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INACTIVATION OF $(Na^+ + K^+)$-STIMULATED ATPase BY A CYTOTOXIC PROTEIN FROM COBRA VENOM IN RELATION TO ITS LYTIC EFFECTS ON CELLS

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SUMMARY

The mechanism of action of the cytotoxic protein P_6 isolated from cobra venom (*Naja naja*) which shows preferential cytotoxicity particularly to Yoshida sarcoma cells has been studied by its effects on the membrane-bound enzyme $(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3.6.1.3) of a variety of cell systems. Evidence obtained with Yoshida sarcoma cells, dog and human erythrocytes and three tissue culture cell lines KB (human oral carcinoma), Hela (human cervix carcinoma) and L-132 (human lung embryonic) shows that inhibition of $(Na^+ + K^+)$-ATPase by the P_6 protein can be correlated with its lytic activity. $(Na^+ + K^+)$-ATPase of Yoshida sarcoma membrane fragments inactivated by P_6 protein could be reconstituted by the addition of phosphatidylserine and phosphatidic acid. It is conceivable that lysis of cells by the P_6 protein may be due to an imbalance of K^+ and Na^+ in the cell which leads to swelling and disintegration of the membrane structure. Observations indicate that the P_6 protein combines with membrane constituents of susceptible cells. The overall evidence suggests that both the specificity of its protein structure and the highly basic nature of the P_6 protein are factors which enable it to compete with the lipid moiety maintaining the $(Na^+ + K^+)$-ATPase of the susceptible cells in proper conformation for activity.

INTRODUCTION

Previous studies [1] have described the isolation and properties of a cytotoxic protein (P_6) from cobra venom (*Naja naja*) which shows preferential cytotoxicity to certain cells. The Yoshida sarcoma cells were most susceptible to the destructive effects of this cytotoxin in experiments in vitro. It was also found to prevent the growth of this tumour in rats in nontoxic doses. Experiments with a fluorescent labelled cytotoxin [2] have revealed that it binds with the plasma membrane of susceptible cells. In the present paper the mechanism of the cytotoxic action of the P_6 protein on cells was investigated by a study of its effects on the membrane bound enzyme $(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3.6.1.3) which is involved in the transport of Na^+ and K^+ across cell membranes [3]. The overall evidence

suggests that P_6 protein more actively inhibits the $(Na^+ + K^+)$-ATPase of those cells which are susceptible to its lytic effects.

MATERIALS AND METHODS

ATP and ouabain were obtained from E. Merck (G.F.R.). Eagle's medium modified according to Dulbecco was obtained from BIOS Laboratories, Bombay. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidic acid were from Koch-Light Laboratories Ltd. Phospholipids used in these studies were purified where necessary and showed a single spot in thin layer chromatograms.

Preparation of cells

Yoshida sarcoma cells. The tumour was regularly maintained in Wistar rats by biweekly transplantation of 0.8 ml of the fluid derived from the intraperitoneal cavity. The ascitic fluid containing Yoshida sarcoma cells was processed as described earlier [1].

Erythrocytes. Blood was collected in citrate and centrifuged to separate the plasma. The packed cells were washed 3 times with Tyrode's or isotonic NaCl as per experimental requirements.

Tissue culture cells. The tissue culture cell lines KB (human oral carcinoma), Hela (human cervix carcinoma) and L-132 (human lung embryonic) were obtained from the Virus Research Centre, Poona. These were maintained as monolayers adherent to glass in Eagle's medium as modified by Dulbecco. The medium was supplemented with 15% Human AB serum and was replaced twice a week. For use in experiments the bottles were generally seeded with $2.5 \cdot 10^6$ cells and allowed to grow at 37 °C for 48 h. The cells were removed from the glass surface by 0.3% trypsin treatment for 2 min at 37 °C.

Assay of cytotoxicity

Yoshida sarcoma cells. The cytotoxicity of cobra venom cytotoxic protein (P_6) on Yoshida sarcoma cells was determined by increase in absorbance at 260 nm as described earlier [2], as well as by a dye exclusion test [1] using lissamine green.

Erythrocytes. Haemolytic action of P_6 protein on erythrocytes was determined in tubes containing 0.6 ml of a 3% erythrocyte suspension, (this concentration of erythrocytes on complete haemolysis in a system of 5.0 ml produced an absorbance of approximately 1.00 at 540 nm) and varying amounts of P_6 protein in a total volume of 5.0 ml of Tyrode's (pH 7.4). The tubes were incubated for 30 min at 37 °C. A measure of haemoglobin released as a result of lysis was obtained by increase in absorbance at 540 nm at the end of the incubation period.The absorbance at 540 nm in the system without P_6 protein at the end of incubation period was negligible.

Tissue culture cells. Percent inhibition of growth of tissue culture cells by P_6 protein was determined as described [4] except that the cells were grown as monolayers in Leighton tubes. For this purpose 80 000 cells in 1 ml of growth medium were seeded in each Leighton tube. 16 tubes were used for each group containing varying amounts of P_6 protein and a control group without P_6 protein. The cells were grown for 48 h. At the end of this period half the number of tubes in each group

were trypsinized and cells counted in a haemocytometer. The medium and P_6 protein in the remaining tubes were replaced and cells allowed to grow another 48 h. The cultures were then trypsinized and counted as before.

($Na^+ + K^+$)-ATPase preparation

($Na^+ + K^+$)-ATPase of Yoshida sarcoma cells used in the experiments described here consisted of partially purified plasma membrane fragments prepared according to Wallach and Kamath [5]. For this purpose a suspension of Yoshida sarcoma cells ($2.5 \cdot 10^6$ cells/ml) were sonicated in Raytheon to the extent of 100% breakage (as estimated microscopically). There sidue obtained by centrifuging at $8000 \times g$ was discarded and the supernatant was recentrifuged at $105000 \times g$ for 60 min (Pellet 1). This process was repeated 3 times. The final pellet (Pellet 4) was suspended in 5 mM Tris · HCl buffer (pH 7.4).

($Na^+ + K^+$)-ATPase preparation of human and dog blood erythrocytes was carried out according to the method of Marchesi and Palade [6]. ($Na^+ + K^+$)-ATPase of tissue culture cells was prepared according to the method of Charalampous [7].

Enzyme assay

($Na^+ + K^+$)-ATPase activity was determined as described by Marchesi and Palade [6]. It was calculated from the difference in the inorganic phosphorus liberated in the presence and absence of K^+. The activity of ($Na^+ + K^+$)-ATPase could also be derived by subtracting the rate of ATP hydrolysis in the presence of ouabain (0.1 mM) from that obtained in absence of ouabain. Ouabain abolished the stimulation produced by K^+. Thus both methods gave equal results.

Phosphate estimation

Inorganic phosphorus was assayed by Ames' method [8].

Protein determination

Protein was assayed by the method of Lowry et al. [9] and also by the absorbance method described by Kalckar [10].

Electrophoresis in acrylamide

The method used was as described by Johns [11].

RESULTS

The purity of P_6 protein used in these studies was as shown in the acrylamide gel electrophoretic pattern as given in Fig. 1. The detailed studies on the effect of the P_6 protein on ($Na^+ + K^+$)-ATPase were carried out using a particulate preparation of membrane fragments made from Yoshida sarcoma cells (preparation and assay of enzyme are given in Materials and Methods). Preliminary experiments had shown that for a range of 10–15 μg of enzyme protein, the optimum enzyme activity was obtained with 1 μmol/ml of ATP in the system. Larger amounts of ATP were inhibitory, which has been reported to occur possibly due to accumulation of ADP in the reaction mixture [12]. Ouabain (0.1 mM) produced 100% inhibition of the

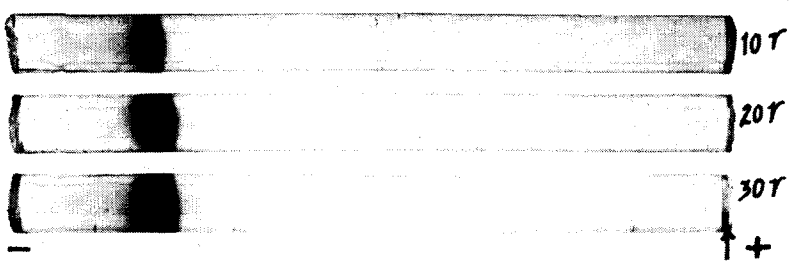

Fig. 1. Electrophoretic pattern of cytotoxin P_6 in acrylamide gel. 0.01 M acetic acid, pH 2.4, 300 V, 3 h. Stained with amido black.

TABLE I

$(Na^+ K^+)$-ATPase ACTIVITY OF YOSHIDA SARCOMA CELLS

Reaction mixture contained, in a total volume of 2.0 ml, 1 mM ATP, 100 mM NaCl, 20 mM KCl, 4 mM $MgSO_4$, 50 mM Tris.HCl buffer (pH 7.0) and 10 μg of enzyme protein. Incubation period 30 min. Temperature 37 °C. The reaction was terminated by addition of 1 ml of 10 % trichloroacetic acid. P_i was determined in 0.5 ml aliquots of the supernatant.

Enzyme preparation	$(Na^+ - K^+)$-ATPase activity, μmol P_i/mg protein per h
Pellet 1	1.75
Pellet 2	4.50
Pellet 3	6.75
Pellet 4	15.00

TABLE II

INACTIVATION OF $(Na^+ + K^+)$-ATPase OF YOSHIDA SARCOMA CELLS BY P_6 PROTEIN

See Table I for $(Na^+ + K^+)$-ATPase assay system. Total volume 2 ml. Percent inhibition calculated on the basis of activity in the absence of P_6 protein/ouabain.

System with or without P_6 protein/ouabain	Concentration of inhibitor	$(Na^+ + K^+)$-ATPase activity (Pellet 4), μmol P_i/mg protein per h	Inhibition %
Control	—	10.00	—
P_6 protein (μg)	5.0	6.50	45
	10.0	4.10	59
	20.0	1.8	82
	30.0	0.7	94
Control	—	13.5	—
Ouabain (μmol)	0.025	7.5	44.4
	0.05	4.5	67
	0.075	1.5	89
	0.10	0.0	100

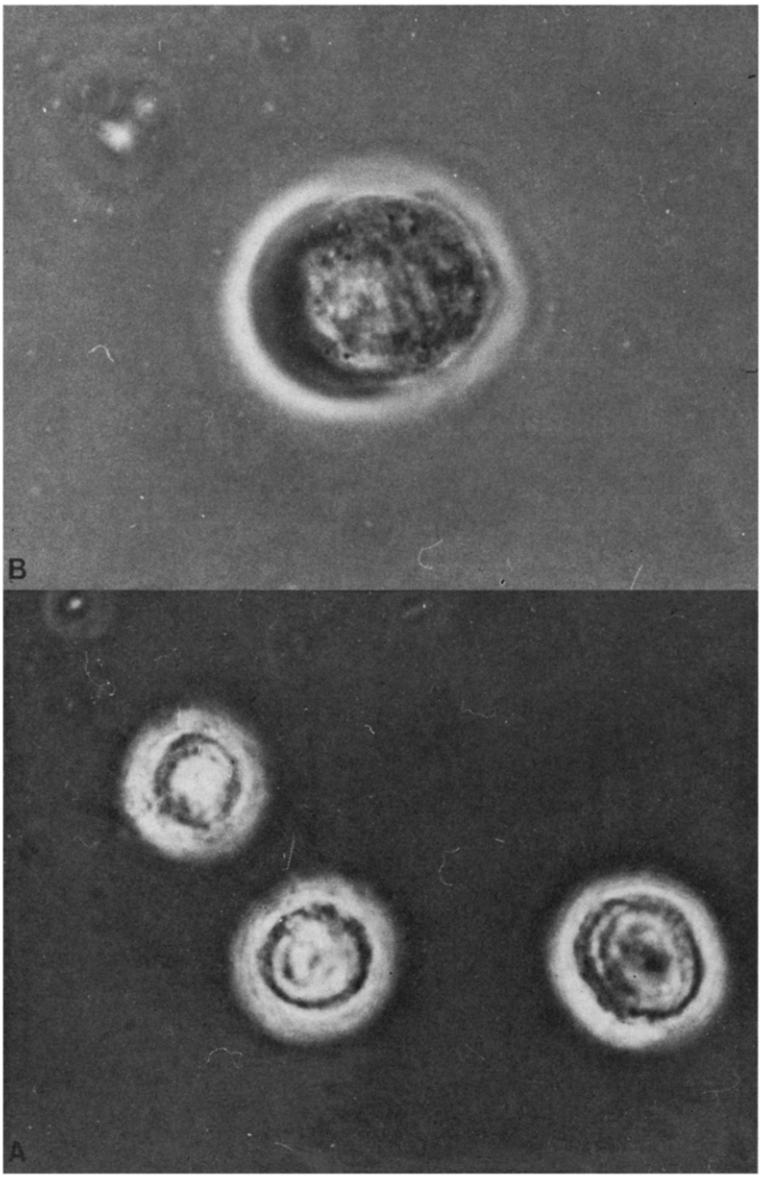

Fig. 2. Effect of ouabain on Yoshida sarcoma cells. Phase contrast photomicrographs of Yoshida sarcoma cells × 1060. a, Control cells; b, Cells treated with ouabain.

enzyme indicating that in the experimental system the release of phosphorus from ATP was entirely due to $(Na^+ + K^+)$-ATPase activity. Table I shows the enrichment in ATPase activity obtained during the intermediate stages of the preparation. The activity of the particulate enzyme preparation (Pellet 4) used in various experiments described here ranged from 10–15 μmol P_i per mg protein per h. The effect of various concentrations of P_6 protein as well as ouabain on $(Na^+ + K^+)$-ATPase is given in Table II.

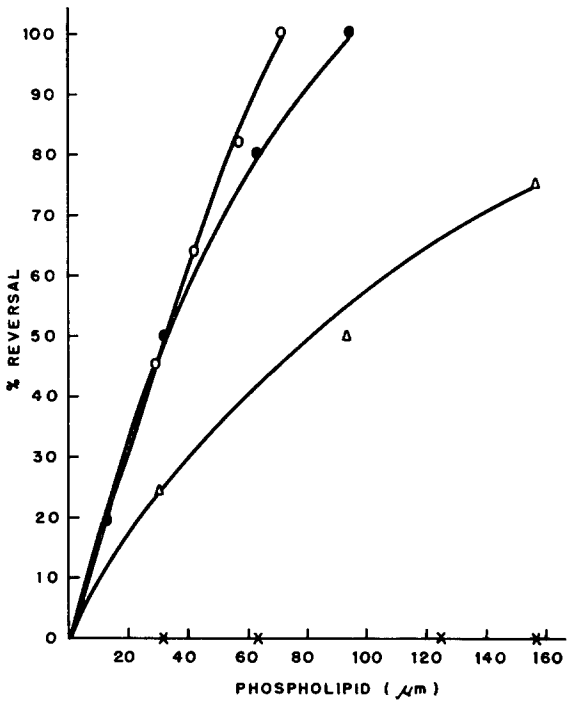

Fig. 3. Effect of phospholipids on $(Na^+ + K^+)$-ATPase of Yoshida sarcoma cells (Pellet 4) partially inactivated by the P_6 protein. Experimental conditions as in Table I. ○—○, Phosphatidic acid; ●—●, Phosphatidylserine; △—△, Phosphatidylethanolamine; ×—×, Phosphatidylcholine.

It is apparent from the results that the activity decreased with increasing amounts of added P_6 protein as well as ouabain. Concentration of 10 μg/ml P_6 protein produced 59 % inhibition and 30 μg/ml P_6 protein almost completely inactivated the $(Na^+ + K^+)$-ATPase of Pellet 4. Ouabain (0.025 μmol) inhibited the enzyme to the extent of 44.4 % and 0.1 μmol caused complete inactivation. Ouabain (0.013 μmol) also produced 50 % lysis of Yoshida sarcoma cells as measured by the dye exclusion test [1]. The lysis was accompanied by swelling of the cells as seen in Fig. 2.

A number of reports have suggested that the $(Na^+ + K^+)$-ATPase of various tissues can be inactivated by phospholipases [13] and detergents [14] and can be reconstituted by addition of phospholipids [13, 15]. Fig. 3 illustrates the effects of various phospholipids on the $(Na^+ + K^+)$-ATPase of Yoshida sarcoma cells (Pellet 4) which was partially inactivated by P_6 protein. In these experiments the reconstitution of the enzyme in presence of phospholipids was examined in a $(Na^+ + K^+)$-ATPase system inhibited by 9 μg/ml P_6 protein. This was the concentration of the cytotoxin required to produce about 50 % inhibition of enzyme activity.

Phosphatidic acid (71 μM) and phosphatidylserine (91 μM) produced complete reversal of enzyme inactivation brought about by P_6 protein. Phosphatidylethanolamine (156 μM) produced 75 % reversal and phosphatidylcholine (156 μM) had no effect. Although no phospholipid degrading activity could be detected in the P_6 protein preparation, phospholipids which are known to reverse the inhibitory effects of phospholipases on $(Na^+ + K^+)$-ATPase of tissues were also effective in

TABLE III

EFFECT OF PHOSPHOLIPIDS ON THE LYSIS OF YOSHIDA SARCOMA CELLS BY P_6 PROTEIN

Assay system: Phospholipid, 25 μg/ml; P_6 protein, 4 μg/ml; Yoshida sarcoma cells, 1.6 ml ($2.5 \cdot 10^6$ cells/ml), total volume made up to 5 ml with Tyrode's (pH 7.4). Incubation period 30 min. Temperature 37 °C. The percentage inhibition of lysis was calculated on the basis of absorbancy in absence of the phospholipid. In absence of P_6 protein and phospholipid, absorbance = 0.11.

Phospholipid	Net absorbance at 260 nm		Inhibition %
	P_6 protein	P_6 protein + phospholipid	
Phosphatidic acid	0.56	0.28	50
Phosphatidylserine	0.56	0.34	39
Phosphatidylethanolamine	0.56	0.54	4
Phosphatidylcholine	0.56	0.56	0

reconstituting the enzyme inactivation by P_6 protein. It is of interest that results given in Table III reveal that phosphatidic acid, phosphatidylserine and phosphatidylethanolamine were also able to prevent the lytic action of P_6 protein on Yoshida sarcoma cells whereas phosphatidylcholine had no effect. In view of the data which suggest that factors which reverse the inactivating action of P_6 protein on $(Na^+ + K^+)$-ATPase are able to prevent its cytotoxicity, the effect of P_6 protein was examined on $(Na^+ + K^+)$-ATPase of cell systems which are resistant and susceptible to its lytic action.

TABLE IV

LYTIC ACTION OF P_6 PROTEIN ON ERYTHROCYTES OF DIFFERENT SPECIES

System, see Materials and Methods

Species	Absorbance at 540 nm in the presence of P_6 protein	
	20 μg/ml	40 μg/ml
Cow	0.04	0.05
Buffalo	0.05	0.05
Bull	0.05	0.06
Horse	0.06	0.07
Rat	0.08	0.09
Sheep	0.09	0.11
Hamster	0.09	0.11
Rabbit	0.09	0.13
Mouse	0.10	0.12
Goat	0.11	0.19
Human	0.19	0.23
Chicken	0.21	0.33
Cat	0.29	0.35
Guinea pig*	1.00	–
Dog**	1.00	–

* 9 μg/ml P_6 protein for 0.5 absorbance.

** 5.8 μg/ml P_6 protein for 0.5 absorbance.

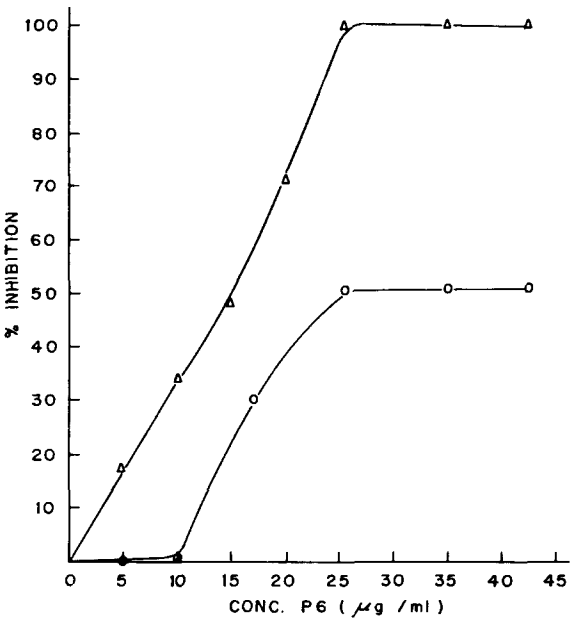

Fig. 4. Inactivation of $(Na^+ + K^+)$-ATPase of human and dog erythrocyte ghosts by P_6 protein. Experimental conditions as in Table I. ○ – ○, human erythrocyte ghosts; △ – △, dog erythrocyte ghosts.

Observations have shown that the P_6 protein displays a wide spectrum of lytic activity towards erythrocytes from different species. Whereas human erythrocytes are very resistant, dog erythrocytes were found to be relatively more susceptible to the cytotoxic action of this protein (Table IV). It was of interest to study the inhibitory effects of P_6 protein on the $(Na^+ + K^+)$-ATPase of these two species of erythrocytes. The enzyme preparations from human and dog erythrocyte ghosts were made under identical conditions (Materials and Methods). It is evident from results given in Fig. 4 that 25.5 μg of the P_6 protein caused 50 % inhibition in the human erythrocyte system whereas this amount produced 100 % inhibition in the enzyme prepared

TABLE V

EFFECT OF P_6 PROTEIN ON GROWTH OF TISSUE CULTURE CELL LINES

Experimental conditions described in Materials and Methods.

Cell type	100 % growth inhibition P_6 protein (μg/ml)
Hela cells (human cervix carcinoma)	5.0
KB cells (human oral carcinoma)	10.0
L-132 cells (human lung embryonic)	> 15.0

from dog erythrocytes. It is also apparent that in case of human erythrocytes, after about 50 % of the $(Na^+ + K^+)$-ATPase had been inhibited, no further decrease in activity could be detected by increasing the concentration of P_6 protein from 25.5 to 42.5 μg.

Growth inhibition experiments on three different tissue culture cell lines have shown that P_6 protein was more inhibitory to the growth of KB and Hela cells than to the L-132 cell line (Table V). Preparation of $(Na^+ + K^+)$-ATPase made from the three cell lines showed an enzyme activity ranging from 1.6 to 3.0 μmol P_i/mg protein per h. Cytotoxin P_6 inhibited more actively the enzyme from Hela and KB cells than the preparation from L-132. It is evident from the results illustrated in Fig. 5 that 1.5, 1.9 and 4.5 μg/ml P_6 protein produced 50 % inhibition of the enzyme in Hela, KB and L-132 cells respectively.

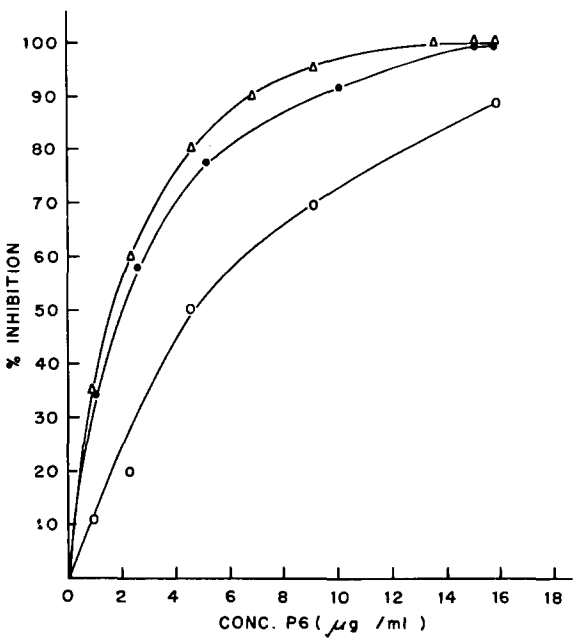

Fig. 5. Inactivation of $(Na^+ + K^+)$-ATPase of three tissue culture cell lines by P_6 protein. For experimental conditions, See Table I and Materials and Methods. ● — ●, KB; △ — △, Hela; ○ — ○, L-132.

DISCUSSION

The basic protein (P_6) isolated from cobra venom has been of interest for study as it displays great variation in its effects on membranes of different cells. The Yoshida sarcoma ascitic tumour cells which were most susceptible to destruction by P_6 protein (1.6 μg/ml P_6 protein caused 50 % lysis of Yoshida sarcoma cells) were employed for the detailed experiments designed to study its mechanism of action on the cell surface. The overall results obtained from a study of the inhibitory effects of P_6 protein on the $(Na^+ + K^+)$-ATPase of membrane fragments from Yoshida sarcoma cells, human and dog erythrocytes and three tissue culture cell lines have

demonstrated that P_6 protein inhibition of $(Na^+ + K^+)$-stimulated ATPase can be correlated with its cytotoxic effects on the intact cells. Thus, the enzyme from Yoshida sarcoma cells was most strongly inhibited and also these cells were highly susceptible to lysis by P_6 protein. The results with human and dog erythrocytes prepared under identical conditions showed a similar relationship between $(Na^+ + K^+)$-stimulated ATPase inhibition by the P_6 protein and susceptibility to haemolysis. Thus, the enzyme from dog erythrocytes was completely inhibited with 25.5 μg/ml P_6 protein whereas this amount produced only 50% inhibition with human erythrocytes. Dog erythrocytes were also much more susceptible to haemolysis than the human erythrocytes (Table IV). Although experiments with three tissue culture cell lines also broadly correlate inhibition of $(Na^+ + K^+)$-ATPase by P_6 protein with its potency as growth inhibitor of these cells, it is necessary to consider that inhibition of growth is the end result of a complex series of events and possibly reflects a number of changes besides the enzyme inhibitions discussed here. Among the three cell lines examined, the tumour cells (KB and Hela) were more susceptible to damage by P_6 protein than the L-132 line derived from human embryonic cells. Microscopic studies have shown that the Yoshida sarcoma cells swell to almost double their size prior to disintegration in presence of the P_6 protein [2]. Ouabain also was able to bring about swelling and lysis in these cells.

$(Na^+ + K^+)$-stimulated ATPase is a membrane bound enzyme concerned with the transport of K^+ and Na^+ in and out of the cell [3]. Lipid protein interactions are known to be involved in maintaining this complex system in proper conformation for activity [16]. Phospholipases have been reported to inactivate $(Na^+ + K^+)$-ATPase which can then be reconstituted by the addition of phospholipids. Phosphatidic acid and phosphatidylserine have been found most effective in reversing the inhibition produced by phospholipase A and C on a variety of cell systems [13]. The nature of the lipid moiety involved in the enzyme protein complex in different tissues is not clearly known. The removal of phosphatidylserine apparently does not lead to loss of enzyme activity in all tissues. In a recent paper, De Pont et al. [17], using brain microsomes, report that phosphatidylserine could be totally converted to phosphatidylethanolamine by phosphatidylserine decarboxylase without loss of enzyme activity, whereas evidence from Roelofsen and Van Deenen quoted in the same paper [17] suggests that in human erythrocytes removal of residual phosphatidylserine brought about complete inactivation of $(Na^+ + K^+)$-stimulated ATPase. It has been observed that the P_6 protein does not degrade phospholipids nor could protease activity be detected. Other experiments not reported here have indicated that P_6 protein complexes with acidic phospholipids and also with membrane components. It is of interest that phosphatidic acid and phosphatidylserine were able to re-establish the enzyme activity and also prevented the lysis of intact Yoshida sarcoma cells by P_6 protein. Considering the preferential lytic effects of this protein on different cells including some tumour cells it is possible that the highly basic nature of P_6 protein (isoelectric point above 9.4) as well as its specificity of structure enable it to compete effectively for the lipid moiety of $(Na^+ + K^+)$-ATPase complex in the susceptible tissues causing a disturbance in the conformation of the specific enzyme protein. The cytotoxin P_6 could be a useful reagent for the study of the nature of the specific lipid moiety in the membrane of different tissues which keeps the $(Na^+ + K^+)$-ATPase enzyme in an active state. Since in the Yoshida sarcoma cell system both Ouabain and P_6 protein cause swelling of the

cells followed by lysis, it is possible that the imbalance of Na^+ and K^+ across the cell membrane, which results from inactivation of the Na pump, may be implicated in the lytic effects of P_6 protein.

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