part of the C-terminal portion of bradykinin is important for the brain kininase A activity and, for both enzymes, the size of the peptide and presumably the residues adjacent to the scissile bond are important in determining the rate of peptide bond hydrolysis by these endopeptidases.

The continuous breakdown and renewal of proteins in the brain of mature animals occurs despite the stability of nervous tissue with respect to cell division and differentiation (Leblond and Walker, 1956; Messier et al., 1958; cf. also Droz, 1969). This high turnover of brain proteins is probably related to the functional activity of neurons. Although this relationship has long been postulated (Hydén, 1943, and cf. Droz, 1969), some of the brain proteolytic enzymes that may participate in the process of intracellular catabolism have only been identified in the last decade (cf. Marks and Lajtha, 1971). The difficulties of isolating the enzymes that regulate intracellular protein degradation reflect the fundamental problem of finding

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