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Protein Kinase and Specific Phosphate Acceptor Proteins Associated with Vaccinia Virus Cores

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Incubation of purified vaccinia virus with γ-^{32}P-adenosine triphosphate resulted in the incorporation of ^{32}P into hot trichloroacetic acid-insoluble material. Enzymatic activity was completely dependent on the addition of divalent cations and was stimulated by nonionic detergents and dithiothreitol. Chemical studies demonstrated that serine and threonine residues of 15,000 molecular weight viral polypeptides were phosphorylated. In contrast, the major structural proteins were not phosphorylated or were phosphorylated to a much lesser extent. Added histones and protamine, but not serum albumin, casein, or phosvitin were phosphorylated by the partially disrupted vaccinia virus preparations. The protein kinase was tightly associated with vaccinia virus particles since the specific enzymatic activity remained constant during the final steps of virus purification, the specific activities of many different preparations of virus were similar, and the enzymatic activity cosedimented with vaccinia virus during rate zonal sucrose gradient and potassium tartrate gradient equilibrium centrifugations. Controlled degradation of vaccinia virus, with nonionic detergents and dithiothreitol, indicated that both the protein kinase and the specific phosphate acceptor proteins were located in the virus core.

An enzymatic activity, which phosphorylates the major viral structural proteins, was found by Strand and August (26) to be associated with Rauscher murine leukemia virus, avian myeloblastosis virus, and vesicular stomatitis virus. They considered that the protein kinase may be a virus-specific or host-derived envelope component of budding or membrane-maturing viruses. Subsequent reports suggest the presence of similar enzymes in other enveloped viruses (21; Rubinstein, Gravell, and Darlington, Abst. of the Ann. Mtg. of the Amer. Soc. of Microbiol., 1972; and Silberstein, McAuslan, and August, Fed. Proc., 1972). We now describe the protein kinase activity associated with purified vaccinia virus particles. This study is of particular interest since the envelope of vaccinia virus is not derived from cellular membranes (3) and the protein kinase is located in the virus core. In addition, serine and threonine residues of specific protein components of the virus core are phosphorylated.

MATERIALS AND METHODS

Virus purification. Vaccinia virus (strain WR) was purified from HeLa cells by the procedure of Joklik (10) as previously described (17). Infected cells were disrupted by Dounce homogenization, nuclei were removed by low speed centrifugation, and the virus was sedimented through a 36% (w/v) cushion of sucrose. This step was followed by zonal centrifugation two times on 25 to 40% (w/v) sucrose gradients. In some experiments, an additional equilibrium centrifugation in a potassium tartrate gradient was performed. After each gradient centrifugation, the virus band was diluted threefold with 1 mM tris(hydroxymethyl)aminomethane(Tris)-hydrochloride, *p*H 9, and sedimented. At each stage of the purification, following removal of nuclei, the virus was dispersed with the microtip of a Branson model 140 sonifier at 20% of maximum power. Sonic vibrations were given for five intervals of 15 sec while the tube containing the virus was cooled with crushed ice. All solutions used for the purification were sterilized by filtration, and all tubes were autoclaved or irradiated with ultraviolet light. The purity of the virus preparations was monitored by electron microscopy.

Protein kinase assay. The basic reaction mixture (0.1 ml) contained 50 mM Tris-hydrochloride (*p*H 10.25), 3 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.01% Nonidet P-40, and 0.5 mM γ-^{32}P-adenosine triphosphate (ATP). The *p*H of the complete reaction mixture was 8.3 at 37 C. After equilibration at 37 C, 20 μg of virus protein was added. The reaction was terminated 5 min later by the addition in rapid succession of 0.1 ml of 0.1 M ethylenediaminetetraacetic acid containing 250 μg of bovine serum albumin and 2.5 ml of cold 25% trichloroacetic acid containing 0.05 M sodium pyrophosphate ($NaPP_i$). After standing at 0 C for 15 min, the precipitate was collected by centrifugation

and redissolved in 0.1 ml of 1 N NaOH. The material was reprecipitated and collected and washed on HA 0.45-μm Millipore filters. Reactions were done in duplicate and the average values recorded. Zero time blanks, obtained by addition of virus protein at the end of the incubation, were always subtracted. The specific activity of the γ-^{32}P-ATP was determined for each experiment and the value used to calculate phosphate incorporated.

Source of proteins. Purified lysine-rich histone from Chinese hamster cells was provided by R. Lake. Total histone was purchased from Worthington and calf thymus lysine-rich histone, protamine, arginine-rich histone, phosvitin, α-casein, and serum albumin came from Sigma Chemical Co. Deoxyribonuclease I (ribonuclease-free) was obtained from Worthington and Pronase (nuclease-free) was from Calbiochem.

RESULTS

Protein kinase activity. Incubation of purified vaccinia virus with γ-^{32}P-ATP resulted in the incorporation of ^{32}P into trichloroacetic acid-insoluble material which will be identified later as phosphoprotein. Enzymatic activity was dependent on *p*H, divalent cations, DTT, and Nonidet P-40, a nonionic detergent (Fig. 1). The latter requirement suggested that the enzyme was activated by partial disruption of the virus. Under these conditions, no significant incorporation was obtained using γ-^{32}P-guanosine triphosphate (GTP) as the phosphate donor. Protein kinase activity was unaffected by (i) the addition of unlabeled GTP, cytidine triphosphate and uridine triphosphate, (ii) the addition of actinomycin D and proflavine, and (iii) the addition of the cyclic nucleotides adenosine monophosphate, guanosine monophosphate, cytidine monophosphate, and uridine monophosphate at several different concentrations and a variety of conditions.

Although vaccinia virus contains an active nucleotide phosphohydrolase (7, 19), the protein kinase reaction could be saturated with respect to ATP (Fig. 2). Under these conditions kinase activity was proportional to virus protein concentration (Fig. 3). We calculated that several thousand atoms of phosphorus were incorporated per vaccinia virus particle within 5 min.

Stimulation by phosphate acceptor proteins. Incorporation of ^{32}P was stimulated by known phosphate acceptor proteins such as protamine and histones but was unaffected by phosvitin, casein, and serum albumin (Table 1). Maximal activity with lysine-rich histone was also shown to require Mg^{2+}, Nonidet P-40 detergent, DTT, and similar *p*H values. Kinetic experiments indicated that the histone-stimulated reaction proceeded until the supply of ATP became limiting, whereas the endogenous reaction reached a plateau at an earlier time (Fig. 4). These results suggested that acceptor proteins were limiting in the endogenous reaction. In 0.1 ml of reaction mixture, more than 25 nmoles of ATP were hydrolyzed whereas only about 0.2 nmole of ^{32}P was incorporated into protein (Fig. 4). Pre-

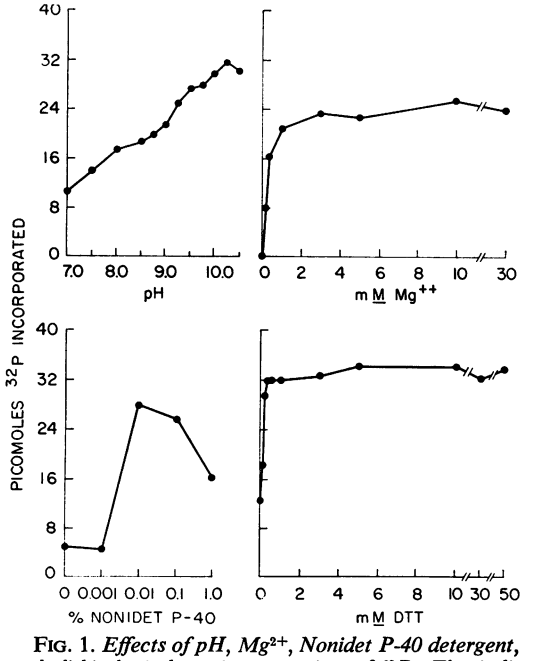

FIG. 1. *Effects of pH, Mg^{2+}, Nonidet P-40 detergent, and dithiothreitol on incorporation of ^{32}P. The indicated pH values are those of 0.5 M tris(hydroxymethyl)aminomethane-hydrochloride at 20 C; the actual pH values of the reaction mixture were lower (see Materials and Methods). The concentration of ATP was 0.1 mM and the specific activities in these four experiments ranged from 100 to 170 counts per min per pmole. The specific activities were used to calculate the picomoles of ^{32}P incorporated in 0.1 ml of reaction mixture.*

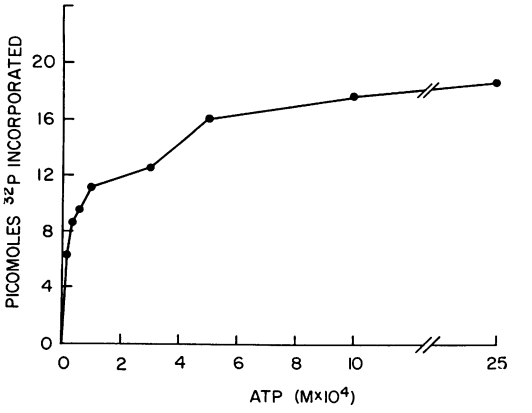

FIG. 2. *Effect of ATP concentration of incorporation of ^{32}P. Enzymatic activity was measured as described in the legend to Fig. 1. The specific activity of γ-^{32}P-ATP was 106 counts per min per pmole.*

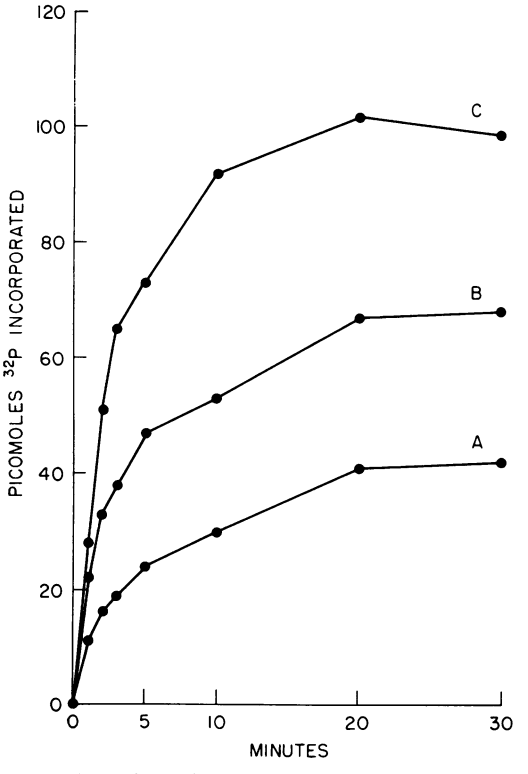

FIG. 3. *Effect of vaccinia virus concentration on incorporation of ^{32}P. At intervals, 0.1-ml samples were removed from 1-ml reaction mixtures containing 20 (A), 40 (B), or 60 (C) μg of virus protein. The specific activity of the γ-^{32}P ATP was 24 counts per min per pmole. In this and all following experiments, the standard reaction mixture contained 0.5 mM ATP.*

sumably, most of the ATP was hydrolyzed by the previously described nucleotide phosphohydrolase activity (7, 19), although some turnover of phosphoprotein has not been ruled out. Apparently, lysine-rich histone partially inhibits the phosphohydrolase activity (Fig. 4). The latter was confirmed more directly by measuring the formation of adenosine diphosphate in the presence and absence of histone.

Identification of P-serine and P-threonine. Chemical studies (26) demonstrated that the ^{32}P-labeled trichloroacetic acid-insoluble product derived from γ-^{32}P-ATP was phosphoprotein. The resistance of the material to hot trichloroacetic acid and deoxyribonuclease (Table 2) indicated that phosphodiester bonds were not involved. Furthermore, after alkaline hydrolysis, (0.3 N NaOH, 16 hr) significant amounts of ^{32}P-labeled adenosine tetraphosphate were not detected by chromatography on thin-layer plates of PEI cellulose. The loss of some ^{32}P-labeled material after treatment of the trichloroacetic acid pellet with chloroform–methanol was irregular and appeared to result from dislodgement of portions of the pellet rather than from lipid extraction. Pronase treatment rendered the labeled material acid soluble (Table 2) indicating that the ^{32}P was linked to polypeptide. The resistance of the ^{32}P-labeled reaction product to hydroxylamine suggested the absence of acyl-phosphate bonds which were considered to be possible intermediates in the nucleotide phosphohydrolase reaction. The alkali lability and acid stability of the ^{32}P product ruled out phosphohistidine. The results suggested the presence of ^{32}P-labeled phosphoserine and phosphothreonine residues.

The presence of ^{32}P-serine and ^{32}P-threonine was demonstrated, after partial acid hydrolysis, by coelectrophoresis with authentic standards (Fig. 5). Phosphoserine and phosphothreonine accounted for 22 to 25% of the applied radioactivity, an amount comparable to that obtained by other workers with a variety of phosphoproteins (22). The most rapidly migrating material in Fig. 5 corresponds to ^{32}P$_i$ which could form from hydrolysis of ^{32}P-serine; the more slowly migrating material presumably represents phosphopeptides (23) and was diminished in amount after longer hydrolysis times.

Polyacrylamide gel electrophoresis of P-proteins. Vaccinia virus contains a large number of polypeptides which can be resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electro-

TABLE 1. *Stimulation of ^{32}P incorporation by phosphate acceptor proteins*[a]

Added protein	^{32}P-incorporation (pmoles/mg vaccinia proteins)	
	− Nonidet P-40	+ Nonidet P-40
None	190	1,465
Protamine	535	5,230
Total histone	1,665	5,000
Arginine-rich histone	665	3,920
Lysine-rich histone[b]	830	7,910
Lysine-rich histone[c]	2,155	7,935
Phosvitin	135	1,200
α-Casein	200	1,760
Serum albumin	195	1,725

[a] Each standard 0.1-ml reaction mixture with or without Nonidet P-40 contained 10 μg of added proteins. All incubations were done in duplicate and separate zero time blanks were subtracted for each protein. The specific activity of the γ-^{32}P-adenosine triphosphate was 59 counts per min per pmole.

[b] Lysine-rich histone from Chinese hamster cells.

[c] Lysine-rich histone from calf thymus.

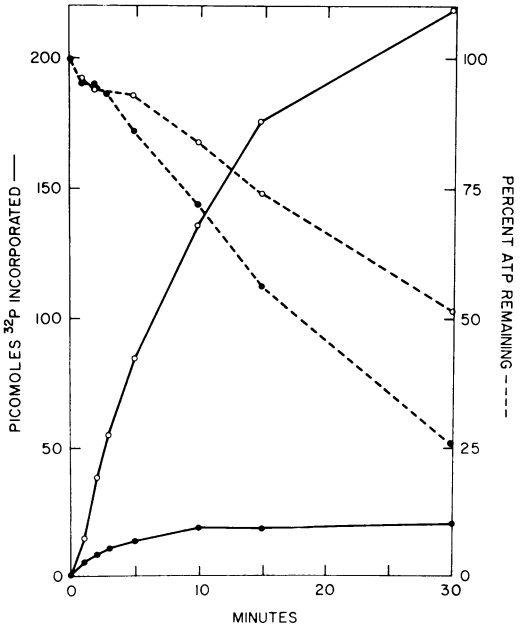

FIG. 4. *Time course of incorporation of ^{32}P and hydrolysis of ^{32}P-ATP in the presence and absence of added phosphate acceptor protein. At intervals, 0.1-ml samples were removed from 1.2-ml reaction mixtures to measure ^{32}P incorporation. Additional 5-μliter samples were removed and spotted on PEI cellulose thin-layer sheets over dried ATP, ADP, and AMP standards. After chromatography with 1 M KH_2PO_4, the ATP spot was cut out and counted. The specific activity of γ-^{32}P-ATP was 40 counts per min per pmole.* (●) *No histone,* (○) *10 μg of lysine-rich histone/0.1 ml.*

phoresis (9, 12, 17). It was therefore of interest to determine which polypeptides were phosphorylated. The phosphorylated proteins were characterized by coelectrophoresis with ^3H-amino acid labeled vaccinia virus proteins on SDS-polyacrylamide gels (Fig. 6). Phosphorylation appeared to be selective in that the most abundant vaccinia virus polypeptides were not significantly labeled with ^{32}P. The molecular weight of the major phosphorylated polypeptide(s) was approximately 15,000 as estimated from electrophoretic mobility on 10% polyacrylamide gels (4, 14, 24). The protein was eluted from the gel and shown to contain both P-serine and P-threonine by acid hydrolysis and thin-layer electrophoresis (Rosemond and Moss, *unpublished observation*). Similar experiments indicated that the stimulation of ^{32}P incorporation by lysine-rich histone resulted from phosphorylation of the histone (Fig. 6). The major peak of radioactive material corresponded to the stained histone band.

Radioautographs, of sliced and dried polyacrylamide gels, also showed that the 15,000 molecular weight polypeptide was most heavily labeled. However, after prolonged exposure times, faint bands corresponding to other viral polypeptides were noted (Fig. 7). Unexpectedly, labeling of these viral polypeptides appeared to be enhanced by the addition of lysine-rich histone (Fig. 7). Nevertheless, the lysine-rich histone itself was by far the most heavily labeled band (Fig. 7). It is possible that the basic histone protein alters or further disrupts the structure of the virus and thus makes certain viral proteins more effective substrates.

Localization of protein kinase activity. The protein kinase appeared to be tightly associated with vaccinia virus particles since the specific

TABLE 2. *Properties of the reaction product*

Treatment	^{32}P incorporation (pmoles/mg vaccinia protein)
1.[a]	
Control	1,130
1 N NaOH, 100 C	15
10% trichloroacetic acid, 90 C	980
Chloroform-methanol (1:1)	800
Succinic acid	1,210
Succinic acid + hydroxylamine	1,105
2.[b]	
Control	1,525
Deoxyribonuclease	1,425
Pronase	75

[a] In part 1, 0.05-ml portions were removed from a reaction mixture and trichloroacetic acid-precipitated two times with 250 μg of carrier serum albumin. Duplicate samples were treated with 0.25 ml of: 1 N NaOH at 100 C for 15 min; 10% trichloroacetic acid at 90 C for 20 min; 1 M succinic acid, *p*H 5.5, at 37 C for 1 hr; 1 M succinic acid + 1 M hydroxylamine, *p*H 5.5, at 37 C for 1 hr. Following these treatments, all samples were precipitated two times with trichloroacetic acid and collected on Millipore filters.

[b] In part 2, 0.05-ml samples were taken from a reaction mixture and heated at 100 C for 3 min to terminate the reaction. Duplicate samples were incubated with 50 μg of deoxyribonuclease I (ribonuclease-free) in 0.50 ml of 0.2 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, *p*H 7, 1.5 mM $MgCl_2$ at 37 C for 30 min. Additional samples were incubated with 50 μg of Pronase (nuclease-free) in 0.5 ml of 0.2 M Tris-hydrochloride, *p*H 7.8, 1.5 mM $CaCl_2$ at 37 C for 30 min. The Pronase had been preincubated for 15 min at 37 C and was found to have no measurable nuclease activity when incubated with ^3H-ribonucleic acid and deoxyribonucleic acid. The samples were trichloroacetic acid-precipitated two times and collected on Millipore filters.

enzymatic activity remained constant during the final steps of virus purification, the specific activities of many different preparations of vaccinia virus were similar, and the enzymatic activity cosedimented with vaccinia virus during rate zonal sucrose gradient and potassium tartrate equilibrium centrifugations (Fig. 8). Additional experiments indicated that the enzymatic activity was associated with the virus core. Envelopes were stripped from purified virus particles with Nonidet P-40 and DTT by a modifi-

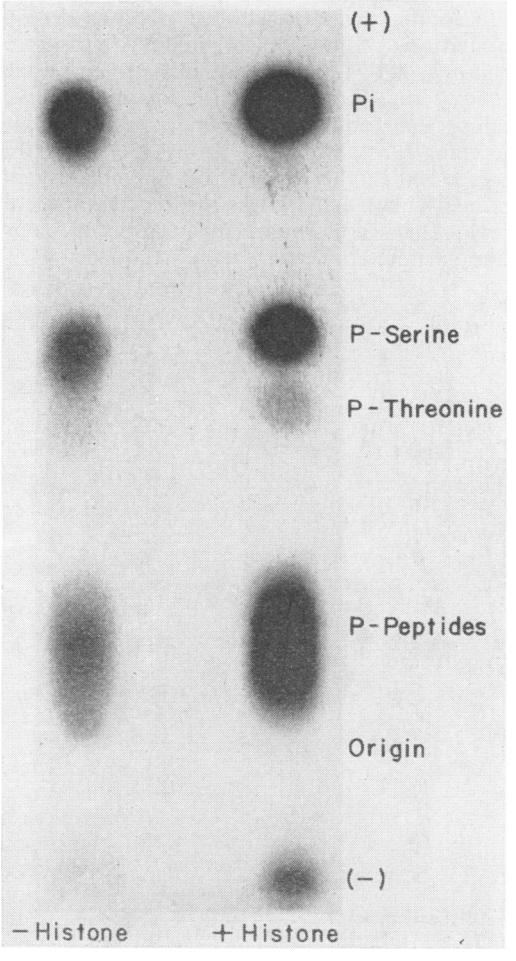

FIG. 5. *Identification of ^{32}P-serine and ^{32}P-threonine by thin-layer electrophoresis. After incubation for 10 min with γ-^{32}P-ATP under standard conditions with or without lysine-rich histone, 250 μg of serum albumin was added and the samples alternately trichloroacetic acid-precipitated and dissolved in 1 N NaOH three times. The final trichloroacetic acid pellets were washed with ethanol-ether (1:1), dried and hydrolyzed at 100 C for 5 hr with 2 N HCl under N_2 in sealed tubes. After removal of HCl, carrier P-serine and P-threonine were added and electrophoresis was carried out with Buffer A of Greenberg and Nachmansohn (8) on 20 by 20 cm thin-layer plates of cellulose for 2 hr at 500 v. Amino acids were localized by spraying with ninhydrin, and radioautographs were prepared. Control experiments demonstrated that γ-^{32}P-ATP was completely converted to $^{32}P_i$ under these conditions.*

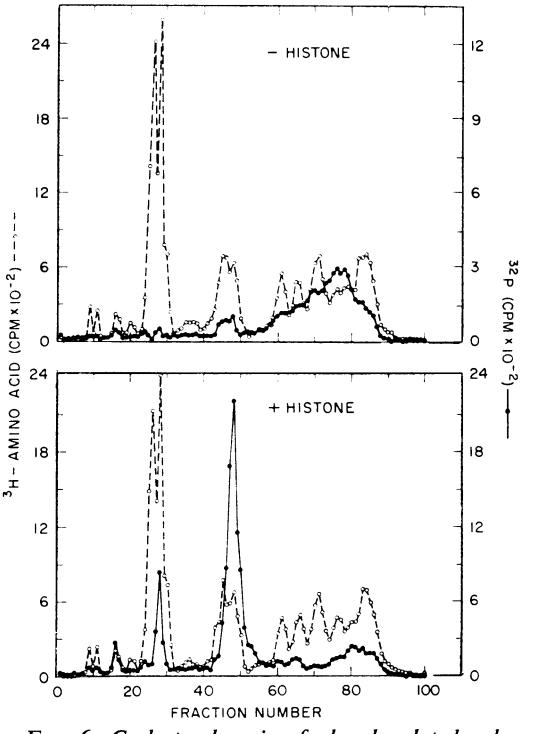

FIG. 6. *Coelectrophoresis of phosphorylated polypeptides and ^{3}H-amino acid-labeled viral polypeptides on polyacrylamide gels. After two cycles of trichloroacetic acid precipitation and an ethanol-ether extraction, the pellets were dissociated with 2% sodium dodecyl sulfate (SDS), 2% mercaptoethanol, 0.01 M sodium phosphate, pH 7, at 100 C for 2 min, and mixed with similarly treated ^{3}H-amino acid labeled polypeptides from purified vaccinia virus. Electrophoresis was carried out with 0.1% SDS, 0.1 M sodium phosphate buffer on 7.5% polyacrylamide gels for 16 hr at 3 ma/gel (13, 16). The gels were fixed with cold 25% trichloroacetic acid containing NaPP$_i$, washed with several changes of cold 10% trichloroacetic acid containing NaPP$_i$, heated at 90 C for 20 min, then washed in the cold overnight. Identical results were obtained without hot trichloroacetic acid treatment. The gels were frozen, sliced into 1-mm sections, dissolved with hydrogen peroxide, and counted in a liquid scintillation counter (16) with appropriate double label corrections. The phosphorylated polypeptides in the upper part of the figure were obtained from the endogenous reaction without added phosphate acceptor proteins; the phosphorylated polypeptides in the lower part of the figure were obtained from a reaction mixture containing lysine-rich histone.*

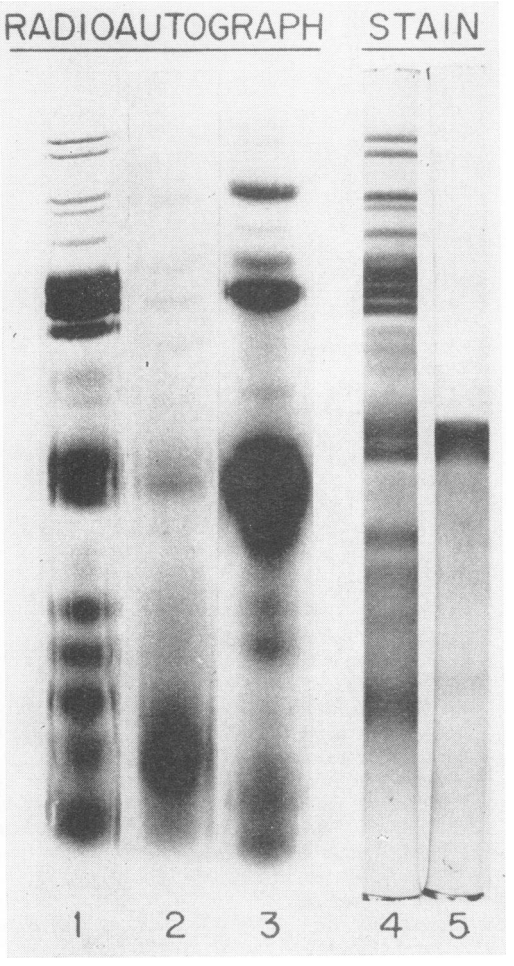

FIG. 7. *Polyacrylamide gel electrophoresis of phosphorylated polypeptides. Electrophoresis of phosphorylated polypeptides was carried out as in Fig. 6 except that ^{3}H-amino acid labeled viral proteins were not added. Instead, electrophoresis of ^{14}C-amino acid labeled vaccinia virus polypeptides was done in a separate gel. The gels were washed and heated in trichloroacetic acid as in Fig. 6, stained with 0.1% Coomassie blue in 10% trichloroacetic acid (2), and then washed overnight in 7.5% acetic acid. The gels were then sliced, dried, and exposed to X-ray film. No radioactive bands were detected when zero time controls were worked up in a similar manner. (1) ^{14}C-amino acid labeled vaccinia virus polypeptides; (2) ^{32}P-labeled polypeptides from a reaction without lysine-rich histone; (3) ^{32}P-labeled polypeptides from a reaction with lysine-rich histone; (4) stained vaccinia virus polypeptides; (5) stained lysine-rich histone. The photographs of the stained gels and radioautographs were made from different gels.*

cation (12) of the Easterbrook procedure (5), and the core and envelope fractions were separated by centrifugation. When examined by electron microscopy, the cores appeared to lack the envelope structure. The removal of specific envelope polypeptides, which account for 20 to 30% of the virus protein (9, 12), was demonstrated by SDS-polyacrylamide gel electrophoresis. All of the endogenous protein kinase activity was recovered in the core fraction indicating that both the enzyme and phosphate acceptor proteins were associated with this component of the virus (Table 3). Similarly, all protein kinase activity was associated with cores purified by equilibrium centrifugation on potassium tartrate gradients.

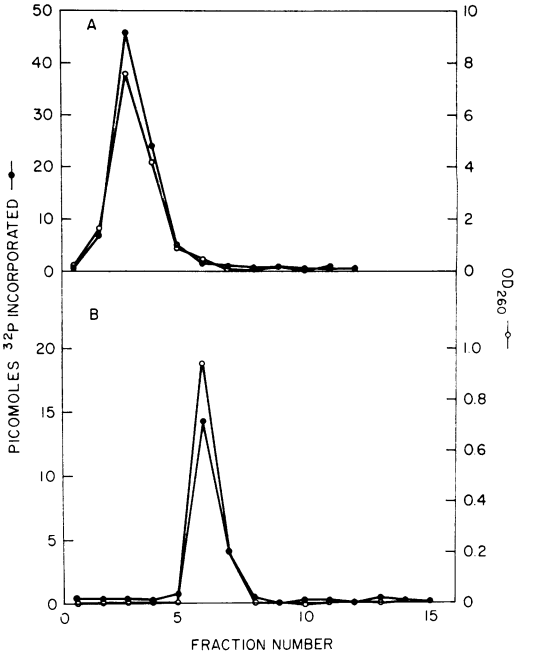

FIG. 8. *Co-sedimentation of protein kinase activity and vaccinia virus particles. A, Purified vaccinia virus particles were sedimented on 25 to 40% (w/v) sucrose gradients in an SW50L rotor at 13,000 rev/min for 25 min, and fractions were assayed for protein kinase activity and optical density. B, Purified vaccinia virus particles were sedimented to equilibrium in a preformed gradient of 25 to 50% (w/v) potassium tartrate in 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.0. Centrifugation was in an SW50L rotor at 36,000 rev/min for 60 min. Fractions were dialyzed overnight to remove tartrate and then assayed for protein kinase activity and optical density. In both A and B, fractions were obtained by piercing the bottom of the tube.*

TABLE 3. *Localization of protein kinase and phosphate acceptor proteins*[a]

Substance	^{32}P incorporation (pmoles/0.1 ml)	
	−Histone	+Histone
Total virus	14.2	35.5
Core fraction	13.3	20.9
Envelope fraction	0.5	0

[a] Envelopes were stripped from purified vaccinia virus particles with Nonidet P-40 and dithiothreitol (DTT), as previously described (12). A sample, designated total virus, was removed and the remainder was centrifuged for 30 min at 30,000 × *g*. The supernatant fluid was designated the envelope fraction and the pellet, after resuspension in an Nonidet P-40–DTT solution of the original volume and composition, was referred to as the core fraction. The kinase activities with endogenous and exogenous (lysine-rich histone) substrates were measured under standard conditions using equal volumes of each virus fraction. The tabulated values are picomoles per 0.1 ml reaction mixture averaged from two separate experiments.

DISCUSSION

The localization of a protein kinase within vaccinia virus cores and its selectivity in phosphorylating viral proteins suggest a specific role for the enzyme in virus replication. We have considered two possibilities: (i) that the enzyme has served its primary function during virus assembly and maturation and remains in the virus particle or (ii) that the enzyme is packaged to function during early stages of infection after partial uncoating of the virus. The first hypothesis is supported by the finding of a phosphorylated polypeptide in vaccinia virus particles (Rosemond and Moss, *manuscript in preparation*). The second hypothesis is supported by the finding that specific proteins are phosphorylated after disruption of the viral envelope with detergents. In contrast, the protein kinase activities of other viruses appear to be less selective (21, 26). Thus the vaccinia virus protein kinase may function at early and late stages of infection.

Four other enzymes, including a deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA)-polymerase (11, 18), a nucleotide phosphohydrolase (7, 19), and DNA endo- and exonucleases (1, 20) are also present in vaccinia virus cores. It is possible that phosphorylation of core proteins plays a role in the regulation of these enzymatic activities. Some studies (13, 25) suggest that protein phosphorylation may be involved in regulating RNA synthesis in nonviral systems. In particular, Martelo et al. (15) report that addition of a protein kinase to the purified DNA-dependent RNA polymerase of *Escherichia coli* stimulates RNA synthesis. Since these two enzymes have not yet been dissociated from vaccinia virus cores, the vaccinia RNA polymerase cannot be measured in the absence of the protein kinase. Although protein phosphorylation precedes the linear synthesis of viral RNA in reaction mixtures suitable for measuring both activities, the significance of this is not known. The possible functional relationships between the five enzymatic activities now known to be associated with vaccinia virus cores may be an interesting subject for future research.

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