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CHARACTERIZATION OF PROTEIN PHOSPHOKINASE ACTIVITIES IN HORSE THYROID NUCLEI

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Summary

The distribution of protein phosphokinase (EC 2.7.1.37) activities has been established in horse thyroid nuclei. The presence of several enzyme activities has been demonstrated, two of which are clearly distinct. The first one acts on histone as substrate and is activated by cyclic AMP. Physico-chemical properties of this nuclear cyclic AMP-dependent histone kinase and of the cytosol histone kinase are different, demonstrating the absence of a contamination from the cytosol. The second enzyme acts on casein as substrate and is not stimulated by cyclic AMP or cyclic GMP.

The findings are consistent with the observation of thyrotropin stimulation of histone phosphorylation in thyroid nuclei.

Introduction

Numerous observations suggest that nuclear histone and non-histone phosphorylation could play an important role in the regulation of cellular growth at both transcriptional and translational levels [1—6]. Protein phosphokinase (ATP: protein phosphotransferase: EC 2.7.1.37) activity has been reported in nuclei isolated from liver [7—14]. We [15] and Roques et al. [16] have previously reported that a thyroid nuclei-enriched fraction also exhibits protein phosphokinase activity, stimulated by adenosine 3′,5′-monophosphate (cyclic AMP). Moreover, the in vitro phosphorylation of nuclear f_1 histones is increased in response to hormonal stimulation [17].

The aim of the present work was to investigate the nuclear localization of protein phosphokinase and to characterize the enzymatic activities found. Parts of this work were presented at the VIth European Thyroid Association Meeting [18] and at the 10th FEBS Meeting [19].

Materials and Methods

Horse thyroid glands, obtained in a local abattoir 3—5 min after slaughter were carried to the cold room of the laboratory in ice-cold isotonic saline solution. All operations were performed at 0—4°C. After removal of fat and connective tissue and mincing with scissors, the glands were homogenized with a motor driven teflon glass homogenizer by five strokes at low speed in a slightly hypertonic medium (0.25 M sucrose, 0.1 M potassium phosphate buffer, pH 7.4, 2 mM EDTA), and filtered through gauze. The resulting homogenate was centrifuged at 1000 × *g*; the pellet (crude nuclear fraction), resuspended in 2.3 M sucrose containing 2 mM $CaCl_2$ and 1 mM $MgCl_2$ was centrifuged for 1 h at 40 000 × *g* in a SW27 rotor [20].

The supernatant obtained from the 1000 × *g* centrifugation was centrifuged at 15 000 × *g* for 20 min. The supernatant was retained and centrifuged at 100 000 × *g* for 1 h to obtain the soluble protein phosphokinase-containing fraction. The enzyme was partially purified on DEAE-cellulose. One major peak of activity was obtained and used in this study.

Nuclear subfractionation is shown in Fig. 1. After five washings with low ionic strength buffer [21], soluble nuclear proteins were separated from the "associated proteins". The acidic nuclear proteins were obtained according to the scheme of Ruddon and Anderson [11] followed by treatment with DNAase and extensive dialysis. The soluble nuclear protein fraction was concentrated by $(NH_4)_2SO_4$ precipitation (90%), redissolved in a buffer containing 5 mM potassium phosphate, pH 6.8, 10% glycerol (v/v), 2 mM EDTA and 5 mM 2-mercaptoethanol and dialysed against the same buffer.

DNA and protein contents were, respectively, assayed by the method of Burton [22] with deoxyribose as standard and of Lowry et al. [23] with bovine serum albumin as standard.

Protein phosphokinase was assayed as previously described [15] at ATP concentration saturating for the soluble enzyme (0.2 mM), with casein

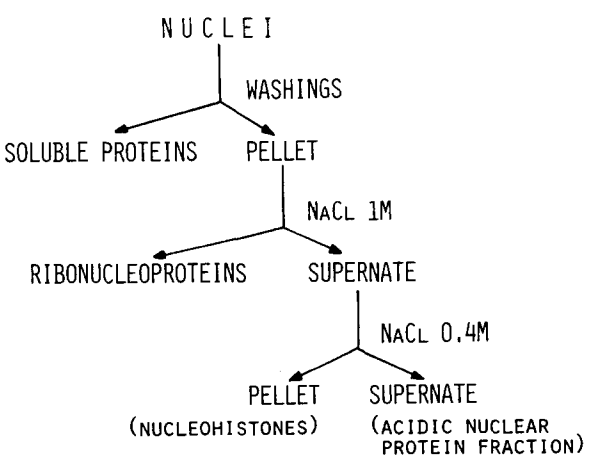

Fig. 1. Nuclear subfractionation procedure according to Ueda et al. [21] and to Ruddon and Anderson [11].

(6 mg/ml) or histones (2 mg/ml, Sigma) as substrate and, when added, cyclic AMP (2 μM). The reaction was proportional to enzyme concentration up to 200 μg of protein and linear over a period of 20 min.

Cytochrome $c : O_2$ oxidoreductase (EC 1.9.3.1) was assayed by following the decrease in absorbance at 550 nm of reduced cytochrome c at room temperature [24], orthophosphoric monoester phosphohydrolase (EC 3.1.3.2) (acid phosphatase) assayed according to Schmidt [25], and 5'-nucleotidase (EC 3.1.3.5) assayed on the basis of inorganic phosphate release according to Matsuzaki et al. [26].

Linear sucrose density gradients (5—20% sucrose) were performed by the method of Martin and Ames [27] using a Beckman SW 50 rotor at 50 000 rev./min for 20 h at 4°C.

The heat-stable protein inhibitor of cyclic AMP-dependent protein phosphokinases was extracted from beef skeletal muscle and purified by the method of Walsh et al. [28] to the trichloroacetic acid precipitation step.

Results

The recovery of DNA, proteins, and protein phosphokinase activity in purified nuclei is presented in Table I. The DNA/protein ratio of 0.3 indicated a satisfactory purification of nuclei [29]. Moreover, the procedure yielded thyroid nuclei of high purity as judged by marker enzyme measurements. Indeed, marker activities represented in the crude nuclear fraction and in the purified nuclei, respectively, 15—30% and 0.2—0.3% of the total activity in the homogenate.

Protein phosphokinase activity measured with casein or a mixture of histones as substrate, represented, respectively, 1.4 and 0.4% of the total activity in the gland, without taking into account the recovery of DNA. Addition of cyclic AMP ($2 \cdot 10^{-6}$ M) increased activity by a factor of 1.5 but only in the presence of histones.

After nuclear subfractionation (Fig. 1, Table II), nuclear protein phosphokinase activities measured with casein were essentially found in the acidic nuclear protein fraction (about 80% of the total activity). Cyclic AMP and cyclic GMP at 10^{-8}, $2 \cdot 10^{-6}$ and $2 \cdot 10^{-4}$ M had no stimulatory effect; an eventual cyclic AMP inhibitory effect was observed in the 1 M NaCl extract and in the acidic nuclear protein fraction. This inhibition was not observed in measurements of protein phosphokinase activity in unfractionated nuclei with the same final concentrations of salts.

In contrast, in the presence of histones as substrate, the kinase activity was distributed between the soluble nuclear protein, the ribonuclear protein and the acidic nuclear protein fractions. The cyclic AMP dependence observed in the acidic nuclear protein fraction was apparently lost after DNAase treatment and dialysis against low ionic strength buffer.

Fractionation of the concentrated soluble nuclear proteins was carried out on DEAE-cellulose as shown in Fig. 2. Protein phosphokinase activity was eluted as one major peak at 0.35 M NaCl using casein or histones as substrate. Under the same conditions, the cytosol histone kinase was eluted as one major

TABLE I

Recovery of DNA, proteins, protein phosphokinase activity and enzyme markers for organelles in horse thyroid nuclei. The protein phosphokinase activity was measured in the absence or presence of 2 μM cyclic AMP.

	DNA (mg/g tissue)	Proteins (mg/g tissue)	DNA / proteins	Protein kinase (nmol/min/g tissue)				Cytochrome oxido-reductase	Acid phospha-tase	5′-Nucleo-tidase	Alkaline-phospha-tase
				Casein		Histones					
				Basal	+cyclic AMP	Basal	+cyclic AMP	(μmol/h/g tissue)			
Homogenate	2.65 (100%)	160 (100%)	0.02	3.60	3.70	2.00	2.20	245	43.5	35	11.5
Crude nuclear fraction	2.12 (80%)	30 (19%)	0.07	0.31	0.36	0.09	0.11	65	7.0	8.7	3.2
Purified nuclei	1.54 (58%)	5.1 (3.2%)	0.30	0.05	0.05	0.006	0.009	0.46	0.13	0.07	0.04

TABLE II

Distribution of protein phosphokinase activity in the subnuclear fractions of horse thyroid. The activity was measured in the absence or presence of 2 μM cyclic AMP.

	Protein recovery (%)	Protein phosphokinase (pmol/min/g tissue) Substrate:			
		Casein —cyclic AMP	Casein +cyclic AMP	Histones —cyclic AMP	Histones +cyclic AMP
Purified nuclei	100	32	32	6	9
Nuclear sub-fractions:					
Soluble proteins	5	4	5	1.2	1.7
Associated proteins	—	35	35	5.6	5.6
Ribonuclear protein fraction	44	5.5	5.5	3.1	3.1
1 M NaCl extract	32	35	23	2.3	3.8
Nucleohistones	4	0.5	0.4	0.1	0.2
Acidic nuclear protein fraction	17	20	16.5	1	1.9
Acidic nuclear protein fraction after DNAase treatment	12	26.3	26.3	1.5	1.5

peak at 0.5 M NaCl, indicated by the arrow in Fig. 2. Further characterization was performed with these partially purified enzymes.

Molecular weight estimations of the protein phosphokinases were performed

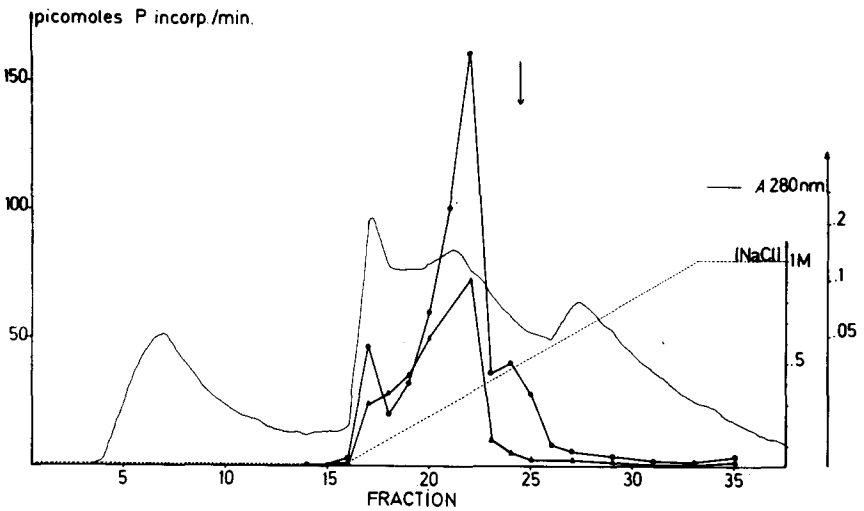

Fig. 2. DEAE-cellulose chromatography of the soluble nuclear protein fraction. The enzyme preparation was concentrated by $(NH_4)_2SO_4$ precipitation redissolved in 5 mM potassium phosphate buffer (pH 6.8) containing 2 mM EDTA and 5 mM 2-mercaptoethanol (EtSH), dialysed against the same buffer and applied to a column of DEAE (Serva) (2.5 × 20 cm) equilibrated with 5 mM potassium phosphate buffer (pH 6.8) containing 2 mM EDTA and 5 mM 2-mercaptoethanol. The column was first eluted with this same buffer, and then with a linear concentration gradient from 0 to 1 M NaCl. Fractions of 2 ml were collected. Protein kinase activity was measured as indicated in Materials and Methods, with casein (●———●) or with histones (▲———▲) in the presence of 2 μM cyclic AMP. The arrow indicates the elution in the same conditions of the cytosol histone kinase.

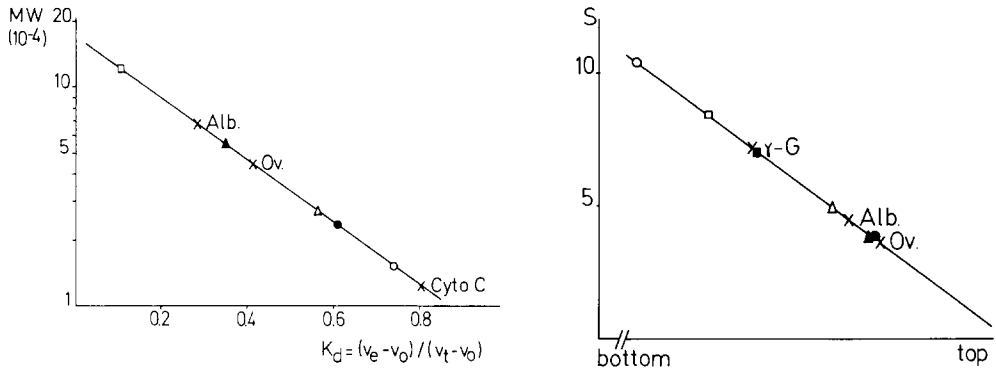

Fig. 3. Molecular weight estimation of the protein phosphokinases of the soluble nuclear protein fraction. The enzyme solution (1.5 mg of protein) was passed over a Sephadex G-200 column (Pharmacia) (1.5 × 90 cm) equilibrated with 5 mM potassium phosphate buffer (pH 6.8) containing 10% glycerol, 2 mM EDTA and 5 mM 2-mercaptoethanol. Protein phosphokinase activity was assayed with casein or a mixture of histones, at pH 6.5 and 7.5, respectively, in the presence of 2 μM cyclic AMP; ○, soluble nuclear casein kinase$_{-1}$; △, soluble nuclear casein kinase$_{-2}$; □, soluble nuclear casein kinase$_{-3}$; ●, soluble nuclear histone kinase$_{-1}$; ▲, soluble nuclear histone kinase$_{-2}$. Alb., bovine serum albumin; Ov., ovalbumin.

Fig. 4. Estimation of the sedimentation coefficient of the protein phosphokinases of the soluble nuclear protein fraction and of the cytosol. The enzyme solutions were applied to sucrose density gradients as described in Materials and Methods. Protein phosphokinase activity was assayed as indicated in the legend of Fig. 3 with or without 2 μM cyclic AMP. ○, cytosol casein kinase; △, cytosol histone kinase a; □, cytosol histone kinase b; ●, soluble nuclear casein kinase; ▲, soluble nuclear histone kinase a; ■, soluble nuclear histone kinase b. γ-G, γ-globulin; Alb., bovine serum albumin; Ov., ovalbumin.

by filtration on Sephadex G-200 using blue-dextran, bovine serum albumin, ovalbumin, and cytochrome *c* as markers. Two peaks of histone kinase were observed, of about 24 000 and 53 000 molecular weight, respectively, (the soluble nuclear histone kinases$_{-1}$ and $_{-2}$ on Fig. 3); three additional peaks of casein kinase of about 15 000, 27 000 and 120 000 molecular weight were also detected (the soluble nuclear casein kinases $_{-1}$, $_{-2}$ and $_{-3}$). Under the same conditions, cytosol histone and casein kinases were eluted just after the void volume, corresponding to a molecular weight between 120 000 and 200 000.

When the same partially purified protein phosphokinases were subjected to

TABLE III

Comparison of the effect of the muscle heat-stable inhibitor [28] on the soluble nuclear histone kinase and the cytosol histone kinase activities. The activity was measured in the absence or presence of 2 μM cyclic AMP.

Muscle heat-stable inhibitor (μg proteins)	Protein phosphokinase activity (pmol/min/mg protein)			
	Soluble nuclear histone kinase		Cytosol histone kinase	
	Basal	+cyclic AMP	Basal	+cyclic AMP
0	107	177	18.8	36
5.6	81	110	18.4	20.1
56	50	60	14.2	14.9

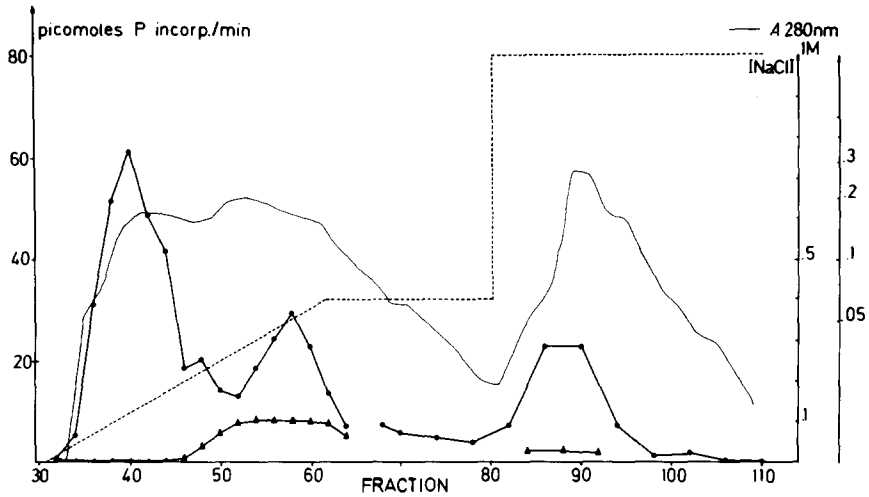

Fig. 5. DEAE-cellulose chromatography of the acidic nuclear protein (ANP) fraction after treatment with DNAase and dialysis against 5 mM potassium phosphate buffer (pH 6.8) containing 2 mM EDTA and 5 mM 2-mercaptoethanol. The fraction was applied to a column of DEAE-cellulose (Serva) (2.5 × 20 cm) equilibrated with the same buffer and a linear gradient from 0 to 0.4 M NaCl followed by 1 M NaCl was used for elution. Protein kinase activity was measured with casein (●———●) or with histones (▲———▲) as substrate in the presence of 2 μM cyclic AMP.

sucrose density gradient centrifugation, soluble nuclear histone kinase had histone kinase (the soluble nuclear histone kinases a and b on Fig. 4) had sedimentation coefficients of 3.8 S and 7.1 S; the former was stimulated by cyclic AMP, while the latter was cyclic AMP independent. Soluble nuclear casein kinase had a sedimentation coefficient of 3.8 S and was unstimulated by cyclic AMP. Under the same conditions, cytosol histone and casein kinases had sedi-

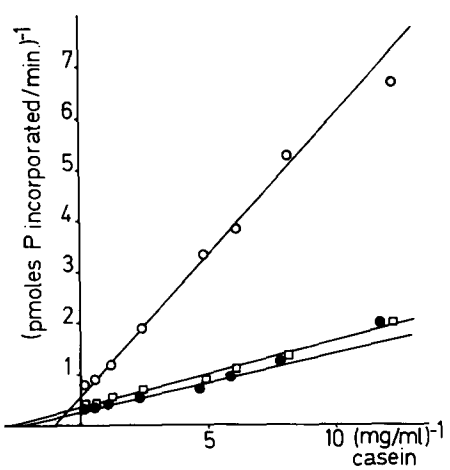

Fig. 6. Reciprocal plot of casein phosphokinase activity as a function of casein concentration. The concentration of protein in the assay is 12—15 μg per ml. □, acidic nuclear protein fraction eluted at 0.15 M on DEAE-cellulose; ○, acidic nuclear protein fraction eluted at 0.35 M; ●, soluble nuclear protein fraction eluted at 0.35 M.

mentation coefficients of 4.9 S and 8.4 S (the cytosol histone kinases a and b), both stimulated by cyclic AMP, and 10.4 S (the cytosol casein kinase), unstimulated by cyclic AMP.

Table III shows the effect of the muscle heat-stable inhibitor of protein kinase on soluble nuclear histone kinase and cytosol histone kinase. It was previously verified that 5.6 μg of the protein preparation inhibited completely the activity of stimulated beef muscle protein kinase (Sand, G., unpublished results). Stimulated soluble nuclear histone kinase and cytosol histone kinase activities were inhibited at the same level, but surprisingly, basal soluble nuclear histones kinase activity was also diminished. No effect of the inhibitor was observed on the soluble nuclear casein kinase and on the cytosol casein kinase.

Optimum pH (not illustrated) was near 6.5 for the soluble nuclear casein kinase and for the cytosol casein and histone kinases and near pH 7.5 for the soluble nuclear histone kinase.

After DNAase treatment and extensive dialysis, the acidic nuclear protein fraction was applied to a DEAE-cellulose column. The protein phosphokinase activity was resolved into three peaks (Fig. 5). The first one, eluted at about 0.12—0.15 M NaCl phosphorylated only casein. A rather broad pH curve with an optimum at about pH 6.5 was observed. Stimulation by cyclic AMP or cyclic GMP at 10^{-8}, 10^{-6}, 10^{-4} M was not significant. A second peak of activity was eluted at 0.35 M NaCl, which phosphorylated both tested substrates. With casein, activity was 5-fold higher at pH 6.5 than at pH 7.5, whereas with histones no difference was observed. Cyclic AMP had no stimulatory effect on casein and histone kinase activities. The third peak had not yet been characterized. As shown in Fig. 6, the acidic nuclear casein kinases eluted at 0.15 and 0.35 M NaCl had different apparent K_m for casein ($1.5 \cdot 10^{-5}$ and $4.2 \cdot 10^{-5}$ M, respectively). The K_m observed using the soluble nuclear casein kinase eluted at 0.35 M NaCl as enzyme source was $1.8 \cdot 10^{-5}$ M.

Discussion

Our findings demonstrate the existence of both cyclic AMP-dependent and apparently cyclic AMP-independent protein phosphokinases in horse thyroid nuclei. This situation appears similar to that observed in beef and rat liver nuclei [10—14].

It is important to exclude the possibility that the observed nuclear protein phosphokinase activities are not due to organelle contamination. The proportion of cyclic AMP-dependent histone kinase associated with thyroid nuclei is similar to the proportion of enzyme markers for mitochondria (cytochrome oxidase), lysosomes (acid phosphatase), plasma and endoplasmic reticulum (5′-nucleotidase, alkaline phosphatase). However, these enzymatic activities have also been demonstrated in nuclei (for a review of the subject, see ref. 30). In addition, when comparing the recovery of protein phosphokinase and enzyme marker activities in the crude nuclear pellet and in the purified nuclei, protein kinase recovery is 10-fold higher than marker enzyme recovery.

As the existence of a nuclear cyclic AMP-dependent protein kinase has important physiological implications, it is crucial to establish that the soluble nuclear histone kinase activity is not simply a contamination by cytosol histone

kinase. Comparison of the physico-chemical properties of these two enzymes in horse thyroid shows differences at several levels: the soluble nuclear histone kinase and the cytosol histone kinase are eluted from DEAE-cellulose at different salt molarities; they also differ by their respective elution on Sephadex G-200, their sedimentation coefficient in sucrose gradient, their response to the muscle heat-stable inhibitor and their optimum pH. Therefore the nuclear histone kinase is clearly distinct from the cytosol histone kinase. Nevertheless, it is not excluded that the cytosol histone kinase may associate with the nucleus with an accompanying change in its properties [31]. In a recent paper, Desjardins et al. [32] have discussed this controversial problem and have concluded that the cyclic AMP-dependent histone kinase released by washings of purified liver nuclei behaves as a cytoplasmic rather than a nuclear enzyme. However, their study was limited to the substrate specificity of the enzymes and compared a soluble nuclear enzyme isolated from liver to a cytosol enzyme from heart. Our results, obtained by comparison of some physico-chemical properties of the thyroid isolated from the soluble nuclear protein fraction and from the cytosol cyclic AMP-dependent histone kinases demonstrate that the soluble nuclear enzyme cannot be considered as a cytosol contamination. We agree with the suggestion of Desjardins et al. [32] that an additional demonstration of the nuclear localization might be obtained by the use of fluorescent antibodies prepared against purified cyclic AMP-dependent histone kinase. The demonstration obtained by Jungmann et al. [31] of the in vitro phosphorylation of calf ovarian chromatin by a cytoplasmic protein phosphokinase does not conclusively prove either the translocation of cytoplasmic protein phosphokinases to intranuclear acceptor sites or the absence of specific nuclear protein phosphokinases.

Our data indicate several acidic nuclear protein phosphokinase activities unstimulated by cyclic AMP or cyclic GMP. Similar results have been reported in studies devoted entirely to chromatin-bound protein phosphokinases [10—13]. In beef liver nuclei [13] and in calf ovary nuclei [31] at least 12—15 distinct protein kinases have been found closely associated with the nucleus, several of them being cyclic AMP dependent. As pointed out by Rikans and Ruddon [33], the failure to demonstrate an activation by cyclic AMP of nuclear protein kinases does not exclude the general model in which the activity of nuclear kinases is controlled by a cyclic AMP-binding subunit. Our present results only differentiate two casein kinases in the acidic nuclear protein fraction by the elution pattern on DEAE-cellulose and the apparent K_m for casein. More pooled material from several isolations of horse thyroid nuclei is needed to continue the characterization of the protein phosphokinase activities.

In conclusion, although it is not possible from our data to differentiate all protein phosphokinase activities in horse thyroid nuclei, we have demonstrated the existence of at least two distinct enzymes and suggested the presence of several others. The comparison of the physico-chemical properties of the cyclic AMP-dependent histone kinase from the soluble nuclear protein fraction and the cyclic AMP-dependent histone kinase from the cytosol demonstrates that the soluble nuclear histone kinase is distinct from the cytosol histone kinase. These findings are consistent with the observation of thyrotropin stimulation of histone phosphorylation in thyroid nuclei [17].

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