

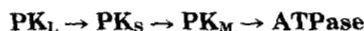
Regulation of Phosphorylation of the β -Subunit of the Ehrlich Ascites Tumor Na^+K^+ -ATPase by a Protein Kinase Cascade*

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The low efficiency of the Na^+K^+ -ATPase from Ehrlich ascites tumor cells is caused by phosphorylation of the β -subunit by an endogenous protein kinase (Spector, M., O'Neal, S., and Racker, E. (1980) *J. Biol. Chem.* 255, 8370-8373). We have now characterized a pathway consisting of three endogenous protein kinases that regulates the pumping efficiency (expressed as the ratio of Na^+ transported/ATP hydrolyzed) of the Na^+K^+ -ATPase in these tumor cells. Starting with plasma membranes, the tumor kinases have been purified to homogeneity (about 400-fold). The β -subunit of the Na^+K^+ -ATPase is phosphorylated by protein kinase M, which is active only after phosphorylation by another kinase, protein kinase S. A third enzyme, protein kinase L, phosphorylates and activates protein kinase S. The pathway is summarized as follows.



Protein kinase M consists of two polypeptide subunits ($M_r = 40,000$ and $20,000$) as does protein kinase L ($M_r = 44,000$ and $48,000$), while protein kinase S is monomeric ($M_r = 57,000$). The β -subunit of the Na^+K^+ -ATPase is phosphorylated on a tyrosine residue by protein kinase M as is the $20,000$ -dalton subunit of protein kinase M, but protein kinase S is phosphorylated on serine and tyrosine residues.

Double immunodiffusion and two-dimensional crossed immunoelectrophoresis of monospecific antisera show that the three kinases are immunologically unrelated. Peptides produced by cleavage with cyanogen bromide show that the kinases are structurally dissimilar. All of the kinases are found at elevated levels in Ehrlich ascites tumor cells compared with mouse brain, as determined by competition radioimmunoassays. Most notably, the level of protein kinase S is about 30-fold higher in the tumor cells. Immunoprecipitates from brain membrane lysates contain Na^+K^+ -ATPase and the kinases mainly in the unphosphorylated form, suggesting that the kinases are controlled in brain membranes. However, after solubilization and partial purification, the kinases from brain show some activity indicating removal of an inhibitor. In contrast, protein kinases M and S are phosphorylated and active in tumor membranes thereby causing

the phosphorylation of the β -subunit of the Na^+K^+ -ATPase and rendering its pumping activity inefficient.

The Na^+K^+ -ATPase catalyzes the hydrolysis of ATP and the transport of sodium and potassium across the plasma membranes of most cells. Indirect evidence suggests that the Na^+K^+ -ATPase in several tumors is operating inefficiently and contributes to the high aerobic glycolysis of these cells (1). Recent work in this laboratory has demonstrated that purified and reconstituted Na^+K^+ -ATPase from Ehrlich ascites tumor cells is an inefficient sodium pump (2). In these tumor cells, the β -subunit of the Na^+K^+ -ATPase is phosphorylated by protein kinase M, an endogenous protein kinase, and this decreases the net efficiency of sodium pumping (3). The inefficiency of the purified ATPase is reversed by phosphatase and the level of phosphorylation of the β -subunit parallels the degree of efficiency of sodium pumping.

We now wish to report that PK_M , which phosphorylates the β -subunit, is itself phosphorylated by a kinase called protein kinase S. Furthermore, PK_S is phosphorylated by yet another endogenous protein kinase called protein kinase L. In order to study this cascade in detail, we have purified the three kinases and, with the aid of monospecific antisera, we have measured their contents in tumor and brain membranes. Some of the properties of the purified kinases will be described.

MATERIALS AND METHODS

Casein-Sepharose was prepared according to the method outlined in Reference 4 and [$\gamma\text{-}^{32}\text{P}$]ATP was prepared as described in Reference 5. ^{125}I was conjugated to 3-(4-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide ester by the chloramine-T method (6). Phosphotyrosine was synthesized from tyrosine, phosphoric acid, and phosphorus pentoxide as described (7). Carrier-free $^{32}\text{P}_i$ and ^{125}I (100 mCi/ml) were purchased from New England Nuclear. Enzyme grade sucrose and ultrapure ammonium sulfate were from Schwarz/Mann. NP-40, Lubrol PX, deoxycholate, polyethylene glycol (average molecular weight = 4,000), agarose (low electroendosmosis), cyanogen bromide, casein, histones, phosvitin, soybean trypsin inhibitor, subtilisin BPN', phosphoserine, phosphothreonine, *Staphylococcus* cells, and chloramine-T were from Sigma. 3-(4-Hydroxyphenyl)propionic acid

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¹ The abbreviations used are: PK_M , protein kinase M; PK_S , protein kinase S; PK_L , protein kinase L; buffer A, 25 mM Hepes-KOH (pH 7.5), 20 mM KH_2PO_4 (pH 7.5), 15 mM 2-mercaptoethanol, 10 mM NaF, and 2 mM PMSF; buffer B, 25 mM Tris-HCl (pH 6.8), 20 mM NaF, 15 mM 2-mercaptoethanol, and 2 mM PMSF; buffer C, 25 mM Tris-HCl (pH 8.3), 15 mM 2-mercaptoethanol, 20 mM NaF, and 2 mM PMSF; TEN buffer, 20 mM Tris-HCl (pH 7.6), 1 mM EDTA, 100 mM NaCl, 0.01% Triton X-100, and 2 mg/ml of BSA; NP-40, Nonidet P-40; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin, SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; IgG, immunoglobulin G; Tricine, *N*-tris(hydroxymethyl)methylglycine.

N-hydroxysuccinimide ester was supplied by Pierce. Ampholytes, octyl Sepharose, and Ficoll were purchased from Pharmacia. Goat anti-rabbit IgG was obtained from Miles Laboratories, Freund's adjuvant was from Difco, and rabbit muscle aldolase, beef liver catalase, phosphorylase α , and bovine serum albumin were from Boehringer Mannheim.

Assays for Protein Kinase Activity—Substrates and fractions containing protein kinase activity were incubated at 30 °C for various lengths of time in kinase assay buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM KH_2PO_4 (pH 7.5), 10 mM MgCl_2 , 15 mM 2-mercaptoethanol, and 2 mM PMSF in a final volume of 0.09 ml. The reaction was started by addition of 0.01 ml of 10 mM [γ - ^{32}P]ATP (50–150 cpm/picomole), and the extent of phosphorylation was determined by the filter paper method after acidification (8). The sample was applied to Whatman glass filter (2.3 cm diameter) and washed 3 times for 30 min with ice-cold 10% trichloroacetic acid. Generally, 500 ml was used for a batch of 25 filters, and a final 12-h trichloroacetic acid wash was followed by one rinse with 250 ml of 95% ethanol and 250 ml of ethyl ether. Filters were dried, placed in scintillation vials with 5 ml of Liquiscint, and counted in a Beckman model LS-7000 liquid scintillation spectrometer. Alternatively, reactions were ended by addition of 0.1 ml of SDS electrophoresis sample buffer containing 2% SDS, 25% sucrose, 25 mM Tris-HCl (pH 6.8), 3% 2-mercaptoethanol, and 0.05% bromophenol blue. The polypeptides were fractionated on SDS polyacrylamide slab gels using a 12% acrylamide separating gel, a 5% acrylamide stacking gel, and a discontinuous buffer system. Polypeptide bands were visualized by soaking the gel in 250 ml of ice-cold 0.5 M KCl, and the desired bands were cut from the gel with a razor blade. Each gel slice was solubilized with 0.1 ml of 30% hydrogen peroxide at 50 °C for 5 h (9). Liquiscint (5 ml) was added, and radioactivity was measured as described above. PK_M kinase activity was measured by incubation of 25 μg of Na^+K^+ -ATPase with fractions containing PK_M activity for 1 to 2 h in a final volume of 0.025 ml. PK_S and PK_L activities were likewise determined by incubating 5 μg of PK_M or 5 μg of PK_S , respectively, together with fractions containing activity in a final volume of 0.025 ml. When ATPase, PK_M , or PK_S were used as substrates, they were first dephosphorylated (3) by incubating with 50 mg of alkaline phosphatase-agarose in a medium containing 25 mM glycyl-glycine (pH 7.0), 1 mM MgCl_2 , 100 mM ZnCl_2 , 2 mM PMSF, and 100 mM sucrose for from 1.5 to 4.5 h at 30 °C. The final volume was 0.05 ml, and the alkaline phosphatase was removed by centrifugation ($2,500 \times g$, 5 min). Fifty micromolar cAMP was included in all assays of PK_M phosphorylation by PK_S unless otherwise indicated.

Extraction of Kinases—All steps were carried out at 4 °C unless otherwise noted. Plasma membranes were isolated from tumor cells according to the method described in Reference 10 as modified in accordance with Reference 2. Generally, 9-day cultures from 30 mice yielded 90 g wet weight of cells, from which 100 mg of plasma membrane were isolated. Membranes (100 mg) were pelleted by centrifugation at $35,000 \times g$ for 30 min and were extracted in 10 ml of buffer A (25 mM Hepes-KOH (pH 7.5), 20 mM KH_2PO_4 (pH 7.5), 15 mM 2-mercaptoethanol, 10 mM NaF, and 2 mM PMSF) containing 1.25% (w/v) deoxycholate and 200 mM KCl for 30 min. The suspension was centrifuged at $144,000 \times g$ for 35 min in a Beckman type 60 rotor. The pellets were resuspended in 8 ml of ice-cold buffer A to give a protein concentration of about 10 mg/ml. About 12 mg of deoxycholate-extracted membranes were layered on each of six 2.5 to 25% continuous glycerol gradients (35 ml) containing 20 mM EDTA-KOH (pH 7.5) in buffer A and centrifuged at $121,000 \times g$ in a SW27 rotor for 17 h at 2 °C. Membrane fragments having protein kinase activity and banding at 12.5% glycerol were pooled from six gradients to give about 20 mg of protein in 20 ml. After concentration with Ficoll (11) to about 4 mg/ml, the membrane fragments (now in a volume of about 5 ml) were diluted with an equal volume of 1% NP-40 dissolved in 2 times concentrated buffer A. After 10 min, the mixture was centrifuged at $50,000 \times g$ for 90 min in a 50Ti rotor. The clear supernatant contained about 18 mg of protein and was concentrated with Ficoll to 10 mg/ml, and then was stored in liquid nitrogen.

Purification of PK_M —Frozen NP-40 extracts (20 mg) were thawed and diluted to 5 mg/ml with 2 ml of 2 times concentrated buffer A, and an equal volume of 2.8 M ammonium sulfate freshly diluted from a 4 M stock solution (pH 8.0) was added. After 30 min, the suspension was centrifuged at $15,000 \times g$ in a SS-34 rotor for 30 min. The pellet (about 8 mg of protein) was washed twice with buffer A containing 2 M ammonium sulfate and then resuspended in 0.5 ml of buffer A containing 0.05% NP-40. After dialysis overnight against 1000 ml of buffer A plus 0.05% NP-40, the preparation (7.5 mg of protein) was

fractionated on two 4 to 25% sucrose gradients (3.5 ml) containing buffer A and 0.05% NP-40. The sucrose gradients were centrifuged at $310,000 \times g$ for 17 h in a SW60 rotor and the active fractions, which banded at about 8% sucrose, were pooled to give about 2.4 mg of protein in 1.2 ml.

Further purification of PK_M was achieved by adsorption to casein-Sepharose (12) as follows. Well washed casein-Sepharose (1 ml) was suspended in 1 ml of buffer B (25 mM Tris-HCl (pH 6.8), 20 mM NaF, 15 mM 2-mercaptoethanol, 2 mM PMSF) containing 0.05% NP-40, and 1 ml of protein (1 mg) was added. The casein-Sepharose/protein slurry was stirred for 30 min, the supernatant were decanted, and the gel was washed twice (15 min each) with 5 ml of buffer B containing 0.1 M NaCl. PK_M was eluted by resuspending the casein-Sepharose gel in 0.5 ml of buffer B containing 0.05% NP-40 and 2.5 M NaCl. The pooled eluate contained 105 μg in 0.5 ml and was dialyzed against 1000 ml of buffer B for 2 h. Immediately following dialysis, the sample was isoelectrofocussed on an LKB column (110 ml) using 2% carrier ampholytes (Pharmalytes) in the pH range of 3.5 to 10 in the presence of 0.5% NP-40 and sucrose to form a density gradient from 0 to 50% as described (13). Isoelectric focusing was conducted at 400 V for 48 h, and fractions of 0.5 ml were collected for kinase assays and pH measurements. Active fractions were pooled, concentrated with Ficoll, quick-frozen in liquid nitrogen, and stored at -80 °C.

Purification of PK_S and PK_L —The NP-40 extract (20 mg in 2 ml) was diluted with 2 ml of 2 times concentrated buffer A containing 0.1% NP-40 and 20% (w/v) sucrose. After addition of 2 ml of 4 M $(\text{NH}_4)_2\text{SO}_4$ (pH 8.0) to give a final concentration of 1.33 M ammonium sulfate, the entire sample was loaded onto a 20-ml column (1×20 cm) of well washed octyl-Sepharose equilibrated with buffer C (25 mM Tris-HCl (pH 8.3), 15 mM mercaptoethanol, 20 mM NaF, and 2 mM PMSF) containing 1.2 M ammonium sulfate and 0.5% NP-40. PK_S and PK_L activities were eluted in separate fractions with a decreasing gradient of ammonium sulfate (1.3 to 0 M) and an increasing linear gradient of 0 to 35% ethylene glycol in buffer C plus 0.05% NP-40. PK_S eluted at 0.95 M ammonium sulfate and PK_L eluted at 0.6 M ammonium sulfate. Active fractions (0.25 ml) were pooled and dialyzed against 3500 ml of buffer A containing 250 mM KCl. Upon dialysis, the proteins precipitated and were sedimented by centrifugation at $10,000 \times g$ for 30 min. PK_S (3 mg) was solubilized with 0.3 ml of buffer A containing 0.05% NP-40, and PK_L (4.6 mg) was resuspended in 0.46 ml of the same buffer. Pooled fractions containing PK_S or PK_L activity were fractionated on 4 to 25% sucrose gradients as described above for PK_M . In the case of PK_S , 1.1 mg of protein was recovered in 0.75 ml, and 1.7 mg of PK_L was recovered in 1.0 ml. Further purification on casein-Sepharose followed by isoelectric focusing was done as described for PK_M .

PK_M , PK_S , and PK_L were purified from mouse brain membranes as described above for the tumor membranes.

Molecular Weight Determinations—The molecular weights of the purified kinases were estimated by gel filtration on a Sephadex G-200 column (1.5×50 cm) as described by Andrews (14). The elution buffer contained 35 mM phosphate (pH 7.5), 20 mM KCl, and 0.05% NP-40. Catalase ($M_r = 210,000$), aldolase ($M_r = 158,000$), bovine serum albumin ($M_r = 68,000$), and soybean trypsin inhibitor ($M_r = 20,000$) were used as markers. The molecular weights of kinase subunits were determined by SDS-polyacrylamide gel electrophoresis in 12% discontinuous polyacrylamide gels as described (2). Phosphorylase α ($M_r = 94,000$), bovine serum albumin, catalase ($M_r = 58,000$), and soybean trypsin inhibitor served as markers.

Electrophoresis and Fluorography—SDS-polyacrylamide gel electrophoresis was done in an apparatus modified from the design of Studier (15) using the buffer system described by Laemmli (16) modified as described previously (2). Except where noted in the figure legends, 12% and 5% acrylamide was used in the separating and stacking gels, respectively. After electrophoresis at 35 mA for 4 h, gels were stained with Coomassie brilliant blue R as described by Fairbanks *et al.* (17). For fluorography, acrylamide gels containing protein bands were soaked 30 min in 1.4 M sodium salicylate (18), rinsed with water, dried, and exposed to X-Omat RP-1 x-ray film (Kodak, Rochester) for 12 h to 4 days with Cronex intensifying screens (E.I. duPont de Nemours and Co., Wilmington, DE).

Determination of Phosphorylated Amino Acid Residues—Purified ^{32}P -proteins (25 μg) were hydrolyzed with 0.5 ml of 6 N HCl for 2 h at 110 °C, and HCl was removed *in vacuo*. The hydrolyzed protein samples were dissolved in pH 2 electrophoresis buffer (glacial acetic acid:formic acid (88%):water, 78:25:897) containing 1 mg/ml of phosphoserine, phosphothreonine, and phosphotyrosine. Hydrolysates were electrophoresed for 60 min at 1.5 kV on 20×20 cm cellulose

(250 micrometer thick) thin layer plates. In the second dimension, chromatography was performed in isobutyric acid:0.5 M NH_4OH , 5:3 (19). ^{32}P -phosphoamino acids were detected by autoradiography on X-Omat RP-1 x-ray film with Cronex intensifying screens.

Preparation of Antisera—New Zealand white rabbits were immunized subcutaneously with each antigen (PK_M , PK_S , PK_L , or $\text{Na}^+\text{K}^+\text{-ATPase}$), purified from tumor cells as described above, on the following schedule: week 1, 50 μg of antigen in 1 ml of 100 mM KPi (pH 7.0) emulsified in 1 ml of Freund's complete adjuvant; week 2, 50 μg of antigen in 1 ml of 100 mM KPi (pH 7.0) emulsified in 1 ml of Freund's incomplete adjuvant; week 4, 50 μg of antigen in 1 ml of 100 mM KPi (pH 7.0). Rabbits were bled for preimmune sera before immunization, and bleedings were done from the left marginal vein of the ear on week 6 and monthly thereafter. The blood was allowed to clot at 8 °C for 1 h, and the clot was removed by centrifugation at $5000 \times g$ for 4 min. Sera were immediately treated with either 100 units/ml of Trasylol or 2 mM PMSF. The crude antiserum was used for competition radioimmunoassays and other experiments described.

TABLE I

Purification of protein kinases from the plasma membranes of Ehrlich ascites tumor

A. Purification of PK_M					
Purification step	Total protein mg	Total activity moles $\text{P} \times 10^{-11}$ incorporated \times $\text{min}^{-1} \times$ mg protein^{-1}	Recov- ery %	Specific activity moles $\text{P} \times 10^{-11}$ in- corporated \times $\text{min}^{-1} \times$ mg protein^{-1}	Purifi- cation -fold
Plasma membranes	100	88	100	0.88	
Deoxycholate-ex- tracted plasma mem- branes	81	85	96.6	1.05	1.2
Glycerol density gra- dient	22	51	58	2.30	2.6
NP-40 extraction	18	50	57	2.78	3.2
1.4 M $(\text{NH}_4)_2\text{SO}_4$ pre- cipitate	8	30	34	3.75	4.3
Sucrose density gra- dient	2.5	21	24	8.4	9.5
Casein affinity chroma- tography	0.105	18	20.5	170	193
Isoelectric focusing	0.048	16	18.2	350	398
B. Purification of PK_S					
Plasma membranes	100	21	100	0.21	
Deoxycholate-ex- tracted plasma mem- branes	80	19	90.5	0.238	1.1
Glycerol density gra- dient	19.5	12	57.1	0.615	2.9
NP-40 extraction	17.1	11	52.4	0.64	3
Octyl Sepharose chroma- tography	4.6	9.2	43.8	2	9.5
Sucrose density gra- dient	1.7	8.3	39.5	4.88	22.9
Casein affinity chroma- tography	0.096	6.9	32.9	71.9	342
Isoelectric focusing	0.061	6.7	31.9	110	524
C. Purification of PK_L					
Plasma membranes	100	17	100	0.17	
Deoxycholate-ex- tracted plasma mem- branes	78	15.2	89.4	0.195	1.1
Glycerol density gra- dient	19	11	64.7	0.579	3.4
NP-40 extraction	16	9.4	55.3	0.588	3.5
Octyl Sepharose chroma- tography	3	7.3	42.9	2.2	12.9
Sucrose density gra- dient	1.1	6.2	36.5	6.82	40.1
Casein affinity chroma- tography	0.152	4.1	24	62	364.7
Isoelectric focusing	0.050	3.75	22.1	75	441

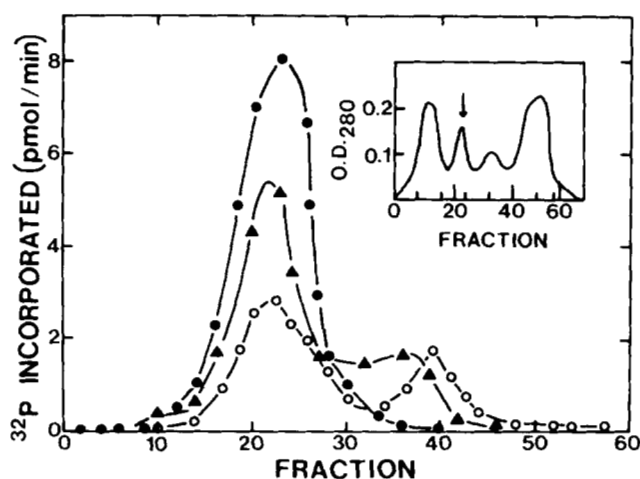


FIG. 1. Elution profile of PK_M , PK_S , and PK_L activities of the glycerol density gradient centrifugation. Deoxycholate-extracted membranes (12.5 mg) were layered on a 35-ml 2.5 to 25% glycerol gradient containing 20 mM EDTA-KOH (pH 7.5) in buffer A and centrifuged at $121,000 \times g$ in a SW27 rotor for 17 h at 2 °C. The gradient was fractionated with a Büchler piercing apparatus, and absorbance at 280 nm was followed with a Pharmacia model UV-1 monitor. Fractions (0.5 ml) were collected in a Gilson micro fractionator, and 0.02 ml of each fraction was assayed for PK_M , PK_S , and PK_L activity by the filter paper technique described under "Materials and Methods." ●—●, PK_M activity; ▲—▲, PK_S activity; ○—○, PK_L activity.

Immunoglobulin G was purified from the crude antisera by ammonium sulfate precipitation followed by DEAE-Sephadex G-50 chromatography (20). The purified IgG fractions were concentrated and extensively dialyzed against 80 mM Tris-HCl (pH 8.6) and 0.15 M NaCl. The concentration of IgG was determined by absorbance at 280 nm using the relationship, $1.4 A_{280}$ units of IgG = 1 mg (21). The purified IgG was lyophilized and stored at -80 °C or stored in solution at 4 °C with 5 mM PMSF.

Double Immunodiffusion Assays—Immunodiffusion assays were performed as described by Ouchterlony and Nilsson (22) at 30 °C for 24 h in 1.2% agarose containing 80 mM Tris-HCl (pH 8.6), 0.1 M NaCl, and 1% Triton X-100. Immunoprecipitates were washed for 2 days in 0.15 M NaCl and stained for 15 min with 0.05% naphthol blue-black (amido black), 10% (v/v) glacial acetic acid, and 0.01% mercuric chloride. Excess stain was removed by washing with 10% acetic acid until the background was clear.

Crossed Immunoelectrophoresis—Two-dimensional crossed immunoelectrophoresis was performed by the method of Chua and Blomberg (23) except that electrophoresis with SDS in the first dimension was performed in 12% acrylamide/0.5% polyacrylamide slabs.

Immunoprecipitation and Radioimmunoassay—Purified tumor and brain proteins were conjugated with ^{125}I [3-(4-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide ester] according to the method described in Reference 6. The ^{125}I -ester (250 μCi) was incubated with 10 μg of purified protein in 40 mM KPi (pH 7.6), 2 mM EDTA, and 200 mM NaCl in a total volume of 0.05 ml for 24 h at 2 °C. After the reaction was terminated (6), 1 mg of crystalline BSA was added to enhance recovery when the ^{125}I -labeled protein was separated from other reaction products on a Sephadex G-25 column (2 ml). For immunoprecipitation of tumor and brain proteins and for titration of antisera, 0.005 ml of normal rabbit serum, 1 to 10 mg of ^{125}I -labeled protein (3.9 to 5.5×10^4 cpm/mg), and 0.01 ml of diluted antiserum were mixed in a siliconized glass tube. The volume was adjusted to 0.06 ml with TEN buffer (20 mM Tris-HCl (pH 7.6), 1 mM EDTA, 100 mM NaCl, 0.01% Triton X-100, and 2 mg/ml of BSA) (24) and after incubation at 37 °C for 4 h, 0.03 ml of goat anti-rabbit IgG was added to precipitate the antigen-antibody complexes. After 12 h at 4 °C, 0.5 ml of ice-cold TEN buffer was added, and the precipitates were washed two times with 0.5 ml of the same buffer. For radioimmunoassay, 0.15-ml cell extracts diluted in TEN buffer with 0.2% Triton X-100 and 20 mg/ml of BSA, 15,000 counts/min of ^{125}I -labeled protein, 0.005 ml of diluted antiserum were mixed, adjusted to a final volume of 0.2 ml with TEN buffer plus 0.2% Triton X-100, and

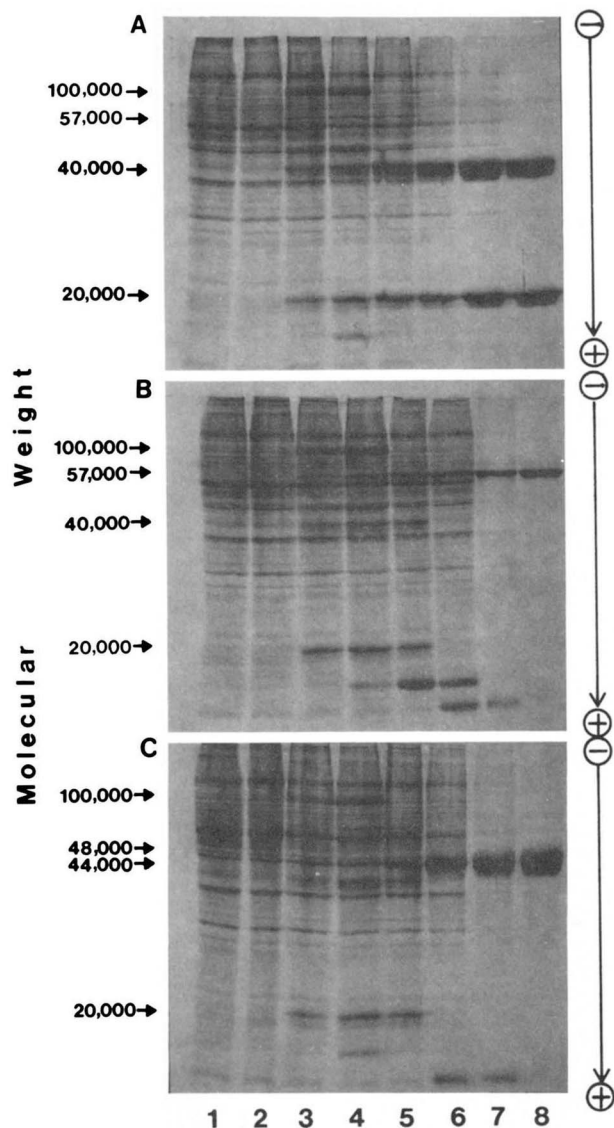


FIG. 2. Polypeptide profiles after SDS-polyacrylamide gel electrophoresis of kinase-containing fractions from various stages of purification of the kinases from tumor cells. The purification of the kinases, electrophoresis in SDS-containing polyacrylamide slab gels, and staining with Coomassie blue are described under "Materials and Methods." Gels were photographed with Polaroid type 52 film and a 99 Wratten filter (Kodak). A, fractions from PK_M purification: lane 1, plasma membranes, 50 μg ; lane 2, deoxycholate-extracted plasma membranes, 50 μg ; lane 3, kinase-rich band after glycerol density gradient centrifugation, 50 μg ; lane 4, NP-40 solubilized proteins, 50 μg ; lane 5, 1.4 M ammonium sulfate-precipitated proteins, 50 μg ; lane 6, pooled active fractions after sucrose density gradient centrifugation, 50 μg ; lane 7, proteins adsorbed by casein-Sepharose, 25 μg ; lane 8, PK_M after isoelectric focusing, 25 μg . B, fractions from PK_S purification: lane 1, plasma membranes, 50 μg ; lane 2, deoxycholate-extracted plasma membranes, 50 μg ; lane 3, kinase-rich band after glycerol density gradient centrifugation, 50 μg ; lane 4, NP-40-solubilized proteins, 50 μg ; lane 5, PK_S-rich fraction after octyl Sepharose chromatography, 50 μg ; lane 6, active fractions after sucrose density gradient centrifugation, 50 μg ; lane 7, proteins adsorbed by casein-Sepharose, 25 μg ; lane 8, PK_S after isoelectric focusing, 25 μg . C, fractions from PK_L purification: lane 1, plasma membranes, 50 μg ; lane 2, deoxycholate-extracted plasma membranes, 50 μg ; lane 3, kinase-rich band after glycerol density gradient centrifugation, 50 μg ; lane 4, NP-40 solubilized proteins, 50 μg ; lane 5, PK_L-rich fraction after octyl Sepharose chromatography, 50 μg ; lane 7, proteins adsorbed by casein-Sepharose, 25 μg ; lane 8, PK_L after isoelectric focusing, 25 μg . The specific activity of each fraction is shown in Table I, and the molecular weight standards used to calibrate the polyacrylamide gels are described under "Materials and Methods."

incubated 14 h at 37 °C (24). Goat anti-rabbit IgG (0.04 ml) was added, and incubation at 37 °C was continued for 2 h and followed by 17 h at 4 °C. Immunoprecipitates were washed with ice-cold TEN buffer (0.5 ml) three times, and radioactivity was counted on a Beckman model 4000 γ counter. Antisera were used at concentrations that bound 80% of the precipitable ^{125}I -labeled protein.

Preparation of Tumor Cells and Brain Tissue for Radioimmunoassay—Ehrlich ascites tumor cells were harvested and washed in 40 mM Tricine-NaOH (pH 7.5), 10 mM NaP_i (pH 7.5), 100 mM NaCl, 5 mM KCl, and 2 mM MgCl₂. The cells were lysed with 2.5% deoxycholate, 1% Triton X-100, and 5 mM PMSF in the same buffer and homogenized with a Teflon and glass homogenizer. The suspension was clarified by centrifugation at 10,000 $\times g$ for 10 min. Brain tissue homogenates were prepared in the same way, and both tumor cells and brain tissue homogenates were stored at -80 °C.

Protein Assays—The concentration of protein was determined according to Lowry *et al.* (25) or by absorbance at 280 nm (26).

RESULTS

Extraction and Purification of the Three Protein Kinases—Extraction of tumor plasma membranes with 1.25% deoxycholate, 200 mM KCl removed about 20% of the protein and solubilized less than 10% of PK_M, PK_S, and PK_L activities. The membrane fragments were fractionated on a glycerol density gradient which resulted in a 2- to 3-fold enrichment of the kinase activities compared to the deoxycholate-extracted membranes (Table I). As can be seen in Fig. 1, the major fraction of the kinase activities co-migrated together, and the smaller peaks of apparent PK_S and PK_L activity were discarded.

NP-40 was chosen over several other detergents for solubilization because it gave the best yield of solubilized kinase activities. The detergent:protein ratio of 2:1 was optimal for solubilization. Yields and specific activity of the fractions during purification are summarized in Table I. The polypeptide patterns at the various stages of purification are shown in Fig. 2. Starting with plasma membranes, the kinases were purified 400- to 500-fold with a yield of about 20 to 30%. Since the yield of plasma membranes was about 5%, the overall purification from intact cells was about 8000-fold. The three kinases were also purified from mouse brain plasma membranes with similar results except that the starting activity was much lower, as will be shown later.

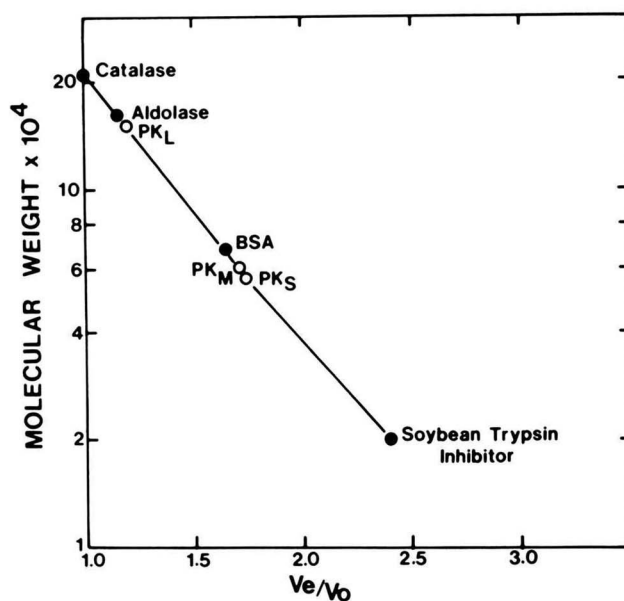


FIG. 3. Determination of the molecular weights of the tumor kinases by gel filtration chromatography on Sephadex G-200 according to Andrews (14). Chromatography was done as described under "Materials and Methods."

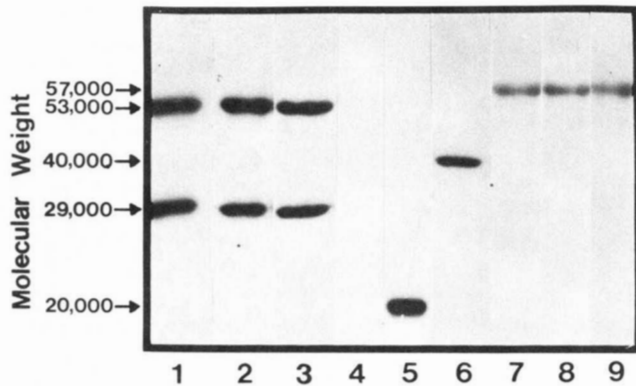


FIG. 4. Phosphorylation reactions catalyzed by PK_M , PK_S , and PK_L . Substrates for each kinase were dephosphorylated by phosphatase as described under "Materials and Methods." The kinases (10 ng) were incubated with either 25 μg of $\text{Na}^+\text{K}^+\text{-ATPase}$, 5 μg of PK_M , or 5 μg of PK_S in kinase assay buffer and 0.5 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (145 cpm/picomole) in a final volume of 0.025 ml for 1 h at 30 °C as described under "Materials and Methods." The phosphorylated products were separated by electrophoresis on 12% acrylamide slab gels and autoradiographed. Lanes 1-3, phosphorylation of $\text{Na}^+\text{K}^+\text{-ATPase}$ (25 μg) by PK_M (10 ng): lane 1, control; lane 2, 50 μM cAMP; lane 3, 5 μM cGMP. Lanes 4-6, phosphorylation of PK_M (5 μg) by PK_S (10 ng): lane 4, control; lane 5, 50 μM cAMP; lane 6, 5 μM cGMP. Lanes 7-9, phosphorylation of PK_S (5 μg) by PK_L (10 ng): lane 7, control; lane 8, 50 μM cAMP; lane 9, 5 μM cGMP.

TABLE II

Activation of PK_M and PK_S by phosphorylation

PK_M (5 μg) and PK_S (5 μg) were dephosphorylated with phosphatase as described under "Materials and Methods." PK_M (5 μg) was phosphorylated with PK_S (10 ng) and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (125 cpm/picomole), and PK_S (5 μg) was phosphorylated with PK_L (10 ng) and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described under "Materials and Methods." The extent of ^{32}P -incorporation was determined by the filter paper method. The specific activities of $^{32}\text{P}\text{-PK}_M$ and $^{32}\text{P}\text{-PK}_S$ were measured by incubating PK_M (50 ng) with 20 μg of $\text{Na}^+\text{K}^+\text{-ATPase}$ and by incubating PK_S (50 ng) with 20 μg of PK_M , both with 0.2 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (125 cpm/picomole) as described under "Materials and Methods." The extent of ^{32}P incorporation into $\text{Na}^+\text{K}^+\text{-ATPase}$ β -subunit and into PK_M was measured, after isolation of the polypeptides electrophoretically and solubilization of the gel slices, by liquid scintillation spectrometry as described under "Materials and Methods."

Enzyme	Substrate	Specific activity	Moles of P bound per mole of enzyme
<i>nmoles P incorporated $\times \text{min}^{-1} \times \text{mg}^{-1}$</i>			
Dephosphorylated PK_M	β -subunit	0.06	<0.05
Phosphorylated PK_M	β -subunit	3.5	0.92
Dephosphorylated PK_S	PK_M	0.08	<0.07
Phosphorylated PK_S	PK_M	1.1	0.94

TABLE III

Substrate specificity of PK_M , PK_S , and PK_L

The kinases (20 ng) were incubated separately in 0.1 ml of kinase assay buffer containing 0.2 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (130 cpm/picomole) with either 100 μg of casein, 100 μg of phosphatase, 100 μg of lysine-rich histones (III from calf thymus), 100 μg of arginine-rich histones (VIII S subgroup F₃), or 25 μg of endogenous substrate for 30 min at 30 °C. The proteins were precipitated with 20% trichloroacetic acid, and the incorporation of ^{32}P was measured by the filter paper method as described under "Materials and Methods."

Enzyme	Endogenous substrate	Casein	Phosvitin	Histones (lysine)	Histones (arginine)
<i>nmoles P incorporated per mg substrate $\times \text{h}^{-1}$</i>					
PK_M	150	15	0.95	0.09	0.21
PK_S	45	5.2	0.21	<0.02	0.09
PK_L	39	3.8	0.23	<0.02	0.07

Subunit Composition and Molecular Weight—As can be seen from Fig. 2, PK_M after polyacrylamide gel electrophoresis yielded two major bands ($M_r = 40,000$ and $20,000$), PK_S yielded a single band ($M_r = 57,000$), and PK_L yielded two bands ($M_r = 44,000$ and $48,000$). The molecular weights of the active forms of the kinases were estimated to be $M_r = 60,000$ for PK_M , $M_r = 57,000$ for PK_S , and $M_r = 150,000$ for PK_L by gel filtration chromatography on Sephadex G-200 (Fig. 3).

Phosphorylation Reactions Catalyzed by the Purified Kinases—The purified preparations of protein kinases phos-

TABLE IV

Cation requirements of PK_M , PK_S , and PK_L

The kinases (2.5 μg) were incubated with endogenous substrates in kinase assay buffer with 0.2 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (115 cpm/picomole) as described under "Materials and Methods" with Mg^{2+} (15 mM), Ca^{2+} (10 mM), or Mn^{2+} (15 mM) as shown. Incorporation of ^{32}P into protein was measured by the filter paper method.

Enzyme	Specific activity					
	+ Mg^{2+} (15 mM)	- Mg^{2+}	+ Ca^{2+} (10 mM)	+ Mg^{2+} + Ca^{2+} (10 mM)	+ Mg^{2+} + Mn^{2+} (15 mM)	+ Mn^{2+} (15 mM)
<i>nmoles P incorporated $\times \text{mg}^{-1} \times \text{min}^{-1}$</i>						
PK_M	344	3.17	2.9	336	341	226
PK_S	102	0.64	0.71	105	101	49
PK_L	78	0.21	0.05	72	81	30

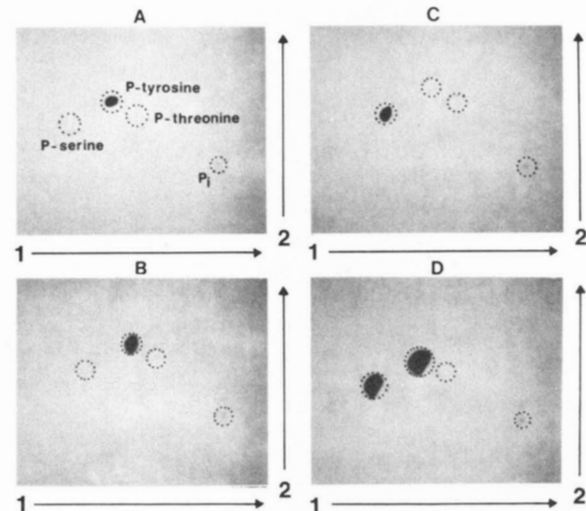


FIG. 5. Identification of the phosphorylated amino acid residues in the tumor $\text{Na}^+\text{K}^+\text{-ATPase}$, PK_M , and PK_S . $\text{Na}^+\text{K}^+\text{-ATPase}$ (75 μg), PK_M (25 μg), and PK_S (25 μg) were dephosphorylated with phosphatase followed by phosphorylation with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (136 cpm/picomole) as described under "Materials and Methods." ATPase was phosphorylated by PK_M (20 ng), PK_M -20,000 subunit by PK_S (20 ng), and PK_S by PK_L (20 ng). In addition, PK_M was incubated with 5 μM cGMP and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Polypeptides from the ^{32}P -proteins were fractionated by SDS-polyacrylamide gel electrophoresis, visualized with ice-cold 0.5 M KCl, and cut from the gels as described under "Materials and Methods." ^{32}P -proteins were eluted from the gel slices by shaking them for 12 h at 37 °C in 1 ml of 0.1 M NH_4HCO_3 containing 0.1% SDS and 5% 2-mercaptoethanol. BSA (1 mg) was added to each 1-ml solution of ^{32}P -proteins, the proteins were precipitated with ice-cold 15% trichloroacetic acid, and proteins were pelleted by centrifugation at $6,000 \times g$ for 10 min. The pellets were washed with organic solvents (8), resuspended in 80% formic acid, and lyophilized. The ^{32}P -proteins were subjected to acid hydrolysis, fractionated in two dimensions, and autoradiographed as described under "Materials and Methods." The dotted circles indicate the position of amino acid standards on the original thin layer plates, A, β -subunit of $\text{Na}^+\text{K}^+\text{-ATPase}$ containing 550 cpm of ^{32}P ; B, 20,000 subunit of PK_M containing 672 cpm of ^{32}P ; C, 40,000 subunit of PK_M containing 510 cpm of ^{32}P ; D, PK_S containing 875 cpm of ^{32}P .

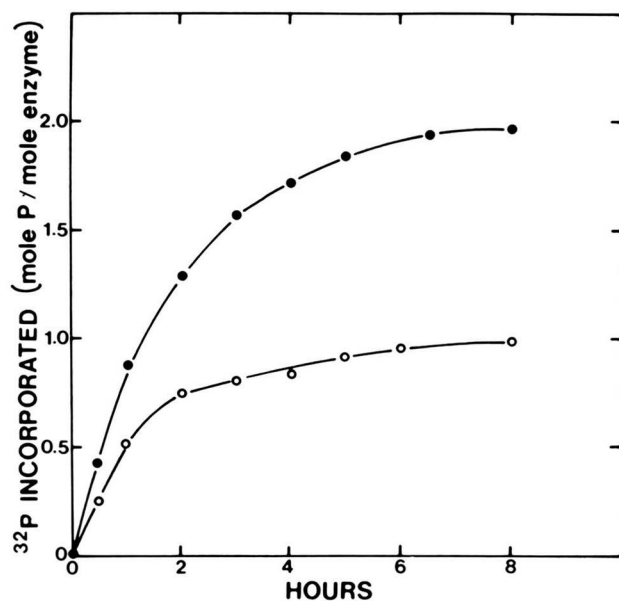


FIG. 6. Stoichiometry of PK_M (○—○) and PK_S (●—●) phosphorylation. PK_M (25 μg) was incubated with 10 ng of PK_S and 10 μM cAMP in kinase assay buffer with 0.2 mM [$\gamma\text{-}^{32}\text{P}$]ATP (150 cpm/picomole) in a final volume of 0.025 ml at 30 °C for the indicated length of time. PK_S (25 μg) was incubated with 10 ng of PK_L in the same manner but without cAMP. The incorporation of ^{32}P into proteins was measured by the filter paper method described under "Materials and Methods."

phorylated specific endogenous substrates. As shown in Fig. 4, PK_M catalyzed the phosphorylation of the β -subunit (and the 29,000-dalton proteolytic fragment (3)) of the $\text{Na}^+\text{K}^+\text{-ATPase}$; PK_S catalyzed the phosphorylation of the 20,000-subunit of PK_M, and PK_L catalyzed the phosphorylation of PK_S. Also, as shown in Fig. 4, cAMP stimulated the phosphorylation of the 20,000-dalton subunit of PK_M. In addition, cGMP stimulated the phosphorylation of the 40,000-subunit of PK_M. We observed only the following order of phosphorylation reactions: PK_L \rightarrow PK_S \rightarrow PK_M \rightarrow ATPase, which suggested that the phosphorylation of PK_M and PK_S might directly affect their kinase activities. To test this hypothesis, PK_M and PK_S were dephosphorylated by phosphatase as described under "Materials and Methods" and subsequently assayed for kinase activity before and following phosphorylation by PK_S and PK_L, respectively. As shown in Table II, only the phosphorylated forms of both PK_M and PK_S were catalytically active.

Table III shows that casein, phosvitin, and histones served very poorly as substrates for PK_M, PK_S, and PK_L, suggesting a high degree of specificity of the kinases. However, PK_S and PK_L do phosphorylate several unidentified proteins in tumor cells (not shown). PK_M, PK_S, and PK_L require Mg^{2+} or Mn^{2+} and are not affected by Ca^{2+} (10 mM), as shown in Table IV.

Analysis of Phosphoamino Acids—Two-dimensional thin layer electrophoresis and chromatography of 6 N HCl hydrolysates of ^{32}P -proteins from tumor cells are shown in Fig. 5. The β -subunit of the $\text{Na}^+\text{K}^+\text{-ATPase}$ and the 20,000-subunit of PK_M were phosphorylated only on a tyrosine residue. The 40,000-subunit of PK_M was phosphorylated on a serine residue (in the presence of cGMP), and PK_S was phosphorylated on both serine and tyrosine residues. The same amino acid residues were phosphorylated in the kinases and ATPase purified from mouse brain (data not shown).

Stoichiometry of Phosphorylation—The number of phosphorylated residues on each kinase was determined by prolonged incubation with [$\gamma\text{-}^{32}\text{P}$]ATP and the necessary enzymes

for phosphorylation. PK_S catalyzed the incorporation of approximately 1 mol of ^{32}P /mol of PK_M, and PK_L phosphorylated PK_S to the extent of 2 moles of ^{32}P /mol of PK_S. The extent of phosphorylation is shown in Fig. 6.

Double Immunodiffusion and Crossed Immunoelectrophoresis—Antisera raised against PK_M gave a single precipitin line with purified PK_M, and no lines were observed between anti-PK_S and PK_M, anti-PK_L and PK_M, or anti-ATPase and PK_M after double immunodiffusion (Fig. 7). Likewise, PK_S gave a single precipitin line only with anti-PK_S, and PK_L gave a single precipitin line with anti-PK_L. This lack of cross-reactivity between the kinases suggests they are immunologically distinct. PK_M (tumor) and PK_M (brain) showed complete immunological identity, as did PK_S (tumor) and PK_S (brain), and PK_L (tumor) and PK_L (brain). Since the resolution of the double immunodiffusion technique is limited, the specificities of the antisera were examined by two-dimensional crossed immunoelectrophoresis. Tumor membrane polypeptides were separated in the first dimension on SDS-polyacrylamide gels and were then electrophoresed at a 90° angle from the direction of the first separation into a gel containing purified IgG against either $\text{Na}^+\text{K}^+\text{-ATPase}$, PK_M, PK_S, or PK_L. As shown in Fig. 8, anti-ATPase cross-reacted only with

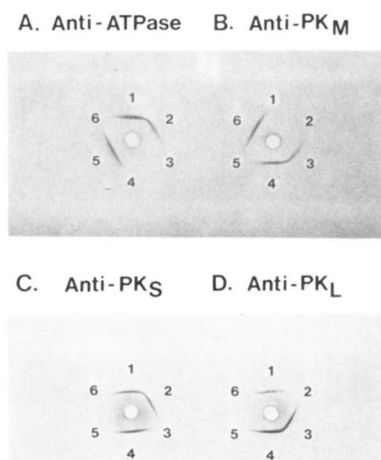


FIG. 7. Immunodiffusion of purified tumor and brain kinases, $\text{Na}^+\text{K}^+\text{-ATPase}$, and tumor plasma membranes with various antisera. Immunodiffusion was performed as described under "Materials and Methods." Triton X-100 extracts of tumor membranes were made by adding 1 ml of 4% Triton X-100 in 20 mM Tris-HCl (pH 6.8) to 35-mg membranes in 1 ml of distilled water and incubating for 2 h at 4 °C. Insoluble material was removed by centrifugation at $144,000 \times g$ for 1 h after which 80% of the protein remained in the supernatant. All of the antigen solutions contained 2% Triton X-100 in 10 mM Tris-HCl (pH 6.8). A, the central well contained 10 μl of anti-tumor $\text{Na}^+\text{K}^+\text{-ATPase}$ (1:16 dilution), and the peripheral wells contained 10 μl each of: 1, tumor $\text{Na}^+\text{K}^+\text{-ATPase}$ (2.1 mg/ml); 2, brain $\text{Na}^+\text{K}^+\text{-ATPase}$ (2.0 mg/ml); 3, tumor PK_M (1.8 mg/ml); 4, tumor PK_S (2.2 mg/ml); 5, 2% Triton X-100 extract of tumor membranes (15 mg/ml); 6, tumor PK_L (2.0 mg/ml). B, the central well contained 10 μl of anti-tumor PK_M (1:8 dilution), and the peripheral wells contained 10 μl each of: 1, tumor $\text{Na}^+\text{K}^+\text{-ATPase}$ (2.1 mg/ml); 2, tumor PK_S (2.2 mg/ml); 3, tumor PK_M (1.8 mg/ml); 4, brain PK_M (1.9 mg/ml); 5, tumor PK_L (2.0 mg/ml); 6, 2% Triton X-100 extract of tumor membranes (15 mg/ml). C, the central well contained 10 μl of anti-tumor PK_S (1:8 dilution), and the peripheral wells contained 10 μl each of: 1, tumor PK_S (2.2 mg/ml); 2, brain PK_S (1.7 mg/ml); 3, tumor $\text{Na}^+\text{K}^+\text{-ATPase}$ (2.1 mg/ml); 4, 2% Triton X-100 extract of tumor membranes (15 mg/ml); 5, tumor PK_M (1.8 mg/ml); 6, tumor PK_L (2.0 mg/ml). D, the central well contained 10 μl of anti-PK_L (1:8 dilution), and the peripheral wells contained 10 μl each of: 1, 2% Triton X-100 extract of tumor membranes (15 mg/ml); 2, tumor $\text{Na}^+\text{K}^+\text{-ATPase}$ (1.8 mg/ml); 3, brain PK_L (2.0 mg/ml); 4, tumor PK_L (2.0 mg/ml); 5, tumor PK_S (2.2 mg/ml); 6, tumor PK_M (1.8 mg/ml).

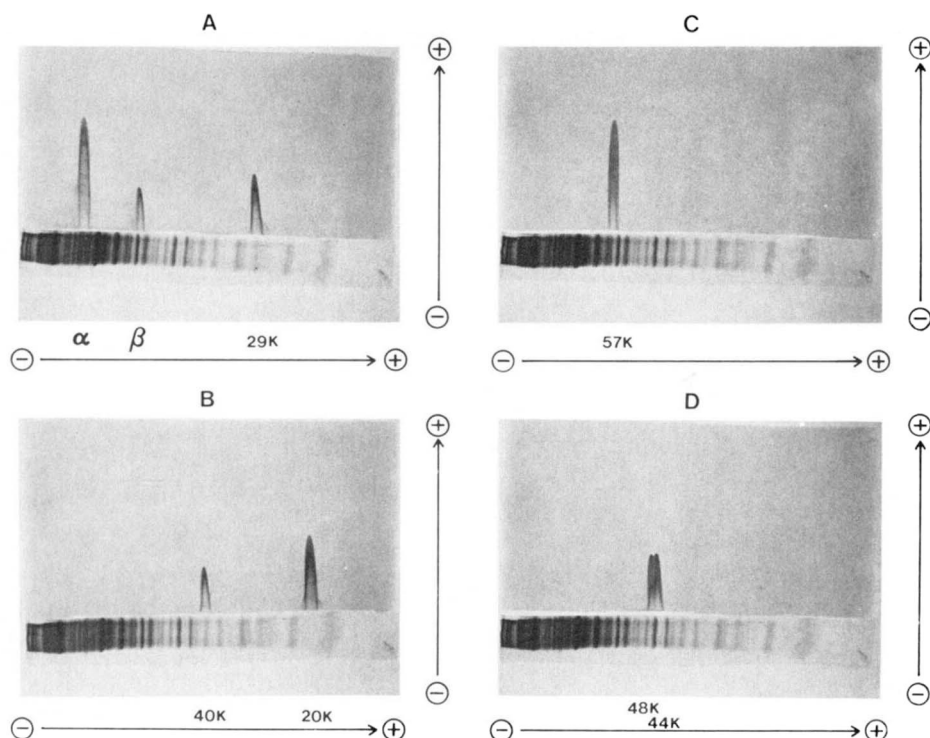


FIG. 8. Crossed immunoelectrophoresis of tumor plasma membrane polypeptides with antibodies directed to tumor $\text{Na}^+\text{K}^+\text{-ATPase}$, PK_M , PK_S , and PK_L . Crossed immunoelectrophoresis was performed as described in Ref. 23. The 1st dimension SDS-polyacrylamide gel contained 200 μg of tumor membranes and the stained reference strip shown contained 75- μg membranes. The SDS-containing polyacrylamide gel strips was 1×12 cm, and the antibody-containing agarose gel measured 6×12 cm and about 3 mm thick. A, anti- $\text{Na}^+\text{K}^+\text{-ATPase}$ (0.67 mg of IgG/ cm^2); B, anti- PK_M (0.48 mg of IgG/ cm^2); C, anti- PK_S (0.72 mg of IgG/ cm^2); D, anti- PK_L (0.82 mg of IgG/ cm^2).

the α -subunit, β -subunit, and the 29,000-proteolytic fragment of the β -subunit. Anti- PK_M formed precipitin arcs with the 40,000- and 20,000-subunits, anti- PK_S cross-reacted only with the 57,000- PK_S , and anti- PK_L formed precipitin arcs with both the 44,000- and 48,000- PK_L subunits. The results show that the antisera are monospecific for the ATPase or protein kinases within the limits of detection of the assay method.

Peptide Maps—Cyanogen bromide digests of the kinases were analyzed by SDS-urea polyacrylamide gel electrophoresis.² As shown in Fig. 9, the peptide maps for each kinase were distinct.

Radioimmunoassays—¹²⁵I-labeled antigens were prepared as described under "Materials and Methods" at the specific activities shown in Table V. The antisera titers were detectable up to the dilutions given in Table V. Antibody titration curves were linear in the range between 10 and 90% of maximum precipitation. Competitive radioimmunoassay was used to estimate the concentration of the kinases in tumor cells and brain tissue. Rabbit anti-kinases and anti-ATPase were used at a concentration that bound 80% of the maximum precipitable ¹²⁵I-labeled antigen in the radioimmunoassay. The concentration of the kinases and $\text{Na}^+\text{K}^+\text{-ATPase}$ in tumor cells and brain tissue is shown in Table VI. Brain tissue was slightly more enriched in ATPase than the tumor cells, but all of the kinases were at higher levels in the tumor cells. PK_S was 31-fold higher in the tumor cells.

Immunoinactivation Studies of Protein Kinases—Antisera specifically inhibited the function of the kinases in membranes (Fig. 10). Anti- PK_M inhibited the phosphorylation of the β -subunit of the ATPase, anti- PK_S inhibited the phosphorylation of PK_M , and anti- PK_L prevented the phosphorylation of PK_S .

Activities of Tumor and Brain Kinases with Brain and Tumor Substrates—Tumor and brain substrates were exposed in various combinations to purified tumor and brain kinases to assess their interactions. As shown in Table VII, the kinases from tumor cells phosphorylated brain substrates to the same

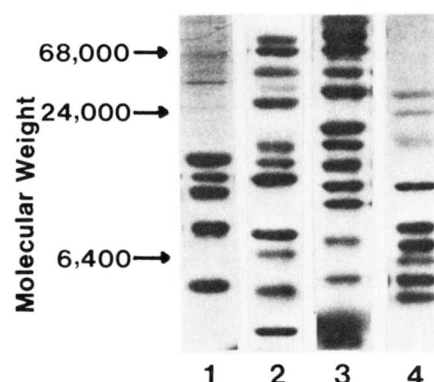


FIG. 9. Cyanogen bromide partial cleavage maps of PK_M , PK_S , and PK_L . The kinases were labeled with [¹²⁵I]3-(4-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide ester (2,400 ci/mmol), separated on SDS-polyacrylamide gels, visualized with cold 0.5 M KCl, and cut from gels as described under "Materials and Methods." The gel slices containing individual kinase polypeptides were treated with cyanogen bromide, and the resulting peptides were fractionated on a 20% acrylamide gel containing SDS and urea.² The gel was dried and autoradiographed against X-Omat RP-1 x-ray film for 12 h. Lane 1, 20,000 subunit of PK_M (10 μg); lane 2, 40,000 subunit of PK_M (12 μg); lane 3, PK_S (10 μg); lane 4, PK_L (9 μg).

extent as tumor substrates. Likewise, the kinases isolated from brain phosphorylated tumor substrates equally as well as brain substrates. This and the similar isolation procedures suggest that the tumor and brain enzymes are very similar.

Analysis of Phosphorylation by Kinases in Crude Membranes—In tumor membranes, PK_M , PK_S , and PK_L actively phosphorylated their endogenous substrates. This was observed by incubation of membranes with [γ -³²P]ATP, lysis, and solubilization of the membranes and immunoprecipitation of the endogenous substrates which were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Fig. 11 illustrates that from tumor membranes the phosphorylated forms of the ATPase, PK_M (20,000) and PK_S , can be immu-

² R. B. Pepinsky and V. Vogt, unpublished procedure.

TABLE V

Specific activities of ^{125}I -antigens and titers of rabbit antisera raised against kinase antigens

The titers are given as the reciprocal of the highest dilution that precipitated 80% of the ^{125}I -antigen (approximately 15,000 cpm).

Antigen	Specific activity $\mu\text{Ci/ng}$	Antisera	Titer
$\text{Na}^+\text{K}^+\text{-ATPase}$	0.028	anti-ATPase	7,680
PK_M	0.019	anti- PK_M	61,440
PK_S	0.029	anti- PK_S	10,240
PK_L	0.024	anti- PK_L	30,720

TABLE VI

Levels of kinases and $\text{Na}^+\text{K}^+\text{-ATPase}$ in Ehrlich ascites tumor cells and mouse brain tissue as determined by competition radioimmunoassay

Tumor cells and brain tissue were prepared and competition radioimmunoassay was done as described under "Materials and Methods." ^{125}I -antigens (0.1 to 0.5 ng) containing 2.5 to 6×10^4 cpm/ng were used.

Enzyme	Content of enzyme		Ratio tumor: brain
	Tumor cells	Brain tissue	
	$\text{mg}/100 \text{ mg cell protein}$		
$\text{Na}^+\text{K}^+\text{-ATPase}$	0.05	0.12	0.42
PK_M	0.015	0.0055	2.7
PK_S	0.11	0.0035	31.4
PK_L	0.043	0.0082	5.2

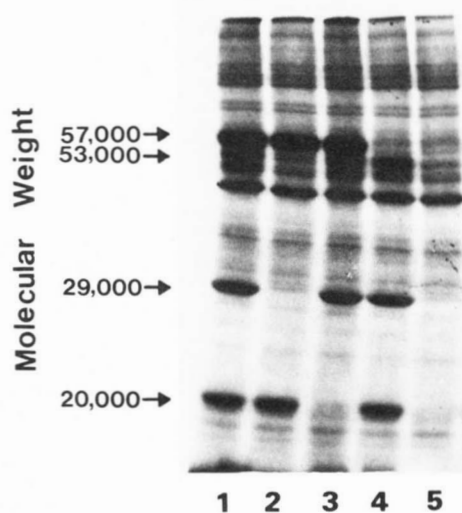


FIG. 10. Immunoinactivation of kinases in membranes from tumor cells. Deoxycholate-extracted membranes (50 μg) purified on the glycerol density gradient were incubated in kinase assay buffer (without ATP) in a total volume of 0.1 ml. Antisera against the kinases (0.03 ml) were added and after 0.5 h at 30°C , 0.2 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (125 cpm/picomole) was added, and incubation was continued for 1.5 h. The membranes were solubilized with 0.1 ml of electrophoresis sample buffer, fractionated on SDS-polyacrylamide gels, and autoradiographed as described under "Materials and Methods." Lane 1, 50- μg membranes; lane 2, 50- μg membranes and 30- μl anti- PK_M ; lane 3, 50- μg membranes and 30- μl anti- PK_S ; lane 4, 50- μg membranes and 30- μl anti- PK_M ; lane 5, 50- μg membranes and 30- μl anti- PK_S , 30- μl anti- PK_M and 30- μl anti- PK_L .

noprecipitated. By the same method, no phosphorylated forms of the ATPase, PK_M , or PK_S were detected in brain membranes.

DISCUSSION

The original goal of this investigation was to determine the nature of the lesion that causes inefficient sodium pumping

TABLE VII

Activities of tumor and brain kinases with brain and tumor substrates

In each experiment 5 μg of substrate and 10 ng of the kinase were assayed by incubation in kinase assay buffer containing 0.5 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (130 cpm/picomole) for 30 min at 30°C as described under "Materials and Methods." Specific activities of the kinases were determined by the filter paper method.

Enzyme	Substrate	Specific activity $\text{nmoles P incorporated} \times \text{mg}^{-1} \times \text{min}^{-1}$
PK_M (brain)	β -subunit (brain)	346
PK_S (brain)	PK_M (brain)	97
PK_L (brain)	PK_S (brain)	72
PK_M (tumor)	β -subunit (tumor)	351
PK_S (tumor)	PK_M (tumor)	106
PK_L (tumor)	PK_S (tumor)	74
PK_M (brain)	β -subunit (tumor)	353
PK_S (brain)	PK_M (tumor)	102
PK_L (brain)	PK_S (tumor)	78
PK_M (tumor)	β -subunit (brain)	345
PK_S (tumor)	PK_M (brain)	94
PK_L (tumor)	PK_S (brain)	69

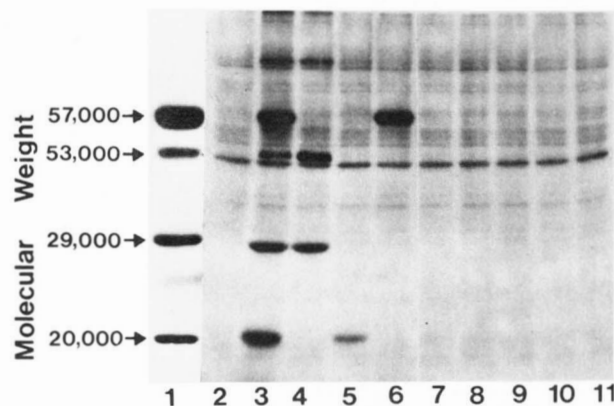


FIG. 11. Immunoprecipitation of kinases and $\text{Na}^+\text{K}^+\text{-ATPase}$ from tumor and brain plasma membranes. Membranes (100 μg) were incubated in kinase assay buffer with 0.5 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (120 cpm/picomole) for 1 h at 30°C . The membranes were solubilized with 0.1 ml of ice-cold buffer containing 0.5% Triton X-100, 0.5% deoxycholate, 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1% Trasylol, 2 mM PMSF, and 20 mM NaF, and 0.03 ml of antisera was added. After 30 min of incubation on ice, glutaraldehyde-fixed *Staphylococcus* cells (25 μl of packed cells) were added. After 30 min, the immunoprecipitates were washed and solubilized as described previously (3). The solubilized proteins were fractionated by electrophoresis on a 12% acrylamide gel and autoradiographed as described under "Materials and Methods." Lane 1, purified $\text{Na}^+\text{K}^+\text{-ATPase}$ (1.5 μg), PK_M (0.5 μg), PK_S (0.5 μg), and PK_L (0.5 μg) incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (120 cpm/picomole) to show positions of kinases on the autoradiograph; lane 2, immunoprecipitate from 100 μg of mouse brain membranes using 30 μl each of anti-ATPase, anti- PK_M , anti- PK_S , and anti- PK_L ; lane 3, immunoprecipitate from 100 μg of tumor cell membranes using 30 μl each of anti-ATPase, anti- PK_M , anti- PK_S , and anti- PK_L ; lanes 4-7, immunoprecipitates from 100 μg of tumor membranes using 30 μl of anti-ATPase (lane 4), 30 μl 4 anti- PK_M (lane 5), 30 μl of anti- PK_S (lane 6) or 30 μl of anti- PK_L (lane 7); lanes 8-11, immunoprecipitates from 100 μg of brain membranes using 30 μl of anti-ATPase (lane 8), 30 μl of anti- PK_M (lane 9), 30 μl of anti- PK_S (lane 10), or 30 μl of anti- PK_L (lane 11).

by the $\text{Na}^+\text{K}^+\text{-ATPase}$ of Ehrlich ascites tumor cells. The inefficiency was associated with the phosphorylation of the β -subunit of the $\text{Na}^+\text{K}^+\text{-ATPase}$ by an endogenous protein kinase that was active in the tumor membranes, but not in mouse brain membranes (3).

The protein kinase (PK_M) that phosphorylates the β -subunit of the $\text{Na}^+\text{K}^+\text{-ATPase}$ was followed during purification by its ability to phosphorylate purified $\text{Na}^+\text{K}^+\text{-ATPase}$ from mouse brain (2). After purification of PK_M (Table I, Figs. 1 and 2) from ^{32}P -labeled tumor membranes, there was considerable radioactive phosphate (about 0.5 mole/mole of enzyme) that remained associated, even after electrophoresis in the presence of SDS (not shown). It was apparent that PK_M was itself phosphorylated. Therefore, purified PK_M , that was first dephosphorylated with phosphatase, was used as substrate to follow the purification of another protein kinase (PK_S). This same experimental logic led to the identification of yet another protein kinase (PK_L) that phosphorylated PK_S . It should be mentioned here that still another protein kinase that phosphorylates PK_L is involved in this cascade.³

The functional order of PK_M , PK_S , and PK_L has been established on the basis of the fact that the $\text{Na}^+\text{K}^+\text{-ATPase}$ serves as substrate for phosphorylated PK_M , PK_M is phosphorylated by PK_S , and PK_S serves as substrate for PK_L (Fig. 4). Furthermore, $\text{Na}^+\text{K}^+\text{-ATPase}$ is not phosphorylated by PK_S or PK_L , and PK_M is not phosphorylated by PK_L (not shown). As shown in Fig. 4, cAMP stimulated the phosphorylation of the 20,000-dalton subunit of PK_M , and cGMP stimulated the phosphorylation of the 40,000-dalton subunit. Clearly, further experiments are necessary to characterize these unusual effects of cAMP and cGMP. Until now all known effects of these cyclic nucleotides are mediated by the cyclic nucleotide-dependent protein kinases (27). Since the protein kinases reported here do not seem to be related to those well characterized kinases, on the basis of molecular weight and phosphorylation of tyrosine residues, these results suggest an additional mode of action of cAMP and cGMP. A detailed study of these effects of cyclic nucleotides on PK_S and PK_M is the subject of a future paper.⁴ The immunoinactivation experiment shown in Fig. 10 suggests that this order ($\text{PK}_L \rightarrow \text{PK}_S \rightarrow \text{PK}_M \rightarrow \text{Na}^+\text{K}^+\text{-ATPase}$) is operative *in vivo*, since anti- PK_L prevents the phosphorylation of PK_S , anti- PK_S inhibits the phosphorylation of PK_M , and anti- PK_M blocks the phosphorylation of the β -subunit. During a pulse label with [$\gamma\text{-}^{32}\text{P}$]ATP and intact membranes, ^{32}P appears first in PK_S followed by PK_M and the β -subunit (not shown). This is again consonant with the functional order we have proposed.

An analysis of phosphoamino acids and the stoichiometry of phosphorylation of these kinases suggests that the β -subunit of the $\text{Na}^+\text{K}^+\text{-ATPase}$ and PK_M are each phosphorylated on a single tyrosine residue, and PK_S is phosphorylated on a tyrosine and a serine residue. Here again, these results must be interpreted with caution. During the preparation of the plasma membranes and during kinase purification, other kinases as well as phosphatases are present.⁴ These may modify the phosphorylated forms of the protein kinases and thus further work is necessary to determine if multiple site phosphorylations take place *in vivo*. We have preliminary evidence to suggest that PK_L and PK_S exist in several phosphorylated forms and that the site of phosphorylation (tyrosine or serine) activates or inhibits kinase activity.⁴

The data presented in this report indicate that the lesion affecting the efficiency of the $\text{Na}^+\text{K}^+\text{-ATPase}$ is at the level of the regulation of the protein kinase cascade. The finding that the β -subunit of the brain $\text{Na}^+\text{K}^+\text{-ATPase}$ is not phosphorylated in the native brain membrane, in spite of the fact that the kinases are present (Fig. 11), suggests the presence of a

superimposed control mechanism. Moreover, the fact that the brain protein kinases are active after solubilization and purification suggests the possibility of an inhibitor. In preliminary experiments we have found that brain membranes contain a relatively heat-stable inhibitor of PK_S .

It has been reported that pp60^{src}, the transforming gene product of avian sarcoma virus, phosphorylates tyrosine residues of substrate proteins (28). With this in mind, it is important to determine if the protein kinases reported here serve as substrate for pp60^{src}, and this is the subject of a future paper.³

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Note Added in Proof—Since this manuscript was submitted we have found a fourth enzyme: protein kinase F (Spector, M., Pepinsky, R. B., Vogt, V., and Racker, E., submitted for publication) which phosphorylates PK_L and becomes phosphorylated by PK_S . PK_F is immunologically related to pp60^{src}. Not only PK_S , but also PK_L and PK_F can be phosphorylated on a serine residue by cAMP-dependent protein kinase. Only the tyrosine monophosphorylated species is active in the cascade.

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³ M. Spector, R. B. Pepinsky, V. Vogt, and E. Racker, manuscript in preparation.

⁴ M. Spector and E. Racker, manuscript in preparation.