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NUCLEOSIDE-DEPENDENT PROTEIN KINASE FROM *TRYPANOSOMA GAMBIENSE*

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Summary

A nucleoside-dependent protein kinase (EC 2.7.1.37) was partially purified from *Trypanosoma gambiense*, the pathogenic agent of sleeping sickness. This enzyme catalyzes the phosphorylation of histone and protamine. Various nucleosides at the concentration of 10^{-4} M stimulated the histone kinase activity about two-fold, whereas cyclic AMP and cyclic GMP were without effect. The pH-optimum for histone phosphorylation was at about pH 7.0. The enzyme activity absolutely depends on Mg^{2+}, Mn^{2+} or Co^{2+}. The apparent K_m-value for histone was 0.3 mg/ml and those for ATP were $2 \cdot 10^{-4}$ M and $6 \cdot 10^{-5}$ M in the absence or presence of 10^{-4} M adenosine, respectively. IDP and ADP compete with ATP. The inhibition constants were calculated to be $2 \cdot 10^{-4}$ M and $2.5 \cdot 10^{-4}$ M, respectively. The molecular weight of the histone kinase was found to be 95 000 by gel filtration and 88 000 by sedimentation in a sucrose gradient.

Introduction

Protein kinases (EC 2.7.1.37) which catalyze the phosphorylation of a variety of protein substrates, can be devided into a cyclic AMP-dependent and a cyclic AMP-independent sub-group. The regulatory role of cyclic AMP on the protein kinase activity of mammalian sources, initially described by Walsh et al. [1], has been found in bacteria [2], invertebrates [3—7], yeast [8], slime moulds [9], *Tetrahymena* [10] and also *Euglena* [11]. The group of cyclic nucleotide-independent protein kinases is just as widespread among organisms. Protein kinases, independent of cyclic AMP or only slightly stimulated, are reported from mammalian sources [12—16] as well as from *Tetrahymena* [10,17], *Neurospora* [18], higher plants [19,20], *Euglena* [11], yeast [8], and sea urchin egg cortices [21].

The present paper describes the partial purification and characterization of a

histone kinase from *Trypanosoma gambiense*. The activity of this enzyme is independent of cyclic AMP, but is stimulated by nucleosides.

Materials and Methods

Materials

[8-^{3}H] Adenosine 3′:5′-monophosphate (27 Ci/mmol) and [γ-^{32}P] adenosine triphosphate were purchased from the Radiochemical Centre, Amersham. Protein substrates were obtained from commercial sources, as follows: calf thymus histone (mixture, lysine-rich and arginine-rich), protamine (salmon sperm) and phosvitin (egg vitellin), Sigma, St. Louis; casein, Merck, Darmstadt. Nucleosides and nucleotides were obtained from Boehringer, Mannheim. Bovine serum albumin was purchased from Behringwerke, Marburg, and fluorescamine (Fluram) from Hoffmann-La Roche, Grenzach. Phosphoserine and phosphothreonine were obtained from Sigma, St. Louis. Silica-gel-coated thin layer plates and all other chemicals used were purchased from Merck, Darmstadt. DEAE-cellulose (DE 52) was obtained from Whatman, Kent, Sephadex G-200 superfine from Pharmacia, Uppsala, and Ampholine from LKB-Instruments, Stockholm.

Protein kinase assay

The activity of the protein kinase was assayed in an incubation volume of 0.1 ml. The standard assay contained, unless otherwise indicated, 0.1 M Tris-maleate (pH 7.0), 0.01 M $MgCl_2$, 0.1 mg bovine serum albumin, 0.1 mM [γ-^{32}P] ATP (10^5—$5 \cdot 10^5$ cpm), 0.1 mg histone (mixture) as phosphate acceptor, and enzyme preparation. Incubation was carried out at 37° C for 0 to 30 min and the reaction was terminated by addition of 1 ml 10% trichloroacetic acid solution. The reaction rate was linear within this period. The protein bound ^{32}P was determined according to Miyamoto et al. [22]. The protein kinase activity is expressed in enzyme units (1 unit = 1 μmol/min).

Acid hydrolysis of (^{32}P)-labeled histone

Partial hydrolysis of histone was performed according to Allerton and Perlmann [23]. The enzymatically phosphorylated radioactive histone preparations were hydrolysed for 4 h in 6 M HCl at 100° C in ampules sealed under nitrogen. The hydrolysates were dried in vacuo over KOH and thin-layer electrophoresis was performed on silica gel (1.5 h, 40 V/cm) in formic/acetic buffer, pH 1.9. The radioactivity was determined by using a thin-layer-scanner (Berthold, Wildbad). The position of authentic phosphoserine and phosphothreonine added as carrier was located by staining with ninhydrin.

Assay of cyclic AMP-binding protein

The cyclic AMP-binding activity of the partial purified protein kinase was determined according to Gilman [24]. The incubation mixture contained in a final volume of 0.1 ml: 0.05 M sodium acetate buffer (pH 4.0) and 4.8 pmoles of cyclic [8-^{3}H] AMP. Incubation was started by addition of protein and carried out at 0° C for 60 min.

Protein determination

Protein was estimated by the methods of Lowry et al. [25] and Böhlen et al. [26] with crystalline bovine serum albumin as a standard.

Preparation of Trypanosoma extract

60 NMRI-mice, infected with *T. gambiense*, were decapitated two days after intraperitoneally inoculation of the parasites [27]. The blood was collected in heparin and phosphate/saline/glucose buffer (pH 8.0, ionic strength 0.22) and centrifuged at 750 rev./min in a Sorvall-SW 34 rotor for 15 min to remove most of the red blood cells. The resulting supernate with trypanosoma, white blood cells, platelets, and remaining red blood cells was layered on a DEAE-cellulose column (2.5 × 20 cm), previously equilibrated with phosphate/saline/glucose buffer. The trypanosoma were eluted with the same buffer according to Lanham [28], while the other cells remained on the column. All further steps were carried out at 0—4°C. The trypanosoma were washed four times with phosphate/saline/glucose buffer and in the last step with 0.9% NaCl (5000 rev./min for 15 min) and then suspended in 50 mM potassium phosphate buffer (pH 7.0). The cells were lysed by freezing and thawing three times and homogenized with a glass pestle in a glass Potter homogenizer. The crude homogenate was centrifuged at 100 000 × *g* for 60 min.

Results

Enzyme purification. The bulk of the protein kinase activity from *T. gambiense* was found in the 100 000 × *g* supernate, whereas about 20% of the enzyme activity remained in the particulate fraction. A summary of the partial purification of the soluble histone kinase achieved is demonstrated in Table I. All purification procedures were carried out between 0 and 4°C.

Neutral saturated ammonium sulfate solution was added slowly to 10 ml of the trypanosoma extract up to 45% saturation. The solution was stirred for 1 h and the precipitate was removed by centrifugation at 10 000 × *g* for 30 min. The resulting supernate was retained and then brought to 65% saturation by further addition of saturated ammonium sulfate solution. After stirring for another hour the precipitate was collected by centrifugation, dissolved and dialysed against 2 l of 50 mM potassium phosphate buffer (pH 7.0) for 6 h. After dialysis the solution (2.5 ml) was centrifuged to remove insoluble material.

The protein solution was then placed on a Sephadex G-200 column (2.5 × 32 cm), which had been previously equilibrated with 50 mM potassium phos-

TABLE I

PURIFICATION OF HISTONE KINASE FROM *T. GAMBIENSE*

The standard assay was used except that the concentration of ATP was 1 mM.

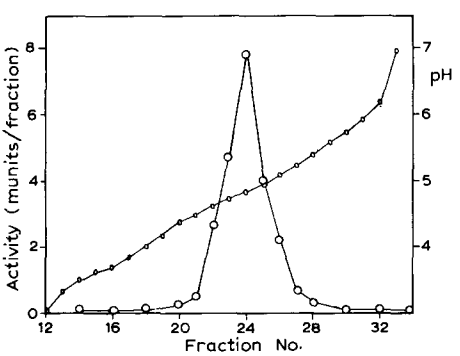
Purification step	Vol. (ml)	Protein (mg)	Activity (munits)	Spec. act. (munits/mg)	Recovery (%)	Purification factor
Extract	10	94.0	199.15	2.12	100	—
$(NH_4)_2SO_4$ precipitate	2.5	37.5	145.80	3.89	73	1.8
Sephadex G-200 (I)	12	4.8	127.60	26.58	64	12.5
Isoelectric focussing	9	—	48.75	—	24	—
Sephadex G-200 (II)	12	0.055	29.13	529.64	15	249

phate buffer (pH 7.0). Elution was achieved by the same buffer at a flow rate of 5 ml/h and fractions of 4 ml were collected. The histone kinase activity was eluted in a single peak, and the peak fractions (tubes 22—24) were combined, concentrated to a volume of 3 ml in an Amicon model 8 MC pressure ultrafiltration apparatus equipped with a Diaflo UM 10 membrane and dialysed overnight against 1% Ampholine (pH 3—6).

For further purification the protein solution was applied to isoelectric focusing in a 100 ml, 0 to 40% linear sucrose gradient with 2.4% in carrier ampholytes (LKB, 40%, 4 ml pH 3—6, 1 ml pH 5—8 and 1 ml pH 3.5—10) [29]. A gradient mixer was used to prepare the density gradient in the electrofocusing column (LKB 8121 and 8101). The enzyme solution was dissolved in the dense solution after a quarter of the gradient had been prepared. The voltage was kept at 500 V, while the current decreased to 2.1 mA during the run. The temperature of the column was maintained at 2°C. After two days the column was eluted and fractions of 3 ml were collected. The pH of every other fraction was measured at 2°C. As shown in Fig. 1 the histone kinase activity appeared as a single peak corresponding to a pH of 4.85. These fractions (tubes 23—25) were pooled, dialysed overnight against 2 l of 50 mM potassium phosphate buffer (pH 7.0) and concentrated by ultrafiltration to reach a volume of 2 ml. This concentrated protein solution was applied to Sephadex G-200 filtration for another time. Flow rate and volume of the fractions collected were as described above. The histone kinase activity was eluted at the same position as in the first run (tubes 22—24). The specific activity of the histone kinase at this stage was about 530 mU and approximately a 250-fold purification was achieved.

Properties of the protein kinase. All further determinations were performed with the partial purified protein kinase. The enzyme solution, after the second Sephadex run, was kept frozen at −76°C in small portions until used. This storage for as long as four weeks did not lead to an appreciable loss of activity.

Effect of variation of pH. The pH optimum of the protein kinase activity



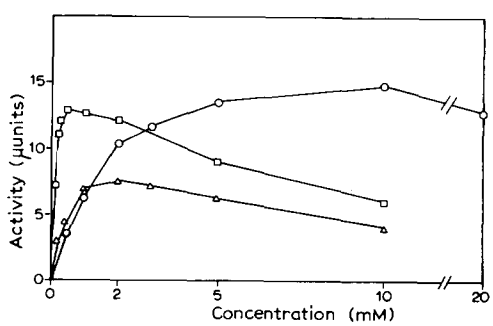


Fig. 1. Electrofocusing of histone kinase in an Ampholine/sucrose gradient. The standard assay was used. ○———○, pH; ○———○, histone kinase activity.

Fig. 2. Effect of divalent cations. The standard assay was used except that $MgCl_2$ was substituted for various concentrations of divalent cations indicated. □———□, $MnCl_2$; ○———○, $MgCl_2$; △———△, $CoCl_2$.

with histone as substrate was at about 7.0, estimated in Tris-maleate buffer, although the activity decreased only slightly at pH values up to 8.0 and down to 6.0.

Effect of ions. The histone kinase showed an absolute requirement for divalent metal ions (Fig. 2); Mg^{2+} as well as Mn^{2+}, and less effectively Co^{2+} supported the protein kinase activity, whereas Ca^{2+}, Ba^{2+}, Ni^{2+}, Zn^{2+}, and Cu^{2+} at concentrations of 0.5 mM and 5 mM were without effect on the enzyme activity. The optimal concentrations for Mg^{2+}, Mn^{2+}, and Co^{2+} were 10 mM, 0.5 mM and 2 mM, respectively. KCl at a concentration of 0.1 M inhibited the protein kinase activity about 30%, if the standard assay containing 10 mM $MgCl_2$ was used.

Effect of cyclic nucleotides. Cyclic AMP and cyclic GMP in the range of 10^{-7} M to 10^{-4} M showed no effect on the enzyme activity, neither stimulating nor inhibiting the protein kinase activity. There was also no stimulation of the histone kinase activity by cyclic AMP or cyclic GMP if crude homogenate was used as source of enzyme. Furthermore, no cyclic AMP-binding activity could be detected in the partial purified histone kinase preparation.

Effect of nucleosides. At the concentration of 10^{-4} M various nucleosides stimulated the activity of the histone kinase, whereas deoxynucleosides, purines and AMP slightly inhibited the enzyme activity (Table II). This stimulation of the enzyme of *T. gambiense* by adenosine, guanosine, inosine, uridine and cytidine was about two-fold. In the case of adenosine the protein kinase was stimulated over a concentration range from 10^{-5} M to $5 \cdot 10^{-4}$ M (Fig. 3). Maximal stimulation was obtained at a concentration of 10^{-4} M. At concentrations above 1 mM the enzyme activity was inhibited by adenosine.

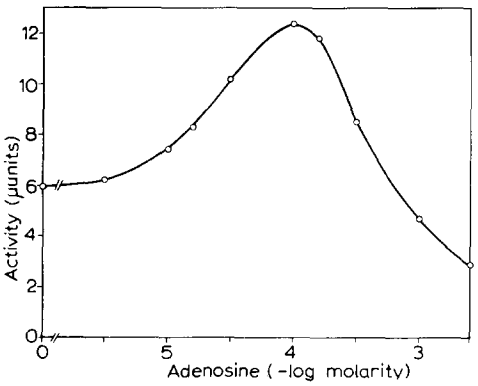
Effect of IDP and ADP. IDP and ADP were found to be potent inhibitors of the protein kinase activity. Fig. 4 shows the competitive inhibition with respect to ATP. The inhibition constants for IDP and ADP were calculated to be $2 \cdot 10^{-4}$ M and $2.5 \cdot 10^{-4}$ M, respectively.

TABLE II

STIMULATION OF PROTEIN KINASE ACTIVITY BY NUCLEOSIDES

Conditions were as described under "Methods". The concentration of ATP was 0.1 mM. Results represent means of triplicate determinations.

Additions (concentration 0.1 mM)	Reaction rate	
	(μunits)	(%)
None	14.4	100
Adenosine	30.7	213
Inosine	33.4	232
Guanosine	32.8	228
Cytidine	28.2	196
Uridine	27.6	192
Deoxyadenosine	12.1	84
Deoxyguanosine	12.8	89
Deoxythymidine	13.8	96
Adenine	12.7	88
Guanine	13.2	92
Adenosinemonophosphate	13.7	95



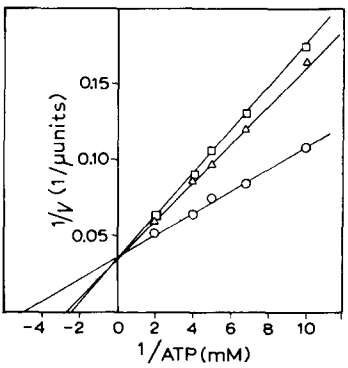


Fig. 3. Effect of adenosine on protein kinase activity. Conditions were as described under "Methods" except for the varying concentration of adenosine. ○———○, histone kinase activity.

Fig. 4. Effect of IDP and ADP. Double reciprocal plots of the rate of phosphorylation of histone as a function of ATP. The standard assay was used except for the addition of 0.2 mM IDP (□———□) or 0.2 mM ADP (△———△) and for various concentrations of ATP.

Effect of ATP. The activity of histone phosphorylation with varying concentration of ATP is shown in Fig. 5. Adenosine causes a decrease in the Michaelis constant for ATP. The K_m-values for ATP, determined both in the absence and in the presence of 10^{-4} M adenosine, were calculated to be $2 \cdot 10^{-4}$ M and $6 \cdot 10^{-5}$ M, respectively.

Acceptor proteins and site of phosphorylation. The protein kinase from *T. gambiense* phosphorylated histone (mixture), arginine-rich histone and protamine. Table III demonstrates the relative effectiveness of protein acceptors. Casein and lysine-rich histone were poor substrates, whereas phosvitin and serum albumin were inactive as substrates. The Michaelis constant for histone was determined to be 0.3 mg/ml as well in the absence as in the presence of 10^{-4} M adenosine (Fig. 6). Thus, the stimulation of the protein kinase was not associated with a change of the K_m-value for histone. The radioactivity incorporated into histone by the trypanosoma enzyme was completely hydrolyzable

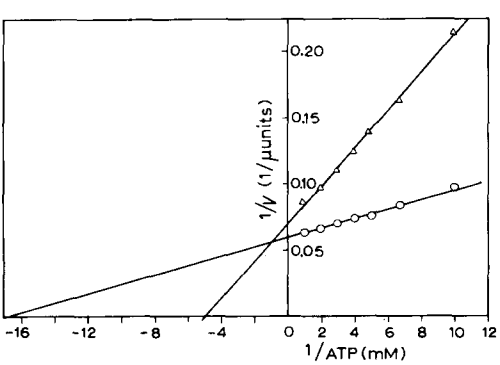


Fig. 5. Effect of ATP concentration. Double reciprocal plots of the rate of phosphorylation of histone as a function of ATP in the absence (△———△) and in the presence of 10^{-4} M adenosine (○———○).

TABLE III

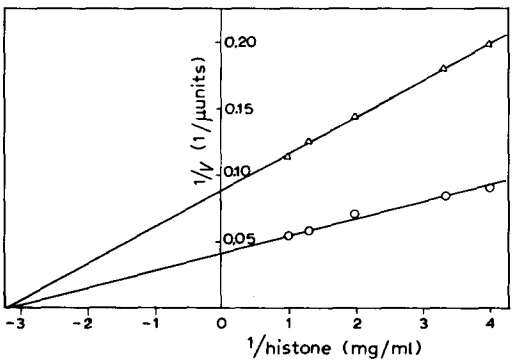
SUBSTRATE SPECIFICITY OF THE *TRYPANOSOMA* PROTEIN KINASE

Conditions were as described under "Methods" except for the variation of acceptor protein (1 mg/ml) and that bovine serum albumin was omitted from the standard assay. Results represent means of triplicate determinations.

Substrate	Reaction rate	
	(μunits)	(%)
Mixed histone	12.2	100
Arginine-rich histone	9.4	77
Lysine-rich histone	0.6	4
Protamine	7.2	59
Casein	0.9	7
Phosvitin	0.1	< 1
Bovine serum albumin	< 0.1	< 1

by incubation for 18 h at 37°C in 1 M NaOH. Under the condition of the standard assay the protein kinase phosphorylated seryl and threonyl residues of histone. When ^{32}P-labeled histone was subjected to partial acid hydrolysis, followed by thin-layer electrophoresis on silica gel, a major radioactive spot was associated with phosphoserine (93%), but a second radioactive spot, identified as phosphothreonine (7%) was present, too.

Molecular weight and sedimentation constant. The molecular weight of the protein kinase was determined with Sephadex G-200 gel filtration by the procedure of Whitaker [30]. In three different runs the activity was eluted in a single peak. The ratio of V_e/V_o indicated a molecular weight of 95 000 (Fig. 7). The sedimentation constant of the protein kinase was determined by sucrose gradient sedimentation according to Martin and Ames [31] to be $s_{20,w}$ = 5.3 S



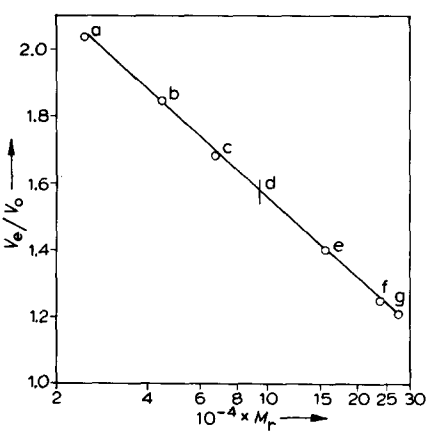


Fig. 6. Effect of histone concentration. Double reciprocal plots of the rate of phosphorylation of histone as a function of histone in the absence (△———△) and in the presence of 10^{-4} M adenosine (○———○).

Fig. 7. Determination of the molecular weight on a Sephadex G-200 superfine column. The column (1.5 × 90 cm) was equilibrated and eluted at a flow rate of 2.5 ml/h with 50 mM potassium phosphate buffer (pH 7.0). Fractions of 1 ml were collected and portions were assayed for histone kinase activity. The standard assay was used. a, chymotrypsinogen; b, ovalbumin; c, bovine serum albumin; d, histone kinase from *T. gambiense*; e, aldolase; f, pyruvate kinase; g, xanthine oxidase.

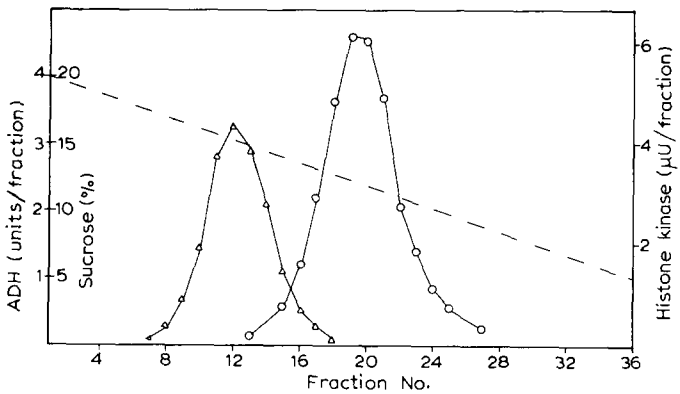


Fig. 8. Sedimentation of histone kinase. 0.2 ml of a solution containing 0.2 mg of yeast alcohol dehydrogenase and 0.5 µg of the partial purified histone kinase were layered on a 4.8 ml linear 5 to 20% (w/v) sucrose gradient in potassium phosphate buffer (pH 7.0). After centrifugation at 5°C in a MSE 59587 rotor at 38 000 rev./min for 19 h, 36 fractions containing 5 drops each were collected and portions were assayed for histone kinase (○———○) and alcohol dehydrogenase (△———△) activity. The standard assay for protein kinase activity was used. The alcohol dehydrogenase activity was assayed according to Racker [37].

(Fig. 8). The molecular weight was calculated according to Schachman [32] to be 88 000. No change of the sedimentation constant occurred if the enzyme was mixed with 10^{-4} M adenosine at 4°C prior to sedimentation in a sucrose gradient containing 10^{-4} M adenosine.

Discussion

The present results demonstrate that a protein kinase occurs in the soluble fraction of *T. gambiense*, which is capable of phosphorylating histone and less efficiently protamine. The activity of this protein kinase is not regulated by cyclic AMP or cyclic GMP, nor does it binds to cyclic AMP. Kuo and Greengard [2] have postulated that most of all effects of cyclic AMP on cellular metabolism are mediated through the stimulation of cyclic AMP-dependent protein kinases. In the extract of *T. gambiense* other protein kinases exist, which prefer phosvitin or protamine as protein substrates, but these protein kinases are independent of cyclic AMP, too (unpublished results). These protein kinases are not stimulated by adenosine.

The occurrence of cyclic AMP and of enzymes regulating its concentration in *T. gambiense* has been reported before [33,34]. The cyclic AMP phosphodiesterase from *T. gambiense* is inhibited by deoxyadenosine and less efficiently by adenosine [35]. It is noteworthy that the histone kinase activity is stimulated about two-fold by 10^{-4} M adenosine, whereas concentrations above 10^{-3} M are inhibitory. This stimulation of the protein kinase activity is associated with a decrease of the Michaelis constant for ATP, whereas the K_m-value for histone remained unaffected. Adenosine and other nucleosides therefore may function as endogenous regulators of histone kinase activity from *T. gambiense*. Yuh and Tao [36] have reported on non-kinase associated adenosine-cyclic AMP-binding protein factors of rabbit erythrocytes. Such binding factors

might be involved in cellular processes by regulating the level of free adenosine and cyclic AMP. With regard to the stimulation by nucleosides the trypanosoma enzyme differs from the protein kinases described, dependent or independent on cyclic nucleotides.

The protein kinase from *T. gambiense* is quite similar to other cyclic AMP-independent protein kinases with respect to the requirement for divalent cations, but it is distinguished from those sources in several respects, including Michaelis constant for ATP, protein substrate specificity and molecular weight. Whereas the protein kinases from sea urchin egg cortices [21], *Tetrahymena* [17], *Neurospora* [18], and yeast [8] prefer casein or protamine as substrate, the trypanosoma enzyme favours histone. The molecular weights of the protein kinases from the former sources were estimated to be 50 000 to 60 000, whereas the molecular weight of the histone kinase from *T. gambiense* was found to be 90 000. The Michaelis constant for ATP of the trypanosoma enzyme decreased in the presence of adenosine from $2 \cdot 10^{-4}$ M to $6 \cdot 10^{-5}$ M, but was nevertheless higher than those values (K_m for ATP, $1.4 \cdot 10^{-5}$ M—$3 \cdot 10^{-5}$ M) reported for sea urchin egg cortices [21], *Tetrahymena* [17], and *Neurospora* [18].

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