External Cell Surface Protein Phosphorylation in Normal and Rous Sarcoma Virus Transformed Chick Embryo Fibroblasts

by

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ABSTRACT

Endogenous protein kinase activity was detected on the external cell surface of both normal and Rous sarcoma virus (RSV)-transformed chick embryo fibroblasts (CEF). Cells growing in plastic dishes were incubated with [γ-^{32}P]ATP for 20 minutes. Under the conditions employed, only proteins located on the external cell surface were labeled, as the radioactivity could be removed by mild trypsin treatment. In addition, exogenous histones were phosphorylated when added to the reaction mixture. The addition of cAMP and cGMP to the reaction had virtually no effect on ^{32}P incorporation, suggesting there is little or no cyclic nucleotide-dependent protein kinase activity present on the external cell surface. Cell surface protein kinase activity was higher in RSV-transformed CEF than in normal CEF, and, using a temperature-sensitive src mutant, this difference was found to be transformation-specific. Several differences were observed in the cell surface proteins phosphorylated in normal and transformed cells, and at least two of these were transformation-specific. These data suggest that changes in external cell surface protein phosphorylation are associated with RSV transformation and thus could play a role in the formation of the transformed cell phenotype.

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
Ad5	adenovirus type 5
CEF	chick embryo fibroblasts
cpm	counts per minute
cAMP	adenosine 3',5'-monophosphate
cGMP	guanosine 3',5'-monophosphate
PBS	phosphate buffered saline
$pp60^{src}$	60,000 dalton src phosphoprotein of RSV
RSV	Rous sarcoma virus
S	Svedberg unit
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SV40	simian virus 40
TCA	trichloroacetic acid
TPB	tryptose phosphate broth
TEMED	N,N,N',N'-tetramethylethylenediamine

INTRODUCTION

One of the major phenotypic differences between malignant cells and their normal counterparts is that malignant cells lose the ability to regulate their growth. The proliferation of normal cells is under stringent regulation and such cells exhibit density-dependent inhibition of growth (Stoker and Rubin, 1967). In vitro, normal cells usually will only multiply to a certain low density to form a monolayer of cells in culture. However, oncogenically transformed cells will multiply to a density far greater than their normal counterparts and form multiple layers of cells in culture. It is clear that malignant cells are no longer under stringent growth regulation and exhibit a loss of density-dependent inhibition of growth. Although many hypotheses have been proposed to explain this altered growth characteristic, little is known of the biochemical processes which underly this phenomenon.

Increasing evidence indicates that the cell surface likely plays some role in the expression of the cancer cell phenotype (Nicolson, 1976). The plasma membrane probably is involved in the regulation of cell growth. Many phenomena which effect cell proliferation, such as the binding of mitogens and plant lectins (Nicolson, 1976; Lis and Sharon, 1973), the action of certain membrane-associated enzymes and molecular transport systems (Holley, 1972), the effects of exogenous proteases (Roblin, et al., 1975) and hormones (Gospodarowicz and Moran, 1976), anchorage dependence (Tooze, 1973), and the regulation of cyclic nucleotide levels (see below) all involve the cell surface.

Thus, in order to understand the basis for the altered cancer phenotype, it is probably important to better understand events associated with cell surface. A major approach to elucidate the role of the plasma membrane in oncogenic transformation has been a straightforward one, namely to determine what biochemical changes take place at the cell surface following transformation. This information could suggest mechanisms by which the transformed cell is phenotypically modified.

The plasma membrane is composed of amphipathic lipids and proteins, some of which contain covalently attached oligosaccharides. It is now well documented that cell membrane lipids are arranged in a bilayer configuration and membrane proteins or glycoproteins are either loosely attached (peripheral proteins) or extend through and/or are tightly attached to the lipid bilayers (Integral proteins) (Singer and Nicolson, 1972; Singer, 1974). There is no doubt that biochemical changes in the plasma membrane do occur following transformation. Alterations have been reported in almost every class of macromolecule at the cell surface (Nicolson, 1976). These include fatty acids, lipids, glycolipids, hyaluronate moities, the sialic acid of fucose-containing glycopeptides, the mobility of surface receptors, antigenic determinants and proteins including cytoskeleton elements (Nicolson, 1976; Pardee, 1975; Oseroff et al., 1973). Some or all of these modifications could play a role in the altered growth properties of transformed cells. However, the understanding of the controlling mechanisms involved is mostly superficial.

There are several problems with cell surface studies. First,

many of these phenomena may be secondary effects and not directly involved in the formation of the transformed state. Second, many experiments have been carried out comparing transformed cells to normal cells which are not growing at the same rate and thus some differences may have been due to differences in cell growth rate rather than transformation. Third, many studies involve the use of cloned virus-transformed cells and therefore observed alterations may be due to clonal variation. And fourth, many biochemical differences are system-specific and therefore their importance is difficult to assess. Thus, although modifications at the cell surface undoubtedly play a role in the altered growth of transformed cells, the exact nature of this role remains unclear.

The system used for the present study, which offers many advantages to overcome these problems, is that of chick embryo fibroblasts (CEF) transformed by Rous sarcoma virus (RSV). Normal and transformed cells can be grown at about the same rate and cloning is not necessary as the transformation efficiency is almost 100%. In addition, excellent mutants which are temperature-sensitive for the transforming gene (src) are readily available (cf. Wyke, 1975).

Rous sarcoma virus was first reported by Peyton Rous in 1911 and bears his name (Rous, 1911). It is a member of the family Retroviride. Four major characteristics define this family of viruses (Fenner, 1976): (i) the architecture of the virion, (ii) a diploid single-strand RNA genome, (iii) the presence of reverse transcriptase in virions, and (iv) the requirement for a DNA intermediate in viral replication. The genome of RSV is apparently diploid; two identical molecules of single-stranded RNA are joined at or near their 5' termini (Beemon et al.,

1976; Bender and Davidson, 1976). The haploid subunits of the genome of RSV have a molecular weight of $2.5\text{-}3X10^6$ and sediment at about 35S.

Four genes have been identified in the genome of RSV (Vogt, 1977): gag, which encodes structural proteins of the viral core; pol, which encodes reverse transcriptase; env, which encodes the glycoproteins of the viral envelope; and src, which is responsible for neoplastic transformation of the host cell. These genes virtually account for the coding capacity of viral genome. The locations of individual genes on the 35S RNA have been mapped. The total genome of Rous sarcoma virus is as follows: 5' gag-pol-env-src-poly(A) 3'.

The gag gene product is translated from a 38S RNA. The primary product of translation from gag is a polyprotein with molecular weight of 76,000 ($Pr76^{gag}$). This polyprotein is subsequently cleaved to yield the major internal virion proteins p19, p12, p27 and p15 (Vogt et al., 1975; Shapiro et al., 1976). A virus-specific RNA with the size and composition expected for a pol messenger has not been identified (Haywood, 1977; Weiss et al., 1977). Instead, pol is apparently expressed by the uninterrupted translation from gag and pol in the 38S RNA. The product of this read-through translation has a molecular weight of 180,000 ($Pr180^{pol}$) (Oppermann et al., 1977) and is then cleaved to yield enzyme reverse transcriptase. The env gene yields a glycosylated precursor protein with a molecular weight of 90,000 - 92,000 ($Pr90^{env}$) (Moelling and Hayami, 1977; England et al., 1977). Upon further processing this precursor yields the mature viral envelope glycoproteins gp 85 and gp 37. The 21S virus-specific RNA encodes only src and the c region which is common to all retroviruses. The results

from genetic studies (Lai, Hu and Vogt, 1977) and from *in vitro* protein synthesis (Beemon and Hunter, 1978; Sefton et al., 1979; Erikson et al., 1978) indicated that only one virus-specific protein is coded for by this gene. The gene has been designated *src* to denote its ability to direct the synthesis of a sarcomagenic protein. It seems remarkable, yet difficult to escape, that a single virus-specific protein is able to cause the widely pleiotropic effects of transformation.

Mutants of RSV that are temperature-sensitive for transformation have been isolated (Wyke, 1975; Manaker and Groupé, 1956). The mutant viruses contain a temperature-sensitive lesion in the *src* gene region and other viral functions are identical to wild-type virus. Thus, the mutant viruses grow equally well at both permissive and nonpermissive temperatures, but they transform only at the permissive temperature. When cells transformed at the permissive temperature are shifted to the nonpermissive temperature, they revert to the normal phenotype. This process is completely reversible, allowing cells to be shifted back and fourth between the transformed and normal states at will. Thus, these mutant viruses provide a powerful tool to examine the function of the transforming *src* gene.

RSV-transformed CEF's are easily distinguished from their normal counterparts. Many of these differences appear to be related to alterations in the plasma membrane. RSV-transformed CEF generally lose their fibroblastic morphology and become round and refractile (Manaker and Groupé, 1956) and poorly attached to the substraturm. Hexose transport dramatically increases to support growth and acid mucopolysaccaride production (Hatanaka and Hanafusa, 1970). Like many other virus trans-

formed and cancerous cells, these cells grow to high densities, they are agglutinated by small amounts of plant lectins (Burger and Martin, 1972), they contain additional sialic acid residues in cell surface fucose-containing glycopeptides (Warren et al., 1972), they have an altered pattern of glycolipids (Hakomori et al., 1977), and they possess new surface antigens (Gelderblom et al., 1972; Kurth and Bauer, 1972). A number of studies have indicated that changes in the pattern of plasma membrane proteins take place after RSV transformation. These include: (i) the loss of the 200-250K dalton external protein fibronectin (Robbins et al., 1974); (ii) the termination of synthesis of a 47K dalton glycoprotein (Wickus, 1974); (iii) a decrease in a glycoprotein of an apparent molecular weight of 140K (Bussell and Robinson, 1973); (iv) increase in proteins of molecular weights 73-79K and 90-95K (Isaka and Yoshida, 1975; Stone et al., 1974); and (v) a decrease in the amount of membrane bound actin (Robbins et al., 1974) caused probably by depolymerization of actin cables as seen in other transformed cells (Pollock et al., 1975). However, the importance of these protein changes with regard to the alteration of growth properties and transformation by RSV is still unknown. It is now becoming apparent that the decrease of the external protein fibronectin appears universal both in this and other virus-cell systems and that the amount of fibronectin present is related to the tumorgenicity of the cell (Hynes, 1976; Spicer and Branton, 1980). Increasing evidence also indicates that fibronectin probably plays an important role in cell morphology, adhesion and movement (Hynes, 1976; Yamada et al., 1976), possibly via an interaction with collagen and actin.

There is now a considerable body of evidence to suggest that cyclic nucleotides may play a role in growth regulation. Intracellular levels of cAMP are higher in density inhibited non-growing cells than in rapidly-growing normal cells (Pastan and Johnson, 1974; Pastan et al., 1975). Transformed cells which are less susceptible to growth inhibition generally have lower cAMP levels than their normal counterparts (Otten et al., 1972; Sheppard, 1972). Also, treatment of some transformed cells with dibutyryl cyclic AMP, an analog of cyclic AMP, was found to induce the flattened appearance characteristic of normal fibroblastic cells (Pastan and Willingham, 1978). Transformation of CEF by RSV results in a reduction of intracellular cAMP which correlates with decreased membrane-bound adenyl cyclase activity (Anderson et al., 1973). Another cyclic nucleotide, cyclic GMP, also may be involved in growth regulation. It has been proposed that high levels of cyclic GMP may be associated with rapidly-growing cells. Goldberg and coworkers (1973) have proposed the Yin-Yang hypothesis and argued that cyclic GMP and cyclic AMP work in opposing fashion, cyclic GMP promoting cell proliferation and cyclic AMP promoting cessation of growth. However, other evidence indicates that cyclic GMP and cyclic AMP both increase as cells commence density-dependent growth inhibition (Nesbitt et al., 1976). It is now generally believed that the relative levels of cyclic AMP and cyclic GMP may play a role in cell proliferation. The mechanism involved in this regulation is not understood, however it is likely that protein phosphorylation is involved since the only known biochemical function of cyclic nucleotides in eukaryotic cells is the activation of protein kinases.

Protein kinases are ubiquitous in eukaryotic cells and are found as both soluble and membrane-bound enzymes. These enzymes catalyze the phosphorylation of hydroxyl groups of serine and threonine through the transfer of the γ-phosphate of ATP (Krebs, 1972; Langan, 1973; Rubin and Rosen, 1975). Many protein kinases function independently from cyclic nucleotides (Krebs and Beavo, 1979). However, the activity of a major class of protein kinases can be stimulated via an interaction with cyclic AMP. The holoenzyme is composed of two types of subunits, a regulatory subunit, which binds on cyclic AMP, and a catalytic subunit, which carries out the phosphorylation reaction. The binding of cyclic AMP to the regulatory subunit results in the release of the highly active catalytic subunit and an increase in enzyme activity (Brostrom et al., 1970), cyclic GMP-dependent protein kinase has also been described (Kuo, 1974) and this enzyme is regulated in a somewhat similar fashion. The regulation of some classes of protein kinases can involve Ca^{2+} or a heat-stable protein-inhibitor, however, the mechanism of regulation of most protein kinases is not yet known.

It is therefore possible that cyclic nucleotides affect cell functions via changes in protein phosphorylation. The biological activities of many proteins have been shown to be altered by phosphorylation (Krebs and Beavo, 1979). The activities of over 20 enzymes are regulated by phosphorylation and more will undoubtedly be found. These enzymes are involved in cell activities such as energy production, carbohydrate metabolism, protein and nucleic acid synthesis and hormone action (Greengard, 1978). One of the classic examples is glycogen phosphorylase (Fischer and Krebs, 1955; Sutherland and Wosilait, 1955),

which has been shown to exist in two interconvertible species, now known to be nonphosphorylated and phosphorylated form of the enzymes. Biological activity of many non-enzymatic proteins is also affected by phosphorylation. The binding of glucocorticoid is dependent upon the phosphorylation of its receptor (Nielson, 1977). The phosphorylation of histones (Lake, 1973; Balhour, 1972) and non-histone chromosomal proteins (Kleinsmith, 1975; Karn et al:, 1974) appear to play a role in the regulation of gene activity (Stein, 1978). Membrane protein phosphorylation is important in the central nervous system in the postsynatic action of neurotransmitters (Greengard, 1976). Furthermore, many viruses code for phosphoproteins and most virions possess protein kinase activity (Rubin and Rosen, 1975). Thus it appears that a great number of biological processes could be controlled via protein phosphorylation.

It is now clear that protein phosphorylation plays an important role in control of many biological processes, some of which may participate in cell proliferation. Changes in nuclear protein phosphorylation (Brade et al., 1974; Sons et al., 1976) and nuclear protein kinase activity (Ahmed, 1971; Jungmann, 1974) are associated with alterations in cell proliferation and these may induce associated changes in gene activity. In studies on cytosol type I and type II cyclic AMP-dependent protein kinase of CHO cells it has been shown that levels of both classes change during the cell cycle, type II increasing under transcriptional control at the G1/S interface (Costa et al., 1976). In the kin A mutant lymphoma cell line it has been shown that the growth

inhibitory effects of cyclic AMP are mediated through a cyclic AMP-dependent protein kinase (Insel et al., 1975; Hochman, 1977). The progesterone-induced meiosis in xenopus oocytes is blocked by the catalytic subunit of a cyclic AMP-dependent protein kinase (Maller and Krebs, 1977). Using the technique of in vitro phosphorylation with [γ-^{32}P]ATP, labeling of a specific soluble 100,000 dalton protein was found to increase upon cell contact (Wehner et al., 1977). The phosphorylation of several other cytosol proteins was found to be altered by cell growth (Kletzien et al., 1977). In these experiments with BHK cells it was found that the cyclic AMP-stimulated phosphorylation of several polypeptides was greater in quiescent cells than in proliferating cells. In studies with chick cells, cell growth was associated with a slight increase in the protein kinase activity of whole cells and associated changes in the phosphorylation of several polypeptides were also observed. In experiments with plasma membranes isolated from chick cells (Branton, 1980), growth inhibition was characterized by increased cyclic AMP-dependent protein kinase activity. Again, phosphorylation changes in specific membrane proteins also took place.

Because of the known regulatory role of protein kinases it is possible that at least some of the phenotypic changes in the cell following oncogenic transformation could result from alteration in protein phosphorylation. It seems logical to propose that protein kinases could be important in transformation, since the function of many proteins and the regulation of many biological processes is known to be controlled by phosphorylation (Rubin and Rosen, 1975; Greengard, 1978; Krebs and Beavo, 1979). Earlier studies on hepatoma and normal rat liver cell homogenates indicated that less cyclic AMP was bound by

hepatoma protein kinase than by normal cell enzymes (Mackenzie and Stellwagen, 1974). This difference was shown to be due to the presence of an inhibitor in hepatoma cells (Mackenzie and Stellwagon, 1977). Studies using plasma membranes isolated from normal and RSV-transformed CEF phosphorylated in vitro with [γ-^{32}P]ATP showed that transformation resulted in both a slight increase in membrane protein kinase activity and changes in the pattern of endogenous membrane protein phosphorylation (Branton and Landry-Magnan, 1978; 1979). Increased phosphorylation of a non-histone chromosomal protein (Pumo et al., 1975) and many other endogenous protein substrates (Epstein et al., 1979) was shown in SV40-transformed human fibroblasts. The level of endogenous protein phosphorylation in non-histone chromosomal and ribosomal wash proteins is 7-10 times greater in SV40-transformed rat cells than in untransformed parental cells and it was caused mainly by increased activity of protein kinase and the nature of protein substrates (Segawa and Oda, 1978).

More recently, Collett and Erikson (1978) found that protein kinase activity was associated with the RSV src transforming protein. Using sera from tumor-bearing rabbits, they were able to immunoprecipitate a protein from both RSV-transformed cells (Brugge and Erikson, 1977) and from proteins synthesized from src-specific mRNA in vitro (Purchio et al., 1978). When the immunoprecipitates containing src protein were incubated with [γ-^{32}P]ATP, phosphorylation of the heavy-chain of the antibody was observed (Collett and Erikson, 1978). These results indicate that the src gene product is associated with protein kinase activity. These experiments have now been repeated in several

laboratories (Levinson et al., 1978; Rubsamen et al., 1979; Sefton et al., 1979) and the src protein is now known as pp60^{src}.

Since the src gene only codes for one protein (Brugge and Erikson, 1977; Purchio et al., 1978) and is also responsible for both the induction and maintenance of neoplastic transformation (Vogt, 1977), the data suggest that RSV transformation is entirely dependent on a viral protein kinase and raise the possibility that phosphorylation of specific cellular targets might account for the pleiotropic effect of src on the host cell

The original experiment by Collett and Erikson (1978) does not determine whether the pp60^{src} kinase activity is coded for by the viral gene or if pp60^{src} is associated with a cellular kinase. However, from a number of considerations it seems likely that the src protein kinase is coded for by the viral gene. The pp60^{src} has been synthesized in vitro from RSV src-specific RNA and that protein kinase activity can be detected by immunoprecipitation (Sefton et al., 1979; Erikson et al., 1978). Substantial support also comes from the observation that temperature-sensitive mutants which render the virus unable to transform, simultaneously reduce the protein kinase activity induced at the nonpermissive temperature and cause this activity to be extremely labile after lysis of infected cells (Levinson et al., 1978; Rubsamen et al., 1979; Collett and Erikson, 1978; Sefton et al., 1979; Owada and Moelling, 1980). Also persuasive is the fact that pp60^{src} and the kinase activity copurify (Erikson et al., 1980; Maness, 1980). Nevertheless, whether pp60^{src} itself is a protein kinase, or binds to a cellular kinase and thus modifies its action, changes in protein phosphorylation appear likely

to play a critical role in RSV transformation. It has now been shown that the pp60^{src} kinase, unlike other known protein kinases, phosphorylates tyrosine instead of serine and threonine (Hunter and Sefton, 1980; Collett et al., 1980). This observation suggests that the phosphorylation of tyrosine may be important in the process of transformation (Langan, 1980; Hunter and Sefton, 1980).

The results of Collett and Erikson (1978) have lead to a number of studies to ask if protein kinase activity is associated with the transformation-specific proteins of other tumor viruses. Branton and coworkers have studied the tumor antigens of human adenovirus type 5 (Ad5). They have immunoprecipitated the tumor antigens from cytoplasmic extracts of Ad5-infected KB cells using a tumor serum, 14b, which is specific for Ad5 transforming proteins. Again, protein kinase activity was observed when such immunoprecipitates were incubated with [γ-^{32}P]ATP. The incubation resulted in phosphorylation of the heavy chain of IgG and the 58K Ad5 tumor antigen (Lassam et al., 1979; Branton et al., 1979; 1981). These results lead to the proposal that perhaps all tumor viruses induce transformation via virus-specific protein kinase.

A number of reports on other viruses tend to support this hypothesis. The T antigens immunoprecipitated from adenovirus type 12 infected and transformed cells demonstrated protein kinase activity (Branton et al., 1979; Raska et al., 1979). SV40 large T antigen possesses both ATPase and protein kinase activities (Tjian and Robbins, 1979; Griffin et al., 1979). There is still some controversy as to which activity is T antigen-specific, Tjian claiming the kinase is

only an associated protein (Tjian and Robbins, 1979; Tjian, 1979), Livingston claiming the ATPase is a contaminant (Griffin et al., 1979). The middle T antigen of polyoma virus has also been reported to be associated with protein kinase activity (Smith et al., 1979; Eckhart et al., 1979; Schaffhausen and Benjamin, 1979) and it also processes an activity phosphorylating tyrosine (Eckhart et al., 1979). There are several reports demonstrating that the transforming proteins of several mammalian RNA tumor viruses, including feline sarcoma virus (Reynolds et al., 1980; Van de Ven et al., 1980), Abelson murine leukemia virus (Witte et al., 1980; Van de Ven et al., 1980; Blomberg et al., 1980) and Molony murine sarcoma virus (Sen and Todaro, 1979), are associated with protein kinase activity. Thus, transformation may be induced in part or in whole by viral phosphoproteins which possess kinase activity.

In view of the important role of both the plasma membrane and protein phosphorylation in growth regulation and viral transformation, the studies in this thesis have focussed on an investigation of protein phosphorylation at the external surface of normal CEF and RSV-transformed CEF. Studies with mammalian cells using 3T3 and SV40-transformed 3T3 cells labeled externally with [γ-^{32}P]ATP indicated that (i) protein kinase activity was detected in the external cell surface, (ii) differences in protein kinase activities were found between normal and SV40-transformed cells, and (iii) the phosphorylation pattern of cell surface proteins varied between normal and SV40-transformed 3T3 cells.

Using techniques similar to Mastro and Rozengurt (1976), intact normal CEF and RSV-transformed CEF were incubated with [γ-^{32}P]ATP while they were still attached to the culture plates. Because ATP is not taken up directly by intact cells, only the external cell surface was expected to participate in the phosphorylation reaction. External cell surface protein kinase activity was measured in normal and RSV-transformed CEF and pattern of protein phosphorylation were determined by SDS-PAGE. In addition, using wild-type RSV and a transformation-defective temperature-sensitive RSV mutant, experiments were done to establish if changes in protein kinase activity at the cell surface and in the pattern of surface protein phosphorylation occur specifically in association with oncogenic transformation.

MATERIALS AND METHODS

A. Cells and Viruses

Primary chick embryo fibroblast cultures were prepared from 11-day old COFAL negative chick helper factor negative embryo essentially according to the procedure of Vogt (1969). The limbs, head and internal organs of the chick embryo were removed. The bodies were minced with scissors. The tissue was washed twice with phosphate buffered saline (PBS) and then it was incubated with 0.25% trypsin for 30 min at 37°C. The cell suspension was filtered through sterilized cheese cloth and then the cells were pelleted by centrifugation for 5 minutes at 1500 rpm in a 50 ml plastic centrifuge tube (Falcon). Normal cells were seeded at a density of $1X10^7$ cells per 100 mm plastic Petri dish (Corning) in medium 199 supplemented with 2% tryptose phosphate broth (TPB), 1% calf serum and 1% heat-inactivated chick serum (2:1:1). To prepare Rous sarcoma virus (RSV) transformed cell cultures, cell pellets were incubated with wild-type Schmidt-Ruppin RSV (subgroup A) at a multiplicity of approximately 1 focus-forming unit per cell at 37°C. After 1 hour, medium 199 (2:1:1) was added and all primary cultures were grown to confluency (approx. 3 days) at 39°C.

Secondary cultures were prepared from trypsinized (0.05% trypsin) infected and uninfected primary cells. They were plated in medium 199 (10:4:1) at a density of approximately $6X10^5$ cell per 60 mm plastic Petri dish (Falcon) and they were cultured at specified

temperatures. Medium was frequently changed (every day or two) to ensure that the normal cells were actively proliferating.

Cultures infected with the temperature-sensitive src mutant NY68 (Kawai and Hanabusa, 1971) were prepared in a similar manner and secondary cultures were grown at either 36° or 41°C.

B. Standard Cell Surface Phosphorylation Procedure

Phosphorylation of the external plasma membrane was performed using a modification of the procedure of Mastro and Rozengurt (1976). The medium was removed and the cultures were washed twice with 4 ml of solution A [0.03M Tris/acetate (pH7.5) containing 0.15M NaCl and 4mM Mg acetate] and then they were preincubated at 37°C for 5 min in 2 ml of standard reaction mixture consisting of 0.03M Tris/acetate (pH 7.5), 0.15M NaCl, 4mM Mg acetate, 0.1mM sodium phosphate, 0.01M NaF and 0.3mM EGTA. The phosphorylation was initiated by the addition of 1.5 nmoles of [γ-^{32}P]ATP (specific activity 3-5 Ci/mmol) and the cultures were incubated for 20 minutes at 37°C. The reaction was stopped by removing the reaction mixture and washing twice with ice-cold solution A containing 0.01M NaF and 1mM ATP (solution B). Five ml of ice-cold 10% TCA in 0.1N phosphoric acid was added and the precipitate was scraped into a centrifuge tube. The suspension was combined with 0.2 ml of 0.1% BSA and then centrifuged at 1500 rpm for 15 min. at 4°C. The supernatant was removed, the pellet was dissolved in 0.2 ml of ice-cold 1N NaOH and precipitation was again carried out with 5 ml of cold 5% TcA containing 0.05N phosphoric acid. This step was repeated once more and the final precipitate was solubilized in 0.2 ml of either 1N NaOH or NCS and the amount of ^{32}P was measured on a Beckman

liquid scintillation counter using Formula 950A triton-toluene scintillation fluid. The amount of phosphate incorporated was calculated from the amount of radioactivity detected and was expressed as the number of pmoles of phosphate.

C. Characterization of Phosphorylated Product

Cultures were phosphorylated under standard phosphorylation conditions and after three washes with ice-cold solution B the cells were scraped into ice-cold water. The cell suspensions from several plates were combined and were lysed by freezing and thawing three times and then they were sonicated until a homogeneous suspension was formed. The lysate was divided into a number of equal aliquots for individual treatments. Samples were digested with pronase (25 µg/ml), crystalline trypsin (50 µg/ml) or ribonuclease A (60 µg/ml) in 0.05M Tris/HCl (pH7.0) and with deoxyribonuclease I in 0.05M Tris/HCl (pH 7.0) containing 1mM $MgCl_2$. Hydroxylamine treatment was also carried out, according to Hokin et al.(1965). For all treatments, incubation was performed using 1N NaOH at 37°C for 1 hour. Acid hydrolysis was at 100°C for 1 hour. For ethanol:ether extraction, samples were precipitated with 10% TCA, centrifuged at 1500 rpm and the precipitates were washed with 10% TCA. The pellets were resuspended in ethanol: ether (1:3, v/v) and incubated at 37°C for 30 min. The samples were centrifuged and the procedure was repeated with a 10 min. incubation. Following treatment all samples were combined with an equal volume of 10% TCA containing 0.1N phosphoric acid, and precipitates were collected by centrifugation and redissolved in 1N ice-cold NaOH. This step was repeated twice and the final precipitates were collected by

filtration and the amount of radioactivity present was determined by scintillation counting. Controls were done by incubation of samples in 0.5M Tris/HCl (pH7.0).

D. Thin Layer Electrophoresis

1. High Voltage Paper Electrophoresis

Cells were phosphorylated using the standard procedure and 10% TCA in 0.1N phosphoric acid was added and the precipitates were pelleted by centrifugation as described above. The pellet was resuspended in ethanol-ether (3:1, v:v) and the mixture was incubated at 37°C for 30 min. The samples were centrifuged and the procedure was repeated with a 10 min incubation. The samples were allowed to air-dry and then they were hydrolysed in 6N double-distilled HCl under vacuum at 110°C for 6 hrs. Samples were lyophilized, combined with unlabelled O-phospho-L-serine and phosphothreonine markers and spotted on Whatman 3mm paper. Electrophoresis was carried out for 3 hours at 400V using 0.1M phosphoric acid (pH2.5) as the electrode buffer. After electrophoresis, the chromogram was dried, stained with ninhydrin and autoreadiography was carried out using Kodak RP Royal X-Omat film.

2. Two-Dimensional Thin Layer Electrophoresis

Cells were phosphorylated and processed as described above. For acid hydrolysis the precipitates were incubated in 6N HCl for 2 hours at 110°C under vacuum. The HCl was removed by freeze-drying and the hydrolyzates were dissolved and combined with a marker mixture containing unlabelled phosphoserine, phosphothreonine, and phosphotyrosine (see appendix 1) each at 1 mg/ml. The acid hydroly-

zates were then analyzed on cellulose thin layer plates (100 μ) by two dimensional electrophoresis. The first dimension was ran at pH1.9 for 2 hours at 1000V in a buffer containing glacial acetic acid, formic acid and water in a ratio 78:25:897 (vol/vol). After rotating the plate 90°, the second dimension was run at pH3.5 for 45 minutes at 1500V in a buffer containing glacial acetic acid, pyridine and water in a ratio 50:5:945 (vol/vol). The amino acid markers were located by ninhydrin staining; the radioactive material was located by autoradiography using Kodak RP Royal X-Omat film. After the autoradiography was done, the ninhydrin-stained spot on the cellulose plate was scraped. The phosphoamino acid was eluted from the cellulose by washing three times with 100 μl of 0.1M HCl. The HCl was then collected and pooled together and the amount of ^{32}P was measured on a Beckman liquid scintillation counter using Formula 950 A triton-toluene scintillation fluid.

E. Sodium dodecyl sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Cells were phosphorylated using the standard procedure except that a higher specific activity of [γ-^{32}P]ATP (30 Ci/mmole) was used, and following two washes with ice-cold solution B, cells were solubilized directly on the plate in 0.8 ml of sample buffer containing 0.05M Tris/HCl (pH6.8), 1% SDS, 1% 2-mercaptoethanol, 2mM PMSF, 10% glycerol and 0.001% bromophenol blue. The samples were heated for 2 min. at 100°C. The labeled proteins were analyzed by SDS-PAGE using a system based on that of Laemmli (1970) consisting of a 5% acrylamide stacking gel and a 7.5 to 15% acrylamide gradient separating gel. The ratio of

acrylamide to N-N'-bis-methylene acrylamide was 30:0.8. In addition, the separating gel contained 0.375M Tris/HCl (pH8.8), 1% glycerol, 0.025% (v/v) TEMED and 0.1% SDS. The gels were polymerized chemically by the addition of ammonium persulfate as was the stacking gel which consisted of 5% acrylamide containing 0.125M Tris/HCl (pH6.8), 0.5% glycerol and 0.1% SDS. The electrode buffer contained 0.025M Tris/HCl (pH8.3) and 0.192M glycine and 0.1% SDS. Electrophoresis was carried out at constant current of 35 mAmps until the bromophenol blue marker reached the bottom of the gel (about 4 hours). Gels were stained with Coomassie Blue, destained with several changes of isopropanol-acetic acid and then dried under vacuum. Autoradiography was carried out using Kodak RP Royal X-Omat film.

RESULTS

A. Characterization of the Phosphorylated Product

Incubation of intact normal and RSV-transformed CEF growing on tissue culture plates with [γ-^{32}P]ATP under standard phosphorylation conditions resulted in the incorporation of ^{32}P into TCA-insoluble material (see table 1). The incorporated radioactivity could have resulted from either the incorporation of the labeled γ-phosphate of ATP, or the incorporation or binding of the entire ATP molecule. In order to distinguish between these two possibilities the incorporation of ^{32}P using [α-^{32}P]ATP and [γ-^{32}P]ATP at the same specific activities and containing an equal amount of labeled ATP were compared. As shown in Table 1, with [α-^{32}P]ATP incorporation of ^{32}P was less than 3% of levels obtained with [γ-^{32}P]ATP. Thus the uptake of ^{32}P appeared to be due to the incorporation of the γ-phosphate of ATP rather than the entire ATP molecule.

In order to assess the nature of the phosphorylated product, the labeled material was analyzed using several enzymatic and chemical treatments. To determine if phospholipids were labeled in the reaction, the TCA-insoluble material was extracted twice with ethanol:ether (1:3, v:v) to remove the phospholipids. As shown in Table 2, this treatment failed to remove any radioactivity, indicating that little ^{32}P was present in phospholipids. The incorporated label was also resistant to DNase and RNase (Table 2), thus showing that nucleic acids were not phosphorylated to any significant degree during the reaction. However,

TABLE 1

Incorporation of ^{32}P using $[\alpha^{32}P]ATP$ and $[\gamma^{32}P]ATP$

Cells	Incorporation of ^{32}P* (cpm)		Ratio
	$[\alpha^{32}P]ATP$	$[\gamma^{32}P]ATP$	$\frac{[\alpha^{32}P]ATP}{[\gamma^{32}P]ATP}$
CEF	65	2573	0.025
RSV-CEF	67	3543	0.019

*External cell surface phosphorylation of normal and RSV-transformed CEF was carried out under standard conditions using either $[\gamma^{32}P]ATP$ or $[\alpha^{32}P]ATP$ present in equal amounts of radioactivity.

TABLE 2

Characterization of Phosphorylated Substrate

Treatment	% ^{32}P remaining (vs controls)*	
	CEF	RSV-CEF
Control	100	100
deoxyribonuclease	97	98
ribonuclease	100	100
pronase	33	21
trypsin	29	27
1N HCl	6	3
1N NaOH	4	2
ethanol-ether	100	100
hydroxylamine	100	100

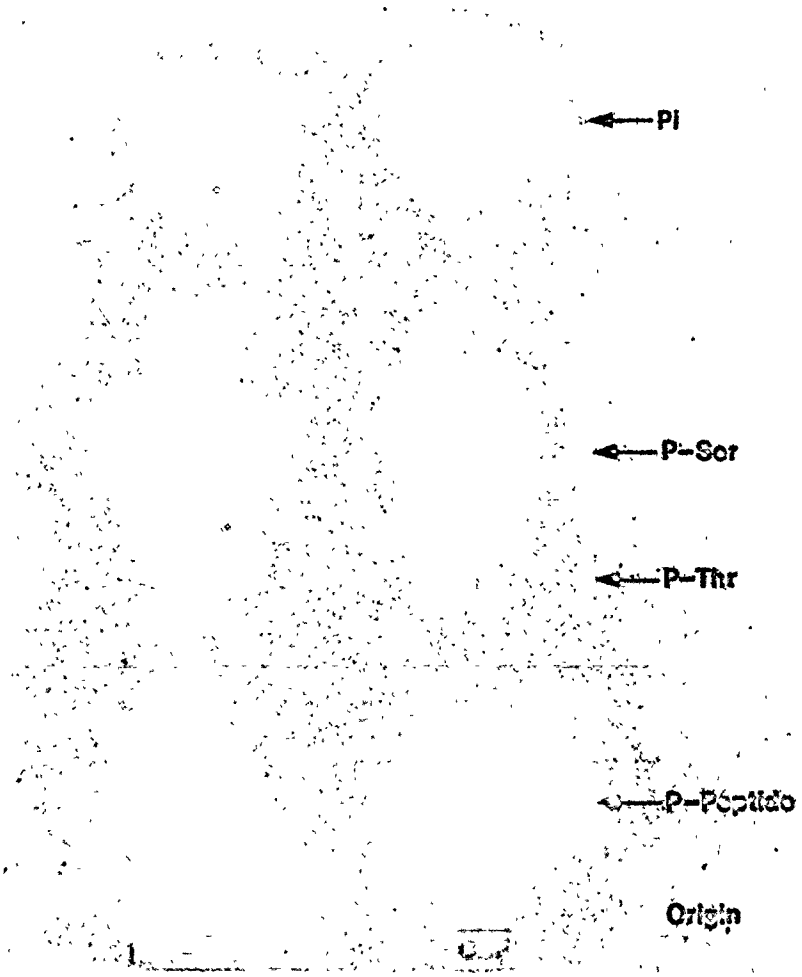
*Cells were phosphorylated using [γ^{32}P]ATP and then equal aliquots were subjected to various treatments, as described in Materials and Methods. The data has been presented as the % of radioactivity remaining after treatment vs controls which were incubated in buffer alone. The amount of radioactivity present in controls was 1169 cpm for CEF and 1589 cpm for RSV-CEF.

the labeled material was sensitive to pronase, trypsin and hot HCl. As shown in table 2, over 70% of the radioactivity was removed after the labeled material was treated with trypsin and pronase and only about 5% of the radioactivity was left after the hot HCl treatment. These results indicated that protein was the major phosphorylated product. Mild alkaline hydrolysis removed over 95% of the radioactivity but hydroxylamine treatment failed to remove any significant quantity of ^{32}P. These data suggested that phosphorylation was not via an acyl-phosphate linkage but rather appeared to be a phosphomonoester bond. This conclusion was strengthened by the results described below involving acid hydrolysis (Fig. 1) and SDS-PAGE analysis (Fig. 12). These processes also would have been expected to remove ^{32}P if phosphorylation had been via an acyl-phosphorylate linkage. Thus the properties of the phosphorylated product suggested a phosphomonoester linkage to a protein.

The nature of the phosphate bonding was further characterized by acid hydrolysis followed by high voltage electrophoresis. Labeled material was hydrolyzed in 6N HCl at 110^{o}C under vacuum for 6 hours and the hydrolysate was analyzed by high voltage paper electrophoresis. As shown in Fig. 1, with both normal and RSV-transformed CEF, four labeled species were detected. The first, which was located furthest away from the origin, migrated in the position of inorganic phosphate. A second species, which did not migrate far from the origin, probably represented incompletely hydrolyzed polypeptides. A third major labeled species comigrated with authentic phosphoserine marker. A fourth minor species, which was barely detectable, comigrated with phosphothreonine marker. Phosphoserine and phosphothreonine have been found to be the major

FIGURE 1

Analysis of hydrolysed ^{32}P-labeled cells by high voltage paper electrophoresis. Normal and transformed cells were phosphorylated, then hydrolysed and subjected to high voltage paper electrophoresis as described in Materials and Methods. The arrows marked P-Ser and P-Thr indicated the positions of ninhydrin-stained phosphoserine and phosphothreonine markers.


Pi
P-Ser
P-Thr
P-Peptido
Origin

phosphoamino acids present in nature, phosphorylation occurring as a result of the action of protein kinase (Rubin and Rosen, 1975). All these results suggested that the incorporation of [^{32}P] phosphate following incubation of intact normal and RSV-transformed cells with [γ-^{32}P]ATP was due to protein kinases.

There are now two reports (Hunter and Sefton, 1980; Collett et al., 1980) which demonstrate that RSV pp60^{src} protein kinase is tyrosine-specific and RSV-transformed CEF contain as much as 8-fold more phosphotyrosine than do normal CEF (Hunter and Sefton, 1980). Since phosphotyrosine is relatively unstable under standard conditions of hydrolysis and also phosphotyrosine and phosphothreonine comigrate under the present conditions of electrophoresis, a further attempt was made to analyze the phosphoamino acids. Labeled material was hydrolysed in 6N HCl at 110^{o}C under vacuum for 2 hours and the phosphoamino acids were separated by two-dimensional electrophoresis on cellulose thin layer plates. As shown in figs. 2 and 3, under these conditions phosphoserine, phosphothreonine and phosphotyrosine were well separated. Again, phosphoserine and phosphothreonine represented the major labeled species in both normal (fig. 2) and RSV-transformed cells (fig. 3). As shown in table 3, phosphoserine, phosphothreonine and phosphotyrosine represented about 65, 35 and less than 0.1%, respectively, in both normal and RSV-transformed cell preparations. No significant increase in labeled phosphotyrosine was observed with RSV-transformed cells, suggesting that tyrosine-specific src-related protein kinase activity is not detected by the present method.

Figure 2

Identification of phosphoamino acid composition of ^{32}P-labeled normal CEF. Normal CEF were phosphorylated and hydrolysed in 6N HCl for 2 hours at 110°C. This material was then combined with unlabeled phosphoserine, phosphothreonine and phosphotyrosine markers and the mixture was analyzed by two dimensional thin layer electrophoresis as described in Materials and Methods. The positions by labeled amino acids and of the markers were determined by autoradiography and ninhydrin-staining, respectively. The dashed lines indicate the positions of ninhydrin-stained reference markers.

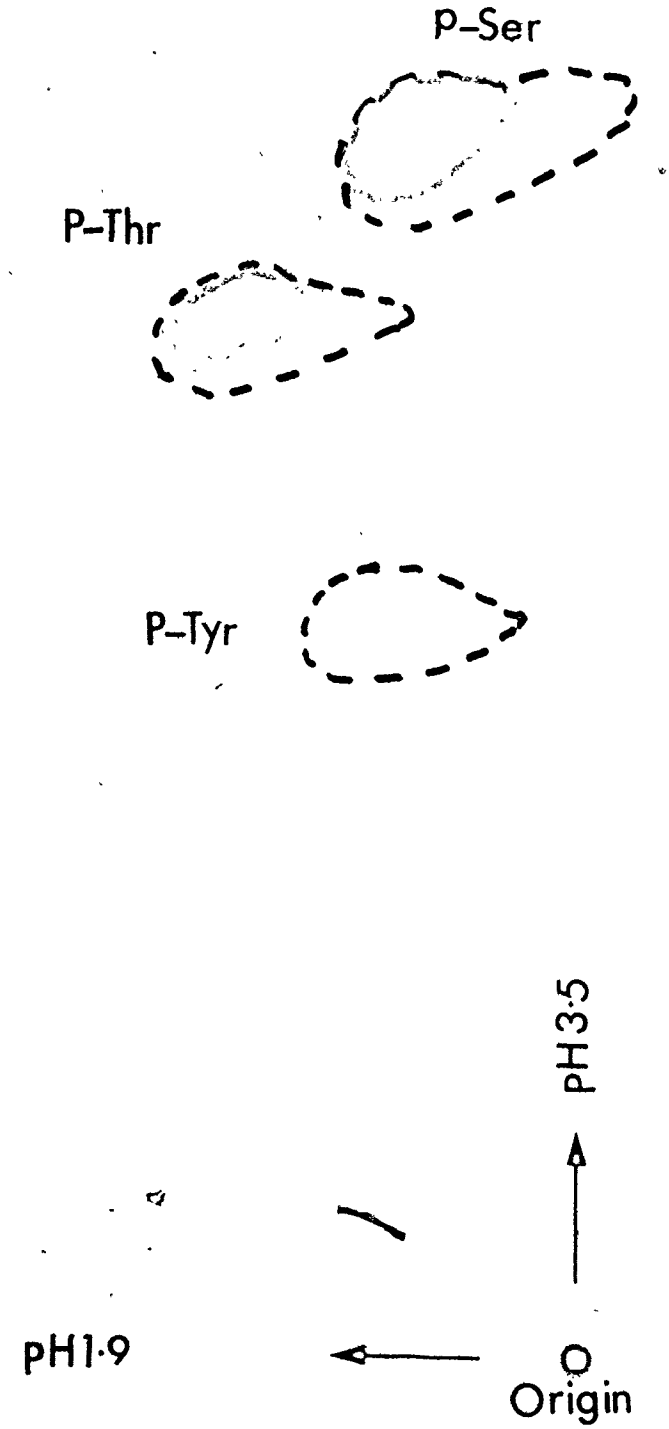

P–Ser
P–Thr
P–Tyr
pH3·5
pH1·9
O
Origin

FIGURE 3

Identification of phosphoamino acid composition of ^{32}P-labeled RSV-transformed CEF. RSV-transformed CEF were phosphorylated and hydrolysed in 6N HCl for 2 hours at 110^{o}C. This material was then combined with unlabeled phosphoserine, phosphothreonine and phosphotyrosine markers and the mixture was analyzed by two-dimensional thin layer electrophoresis as described in Materials and Methods. The positions of labeled amino acids and of the markers were determined by autoradiography and ninhydrin staining, respectively. The dashed lines indicate the positions of ninhydrin-stained reference markers.

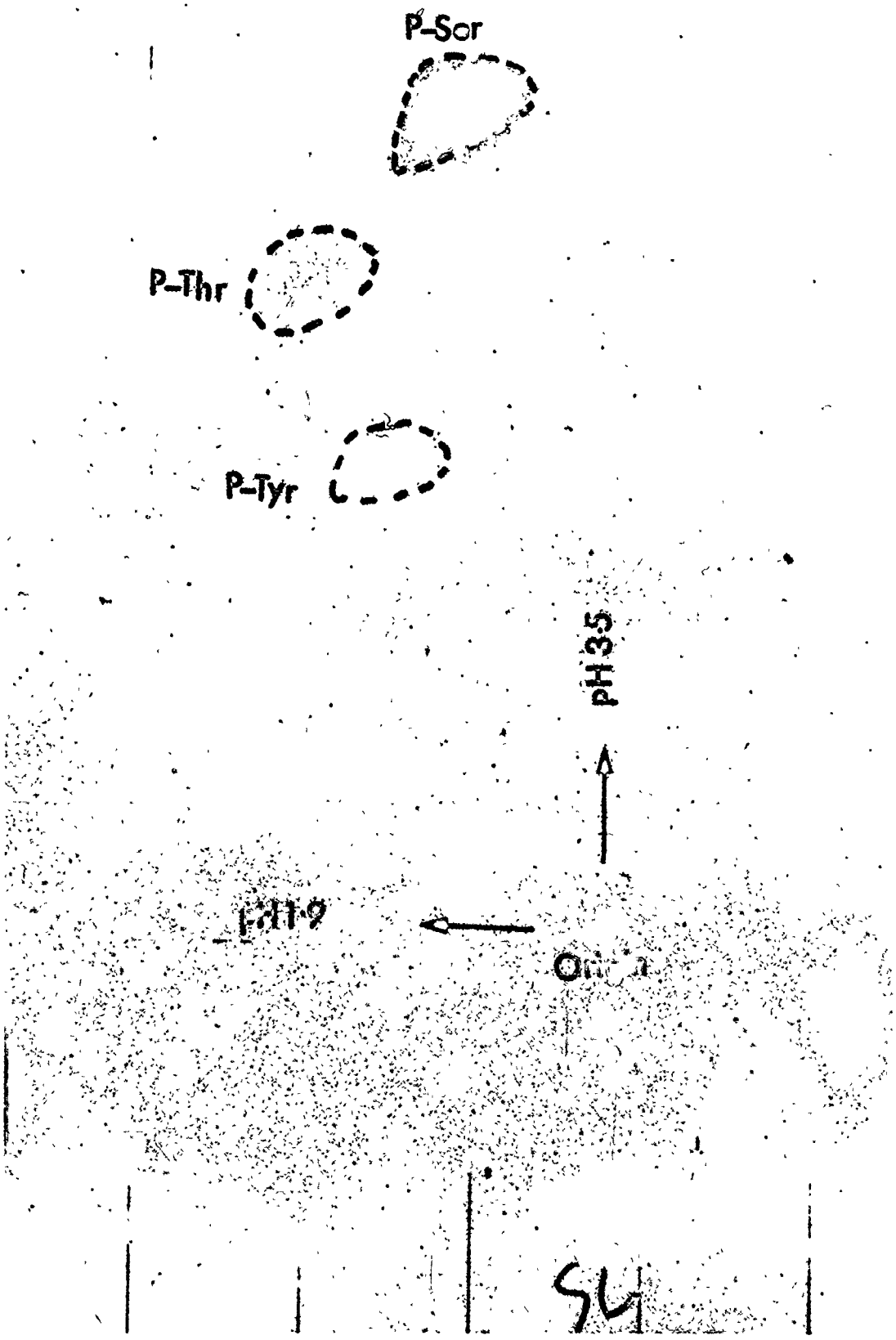

P-Ser
P-Thr
P-Tyr
pH 3.5
pH 9
Origin

TABLE 3

Abundance of Phosphoamino Acids in Cells*

Cells	Phosphoserine	Phosphothreonine	Phosphotyrosine
CEF	64.7	35.2	0.09
RSV-CEF	65.1	34.8	0.09

*The cells were phosphorylated under standard conditions and subjected to two-dimensional electrophoresis as described in Materials and Methods. The phosphoamino acids, which were visible by ninhydrin-staining, were scraped from the thin layer plates and eluted from cellulose by washing three times with 0.1N HCl. The HCl washes were combined and the amount of ^{32}P was measured on a Beckman liquid scintillation counter using Formula 950A triton-toluene scintillation fluid. An arbitrary spot on the thin layer plate was also scraped and the radioactivity was measured as background control. The amount of each phosphoamino acid was calculated as the percentage of total phosphoamino acids detected.

B. Characterization of the Phosphorylation Reaction

Various parameters of the protein kinase reaction with normal and RSV-transformed CEF incubated with [γ-^{32}P]ATP directly on culture plates were analyzed. As shown in fig. 4, under the standard assay conditions the rate of ^{32}P incorporation was approximately constant for at least 60 minutes in normal CEF. With RSV-transformed CEF, there was a rapid initial rate of incorporation for the first 10 minutes, followed by a slower rate similar to normal CEF. The amount of ^{32}P incorporation was measured with different cell densitites. As shown in fig. 5, ^{32}P incorporation increased linearly with cell number with both normal and RSV-transformed CEF. The incorporation of ^{32}P was markedly dependent on the concentration of Mg^{2+} and 10mM appeared to be optimal for both normal and RSV-transformed CEF (fig. 6). The incorporation of ^{32}P was proportional to the amount of ATP present, at least up to 5 nmoles (fig. 7). As shown in fig. 8 and 9, the incorporation of ^{32}P was also affected by temperature and pH, and both normal and RSV-transformed cells showed an optimal temperature and pH of 41^{o}C and pH7.5. The reaction kinetics of the protein kinase detected in both normal and RSV-transformed CEF were found to be very similar, the only difference being that the levels of ^{32}P incorporation were generally higher with transformed than with normal cells.

C. Localization of the Protein Kinase and its Phosphate Acceptors

The data presented so far have shown that proteins are the principle, if not the only, phosphorylated product in the reaction. However, it was still not clear that phosphorylation took place exclusively at the external cell surface. Several kinds of evidence

FIGURE 4

Effect of period of incubation on cell surface protein kinase activity. Normal and RSV-transformed CEF were phosphorylated as described in Materials and Methods except that the period of incubation was varied as indicated. The number of pmoles of phosphate incorporated was determined from the incorporation of ^{32}P into TCA-insoluble material. The values given represent the average two separate determinations.

RSV-CEF (●——●). Normal (o——o).

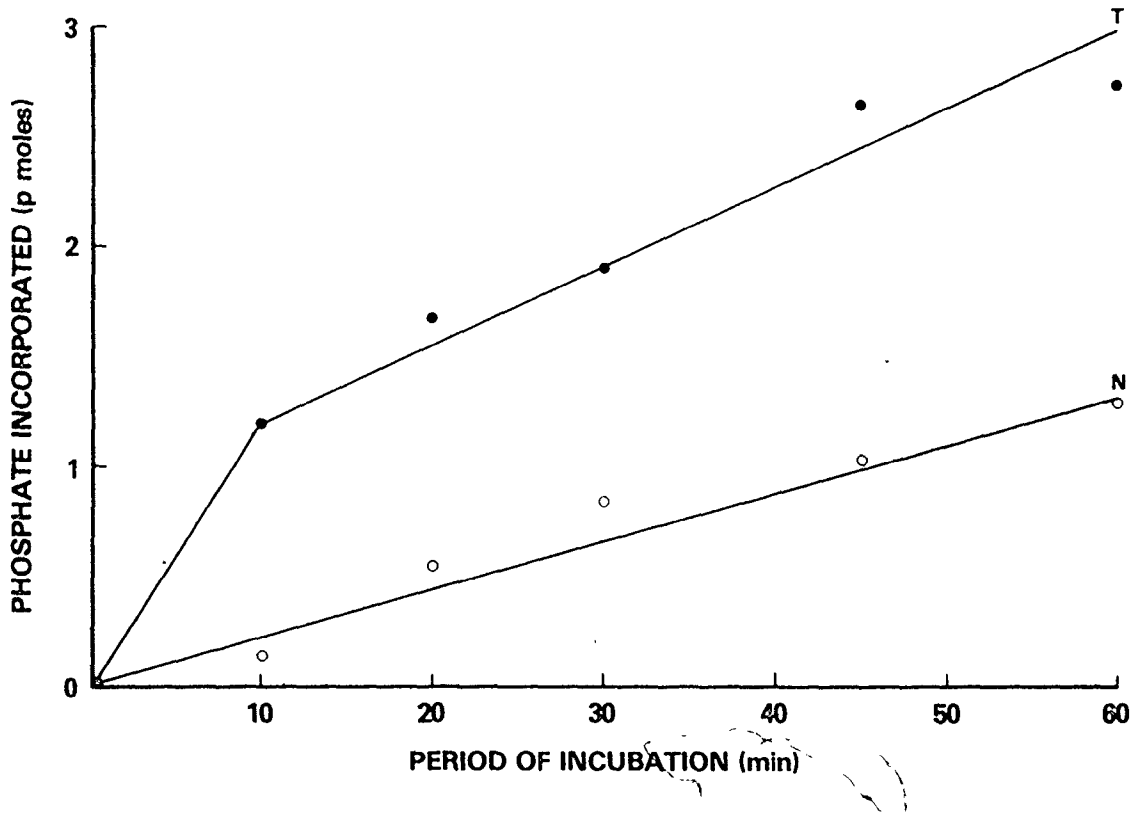

PHOSPHATE INCORPORATED (p moles)
3
2
1
0
T
N
10
20
30
40
50
60
PERIOD OF INCUBATION (min)

FIGURE 5

Effect of cell number on cell surface protein kinase activity. Normal and RSV-transformed CEF were phosphorylated as described in Materials and Methods except that the number of cells per plate was varied as indicated. The number of pmoles of phosphate incorporated was determined from the incorporation of ^{32}P into TCA-insoluble material. The values given represent the average of two separate determinations.

RSV-CEF (●——●). Normal CEF (o——o).

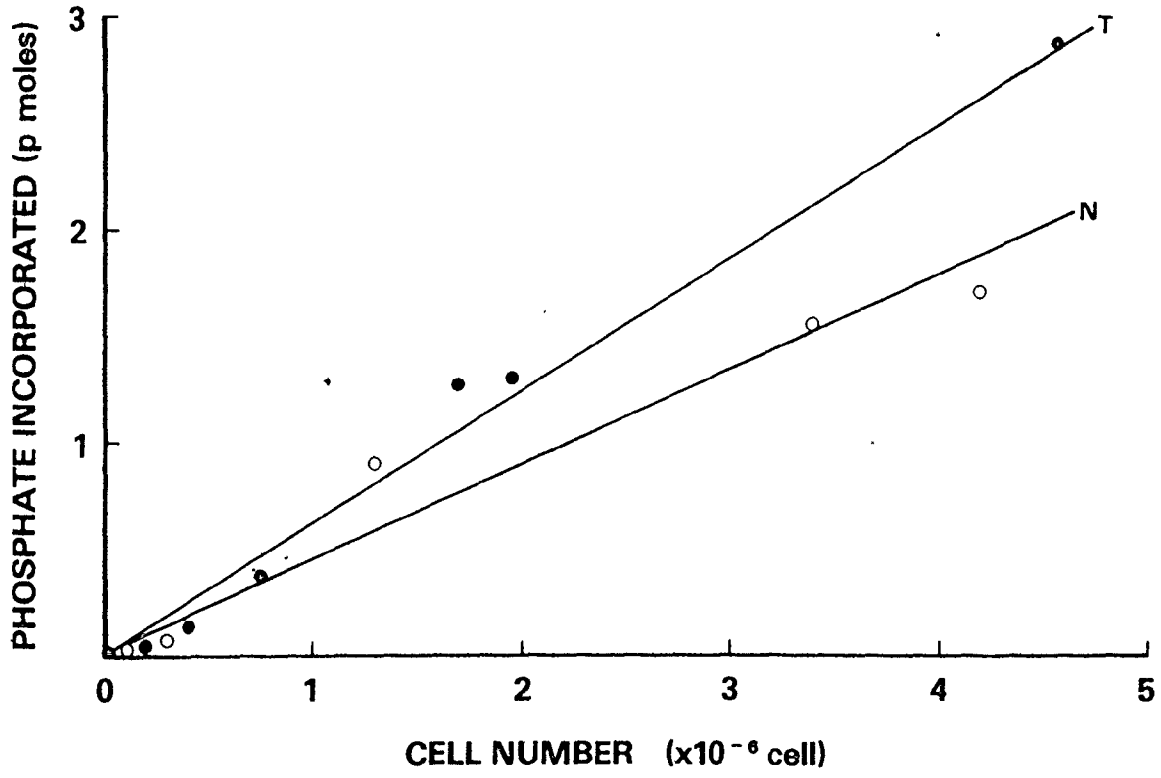

PHOSPHATE INCORPORATED (p moles)
3
2
1
0
1
2
3
4
5
CELL NUMBER (x10⁻⁶ cell)
T
N

FIGURE 6

Effect of Mg^{2+} concentration on cell surface protein kinase activity. Normal and RSV-transformed CEF were phosphorylated as described in Materials and Methods except that the Mg^{2+} concentration was varied as indicated. The number of pmoles of phosphate incorporated was determined from the incorporation of ^{32}P into TCA-insoluble material. The values given represent the average of two separate determinations.

RSV-CEF (●——●). Normal CEF (o——o).

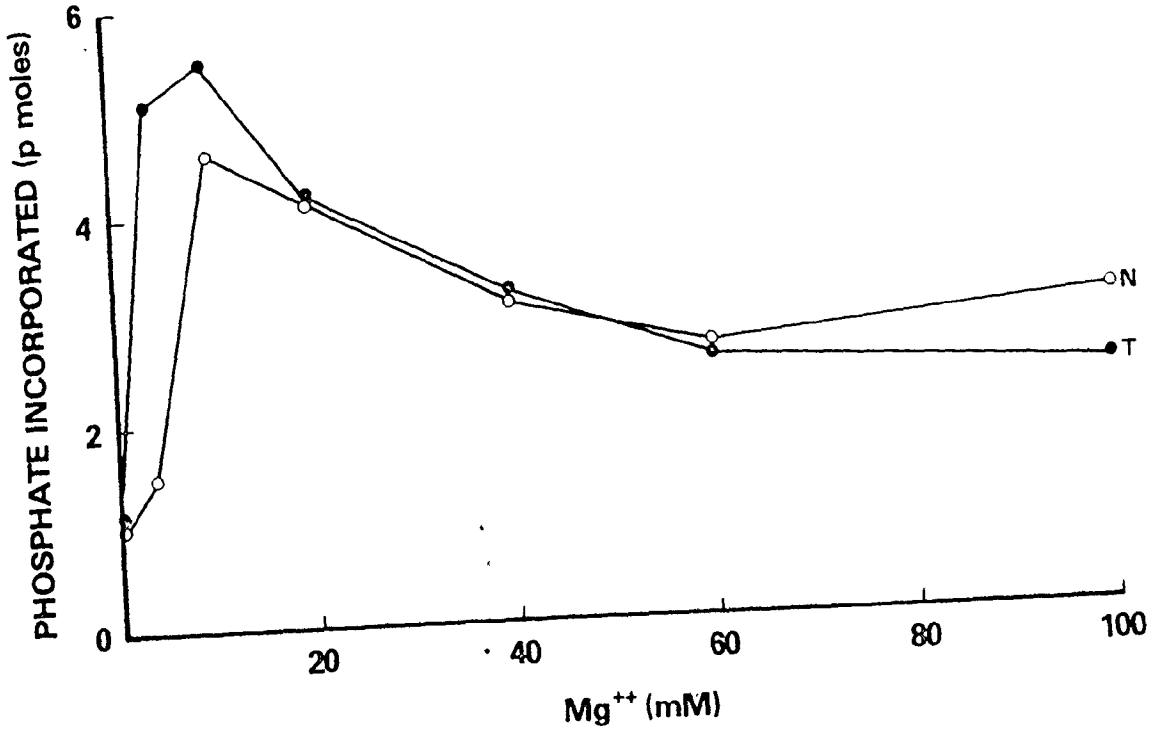

PHOSPHATE INCORPORATED (p moles)
6
4
2
0
20
40
60
80
100
Mg++ (mM)
N
T

FIGURE 7

Effect of ATP concentration on cell surface protein kinase activity. Normal and RSV-transformed CEF were phosphorylated as described in Materials and Methods except that the ATP concentration was varied as indicated. The number of pmoles of phosphate incorporated was determined from the incorporation of ^{32}P into TCA-insoluble material. The values given represent the average of two separate determinations.

RSV-CEF (●——●). Normal CEF (o——o).

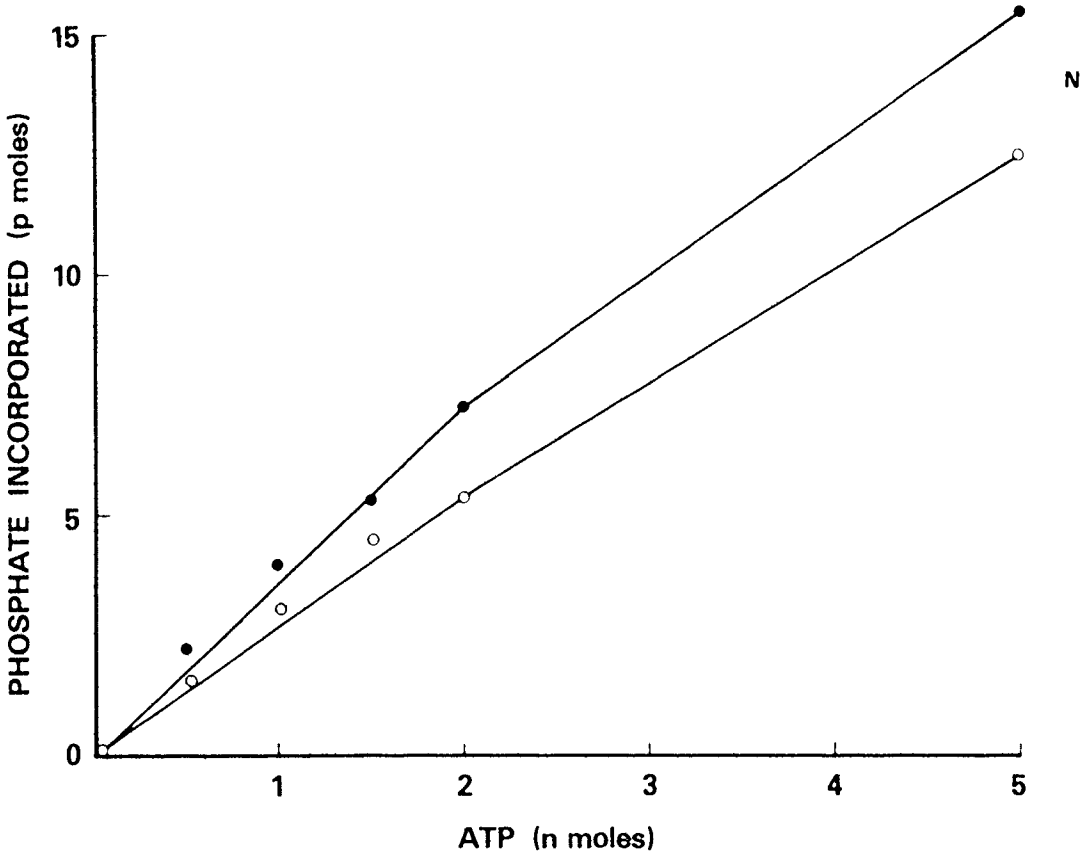

15
10
5
0
PHOSPHATE INCORPORATED (p moles)
1
2
3
4
5
ATP (n moles)
N

FIGURE 8

Effect of temperature on cell surface protein kinase activity. Normal and RSV-transformed CEF were phosphorylated as described in Materials and Methods except that the temperature was varied as indicated. The number of pmoles of phosphate incorporated was determined from the incorporation of ^{32}P into TCA-insoluble material. The values given represent the average of two separate determinations.

RSV-CEF (●——●). Normal CEF (o——o).

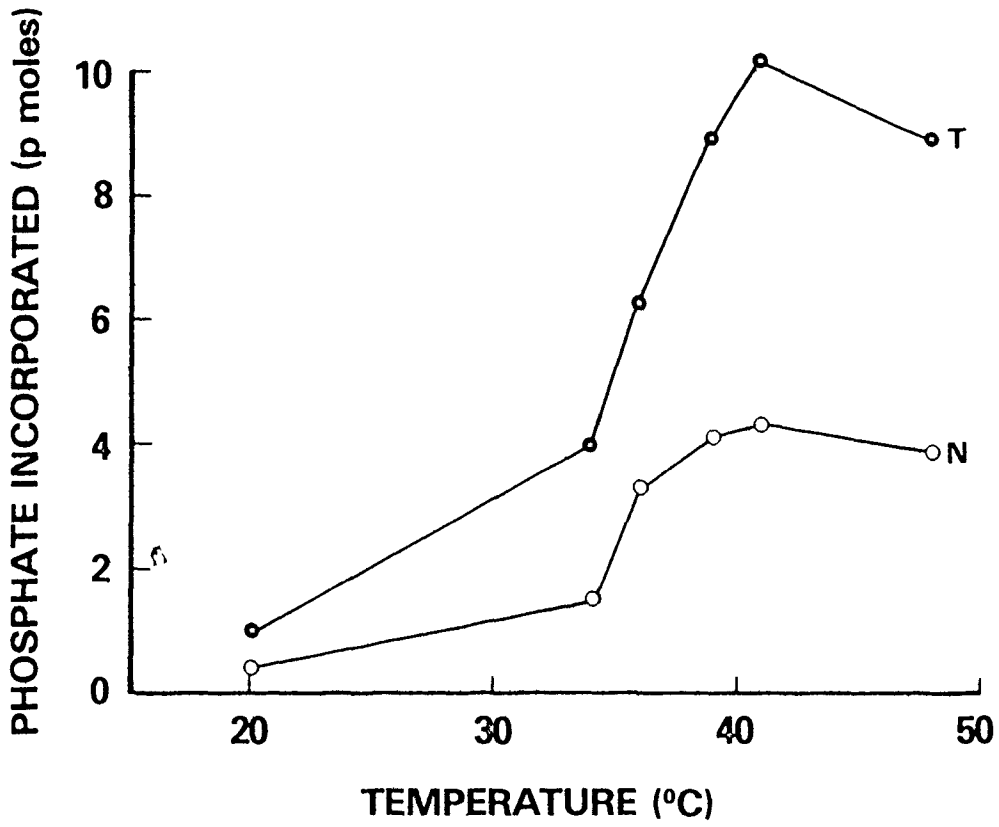
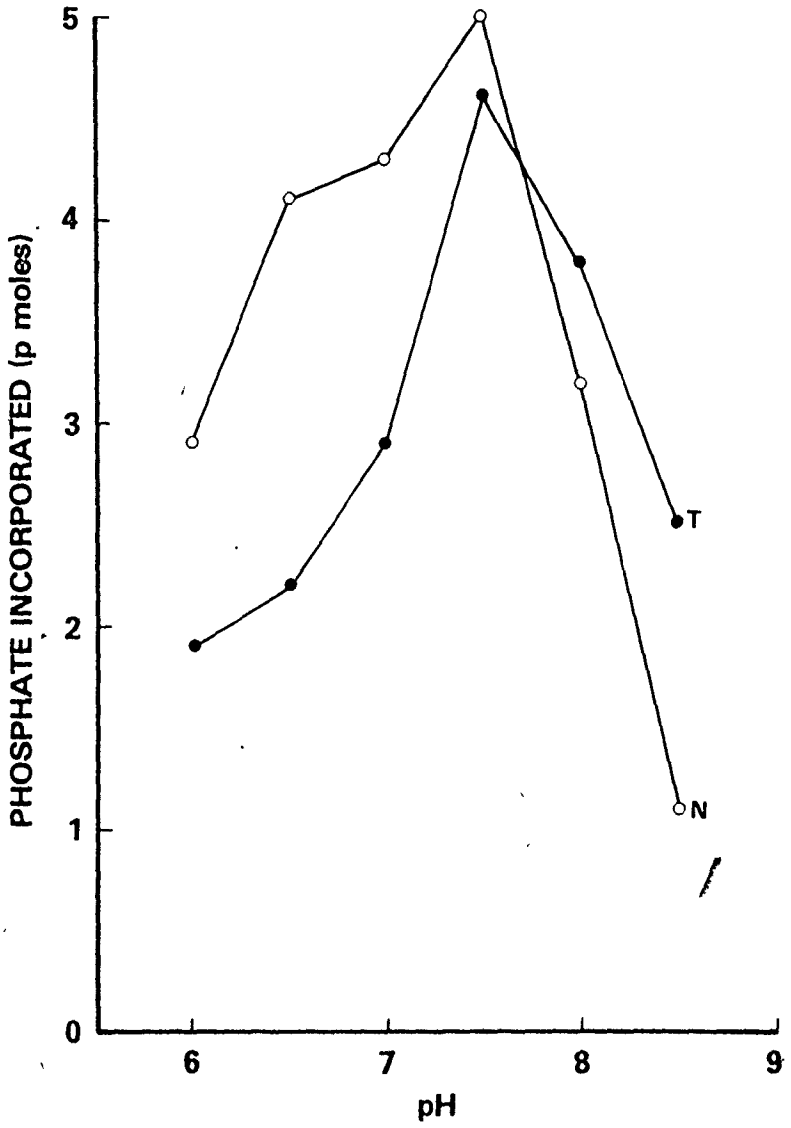

PHOSPHATE INCORPORATED (p moles)
10
8
6
4
2
0
20
30
40
50
TEMPERATURE (°C)
T
N

FIGURE 9

Effect of pH on cell surface protein kinase activity. Normal and RSV-transformed CEF were phosphorylated as described in Materials and Methods except that the pH was varied as indicated. The number of pmoles of phosphate incorporated was determined from the incorporation of ^{32}P into TCA-insoluble material. The values given represent the average of two separate determinations.

RSV-CEF (●——●). Normal CEF (o——o).


PHOSPHATE INCORPORATED (p moles)
5
4
3
2
1
0
6
7
8
9
pH
T
N

suggested that such was the case. While ATP does not enter the cell readily, it was possible that [γ-^{32}P]ATP was hydrolyzed, and internal proteins were phosphorylated from the incorporation of [^{32}P] phosphate, rather than from the direct utilization of [γ-^{32}P]ATP by external cell surface protein kinases. To investigate this possibility, two control experiments were carried out. First, the proportion of free [^{32}P] phosphate generated from [γ-^{32}P]ATP which had been incubated in the standard reaction mixture for 20 minutes at 37^{o}C was measured by ascending chromatography (see appendix II). Less than 10% of ^{32}P migrated away the bulk of the [γ-^{32}P]ATP (appendix II) indicating that hydrolysis occurred in only a minor fraction of the total ATP present. Furthermore, any free $^{32}PO_4$ should have been effectively diluted out by the 10,000 fold excess of cold phosphate present in the reaction mixture. Second, since free phosphate could enter the cell readily, it is possible that internal proteins would utilize the phosphate. In order to examine this possibility, the pattern of protein phosphorylation of RSV-transformed CEF labeled with [γ-^{32}P]ATP and [^{32}P] phosphate (equal amount of radioactivity and under the same conditions) were directly compared by SDS-PAGE. As shown in fig. 10, incorporation of radioactivity was detected in both cases. Over 25 polypeptides were phosphorylated when ATP was used. However, when [^{32}P] phosphate was used, fewer polypeptides were labeled and the amount of incorporation was very much reduced relative to [γ-^{32}P]ATP, and the pattern of phosphorylation was clearly different. Similar results were also obtained using normal CEF (data not shown). These results suggested that the protein phosphorylation found in the present system did not

FIGURE 10

Analysis of ^{32}P-labeled proteins by SDS-PAGE. RSV-transformed CEF were phosphorylated under various conditions and the labeled products were analyzed by SDS-PAGE. A. Standard cell surface phosphorylation (1.5 nmoles [$\gamma^{32}P$]ATP containing 45 µCi). B. Standard cell surface phosphorylation as in A, followed by treatment with 100 µg/ml trypsin for 10 min at 37°C. C. Cells incubated under standard conditions except 45 µCi of $^{32}PO_4$ used instead of [$\gamma^{32}P$]ATP. D. Arginine-rich histone (2 mg) was added to the reaction mixture used for a standard surface phosphorylation reaction. After incubation, the reaction mixture (containing histone) was removed and a small fraction was analyzed. For A to C, and equal amount of cell protein was analyzed.

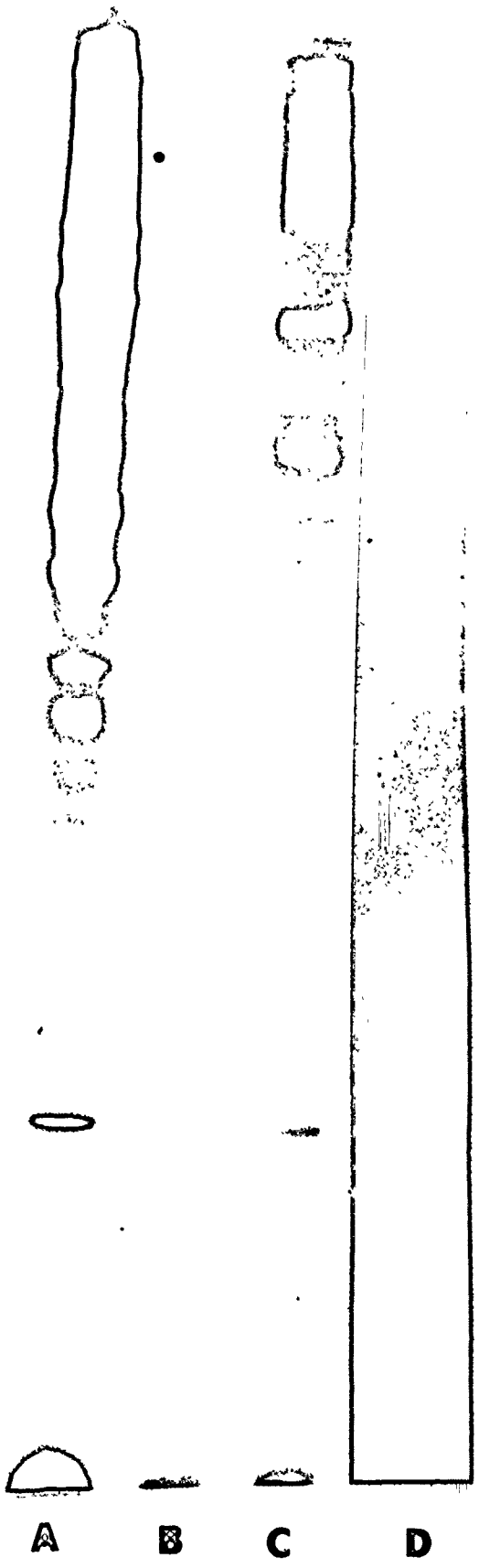


Fig 10

result from the hydrolysis of [γ-^{32}P]ATP and subsequent utilization of liberated $^{32}PO_4$.

Direct evidence also indicated that only external cellular proteins were phosphorylated. If the labeled proteins were located at the external cell surface, the radioactivity should be sensitive by trypsin treatment. As shown in fig. 10, RSV-transformed CEF were treated with crystalline trypsin following the standard phosphorylation procedure using [γ-^{32}P]ATP. Virtually all of the labeled material (>95%) was removed by the trypsin treatment. To demonstrate further that the protein kinase activity was present on the external cell surface, experiments were carried out in which histones were added to the reaction mixture as an exogenous substrate. The histones were then analyzed by SDS-PAGE. As shown in fig. 10, experiments using RSV-transformed CEF indicated that all three major separable arginine-rich histone species were labeled with ^{32}P. Similar results were also obtained using normal CEF (data not shown).

It was also possible that the observed protein kinase activity originated from enzymes that were released from dead cells. This possibility was examined by staining the monolayer with trypan blue following the washing and incubation procedure. There were very few, if any, stainable dead cells in the monolayer (data not shown). Also, the reaction mixture following the incubation procedure was recovered and assayed for protein kinase activities using [γ-^{32}P]ATP and histones as substrates. No detectable protein kinase activity was detected under these conditions (Table 4). These results indicated that significant amounts of protein kinase were not released into the

TABLE 4

Protein Kinase Activity to Conditioned Incubation Mixture

Cells	Assay Conditions*	Incorporation of ^{32}P (cpm)
CEF	+ cells	2453
	- cells	12
RSV-CEF	+ cells	3689
	- cells	9

*Phosphorylation was carried out as described in Materials and Methods except that the incubation mixture contained 2 mg of histone. The incubation mixture was collected after the incubation and any detached cells were removed by centrifugation. The histone was then precipitated by ice-cold TCA and radioactivity incorporated was measured. In another case, 2 ml of reaction mixture containing 2 mg of histone was incubated with cells under standard conditions except that no ATP was added. The incubation mixture was then collected and any detached cells were removed by centrifugation. Protein kinase activity in the conditioned incubation mixture was then assayed in the absence of any cells.

reaction mixture during the incubation period. Finally, protein kinase activity was also assayed with culture medium containing serum. Again, as shown in table 5, no detectable protein kinase activity were detected in culture medium. Thus, it appeared that incubation of normal and RSV-transformed CEF with [γ-^{32}P]ATP under the present conditions resulted primarily in the phosphorylation of external cell surface proteins by protein kinases located at the external surface of the plasma membrane.

D. Effect of Cyclic Nucleotides on External Surface Protein Kinase Acitivities

It is well known that the activities of certain classes of protein kinases can be stimulated by cyclic AMP and cyclic GMP. In order to determine if the external cell surface protein kinases can be stimulated by these cyclic nucleotides, cAMP and cGMP were added to the reaction mixture at various concentrations and phosphorylation was carried with both normal and RSV-transformed CEF, and the amount of ^{32}P incorporation into cell surface proteins was measured. In a companion experiment, histone was also present in the reaction mixture added to cells, and following the incubation period, this mixture was removed to determine the incorporation of ^{32}P into histone. As shown in figs. 11A and 11B, the incorporation of ^{32}P in both normal and RSV-transformed CEF was slightly stimulated by cAMP and cGMP. The maximum stimulation observed was 30% (i.e. 1.3 fold) which was not a great deal more than the level of experimental error. As a positive control, purified beef heart cAMP-dependent protein kinase was incubated with [γ-^{32}P]ATP and arginine-rich histone under the same condi-

TABLE 5

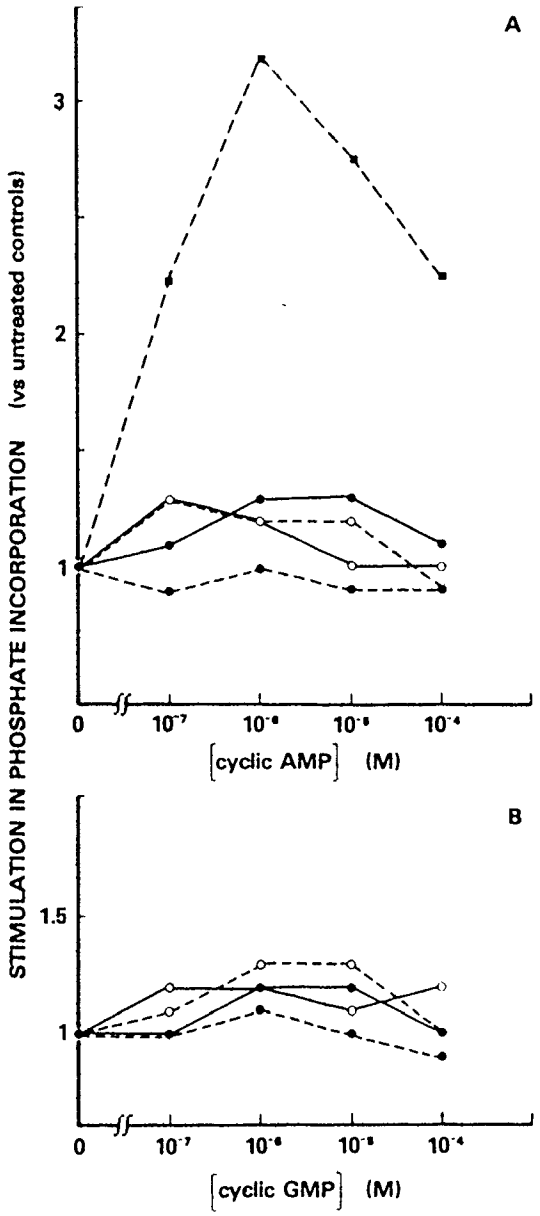
Protein Kinase Activity in Medium 199 (10:4:1)

Material	Incorporation of $^{32}P^{*}$ (cpm)
Medium 199 (10:4:1)	6
CEF	2453

*0.2 ml of medium 199 (10:4:1) was added to 2 ml of reaction mixture containing 2 mg of histone. Phosphorylation was carried out at 37°C for 20 min in the presence of [γ^{32}P]ATP. The reaction was stopped by adding 2 ml ice-cold 20% TCA. The precipitate was then collected and washed and the ^{32}P incorporated was measured by scintillation counting.

FIGURE 11

Effect of cyclic nucleotides on cell surface protein kinase activity. The standard reaction was carried out in the presence or absence of histone (2 mg) using reaction mixture containing various concentrations of either cyclic AMP or cyclic GMP. The amount of ^{32}P incorporated into cells was determined as described in Materials and Methods. With cultures incubated in the presence of histone, reaction mixture was removed, then centrifuged to remove detached cells and the ^{32}P present in histone was measured. The data has been plotted in terms of ^{32}P incorporation relative to samples containing no cyclic nucleotides. As a control, purified cyclic AMP-dependent beef heart protein kinase (5 µg) was reacted under identical conditions using histone substrate. A. Effect of cyclic AMP. (■---■) beef heart protein kinase. Normal CEF without (o——o) and with (o---o) exogenous histone. RSV-transformed CEF without (●——●) and with (●---●) histone. B. Effect of cyclic GMP. Same symbols as A. (No beef heart kinase studies included). The amount of phosphate incorporation for normal and transformed cells in the absence of histone was 3.45 and 3.80 pmoles, and incorporation into histone was 3.64 and 4.86 pmoles respectively. The data represent the average of 4 separate determinations. Experimental error overall was about 8-14%.


A
3
2
1
STIMULATION IN PHOSPHATE INCORPORATION (vs untreated controls)
0
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
[cyclic AMP] (M)
B
1.5
1
0
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
[cyclic GMP] (M)

tions. As shown in fig. 11A, over a 3-fold increase in kinase activity was observed with cAMP. These results suggested that if cyclic nucleotide-dependent protein kinases exist at external cell surface, they are present only in very low quantities. The analysis of the pattern of phosphorylation by SDS-PAGE also confirmed these results. The addition of cAMP and cGMP had no detectable quantitative effect of the phosphorylation of any polypeptides present in normal and RSV-transformed CEF (see below, Fig. 12).

E. External Cell Surface Protein Kinase Activity in Normal and RSV-Transformed CEF

The data described in Fig. 5 suggested that RSV-transformed CEF contained higher protein kinase activity than normal CEF. In order to examine this difference more precisely and determine whether or not it is transformation-specific, experiments were carried out with uninfected, wild-type-infected and temperature-sensitive mutant NY68-infected cells. Cells were cultured at both $36^{o}C$ and $41^{o}C$ and all assays were done at $36^{o}C$. In order to obtain a quantitative comparison, protein kinase activity was expressed in terms of $3X10^{6}$ cells or 500 µg of cell protein. As shown in Table 6, the following results were obtained: (i) culture temperature did not affect the kinase activity in uninfected cells, or in wild-type RSV-infected cells which were phenotypically transformed at both $36^{o}C$ and $41^{o}C$, (iii) approximately 1.5-fold higher kinase activity was found in wild-type RSV-transformed cells than in uninfected cells at both culture temperatures, (iii) with NY68-infected cells cultured at $36^{o}C$ the level of kinase activity was similar to wild-type infected cells, but

TABLE 6

External Cell Surface Protein Kinase Activity in Normal and RSV-Transformed CEF

CEF Infected with	Cell Growth Temperature	Phosphate Incorporation* (cpm per $3X10^6$ cells) [ratio vs uninfected at 36^oC]	Phosphate Incorporation* (cpm per 500 µg protein) [ratio vs uninfected at 36^oC]
uninfected	36^oC	937 ± 48 [1]	694 ± 36 [1]
	41^oC	914 ± 52 [0.98]	692 ± 40 [1.0]
wild-type SR-RSV(A)	36^oC	1426 ± 85 [1.52]	1063 ± 63 [1.53]
	41^oC	1446 ± 79 [1.54]	1007 ± 55 [1.45]
NY68	36^oC	1412 ± 47 [1.51]	1079 ± 36 [1.55]
	41^oC	990 ± 94 [1.06]	657 ± 63 [0.95]

*Uninfected, wild-type RSV-transformed and NY68-infected cells were cultured at both 36 and 41^oC. These cells were then phosphorylated under standard conditions at 36^oC and the amount of ^{32}P incorporation was measured. The results presented were from two completely separate experiments. In one, ^{32}P incorporation was normalized to cell number based on quadruplicate cell counts of identical sister cultures. In the other it was normalized to protein concentration as determined directly by the method of Lowry et al. (1951). The data presented represent the amount of ^{32}P incorporation (± the standard deviation) based on 4 separate determinations. Using Student's t-test, differences in ^{32}P incorporation for uninfected cells grown at 36 and 41^oC were not significant ($P>.25$), nor were they significant for wild-type RSV-transformed cells grown at the two temperatures ($P>.25$). There was a significant difference in incorporation between uninfected and wild-type RSV-transformed cells grown at 36^oC ($P<0.005$) and at 41^oC ($P<0.005$). With NY68-infected cells grown at 36^oC, ^{32}P incorporation was not significantly different from wild-type RSV-transformed cells ($P>.25$) but was significantly higher than with uninfected cultures ($P<0.005$). With NY68-infected cells grown at 41^oC, ^{32}P incorporation was significantly lower than with wild-type RSV-transformed cells ($P<0.005$), but was not significantly different than with uninfected cells ($P>.25$).

significantly (∿ 1.5-fold) higher than in uninfected cells; (iv) with phenotypically normal NY68-infected cells cultured at 41°C, the level of kinase activity was lower than that in wild-type infected cells, but it was similar to that in uninfected cells. Thus these results demonstrated that RSV-transformed cells possess approximately 50% higher external cell surface protein kinase activity than normal CEF and such increase was transformation specific.

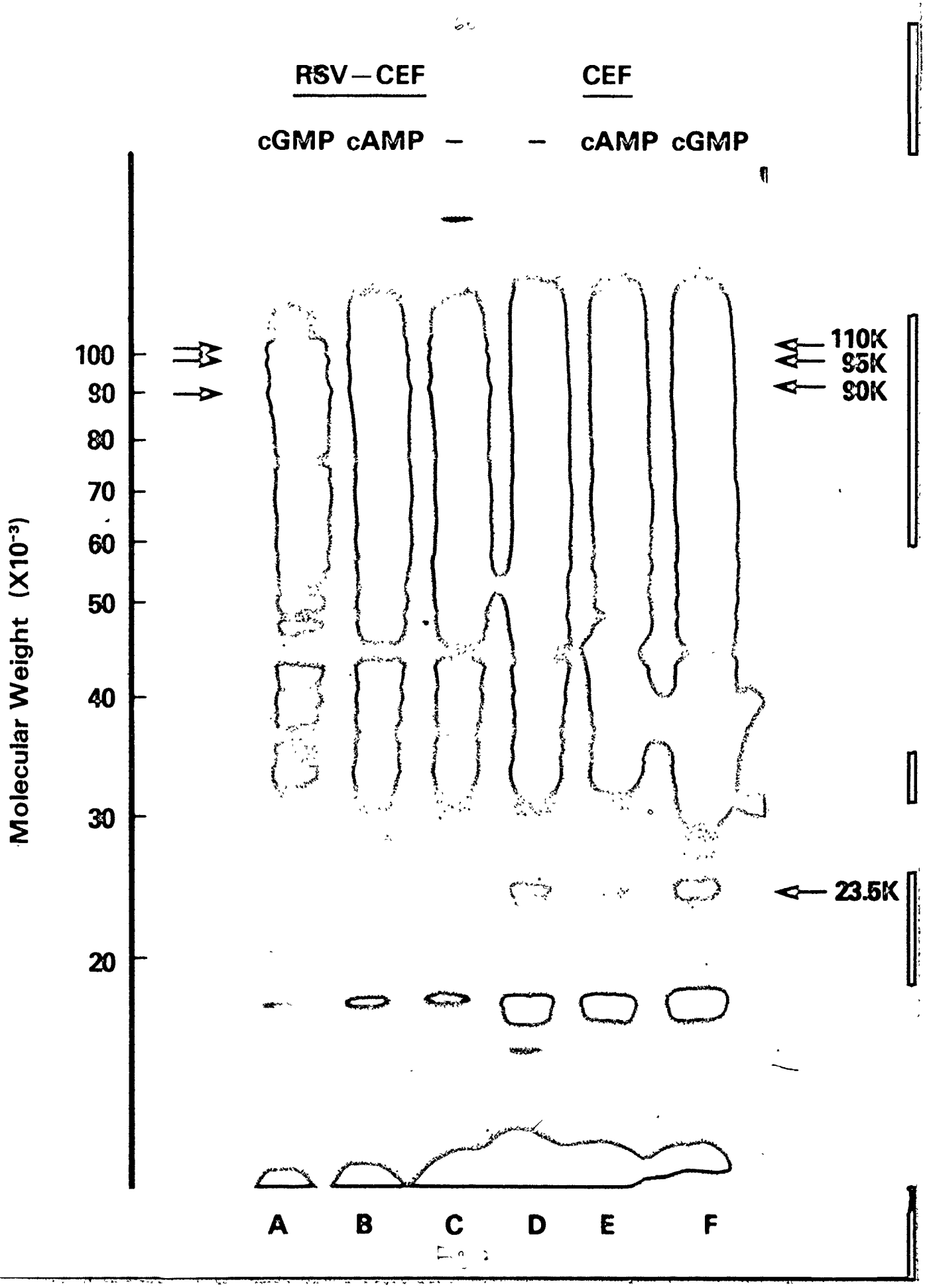
F. Analysis of the Pattern of External Cell Surface Protein Phosphorylation by SDS-PAGE

The pattern of external cell surface protein phosphorylation was analyzed by SDS-PAGE. As shown in Fig. 12, the pattern of labeled polypeptides with both normal and RSV-transformed CEF were quite complex. The phosphoproteins ranged in size from <15,000 to >200,000 daltons as judged by molecular weight markers. As discussed above (Page 52), addition of cAMP or cGMP to the reaction mixture had absolutely no effect on the pattern of phosphorylation with either normal or transformed cells, indicating that little or no cyclic nucleotide-dependent kinase activity was present on the external cell surface.

Although several quantitative differences were detected between the normal and RSV-transformed CEF, four major phosphoprotein alterations were consistently detected over the course of many experiments. Three polypeptides with molecular weights of 110,000, 95,000 and 90,000 (referred as 110K, 95K and 90K) were highly phosphorylated in RSV-transformed CEF but virtually not phosphorylated in normal CEF.

FIGURE 12

Pattern of cell surface protein phosphorylation in normal and RSV-transformed CEF as determined by SDS-PAGE. Normal and transformed CEF were phosphorylated under standard conditions in the absence of cyclic nucleotides or in the presence of cyclic AMP(10^{-6}M) or cyclic GMP(10^{-5}M). An equal amount of radioactivity for each was analyzed by SDS-PAGE. Transformed CEF incubated in the presence of cyclic GMP (A), cyclic AMP (B) or no cyclic nucleotides (C). Normal CEF incubated in the absence of cyclic nucleotides (D) or in the presence of cyclic AMP (E) or cyclic GMP (F).

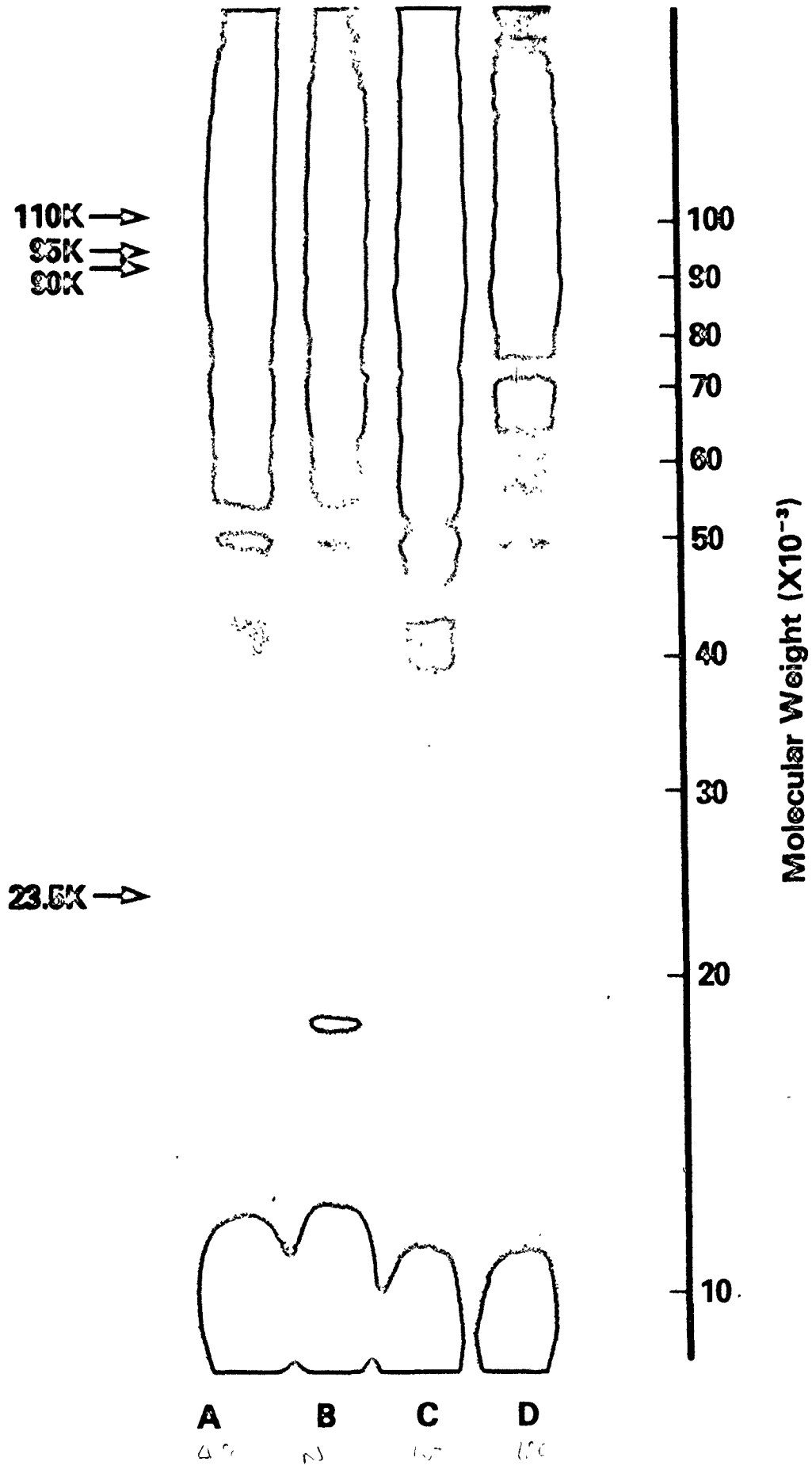

RSV—CEF
CEF
cGMP cAMP — — cAMP cGMP
Molecular Weight (X10⁻³)
100
90
80
70
60
50
40
30
20
110K
95K
90K
23.5K
A
B
C
D
E
F

In addition, a protein with a molecular weight of 23,500 was phosphorylated far more in normal than in RSV-transformed CEF. It was unlikely that these differences in protein phosphorylation resulted from the presence of different amounts of these proteins on the normal and RSV-transformed cells, as Coomassie blue-stained patterns were identical in these regions (data not shown).

In order to examine whether these differences in protein phosphorylation were transformation-specific, uninfected, wild-type RSV-infected CEF and CEF infected with NY58 cultivated at 36°C and 41°C were phosphorylated. As shown in Fig. 13, differences in the phosphorylation of these polypeptides were again detected between normal and RSV-transformed CEF. Again, the phosphorylation of 110K, 95K and 90K was far higher in wild-type RSV-transformed cells than in uninfected cells, and the phosphorylation of 23.5K was much less (Figs. 12A and B). The phosphorylation pattern of NY68-infected CEF cultivated at the permissive temperature was very similar to that found with wild-type RSV-infected CEF (Fig. 13D). With NY68-infected CEF cultured at the non-permissive temperature (Fig. 13A), no phosphorylation of 95K was detected and the phosphorylation of 23.5K was reasonably high. Some phosphorylation of 110K and 90K was detected in these cells. The level of phosphorylation of these two peptides was higher than in uninfected cells but lower than in transformed cells. Thus these results suggested that the phosphorylation differences in 95K and 23.5K appeared to be transformation-specific whereas phosphorylation of 110K and 90K may not be.

FIGURE 13

Cell surface phosphoproteins of normal and wild-type or NY68-infected CEF. Uninfected and wild-type RSV-tranformed CEF grown at 36°C or NY68-infected CEF grown at either 36 or 41°C were phosphorylated under standard conditions. Equal amounts of radioactivity were analyzed by SDS-PAGE. A. NY68-infected cells grown at 41°C. B. Uninfected normal cells. C. Wild-type RSV-transformed cells. D. NY68-infected cells grown at 36°C.


110K
95K
90K
23.5K
100
90
80
70
60
50
40
30
20
10
Molecular Weight (X10⁻³)
A
B
C
D

DISCUSSION

The present study was carried out to determine the effect of RSV transformation on external cell surface protein phosphorylation in chick embryo fibroblasts. By incubating intact cells on culture plates with [γ-^{32}P]ATP, protein kinase activity and phosphate acceptor proteins were detected on the external cell surface of both normal and RSV-transformed CEF. Transformation induces changes in both external surface kinase activity and surface protein phosphorylation.

It was important to ensure that the incorporation of ^{32}P resulted from the phosphorylation of cell surface proteins by external surface protein kinase. Analysis of the labeled material using several enzymatic and chemical treatments indicated that neither nucleic acids nor phospholipids were labeled to any significant degree by ^{32}P. Proteins were indeed found to be the only phosphorylated species and ^{32}P was bound to serine and threonine by a monoester linkage.

Several lines of evidence indicated that the protein kinases and substrates are located at the external surface of the cells. Firstly, the incubation was carried out under conditions in which ATP does not enter the cell. Because ATP is not transported into intact cells, it was likely that only external cell surface protein kinases and substrates could participate in the phosphorylation reaction. However, it was possible that ATP was hydrolyzed by cellular ATPases and that ^{32}P incorporation resulted from liberated $^{32}PO_4$. This possibility seems unlikely as an ATPase inhibitor, NaF, was present in the

reaction mixture throughout the entire incubation period. Nevertheless, small amounts of ATP might still be hydrolyzed as it may not be totally stable under the conditions of incubation. The amount of $^{32}PO_4$ liberated during the course of the incubation period was measured and less than 10% of ATP was hydrolyzed. Also, there was a 10,000-fold excess of cold PO_4 relative to 1.5nm of [γ-^{32}P]ATP present in the reaction mixture. Thus any $^{32}PO_4$ liberated from this [γ-^{32}P]ATP should have been effectively diluted out. In order to further examine whether or not significant ^{32}P incorporation could have resulted from liberated $^{32}PO_4$, cells were incubated with equal amounts of either $^{32}PO_4$ or [γ-^{32}P]ATP. The results indicated that ^{32}P incorporation was considerably reduced using $^{32}PO_4$ and that the pattern of phosphorylation was markedly altered compared with that obtained using [γ-^{32}P]ATP. Again, these data indicated that the cell surface phosphorylation did not result from incorporation of free $^{32}PO_4$. In addition, if significant incorporation of $^{32}PO_4$ resulted from hydrolysis of ATP, nucleic acids and phospholipids should have been highly labeled, and such was not the case. Secondly, virtually all of the incorporated ^{32}P was removed by trypsin treatment of cells following standard incubation with [γ-^{32}P]ATP. This result strongly suggested that all of the labeled proteins were present on the external cell surface. If significant incorporation resulted from $^{32}PO_4$ which can be transported into intact cells readily, it would have been expected that many of the ^{32}P-labeled protein would be resistent to trypsin treatment. Finally, the demonstration that exogenous histone could serve as a substrate suggested that the protein kinase activity being measured

was present on the external surface of the cells. It was possible that ^{32}P incorporation was due to cytoplasmic protein kinases released from cells into the reaction mixture or to protein kinases originating from culture medium trapped in the cell monolayer after the washing procedure. However, experiments carried out to directly test these possibilities indicated that virtually no detectable protein kinase activity was present in culture medium containing serum or in the reaction mixture after the 20 minute incubation period. All these data indicated that chick cells possess external cell surface protein kinases capable of phosphorylating other cell surface proteins or exogenous protein substrates.

Various biochemical properties of chick cell surface kinase were examined. The kinases present on both normal and RSV-transformed chick cells had properties similar to those of other protein kinases including dependence on Mg^{2+} (optimum $\sim$ 10mM) and ATP concentration. The temperature optimum was about, $41^{\circ}C$, the body temperature of chickens. The optimal pH was 7.5. The incorporation of ^{32}P increased linearly with the period of incubation and with the number of cells in the culture. The properties of cell surface protein kinase in normal and RSV-transformed chick cells were very similar, if not identical.

The cyclic nucleotide-dependence of cell surface kinases was also examined. Little effect of cyclic nucleotides was observed either by the measurement of total ^{32}P incorporation or by the detection of increased phosphorylation of specific polypeptides as shown by SDS-PAGE. Thus even though significant levels of cyclic AMP-dependent protein kinases have been detected in purified chick

cell plasma membrane preparations (Branton and Landry-Magnan, 1978; 1979) little such activity is expressed at the outer surface of the cells. It is well known that cyclic AMP does not penetrate the cell readily. Therefore it is possible that cyclic AMP-dependent protein kinases exist at the cell surface, however, both the regulatory and catalytic subunits (cf. Krebs and Beavo, 1979) would have to be present at the external cell surface in order to be detected in the present assay. Dibutyryl-cyclic AMP, which is an analog of cyclic AMP and is transported into intact cells readily, was used in the present assay in order to detect any cell surface cyclic AMP-dependent protein kinases in which the regulatory subunit is not located in the external plasma membrane. The results were identical to those obtained with cyclic AMP (data not shown). These data together suggested that little cyclic AMP-dependent protein kinase was present on the external cell surface. External protein kinase activity has also been detected in normal and SV40-transformed 3T3 cells (Mastro and Rozengurt, 1976), as well as with normal human fibroblasts and fibrosarcoma cells (Chiang, Kang and Kang, 1979). A lack of cell surface cAMP-dependent protein kinase was also observed with these cell types. In contrast, Schlager and Köhler (1976) reported the presence of cyclic AMP-dependent protein kinase activity on the outer surface of c-6 glioma cells. Thus the absence of this class of enzyme may be typical of fibroblasts and not of all cell types.

The levels of external cell surface protein kinase activity were normalized to a per cell or per mg cellular protein basis in order to compare the difference in activity between normal and RSV-

transformed chick cells. The results revealed that a 50% higher activity was found on RSV-transformed chick cells than on normal chick cells. That this difference was specifically related to transformation, and not simply to replication of RSV, was shown in a study carried out with the RSV src mutant, NY68, which is temperature-sensitive for transformation but not virus production. The results from these studies clearly showed that transformation was associated with the increase in kinase activity and such increase was under the control of the RSV src gene. A similar difference was also observed in comparisons made between 3T3 and SV40-transformed 3T3 cells (Mastro and Rozengurt, 1976), and thus viral transformation may generally be associated with increased cell surface kinase activity.

There are several possible explanations for the higher external cell surface protein kinase activity found in RSV-transformed chick cells. It could result from the presence of an increased amount of available substrates on the surface of transformed cells, as was suggested in the case of SV40-transformed 3T3 cells (Mastro and Rozengurt, 1976). However, this possibility seems unlikely in RSV-transformed chick cells since the patterns of protein phosphorylation in normal and RSV-transformed CEF were similar, with some exceptions. Phosphoprotein phosphatase has been detected at the external cell surface of 3T3 cells and such activity is higher than in SV40-transformed 3T3 cells (Makin, 1979). It is possible therefore that such phosphatase are also present at the external cell surface of chick cells and that the increased ^{32}P incorporation observed with RSV-transformed CEF was due to lower levels of external cell surface

phosphoprotein phosphatase.

There are several reports indicating that the RSV src protein ($pp60^{src}$) is a protein kinase (Collett and Erikson, 1978; Levinson et al., 1978). It is tempting to postulate that the increased ^{32}P incorporation in RSV-transformed cells is due to the presence of $pp60^{src}$ at the external cell surface. It is now believed that $pp60^{src}$ is partially, if not entirely, associated with plasma membranes (Brugge et al., 1978; Willingham et al., 1979; Krueger et al., 1980; Courtneidge et al., 1980). It has also been found that plasma membrane purified from RSV-transformed CEF contain both $pp60^{src}$ and src-related protein kinase activity (Branton, unpublished result). However, in studies using immunofluorescence involving src-specific antiserum, Rohrschneider (1979) concluded that $pp60^{src}$ was not found at the outside surface of RSV-transformed cells. It is known that $pp60^{src}$ undergoes an apparent autophosphorylation in the presence of ATP (Erikson et al., 1979). In studies using cell surface phosphorylation as employed in this report and antiserum from tumor-bearing rabbits (supplied by Dr. J. Buchanan, M.I.T.) that contains antibodies to $pp60^{src}$, I have been unable to precipitate a ^{32}P-labeled polypeptide with the characteristics of $pp60^{src}$. Furthermore, unlike other classes of known protein kinases that phosphorylate serine and threonine (Krebs and Beavo, 1979), it has recently been shown that $pp60^{src}$ protein kinase specifically phosphorylates tyrosine (Hunter and Sefton, 1980; Collett et al., 1980). The data in this report indicate that virtually all of the ^{32}P incorporation is alkali labile, while the tyrosine-phosphate bond is relatively alkali stable. In addition, the

majority of ^{32}P was incorporated into phosphoserine and only a small percentage was incorporated into phosphothreonine as revealed by the high voltage paper electrophoresis separation. Since the phosphotyrosine is relatively unstable under standard hydrolysis conditions and also it would comigrate with phosphothreonine at low pH, a second attempt took place to separate these amino acids. The phosphorylated polypeptides were hydrolyzed under milder conditions and the phosphoamino acids were separated using two dimensional electrophoresis. Again the data confirmed the previous result that the majority of ^{32}P was incorporated into phosphoserine and virtually no ^{32}P-phosphotyrosine was detected. Thus these data suggest that pp60$^{\underline{src}}$-specific protein kinase does not contribute significantly to the external surface phosphorylation reaction. However, because the increased level of surface kinase activity in the RSV-transformed CEF appears to be under the control of pp60$^{\underline{src}}$, this virus transforming protein could regulate the activity of other surface kinases (or phosphoprotein phosphatases) directly via phosphorylation. It is also possible that changes in the plasma membrane, including loss of fibronectin or cytoskeletal elements, alterations in membrane fluidity, etc. (cf. Nicolson, 1976) induced by pp60$^{\underline{src}}$, could lead indirectly to altered enzymatic activity through configurational modifications in the membrane.

The patterns of phosphorylation as analyzed by SDS-PAGE indicated four reproducible phosphorylation differences between normal and transformed CEF. Using cells infected by the temperature-sensitive src mutant, NY68, it was shown that the increased phosphory-

lation of two of these proteins 110K and 90K, which were phosphorylated more in transformed than in normal cells, did not appear to be entirely transformation-specific. The level of phosphorylation of these two polypeptides in phenotypically normal NY68-infected cells cultured at 41°C, was higher than with normal infected cells, but considerably less than with transformed CEF. This result could have been due to leakiness of the mutant, however, it is more likely that virus replication which is normal at 41°C, affects their phosphorylation to some extent. Neither the identity nor the function of these polypeptides is known. It is possible that 90K is $Pr90^{env}$, the precursor to RSV envelop antigen (Eisenman and Vogt, 1978), however, this protein is not normally phosphorylated. A third polypeptide, 23.5K was phosphorylated more in normal than in transformed cells and this difference was transformation-specific. The increased phosphorylation of a fourth polypeptide, 95K, also appeared to be transformation-specific. This protein could be related to the 95,000 dalton protein shown to play a role, along with a 75,000 dalton protein, in hexose transport (Banjo and Perdue, 1976; Shiu et al., 1977). In a study in which plasma membranes purified from normal and RSV-transformed CEF were phosphorylated *in vitro* using [γ-^{32}P]ATP, it was found that increased phosphorylation of 75K and 95K polypeptides occurred following transformation (Branton and Landry-Magnan, 1979). Increased hexose transport is an early phenotypic change associated with the onset of transformation. Thus it is possible that phosphorylation of 75K and 95K play a role in altering sugar transport.

It is clear that a number of pathways in the cell are regulated

by protein phosphorylation mediated by protein kinases (Rubin and Rosen, 1975; Krebs and Beavo, 1979; Greengard, 1978). With the presence of both protein kinases and phosphoprotein phosphatases (Makan, 1979) at the cell surface, the possibility is raised that protein phosphorylation-dephosphorylation represents a mechanism in the control of membrane-dependent properties. Cell surface mitogen receptors may be regulated by this phosphorylation-dephosphorylation mechanism in a fashion similar to that proposed for glucocorticoid receptor (Nielson et al., 1977). In addition, external ATP is known to alter a number of cell surface-dependent phenomena. The addition of exogenous ATP will alter the cell volume (Stewart et al., 1969) in ascites tumor cells, cell adhesion, aggregation and movement in fibroblasts (Jones, 1966; Knight et al., 1966) and prevents insulin stimulation of glucose transport in adipocytes (Chang and Cuatrecasas, 1974). External ATP has also been shown to change membrane permeability to various metabolites such as uridine, adneosine and 2-deoxyglucose in a transformed mouse cell line but not in normal mouse cells (Rozengurt et al., 1977; Makan, 1978; Rozengurt and Heppel, 1979; Dicker et al., 1980). Evidence exists that ATP may be translocated from the cytosol to the exterior of the cell (Trams. 1974) and ATP may also be synthesized at the external surface of the cell (Agren and Ronquist, 1969). The present data showing that both kinases and their phosphate acceptor proteins are detected in the external plasma membrane suggest that at least some of the diverse effects of external ATP on the cell may be mediated vira phosphorylation-dephosphorylation of the surface membrane protein.

At present, it is not clear if surface phosphorylation occurs between kinases and substrates on the same cell or if phosphorylation can occur via enzymes on neighboring cells. However, the incorporation of ^{32}P increased linearly as the cell number increased. These data suggested that phosphorylation does not occur via enzymes on neighboring cells alone. If this were the case, a rapid increase in incorporation of ^{32}P would have been expected when cells came in contact with neighboring cells ($\sim$ 0.8-1.0X10^6 cells per 60mm plates) and such was not observed. However, because cell surface kinases are capable of phosphorylating exogenous histone, it is possible that cell surface protein phosphorylation could occur by enzymes on the same cell and by those on neighboring cells. Such external cell surface protein kinases could therefore play a major role in the regulation of cell activity and behavior.

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APPENDIX I

Synthesis and Characterization of Phosphotyrosine

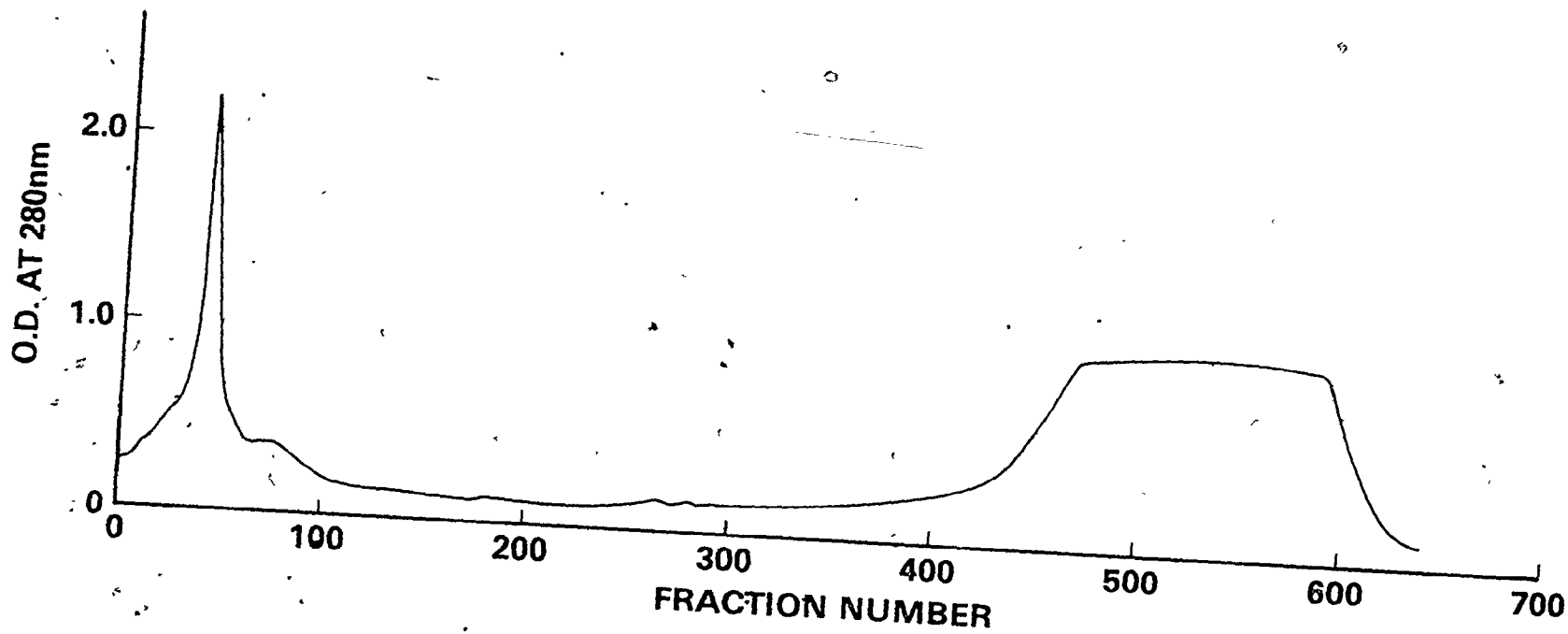
Phosphotyrosine is not commercially available and therefore must be synthesized in the laboratory. Phosphotyrosine was synthesized by a method similar to that of Mitchell and Lunan (1964). Five grams of tyrosine and 15 grams of phosphoric acid anhydride were added to 35 grams of 85% phosphoric acid. The mixture was heated at 110°C for 24 hrs. After addition of a further 5 grams of phosphoric acid anhydride, heating was continued for another 24 hrs after which the process was repeated with an additional 3 grams of phosphoric acid anhydride. The mixture was then dissolved in 150 ml of water containing 50 grams of ice.

The phosphotyrosine was purified on a Dowex 50x8 column (5.0 x 30 cm, H^+ form) and the column was washed with water. Fractions eluting from the column contained two peaks as monitored by absorbance at 280 mm: peak I (beginning at the void volumn of the column) that contained tyrosine and inorganic phosphate, and peak II, eluting over a large number of fractions. Results are shown in fig. 14. Those fractions of peak II with an absorbance of >0.8 were pooled together and mixed with 1500 ml ethanol:ether (1300:200, vol:vol) and stored at 4°C for 48 hrs. The white precipitate was recovered by filtration, washed with the ethanol:ether solution and was dried in air.

Over 3.6 grams of white crystal was recovered. The product was examined to verify whether it was phosphotyrosine or not. The

FIGURE 14

Purification of phosphotyrosine. Phosphotyrosine was synthesized as described in appendix I and was purified on a Dowex 50x8 column (5.0x30 cm, H^+ form). The phosphotyrosine was eluted with H_2O and 5 ml fractions were collected. Each fraction was monitored by absorbance at 280 nm.


O.D. AT 280nm
2.0
1.0
0
0
100
200
300
400
500
600
700
FRACTION NUMBER

melting point was measured and the white crystal melted between 246°-247°C, which is the melting point of phosphotyrosine. The solution of the white crystal with a concentration of 1 mg/ml was treated with alkaline phosphotase (10 μ/ml) for 1 hour at 37°C in 0.05M Tris-HCl (pH8.0). This treatment was carried out to remove the phosphate ester bonding of phosphotyrosine. The mobility in thin-layer electrophoresis of the treated sample was measured and compared with untreated sample and tyrosine, as well as phosphoserine and phosphothreonine. Thus, if the white crystal was indeed phosphotyrosine, the alkaline phosphatase-treated sample should comigrate with tyrosine in the thin-layer electrophoresis.

As shown in fig. 15, while phosphoserine, phosphothreonine and the untreated phosphotyrosine migrated towards the anode, tyrosine and the alkaline phosphatase-treated phosphotyrosine migrated towards cathode. The tyrosine and alkaline phosphatase-treated phosphotyrosine both migrated an equal distance away from the origin. These results indicated that the laboratory synthesized product was indeed phosphotyrosine.

FIGURE 15

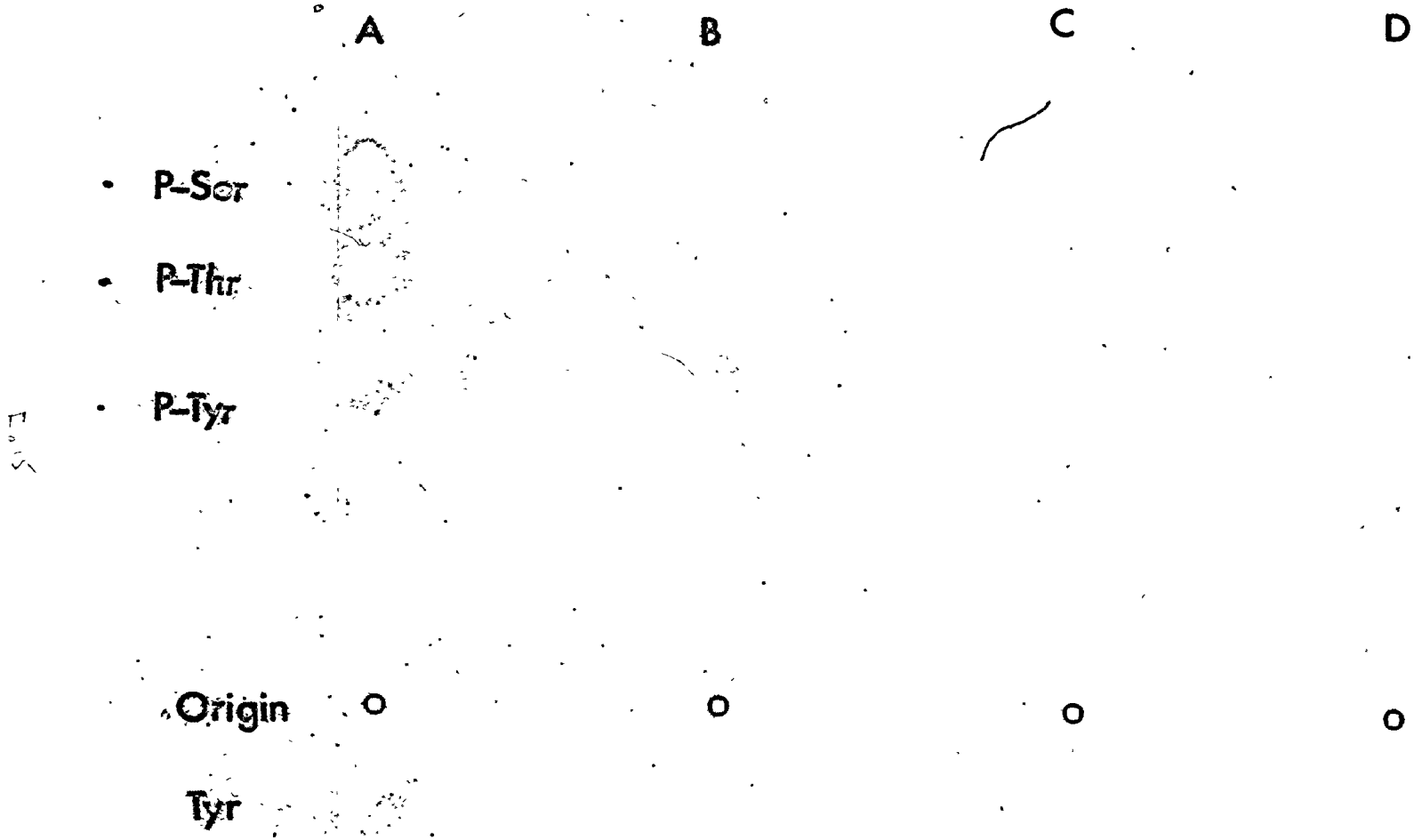
Characterization of phosphotyrosine by thin-layer electrophoresis. The phosphoamino acids were analyzed by thin-layer electrophoresis at pH3.5 for 30 min at 1500 V in a buffer containing glacial acetic acid, pyridine and water in a ratio 50:5:945 (vol:vol). The position of the phosphoamino acids are visualized by ninhydrin-staining and are shown together with the origin. The samples were as follows:

A. A mixture of phosphoserine, phosphothreonine, phosphotyrosine and L-tyrosine.

B. Phosphotyrosine alone.

C. L-tyrosine alone.

D. Phosphotyrosine treated with alkaline phosphatase as described in the text.


A
B
C
D
P-Ser
P-Thr
P-Tyr
Origin
Tyr

APPENDIX II

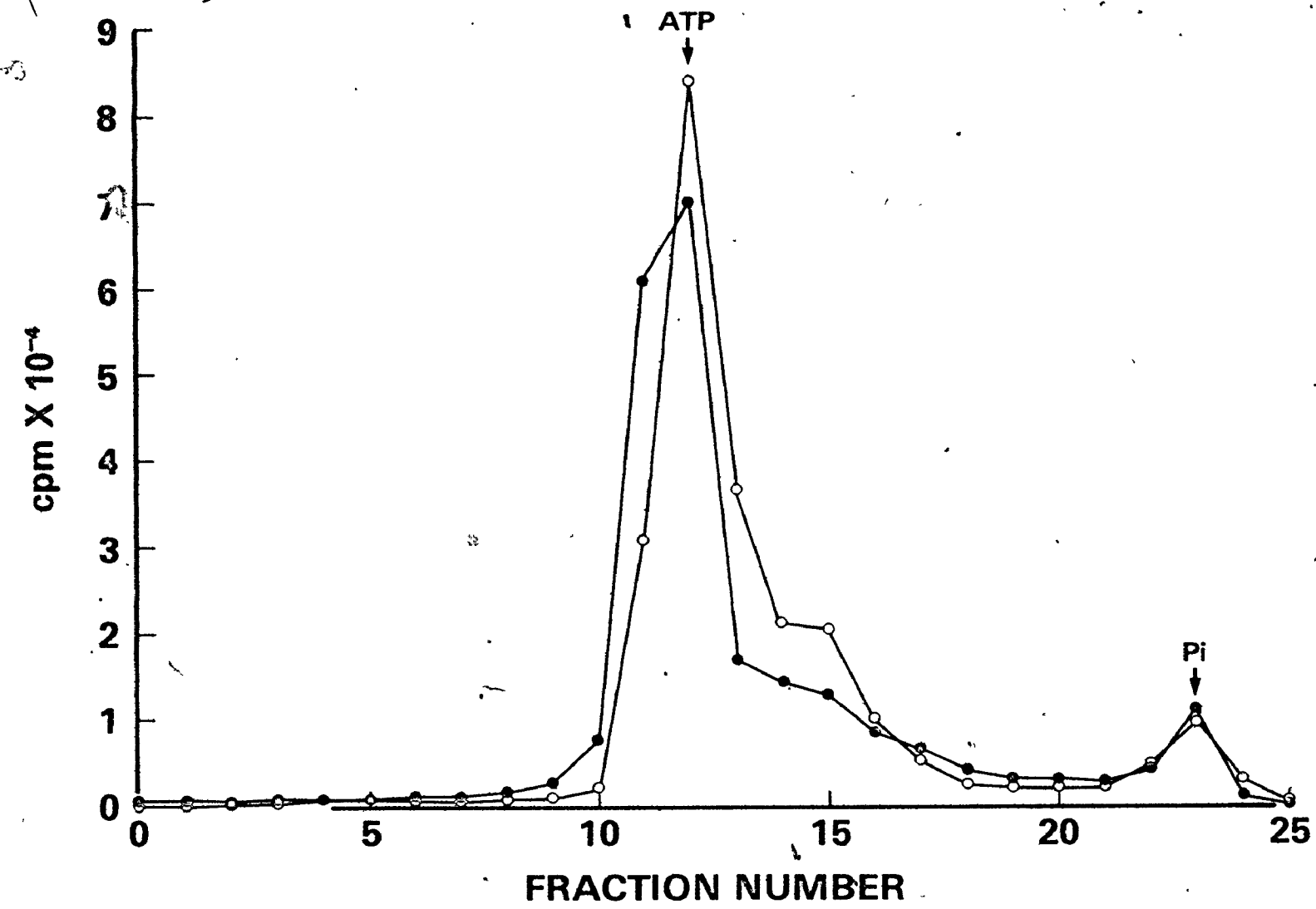
Hydrolysis of ATP

In order to get an estimate of the amount of ATP hydrolysed throughout the incubation period, [γ-^{32}P]ATP was added to incubation mixture and incubated for 20 min. at 37^{o}C. Following the incubation, 2 µl of 0.1M ATP were mixed with 100 µl of the incubation mixture as a carrier and the combination was spotted on DEAE cellulose paper. The ATP and phosphate were separated by ascending chromatography. The result is shown in fig. 16. A small peak of radioactivity was detected close to the solvent front. This peak represented the bulk of ^{32}P-phosphate. The major peak of radioactivity which comigrated with the ATP carrier (visible under UV-light at 280 nm) represented the unhydrolysed ATP. The phosphate and ATP were well separated and represented 8.2% and 91.8% of the total radioactivity detected, respectively.

The phosphate detected in the present study could have resulted from hydrolysis of ATP during the incubation period and/or during the process of separation, or from contaminating phosphate present in the stock [γ-^{32}P]ATP. In order to examine this further, another experiment was done in the same way except that no incubation took place. The result is also shown in fig. 16. Again phosphate and ATP were well separated and represented 7.5% and 92.5% respectively, of the total radioactivity detected. The phosphate detected in the second study probably resulted from ATP hydrolysed during the separation process and/or contaminant phosphate present in the ATP stock solution.

FIGURE 16

Separation of ATP and phosphate by ascending chromatography. Reaction mixture containing [γ^{32}P]ATP was subjected to ascending chromatography using DEAE cellulose paper with a buffer containing 100 mM ammonium formate and 17.5% formic acid. The DEAE cellulose paper was then air dried and cut into 0.5 cm strips. The radioactivity on each strip was then determined by scintillation counting. The direction of the chromatography is from left to right. The value given represents the average of two separate determinations. Reaction mixture containing [γ^{32}P]ATP incubated (o——o) and incubated for 20 min at 37°C (●——●) prior to analysis.


ATP
Pi
cpm X 10⁻⁴
0
1
2
3
4
5
6
7
8
9
0
5
10
15
20
25
FRACTION NUMBER

Nevetheless, the amount of ^{32}P-phosphate present after 20 minutes incubation was at most 8.2% of the total radioactivity present.