Protein Phosphokinase Reactions in Mammalian Testis

STIMULATORY EFFECTS OF ADENOSINE 3':5'-CYCLIC MONOPHOSPHATE ON THE PHOSPHORYLATION OF BASIC PROTEINS

By A. H. REDDI, L. L. EWING* AND H. G. WILLIAMS-ASHMAN

Ben May Laboratory for Cancer Research, University of Chicago, Chicago, Ill. 60637, and Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Md. 21205, U.S.A.

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The soluble fraction of rat testis homogenates is rich in enzymes catalysing the phosphorylation by ATP of many different proteins. The phosphorylation of certain histones, and also of a homogeneous basic protein from guinea-pig seminal-vesicle secretion, is markedly enhanced by 3':5'-cyclic AMP. Various factors affecting these reactions as catalysed by partially purified testicular enzyme preparations are described, and their possible physiological significance is discussed.

Enzymes catalysing the transfer of the terminal phosphate group of ATP to acceptor proteins, of the type first described by Burnett & Kennedy (1954), have since been detected in a wide variety of tissues (Rabinowitz, 1962). Strong impetus for further examination of these reactions was provided by the discovery by Walsh, Perkins & Krebs (1968) in rabbit skeletal muscle of a protein phosphokinase that is activated by 3':5'-cyclic AMP (referred to below as cyclic AMP). Similar enzymes have since been reported to be present in many different eucaryotic and procaryotic cells (Langan, 1968, 1969*a,b*; Corbin & Krebs, 1969; Kuo & Greengard, 1969*a,b*; Miyamoto, Kuo & Greengard, 1969; Gill & Garren, 1970; Jard & Bastide, 1970; Jergil & Dixon, 1970; Kuo, Krueger, Sanes & Greengard, 1970).

During studies on the influence of temperature and development on protein-biosynthetic reactions in rat testis, we noticed that soluble extracts of this organ promoted a rapid phosphorylation of certain exogenous histones by ATP. Three major considerations prompted us to examine these and related reactions in considerable detail. First, elegant studies on the biosynthesis of protamines in fish testis (Dixon & Smith, 1968; Dixon, Ingles, Jergil, Ling & Marushige, 1969) pointed to a possible role of phosphorylation of nascent protamine molecules in their translocation from cytoplasm to nucleus. Secondly, phosphorylation of certain histones has been postulated to influence DNA-histone interactions that may conceivably be

involved in the regulation of RNA polymerase and DNA polymerase reactions (Kleinsmith, Allfrey & Mirsky, 1966; Stevely & Stoken, 1966; Ord & Stocken, 1968; Langan 1969*b*; Buckingham & Stocken, 1970). And thirdly, cyclic AMP has been implicated as an intracellular 'second messenger' in the actions of gonadotrophins on mammalian testes (Murad, Strauch & Vaughan, 1969; Kuehl, Patanelli, Tarnoff & Humes, 1970).

This paper describes the properties of some protein phosphokinase reactions catalysed by soluble preparations from rat testes. The phosphorylation of certain histones, and also of a homogeneous basic protein from guinea-pig seminal-vesicle secretion (Notides & Williams-Ashman, 1967), was found to be markedly enhanced by low concentrations of cyclic AMP. A preliminary account of some of these observations has been reported (Reddi, Ewing & Williams-Ashman, 1970).

MATERIALS AND METHODS

Materials. [γ - ^{32} P]ATP (620 mCi/mmol) was purchased from Amersham/Searle Inc., Des Plaines, Ill., U.S.A. AMP, ADP, ATP and cyclic AMP were obtained from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A. The other cyclic nucleotides were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Mixtures of freeze-dried calf thymus histones (type II-A), salmon sperm protamine, calf thymus DNA, pancreatic deoxyribonuclease, trypsin, creatine phosphokinase and cytochrome *c* were also from Sigma Chemical Co. Calf thymus histone subfractions F1, F2a, F2b and F3 were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. General Biochemical Inc., Chagrin Falls, Ohio, U.S.A., supplied the *Escherichia coli* 'soluble' RNA. Poly(A),

* Present address: Department of Physiology and Pharmacology, Oklahoma State University, Stillwater, Okla. 74074, U.S.A.

poly(U) and poly(C) were from Miles Laboratories Inc., Elkhart, Ind., U.S.A. Mann Research Laboratories Inc., New York, N.Y., U.S.A., supplied the special enzyme-grade $(\text{NH}_4)_2\text{SO}_4$ and casein (Hammersten).

Distilled deionized water was used for the preparations of all solutions. ATP solutions were neutralized by the addition of solid NaHCO_3 . The authenticity of ATP and cyclic AMP was checked by paper chromatography.

Animals. Adult (3–4 months old; 300–350 g) male rats of the Sprague-Dawley strain were used. They were housed in an air-conditioned room and fed on laboratory chow and water *ad libitum*.

Preparation of tissue extracts. Rats were killed by stunning and the testes excised. The tunica albuginea and associated blood vessels were dissected away, and the testicular tissue was placed in ice-cold 20 mM-tris-HCl buffer, pH 7.4 (at 2°C), containing 1 mM-2-mercaptoethanol. The testes were homogenized in 3 vol. of the same buffer in a glass homogenizer with a machine-driven Teflon pestle. The homogenate was centrifuged at 20000g for 15 min in a Sorvall refrigerated centrifuge at 2°C. The supernatant fluid was spun at 42000g for 150 min. The supernatant fluid was saved and is referred to as high-speed supernatant.

Partial purification of protein phosphokinase. All operations were carried out at 0–4°C. The high-speed supernatant fraction, prepared as described above, was fractionated with $(\text{NH}_4)_2\text{SO}_4$ by the addition of the solid salt (17 g/100 ml) with stirring. After standing for 30 min the precipitate was collected by centrifugation at 20000g for 15 min. To the supernatant fluid was added 12 g of $(\text{NH}_4)_2\text{SO}_4$ /100 ml. The precipitate was dissolved in 20 mM-tris-HCl buffer, pH 7.4 (at 2°C), containing 1 mM-2-mercaptoethanol and 0.15 M-NaCl. This solution was dialysed against the same buffer for 14 h with three changes of the medium. The non-diffusible material was centrifuged at 20000g for 20 min. The clear supernatant fluid was used as the enzyme source in most of the studies reported, and is referred to as the 30–50%-saturation- $(\text{NH}_4)_2\text{SO}_4$ fraction. In some experiments the 30–50%-saturation- $(\text{NH}_4)_2\text{SO}_4$ fraction was further subjected to chromatography on a DEAE-cellulose column (2.5 cm × 15.0 cm). The enzyme activity was retained on the column and eluted by a linear gradient of 0.1–0.5 M-NaCl in 20 mM-tris-HCl buffer, pH 7.4, containing 1 mM-2-mercaptoethanol. This procedure afforded an approximately tenfold increase in the histone phosphokinase activity/unit amount of protein in comparison with that of the 30–50%-saturation- $(\text{NH}_4)_2\text{SO}_4$ fraction.

Assay for protein phosphokinase activity. The assay method was based on the transfer of terminal phosphate from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to trichloroacetic acid-precipitable material. The standard assay mixture contained in a final volume of 0.5 ml: sodium phosphate buffer, pH 7.4, 40 μmol ; 2-mercaptoethanol, 5 μmol ; magnesium acetate, 5 μmol ; protein substrate, 3 mg; cyclic AMP, 5 nmol; theophylline, 1 μmol ; $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 1 μmol (0.5×10^6 – 1.5×10^6 c.p.m.). Reactions were initiated by the addition of enzyme and were carried out for 5 min at 37°C unless otherwise stated. The reactions were terminated by the addition of 2 ml of 20% (w/v) trichloroacetic acid containing ATP (0.01%), followed by 0.2 ml of 1.0% (w/v) bovine serum albumin. After the contents had been stirred, the tubes were placed in an ice bath for at least

30 min and then centrifuged at 2000 rev./min in an International clinical centrifuge for 5 min. The precipitate was washed three times with 20% trichloroacetic acid–0.01% ATP solution. A 0.5 ml portion of 88% (w/v) formic acid was used to dissolve the precipitate, to which 10 ml of scintillation-counting fluid was added before radioactivity measurements were made.

In the absence of any added protein substrates there was some incorporation of radioactivity by the enzyme preparations. It was therefore necessary to employ the necessary ‘no-substrate controls’, for which the results expressed throughout the paper have been corrected. Suitable corrections were also applied for ‘zero-time controls’ and background radioactivity.

A modification of the procedure of Dixon & Smith (1968) was used for processing of the reaction mixtures when protamine was employed as substrate. The reaction was halted with 2 ml of 0.5 M-HCl–4.2% (w/v) trichloroacetic acid, followed by 0.2 ml of 1% (w/v) bovine serum albumin. The tubes were centrifuged after standing in an ice bath for 30 min. To the supernatant fluid was added 6 ml of 5% (w/v) trichloroacetic acid containing sodium tungstate (0.25%). The stirred mixtures were kept ice-cold for 30 min to precipitate the protamine. The precipitate was washed four times with the trichloroacetic acid–tungstate solution, the last two times in a boiling-water bath for 5 min. Finally, the precipitate was suspended in 0.5 ml of 88% (w/v) formic acid, to which 10 ml of scintillation-counting fluid was added before measurement of radioactivity.

Preparation of ^{32}P -labelled histone F2b. Radioactive histone F2b was prepared by enzymic phosphorylation by using minor modifications of the method of Meisler & Langan (1969). The reaction mixture contained in a final volume of 2.5 ml: tris-HCl buffer, pH 7.4 (at 37°C), 200 μmol ; 2-mercaptoethanol, 25 μmol ; magnesium acetate, 25 μmol ; histone F2b, 10 mg; $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 2.5 μmol (3.75×10^7 c.p.m.); 30–50%-saturation- $(\text{NH}_4)_2\text{SO}_4$ fraction from rat testis as an enzyme source, 3.6 mg. The tubes were incubated for 4 h at 37°C. The radioactive histone was isolated essentially as described by Meisler & Langan (1969).

Assay for histone phosphatase activity. The assay method was based on that of Meisler & Langan (1969). The reaction mixture contained in a final volume of 0.5 ml: tris-HCl buffer, pH 7.4 (at 37°C), 4 μmol ; 2-mercaptoethanol, 5 μmol ; magnesium acetate, 5 μmol ; the enzyme source was the same protein fraction as that used in the protein phosphokinase assay. The reaction was initiated by the addition of ^{32}P -labelled histone F2b and was allowed to proceed for 5 min at 37°C. The reaction was terminated by addition of 2 ml of 5 mM-silicotungstic acid in 5 mM- H_2SO_4 followed by 0.2 ml of 1% (w/v) bovine serum albumin, and the tubes were placed in an ice bath. After centrifugation of the mixture, 2 ml of the supernatant fluid was treated with 0.5 ml of 5% (w/v) ammonium molybdate in 4 M- H_2SO_4 . The phosphomolybdate complex was extracted in 2.5 ml of 2-methylpropan-1-ol-benzene (1:1, v/v). A sample of the organic phase was pipetted into counting vials containing 10 ml of scintillation-counting fluid.

Measurement of radioactivity. Radioactivity was determined in a Nuclear-Chicago Unilux liquid-scintillation system. The counting efficiency for ^{32}P was approx. 80%. The background was in the range 25–35 c.p.m. The

scintillation-counting fluid had the following composition: xylene-dioxan-ethanol (8:8:9, by vol.) containing naphthalene (7.5%, w/v) 2,5-diphenyloxazole (0.45%) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.0045%).

Preparation of DNA solutions. Calf thymus DNA was dissolved in 20 mM-tris-HCl buffer, pH 7.4 (at 25°C), containing 10 mM-NaCl. The concentration of DNA was determined on the basis of an extinction coefficient of $0.020 \text{ l} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$ at 260 nm. Heat denaturation was achieved by heating the same solution in a boiling-water bath for 10 min followed by rapid quenching in ice.

Isolation of nuclei. Testes were homogenized in 0.25 M-sucrose containing 3 mM-MgCl₂. Then 1 vol. of the homogenate was mixed with 4 vol. of 2.3 M-sucrose-3 mM-MgCl₂ (yielding a solution 1.89 M with respect to sucrose), which was layered on 10 ml of 2.3 M-sucrose. The tubes were centrifuged at 42000g for 2 h at 2°C. With this discontinuous sucrose density gradient nuclei and spermatozoa can be separated to a large extent (J. Jänne, personal communication). However, there remained some degree of cross-contamination of the nuclei, by spermatozoa as observed under a light-microscope. The nuclei and residual spermatozoa were extracted with 20 mM-tris-HCl buffer, pH 7.4 (at 2°C), containing 1 mM-2-mercaptoethanol, and used in enzyme assays.

Sucrose-density-gradient centrifugation. Linear sucrose gradients (5–20%, w/v) were prepared in 20 mM-tris-HCl buffer, pH 7.4 (at 2°C), containing 1 mM-2-mercaptoethanol, in a Beckman density-gradient former. The enzyme preparation [0.1 ml of the 30–50%-saturation-(NH₄)₂SO₄ fraction] containing 2–3 mg of protein was layered on top of the gradient and centrifuged in a Beckman-Spinco model L2-65B ultracentrifuge for 5 h at 60000 rev./min at 2°C. Sedimentation coefficients were calculated by the method of Martin & Ames (1961), with crystalline yeast alcohol dehydrogenase as marker assuming $s_{20,w}$ 7.6S for this protein (Martin & Ames, 1961).

Other methods. The basic protein from the guinea-pig seminal-vesicle secretion was isolated and characterized by methods described by Notides & Williams-Ashman (1967). Protein was determined by the method of Lowry,

Rosebrough, Farr & Randall (1951), with crystalline bovine plasma albumin as standard. pH measurements were made with a glass electrode and refer strictly to the stated temperatures.

RESULTS

Phosphorylation of various proteins by soluble preparations from rat testis. Crude ultracentrifuged extracts of rat testis catalysed the phosphorylation of a number of different histones and also of casein and phosvitin. Preparations of testicular cell nuclei, largely freed from spermatozoa by the methods described above, had only a feeble ability to promote these transphosphorylations. The enzyme or enzymes responsible for phosphorylation of the various proteins examined were largely precipitated from the soluble fraction of homogenates by ammonium sulphate between 30 and 50% saturation at 0°C. Table 1 shows that, at a saturating concentration of 10 μM , cyclic AMP markedly enhanced ³²P transfer to crude mixtures of calf thymus histones, and especially to calf thymus F1 and F2b histones, as classified by the terminology of Johns (1964). The stimulation by cyclic AMP of the phosphorylation of crude mixtures of calf thymus histones varied considerably from one batch of commercially obtained preparations to another. Smaller although clearly significant activation by cyclic AMP of the phosphorylation of calf thymus F3 histone and of protamine was observed. In contrast, ³²P transfer to calf thymus F2a histone, casein and phosvitin was not increased at all by cyclic AMP, although the base-line phosphorylation of these proteins was quite high. It was consistently observed that at low initial concentrations of labelled ATP (0.1 mM), ³²P transfer to casein (but

Table 1. *Phosphorylation of various proteins by soluble rat testicular preparations*

Assays were performed as described in the Materials and Methods section. The different protein substrates used and their concentration is indicated. The enzyme was 300 μg of 30–50%-saturation-(NH₄)₂SO₄ fraction. Reactions were carried out at 37°C for 5 min. [γ -³²P]ATP of specific radioactivity 1080 c.p.m./nmol was used. All histone preparations were from calf thymus.

Substrate	Concn. (mg/ml)	³² P transferred to protein (c.p.m.)	
		Minus cyclic AMP	Plus cyclic AMP (10 μM)
Histone mixture	2	934	4594
Histone mixture	6	1804	8710
Histone F1	2	230	3567
Histone F2a	2	653	633
Histone F2b	2	855	11380
Histone F3	2	1390	2990
Protamine	2	653	992
Casein	2	3156	3100
Phosvitin	2	3025	3220

Table 2. *Phosphorylation of guinea-pig seminal-vesicle-secretion basic protein by testicular enzyme preparations*

Reactions were carried out as described in the Materials and Methods section. The incubations were carried out at 37°C for 5 min unless otherwise stated. The protein substrate (guinea-pig seminal-vesicle-secretion basic protein) was present at a final concentration of 5 mg/ml unless otherwise stated. The enzymes was 270 μ g of 30–50% saturation-(NH₄)₂SO₄ fraction. [γ -³²P]ATP of specific radioactivity 1465 c.p.m./nmol was used.

Experiment	³² P transferred to seminal-vesicle-secretion basic protein (c.p.m.)	
	Minus cyclic AMP	Plus cyclic AMP (10 μ M)
Time-course:		
5 min	777	3270
10 min	1727	5920
20 min	3550	11270
Concn. of substrate:		
2.5 mg/ml	665	1860
5.0 mg/ml	777	3270
7.5 mg/ml	1230	3570

not to phosvitin) was actually inhibited by 10 μ M-cyclic AMP. This inhibition was overcome by raising the ATP concentration to the value (2 mM) normally present in the standard assay system. In all of these experiments theophylline was added to the reaction mixtures so as to diminish the activity of any cyclic AMP phosphodiesterase in the enzyme preparations. Nonetheless omission of theophylline did not affect the results. The following proteins did not appear to be phosphorylated by the soluble testicular preparations in the absence or presence of cyclic AMP: lysozyme, cytochrome c, pancreatic deoxyribonuclease or ribonuclease, bovine serum albumin, trypsin, chymotrypsin, pyruvate kinase and creatine phosphokinase.

Crude mixtures of acetone-dried saline-soluble proteins from rat and guinea-pig seminal-vesicle secretions (Gotterer, Ginsburg, Schulman, Banks & Williams-Ashman, 1955) readily accepted ³²P from the terminal phosphate of ATP in reactions catalysed by soluble testicular extracts, which were stimulated many-fold by cyclic AMP. A homogeneous basic protein from guinea-pig vesicular secretion (Notides & Williams-Ashman, 1967) was an excellent substrate for these cyclic AMP-activated transphosphorylations (Table 2). Recent studies by A. C. Notides (personal communication) have indicated that the amino acid composition of this serine-rich protein is slightly different from that originally reported by Notides & Williams-Ashman (1967). The basic protein, which is clearly different from any known histone, has a molecular weight of about

Fig. 1. Time-course of phosphorylation of calf thymus F2b histone in the absence (○) and in the presence (●) of cyclic AMP (10 μ M). The standard assay system is described in the text. Histone F2b was present at a final concentration of 1 mg/ml.

17900 and the following amino acid composition: (Asp₁₁, Thr₆, Ser₂₃, Glu₂₈, Gly₁₉, Ala₆, Val₁₃, Met₆, Ile₂, Leu₁₄, Phe₆, Lys₁₆, His₂, Arg₁₁, Trp₁). The protein is devoid of tyrosine, proline and cyst(e)ine, and contains 21 amide groups/molecule. As isolated, the protein is devoid of phosphorus.

Kinetic parameters. The time-course of phosphorylation of calf-thymus F2b histone is depicted in Fig. 1. The initial rate of ³²P transfer is strongly enhanced by 10 μ M-cyclic AMP. The reaction is linear for at least 30 min in the absence of cyclic AMP. The gradual tailing off of the reaction and its virtual cessation within 20 min is not due either to exhaustion of the ATP substrate (the adenosine triphosphatase activity of such testicular ammonium sulphate fractions was found to be negligible under the conditions of the histone-phosphorylation assay) or to extensive decomposition of the added cyclic AMP.

At the termination of the reaction shown in Fig. 1, about 20 nmol of ³²P was transferred to the 1 mg of calf thymus F2b histone present. Under standard conditions the initial rates of reaction in the absence or in the presence of cyclic AMP were proportional to the amount of enzyme protein added.

Phosphorylation of histones in the absence or in the presence of cyclic AMP was absolutely dependent on Mg²⁺, which saturated the systems at a concentration of 10 mM. At a final concentration of 5 mM, Mn²⁺ or Co²⁺ was about 20% as effective as 10 mM-Mg²⁺. About the same degree of stimulation of the phosphorylation of calf thymus F2b histone by cyclic AMP was observed with 1 mM-Mn²⁺ or

Table 3. *Effect of cyclic AMP on the phosphorylation of various concentrations of calf thymus F2b histone*

The reactions were carried out as described in the Materials and Methods section, except that various concentrations of histone F2b were used. [γ - ^{32}P]ATP of specific radioactivity 1150 c.p.m./nmol was used.

Concn. of histone (mg/ml)	^{32}P transferred to histone F2b (c.p.m.)		B/A ratio
	Minus cyclic AMP (A)	Plus cyclic AMP (10 μM) (B)	
0.1	235	635	2.7
0.2	264	1296	4.9
0.5	380	3200	8.8
1.0	557	5890	10.6
2.0	1200	9014	7.5

Fig. 2. (a) Effect of ATP concentration on the phosphorylation of guinea-pig seminal-vesicle-secretion basic protein. Assays were carried out as described in the Materials and Methods section with different ATP concentrations in the presence (●) and in the absence (○) of cyclic AMP (10 μM). The protein substrate was present at a final concentration of 5 mg/ml. (b) Double-reciprocal plot of the same results.

10 mM- Mg^{2+} . Ca^{2+} could not substitute for Mg^{2+} , and was very inhibitory to the phosphorylation of histones in the presence of 10 mM- Mg^{2+} .

Maximal rates of ^{32}P transfer to either histones or the basic protein from guinea-pig vesicular secretion occurred at pH 7.4 in the absence or in the presence

of cyclic AMP. A broad optimum in the pH range 7–8 was observed.

Table 3 shows that the stimulatory action of cyclic AMP on the phosphorylation of calf thymus F2b histone was optimum when the final histone concentration was about 1 mg/ml. In other experiments somewhat different values were obtained for the optimum concentration of other proteins whose phosphorylation could be enhanced by cyclic AMP. The effect of alteration of ATP concentration on the phosphorylation of a protein in the absence and in the presence of cyclic AMP was studied with the homogeneous basic protein from guinea-pig seminal-vesicle secretion as acceptor. It is evident from Fig. 2 that the variations in initial linear rates of phosphorylation of this protein due to alteration of the ATP concentration present a complex kinetic picture in which both the apparent K_m and the maximal velocities at high ATP concentrations are influenced by addition of saturating quantities of cyclic AMP. The values obtained for the apparent K_m for ATP in the absence or in the presence of cyclic AMP were somewhat higher than those reported by Kuo *et al.* (1970) for protein phosphokinase preparations from a variety of bovine tissues when crude histone mixtures were used as substrates.

Specificity of activation by 3':5'-cyclic nucleoside monophosphates. Under conditions where cyclic AMP stimulated many-fold the phosphorylation of certain histones or the basic protein from guinea-pig seminal-vesicle secretion, no enhancement of ^{32}P incorporation into various protein substrates was observed with AMP, ADP or 2':3'-cyclic AMP at final concentrations of 10 μM . Table 4 shows, however, that other 3':5'-cyclic ribonucleotides besides cyclic AMP enhanced the phosphorylation of histones by soluble testicular enzyme preparations, although at the high concentrations tested (10 μM) none of these cyclic nucleotides was as effective as cyclic AMP. Fig. 3 shows that the concentration of cyclic AMP needed to evoke half-maximal increases

Table 4. *Specificity of the activation of protein phosphokinase reactions by 3':5'-cyclic nucleotides*

Reactions were carried out as described in the Materials and Methods section. The enzyme was 310 μg of 30–50% saturation- $(\text{NH}_4)_2\text{SO}_4$ fraction. The protein substrate (3mg) was a mixture of calf thymus histones. Cyclic nucleotides were tested at a final concentration of 10 μM . $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ of specific radioactivity 325 c.p.m./nmol was used.

3':5'-Cyclic nucleotide	^{32}P transferred to histone (c.p.m.)
None	536
Cyclic AMP	2085
Cyclic GMP	1500
Cyclic CMP	1355
Cyclic UMP	1096

in phosphorylation of the basic vesicular-secretion protein was about 0.1 μM . Similar values were obtained when mixtures of calf thymus histones were used as substrates. Further experiments will be necessary to determine whether the stimulatory effects of high concentrations of 3':5'-cyclic nucleotides other than cyclic AMP (Table 4) are truly genuine or reflect contamination of the commercially obtained samples with small amounts of cyclic AMP.

Effects of temperature. With either calf thymus F1 histone or F2b histone as substrate the transphosphorylations of ^{32}P from γ -labelled ATP increased markedly as the temperature of incubation was raised over the range from 25 to 45°C. The stimulation by cyclic AMP was about the same at 25 and 37°C, but was considerably diminished at 45°C, although in the absence of cyclic AMP the phosphorylation of these histones was increased to a greater extent by raising the temperature from 37 to 45°C as compared with the temperature differential from 25 to 37°C (Table 5). These experiments were carried out with short periods of incubation (5 min), so that phosphorylation of the histones in the absence and in the presence of cyclic AMP was linear with respect to time at all temperatures examined. Other studies (not shown) revealed that with longer periods of incubation the rates of histone phosphorylation declined markedly with respect to time in the presence of cyclic AMP at higher temperatures, so that the apparent depression of the stimulatory effects of cyclic AMP at 45°C appeared even greater than that shown in Table 5.

Ionic strength and the stimulatory action of cyclic AMP. The total ionic strength of the standard test system used for measurement of protein phosphokinase activity (see the Materials and Methods section) was about 0.15. Increasing the ionic strength of the incubation mixture by the

Fig. 3. Effect of varying the concentration of cyclic AMP on the initial rate of phosphorylation of histone F2b. Reactions were carried out as described in the Materials and Methods section. Histone F2b was present at a final concentration of 2 mg/ml. Phosphate incorporated into histone in the absence of cyclic AMP (0.49 nmol) has been subtracted from values shown on the ordinate.

addition of sodium chloride or ammonium sulphate (neutralized to pH 7.4 with aq. ammonia) decreased the rate of ^{32}P transfer. The effects of high salt concentration were less pronounced in the absence than in the presence of cyclic AMP. With added sodium chloride at a final concentration of 0.6M or with ammonium sulphate at a final concentration of 0.2M there was obliteration of the stimulatory effect of cyclic AMP, but with retention of more than 20% of the base-line histone phosphorylation. At lower increments of ionic strength the inhibitory effect of ammonium sulphate on the enhancement of histone phosphorylation by cyclic AMP was greater than that resulting from comparable increases of ionic strength due to addition of sodium chloride. Table 6 shows that addition of sodium chloride to the standard test system to a final concentration of 0.3M: (a) slightly decreased the phosphorylation of calf thymus F2b histone, and diminished to a small extent its enhancement by cyclic AMP; (b) markedly depressed the phosphorylation of casein and phosvitin, which were unaffected by cyclic AMP in the absence or in the presence of the additional sodium chloride; (c) increased the phosphorylation of protamine, and caused a pronounced rise in its stimulation by cyclic AMP. Jergil & Dixon (1970) reported that high ionic strengths enhanced the cyclic-AMP-stimulatable phosphorylation of protamine by a protein phosphokinase preparation from trout testes.

Effectors. With an ammonium sulphate fraction from rat testis prepared and tested in the absence of 2-mercaptoethanol it was found that *p*-chloro-mercuribenzenesulphonate and *N*-ethylmaleimide inhibited the phosphorylation of crude mixtures of calf thymus histones (Table 7). At high concentra-

Table 5. *Effect of temperature on the phosphorylation of various histones in the absence and in the presence of cyclic AMP*

Reactions were carried out as described in the Materials and Methods section at the stated temperatures. The incubations were carried out for 5 min. The enzyme was 228 μ g of 30–50% saturation- $(\text{NH}_4)_2\text{SO}_4$ fraction. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ of specific radioactivity 1500 c.p.m./nmol was used.

Temp. (°C)	³² P transferred to histone F1 (c.p.m.)		Increase (%)
	Minus cyclic AMP	Plus cyclic AMP (10 μ M)	
25	232	2205	850
37	463	4657	900
45	1170	8585	630

Temp. (°C)	³² P transferred to histone F2b (c.p.m.)		Increase (%)
	Minus cyclic AMP	Plus cyclic AMP (10 μ M)	
25	360	2655	630
37	824	6857	730
45	3940	9770	147

Table 6. *Effect of ionic strength of testicular protein phosphokinase reactions*

Reactions were performed with the standard test system described in the Materials and Methods section. The ionic strength was increased by the addition of NaCl to a final concentration of 0.3 M. Incubations were for 5 min at 37°C. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ of specific radioactivity 1660 c.p.m./nmol was used. All protein substrates were at a final concentration of 6 mg/ml.

Substrate	³² P transferred to protein (c.p.m.)			
	Controls		Plus 0.3 M-NaCl	
	Minus cyclic AMP	Plus cyclic AMP (10 μ M)	Minus cyclic AMP	Plus cyclic AMP (10 μ M)
Histone F2b	1010	9790	705	5110
Protamine	1250	1370	1494	2250
Casein	3160	3380	1440	1350
Phosvitin	4830	4890	770	774

tions (1 mM) these substances depressed the stimulation of histone phosphorylation evoked by cyclic AMP. These effects of thiol-blocking reagents were obscured when 2-mercaptoethanol (10 mM) was present in the standard test system.

A variety of polynucleotides inhibited the phosphorylation of histones and depressed to a greater extent the stimulatory effects of cyclic AMP. A representative experiment is summarized in Table 8, which shows that all the polynucleotides tested, with the exception of native calf thymus DNA, markedly decreased the actions of cyclic AMP. Although the polynucleotides examined all contained 3':5'-monophosphate diester bonds of cyclic AMP, the mechanism by which these polynucleotides so profoundly depress the stimulation of histone phosphorylation by cyclic AMP remains to be determined, and may well involve complexing of the polynucleotides with the histone substrates

and/or the enzyme catalysing these transphosphorylations.

In concentrations of 10 mM the polyamines spermine and spermidine and the diamine putrescine did not effect the phosphorylation of histones in the absence or in the presence of cyclic AMP, and also could not replace the Mg^{2+} requirement for these reactions. Two uncouplers of oxidative phosphorylation, 2,4-dinitrophenol and dicoumarol were also without effect on these reactions at a final concentration of 0.1 mM.

Dephosphorylation of histones. Calf thymus F2b histone was phosphorylated enzymically by the methods described above, and then incubated in the test system used to study protein phosphokinase reactions, but in the absence of labelled ATP. Table 9 shows that the testicular protein phosphokinase preparations dephosphorylated the labelled histone in reactions that were stimulated when the

Table 7. *Effects of thiol-blocking reagents on the cyclic-AMP-dependent phosphorylation of histone*

Reactions were carried out as described in the Materials and Methods section. The enzyme was 260 μg of 30–50%-saturation- $(\text{NH}_4)_2\text{SO}_4$ fraction. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ of specific radioactivity 610 c.p.m./nmol was used. A mixture of calf thymus histones (final concn. 6 mg/ml) was used as substrate. The enzyme was prepared and tested in the absence of 2-mercaptoethanol.

Addition	^{32}P transferred to histone (c.p.m.)		B/A ratio
	Minus cyclic AMP (A)	Plus cyclic AMP (10 μM) (B)	
None	1012	2555	2.5
<i>p</i> -Chloromercuribenzenesulphonate			
1 mM	398	600	1.5
0.1 mM	804	1007	1.25
0.01 mM	940	2340	2.2
<i>N</i> -Ethylmaleimide			
1 mM	513	810	1.6
0.1 mM	793	2040	2.5
0.01 mM	895	2393	2.7

Table 8. *Effect of polynucleotides on the cyclic AMP-dependent phosphorylation of calf thymus histone F2b*

The reaction mixture contained in a final volume of 0.25 ml: sodium phosphate buffer, pH 7.4, 20 μmol ; magnesium acetate, 2.5 μmol ; 2-mercaptoethanol, 2.5 μmol ; histone F2b, 0.25 mg; polynucleotide, 0.25 mg; cyclic AMP 2.5 μmol ; theophylline, 0.5 μmol . The reaction was carried out for 5 min at 37°C. Other procedures were as described in the Materials and Methods section. The enzyme was 188 μg of 30–50%-saturation- $(\text{NH}_4)_2\text{SO}_4$ fraction. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ of specific radioactivity 3840 c.p.m./nmol was used.

Addition	^{32}P transferred to histone (c.p.m.)	
	Minus cyclic AMP	Plus cyclic AMP (10 μM)
None	1078	10801
Native calf thymus DNA	793	5823
Heated calf thymus DNA	390	895
<i>E. coli</i> 'soluble' RNA	495	992
Poly(A)	421	754
Poly(U)	505	950
Poly(C)	545	1212

salt concentration was increased by the addition of sodium chloride to a final concentration of 0.2M. The increases in histone dephosphorylation at lower ionic strengths due to addition of ATP or Mg^{2+} may possibly be ascribed to the contribution of these substances to the ionic strength of the reaction mixture. It is worth noting that Meisler & Langan (1969) found that dephosphorylation of ^{32}P -labelled histones by rat liver preparations was enhanced at high ionic strengths. Cyclic AMP caused only small increases in this enzymic dephosphorylation of calf

Table 9. *Dephosphorylation of preformed ^{32}P -labelled histone F2b by testes enzyme preparation*

Reactions were carried out as described under 'Assay for histone phosphatase activity' in the Materials and Methods section. Incubations were carried out for 5 min, and were initiated by the addition of 0.45 mg of ^{32}P -labelled histone F2b (36000 c.p.m.). The enzyme was 230 μg of 30–50%-saturation- $(\text{NH}_4)_2\text{SO}_4$ fraction.

System	$^{32}\text{P}[\text{P}]_i$ released (c.p.m.)	
	Minus 0.2M-NaCl	Plus 0.2M-NaCl
Complete	2036	4410
Minus Mg^{2+}	1685	4440
Plus ATP (2 mM)	4160	5440
Plus cyclic AMP (10 μM) and theophylline (2 mM)	3310	5100

thymus F2b histone. The precise nature and multiplicity of enzymes catalysing the dephosphorylation of histones in these testicular preparations remains to be clarified.

Sedimentation characteristics. Ammonium sulphate fractions from rat testis were subjected to ultracentrifugation in buffered linear 5–20% (w/v) sucrose density gradients in the absence and in the presence of cyclic AMP. A typical experiment is illustrated in Fig. 4, for which a mixture of calf thymus histones was used for measurement of protein phosphokinase activity, which was in each instance determined either with or without the addition of cyclic AMP to the reaction mixture. When the sucrose-density-gradient preparations were centrifuged in the absence of cyclic AMP

Fig. 4. Sucrose-density-gradient centrifugation of the testicular protein phosphokinase. A sample of the 30–50%-saturation- $(\text{NH}_4)_2\text{SO}_4$ fraction (2.7 mg of protein in a volume of 0.1 ml) was layered on 4.8 ml of linear 5–20% (w/v) sucrose gradient in 20 mM-tris-HCl buffer, pH 7.4 (at 2°C), containing 1 mM-2-mercaptoethanol, in the absence (a) and in the presence (b) of $10\text{ }\mu\text{M}$ -cyclic AMP. The gradients were centrifuged at 60 000 rev./min in a Beckman-Spinco model L2-65B ultracentrifuge for 5 h. The direction of sedimentation is from right to left. Fractions of volume 0.25 ml were collected in the cold and assayed for protein phosphokinase activity, with calf thymus histone mixture (Sigma Chemical Co.) as substrate, in the absence (○) and in the presence (●) of cyclic AMP ($10\text{ }\mu\text{M}$). The recovery of enzyme activity was almost complete when the gradient was run without cyclic AMP; however, there was a recovery of only 65% of the applied enzyme activity when cyclic AMP was present in the gradient. The arrow indicates the sedimentation of yeast alcohol dehydrogenase used as a marker ($s_{20,w}$ 7.6S).

(Fig. 4a) the histone kinase activity sedimented as a single symmetrical peak with a sedimentation coefficient of about 7S. The phosphorylation of histones by this material was markedly enhanced by cyclic AMP. When the sucrose density gradient contained cyclic AMP (Fig. 4b) some of the total histone phosphokinase activity behaved as if the sedimentation coefficient was approx. 5.4S (the activity of this material was still enhanced by the addition of cyclic AMP to the test system); little histone phosphokinase activity sedimented in the 7S region, but a considerable proportion of the total enzyme activity sedimented more quickly (the latter fractions were not so markedly stimulated by cyclic AMP). This suggests that aggregation of some of the histone phosphokinase activity occurred when the sucrose density gradients contained cyclic AMP.

DISCUSSION

Protein phosphokinases that are stimulated by cyclic AMP have been detected in a wide spectrum of procaryotic and eucaryotic organisms (Kuo & Greengard, 1969a,b). Our rat testicular preparations catalysed the phosphorylation of a number of different proteins, but only ^{32}P transfer to certain histones and a homogeneous basic protein from guinea-pig seminal-vesicle secretion was increased by the addition of cyclic AMP. It is worth noting that, unlike a protein kinase from rabbit skeletal muscle described by Walsh *et al.* (1968), the activity of which appeared to be almost completely dependent on exogenous cyclic AMP, all of the cyclic AMP-stimulatable reactions catalysed by our testicular preparations, even those that were further refined by ion-exchange chromatography, proceeded to a

considerable extent without the addition of cyclic AMP. In fact, the reactions exhibited a more prolonged linearity with respect to time at 37°C in the absence than in the presence of saturating concentrations of cyclic AMP. Similar findings have been recorded with protein phosphokinases from liver (Langan, 1968), brain (Miyamoto *et al.* 1969) and adipose tissue (Corbin & Krebs, 1969). Unlike some of the latter enzyme preparations, the phosphorylation of casein or phosvitin by the testicular extracts we have studied was not increased by cyclic AMP, and at low ATP concentrations cyclic AMP significantly inhibited ^{32}P transfer to casein. The precise multiplicity of soluble protein phosphokinases in rat testis remains to be determined. There are indications that skeletal muscle contains a single enzyme catalysing the cyclic AMP-stimulated phosphorylation of both phosphorylase *b* kinase and of glycogen synthetase, resulting in the respective activation and inactivation of these two enzymes (Soederling & Hickenbottom, 1970). After our experiments had been completed, Yamamura, Takeda, Kumon & Nishizuka (1970) reported that two protein phosphokinases could be separated from rat liver cytosol by chromatography on hydroxyapatite; both enzymes catalysed the phosphorylation of a number of different histones, but one enzyme was strongly activated by cyclic AMP whereas the other one was not. Whether the two catalytic proteins separated by Yamamura *et al.* (1970) represent two entirely distinct enzymes or are comprised of different combinations of subunits (conceivably different ones determining catalytic and regulatory activities) cannot be decided from the limited experimental evidence available.

From experiments with rat thymus, and with human lymphocyte cultures dividing in response to phytohaemagglutinin (Kleinsmith *et al.* 1966; Stevely & Stocken, 1966), it has been concluded that histone phosphorylation may be involved in regulation of RNA transcription and possibly DNA replication. Langan (1969*b*) obtained convincing evidence that administration of glucagon to rats enhances the phosphorylation of histone F1 in liver. However, it is not altogether clear whether histone F1 in rat liver nuclei was increasingly phosphorylated under these conditions. There is no evidence for or against the possibility that histone phosphorylation plays a role in the control of gene expression or DNA synthesis in testicular cells. Experiments on hormonal influences on histone phosphokinase reactions in testis and other reproductive organs are described in the Appendix.

The comprehensive investigations by Dixon *et al.* (1969) and Jergil & Dixon (1970) have shown that in trout testis protamines are phosphorylated soon after their manufacture on cytoplasmic polyribo-

somes. It may well, be as these authors point out, that such phosphorylation of protamines plays a control role in the transport of these basic proteins from the cytoplasm to nuclei of spermatid cells, followed by dephosphorylation of the protamines before their combination with the DNA of cell nuclei that are ultimately destined to become part of the heads of spermatozoa. One could imagine that analogous reactions might be involved in the intracellular translocation of basic proteins that eventually come in union with the DNA of the heads of mammalian spermatozoa. As discussed above, our knowledge of the chemistry of the latter basic proteins in mammalian sperm-cell heads remains in a rudimentary state.

It has been suggested that administration of both follicle-stimulating hormone and interstitial-cell-stimulating (luteinizing) hormone (Murad *et al.* 1969; Kuehl *et al.* 1970) can increase the formation of cyclic AMP in mammalian testes. Whether cyclic AMP-stimulated phosphorylation of histones or other proteins plays a part in the train of biochemical events underlying the biological actions of these gonadotrophins remains an open question.

A rather unexpected finding was the rapid rate of phosphorylation, markedly enhanced by cyclic AMP, of a homogeneous basic protein obtained from the seminal-vesicle secretion of the guinea pig. This protein, which has a rather unusual amino acid composition lacking proline, tyrosine and cyst(e)ine, contains 23 serine residues/molecule. The protein is clearly not identical with any known histone, and appears to have a clear-cut physiological function, namely to serve as a precursor of the seminal clot formed after ejaculation by the action of the prostatic enzyme vesiculase on the basic protein in vesicular secretion (Notides & Williams-Ashman, 1967). When isolated this protein is totally devoid of phosphorus. But it is conceivable that it may be phosphorylated in seminal-vesicle epithelial cells, but subsequently dephosphorylated before its secretion into the lumina of the gland. The available evidence suggests that this protein is readily subject to phosphorylation in reactions stimulated by cyclic AMP that are catalysed by the same enzyme or enzymes responsible for histone phosphorylation. The ease of isolation of relatively large quantities of this basic protein is a completely homogeneous state suggests that it may prove to be a useful substrate for further studies on the mechanism of protein-phosphorylating enzymes that are directly activated by cyclic AMP.

From considerations of the enthalpy and free energy of hydrolysis of cyclic AMP Greengard, Hayaishi & Colowick (1969) have postulated that cyclic AMP could actually adenylylate certain proteins, leading to the formation of covalent linkages between the elements of 5'-AMP and one

or more amino acid residues. None of our observations hint that cyclic AMP enhances the phosphorylation of histones or the basic protein of vesicular secretion by truly adenylating the enzyme or enzymes catalysing these reactions. In many experiments performed under a variety of experimental conditions we did not notice any increase in the activating effect of cyclic AMP due to prior incubation of the cyclic nucleotide with the enzyme in the absence of protein substrate and/or ATP. However, it is evident from the experiments on the sedimentation behaviour of protein phosphokinase activity in sucrose density gradients in the absence and in the presence of cyclic AMP that interaction of cyclic AMP with the enzyme can result in complex changes in its physical properties.

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APPENDIX

Hormonal Influences on Adenosine 3':5'-Cyclic Monophosphate-Stimulated Protein Phosphokinase Reactions in Mammalian Reproductive Organs

It is now generally accepted that cyclic AMP serves as an intracellular 'second messenger' in the action of a variety of polypeptide and catecholamine hormones (Butcher, Robison & Sutherland, 1970). A number of hormones influencing the size and functions of mammalian reproductive organs have been reported to influence the steady-state con-

centration of cyclic AMP in these tissues. For example, administration of follicle-stimulating hormone and interstitial-cell-stimulating hormone increases adenylate cyclase activity by particulate preparations from canine and rat testes (Murad, Strauch & Vaughan, 1969; Kuehl, Patanelli, Tarnoff & Humes, 1970). The concentration of

Table 1. *Effect of age on soluble protein phosphokinase activities of rat testes*

Results are expressed as c.p.m. of ^{32}P transferred to acceptor protein/5 min per mg of enzyme protein at 37°C , measured by the standard assay system. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ of specific radioactivity 1460 c.p.m./nmol was used. High-speed supernatant fluids from rat testes were used as enzyme source. There were 6–12 rats per group and assays were carried out in duplicate. Other details are as described in the Materials and Methods section of the main paper. Histone F2b was present at a final concentration of 2 mg/ml; casein and phosvitin were present at 6 mg/ml. The final concentration of cyclic AMP was $10\text{ }\mu\text{M}$.

Age (days)	Histone F2b		Casein		Phosvitin	
	Control	Plus cyclic AMP	Control	Plus cyclic AMP	Control	Plus cyclic AMP
10	4150	19575	10340	9395	20650	22050
21	2303	14280	9606	8424	15340	13990
42	3300	21310	12230	12040	16600	14100
70	4574	20770	11170	11460	13490	13130

cyclic AMP in immature rat uterus was found by Szego & Davis (1967) to be elevated as quickly as 15 s after oestrogen injection. According to Hechter, Yoshinaga, Halkerston & Birchall (1967), cyclic AMP affects several metabolic processes carried out by isolated rat uteri which are known to be influenced by oestrogenic hormones *in vivo*. Stimulation by cyclic AMP of the uptake of labelled amino acids into proteins (Griffin & Szego, 1968) and of tritiated uridine and leucine into RNA and protein (Sharma & Talwar, 1970) by rat uterine preparations *in vitro* has been reported. Increases in the adenylate cyclase activity of chick oviduct by administration of progesterone were found by Rosenfeld & O'Malley (1970) and by Kissel, Rosenfeld, Chase & O'Malley (1970). The experiments of Singhal, Vijayvargiya & Ling (1970) led these authors to conclude that cyclic AMP mimics some of the actions of androgens on the growth and enzyme content of rat seminal vesicle.

The aforementioned observations together with the findings described above made us decide to examine various cyclic AMP-dependent protein phosphokinase reactions in rat testis, ventral prostate and uterus in relation to the hormonal status of the host animals. Ultracentrifuged extracts of these organs were found to catalyse the phosphorylation of a variety of proteins by ATP in reactions that were completely dependent on added Mg^{2+} and were saturated with ATP at a final concentration of 2 mM. Phosphorylation of calf thymus F2b histone by uterine extracts was enhanced nearly sixfold by a saturating concentration of cyclic AMP of $10\text{ }\mu\text{M}$; the same concentration of 5'-AMP was ineffective. By contrast, the phosphorylation of casein by uterine extracts was depressed to a small but significant extent by cyclic AMP, the inhibition being substantially greater when the concentration of ATP was lowered to 0.1 mM from the standard one of 2 mM.

Table 1 of this Appendix illustrates the phos-

phorylation of calf thymus F2b histone, casein and phosvitin in the absence and the presence of cyclic AMP by soluble extracts of the testes of rats of different age groups. Over the period examined, the rat testis develops from its immature state into one in which is attained, at age 70 days, the adult ability to carry out complete spermatogenesis as well as secretion of adult concentrations of androgens by the Leydig cells, largely as a result of increased output of gonadotrophins by the anterior pituitary gland. The base-line rates of phosphorylation of all three proteins in the absence of cyclic AMP, and the degree of stimulation of phosphorylation of calf thymus histone F2b by cyclic AMP, were not grossly altered at 21, 42 and 70 days, compared with values at 10 days of age of the animals. Similar findings were obtained in two other large-scale experiments, and in every instance a small decrease in phosphorylation of phosvitin (with or without addition of cyclic AMP) was noted after 10 days of age.

Table 2 (of this Appendix) summarizes experiments on effects of orchietomy and subsequent administration of testosterone propionate on phosphorylation of calf thymus F2b histone and protamine by soluble extracts of rat ventral prostate. Withdrawal or administration of androgen had little influence on the rates of these protein phosphokinase reactions or their response to cyclic AMP. Other studies (not shown) revealed that over the first 24 h after injection of $1\text{ }\mu\text{g}$ of 17β -oestradiol into immature (23 days old) female rats, there was little change in the ability of soluble uterine extracts to catalyse phosphorylation of a mixture of calf thymus histones, of calf thymus F2b histone, or of casein, measured in the absence or presence of cyclic AMP.

These findings suggest that in their immature state, rat testis, ventral prostate and uterus are replete with soluble enzymes catalysing cyclic AMP-stimulated phosphorylation of histones. It

Table 2. *Effects of orchiectomy and androgen administration on the phosphorylation of histones and protamine by rat ventral prostate*

Assays were performed as described in the Materials and Methods section of the main paper. Results are expressed as c.p.m. of ^{32}P transferred to acceptor protein/15 min per mg of enzyme protein at 25°C . The specific radioactivity of the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ used was 970 c.p.m./nmol. The enzyme source was high-speed-supernatant fraction. There were at least five or six animals per group. Adult sexually mature rats (300–350 g body wt.) were castrated and killed 96 h later. The androgen-treated castrated animals were administered 1 mg of testosterone propionate in sesame oil 18 h before being killed. The control castrated animals received the same volume of oil. The final concentration of cyclic AMP was $10\text{ }\mu\text{M}$.

	Histone F2b		Protamine	
	Control	Plus cyclic AMP	Control	Plus cyclic AMP
Normal	480	1715	188	380
Castrated after 96 h	496	2680	308	500
Castrated, 18 h after androgen administration	672	2220	280	680

remains to be determined whether these histone phosphokinase reactions in these reproductive organs are in any way pertinent to the hormonal regulation of RNA transcription, as has been postulated by Langan (1969) and others, in regard to other tissues. It is worth noting that King & Gordon (1969) stated that the protein phosphokinase activity of isolated rat uterine nuclei, determined with dephosphorylated phosvitin as substrate, was diminished after oophorectomy and enhanced in the spayed creatures by injection of 17-oestradiol; however, these nuclear preparations did not phosphorylate exogenous histones.

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