THE POST-TRANSLATIONAL PROCESSING OF SINDBIS VIRUS GLYCOPROTEINS

Thesis by

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"We know all about the habits of the ant, we know all about the habits of the bee, but we know nothing at all about the habits of the oyster. It seems almost certain that we have been choosing the wrong time for studying the oyster."

Mark Twain

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ABSTRACT

A small glycoprotein (E3) was purified from the culture fluid of Sindbis virus infected chicken cells and shown to be produced from the cleavage of PE2 to produce E2. The N-terminal sequence of E3 is identical to that of PE2. The first 19 amino acids are hydrophobic and presumably serve as the signal sequence for PE2. This sequence is unusual in that it is not immediately cleaved from PE2 and is glycosylated at position 14. Labeling studies imply that the PE2 → E2 + E3 cleavage is not closely coupled to budding. E3 is cleaved and released into the culture fluid under conditions where no virions bud, and the kinetics of appearance of E3 in the culture fluid and E2 in virions are dissimilar. The maturation of E3 is discussed as it relates to the processing of cellular membrane glycoproteins.

Hybridomas were selected by the fusion of NSI/1 myeloma cells with spleen cells from mice inoculated with Sindbis specific antigens. Ten stable hybridomas were obtained, seven producing E1-specific antibodies and three producing capsid-specific antibodies. The seven E1 specific antibodies were divided into two classes, which reacted with diffferent E1 antigenic domains. The two classes of antibodies differed in several tested properties. Two E1 clones inhibited viral infectivity, and one of these precipitated E2 along with E1 in Triton-treated preparations. These properties are discussed with regard to the known relationships between the viral structural proteins.

The tryptic glycopeptides of E1 and E2 grown in BHK or chick cells were purified and analyzed by N-terminal sequencing, pronase digestions and labeling with various radioactive sugars. We found that the glycosylation

patterns for the two proteins were essentially identical in the two hosts. E2 contains exclusively complex chains attached to Asn_{196} and simple chains attached to Asn_{398}. In E1, the Asn_{135} glycosylation site contained only complex chains, but the Asn_{245} site contained a mixture of simple and complex chains. A prediction as to the relative importance of the different glycosylation sites to protein function is offered.

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CHAPTER 1

Sindbis virus glycoproteins: A model for the maturation of cellular membrane glycoproteins

Several small enveloped RNA containing viruses, including Sindbis virus, Semliki Forest virus, and vesicular stomatitis virus, have been used as model systems for the study of the maturation of eukaryotic membrane glycoproteins (for reviews, see Lodish et al., 1981; Simons and Warren, 1983). These systems possess several advantages that make them easier to study than cellular systems. Since these viruses are able to effectively shut off host cell protein synthesis, the only proteins being synthesized during infection are large quantities of a few viral proteins, thus facilitating the study of the synthesis and maturation of individual proteins unobscured by the large background of host cell proteins. Since these simple viruses have very limited coding capacities and depend on cellular machinery for much of their protein synthesis and processing, the results found with viral systems are representative of the events occurring in cellular proteins. Vesicular stomatitis virus has often been chosen because of its simplicity, since it has only one membrane glycoprotein, the G protein (Knipe et al., 1977). On the other hand, Sindbis virus and Semliki Forest virus[1] are somewhat more complicated, but also potentially more interesting than vesicular stomatitis virus. They contain two integral membrane proteins, one of which follows a more complicated maturation scheme involving a proteolytic cleavage late in maturation (Schlesinger and Schlesinger, 1972). As such, they provide models for a broader range of cellular glycoproteins.

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[1]This virus is very similar to Sindbis virus in most of the properties discussed in this chapter. For the sake of this chapter, it can be assumed that their properties are the same, except where noted. In cases where studies have been done in both Sindbis and Semliki Forest virus, the Sindbis virus literature is preferentially cited.

Several recent reviews detailing the molecular biology and biochemistry of Sindbis and Semliki Forest virus are available (Strauss and Strauss, 1977; Schlesinger, 1980; Simons and Warren, 1983). Briefly, Sindbis virus (an alphavirus) is a small enveloped virus containing a single plus stranded, 49S RNA (4.3×10^6 daltons) (Simmons and Strauss, 1972a) and three structural proteins, the two membrane glycoproteins E1 and E2, and the nucleocapsid protein (Schlesinger and Schlesinger, 1972). Semliki Forest virus contains a fourth structural protein (E3) that is associated with the outside of the virion (Garoff et al., 1974), whereas in Sindbis virus this small protein is released into the culture fluid (Welch and Sefton, 1979). The viral 49S RNA codes for a polymerase which produces, via a negative stranded intermediate, both more full length 49S RNA and a subgenomic 26S mRNA (Simmons and Strauss, 1972b, 1974). The 26S mRNA, which comprises the 3' third of the genome (Kennedy, 1976), codes for the three to four viral structural proteins (Clegg, 1975). It is translated from one initiation site into a single polyprotein (Cancedda et al., 1975), which is then processed to produce the structural proteins (Clegg, 1975). Under normal circumstances the complete polyprotein is never present, but is cleaved sequentially as it is translated, producing the proteins in the order C/PE2(E3+E2)/6K/E1 (Rice and Strauss, 1981), where PE2 is the precursor to E2 and E3 and the 6K protein is a small nonstructural protein. The full length polyprotein is only present as an aberrant by-product in some temperature sensitive mutants that do not properly cleave it at the nonpermissive temperature (Schlesinger and Schlesinger, 1973).

Insertion into the Cell Membrane

Blobel and coworkers first proposed a model for the translocation of secretory proteins across the endoplasmic reticulum membrane in 1971 (Blobel and Sabatini, 1971), and a few years later they described this "signal hypothesis" in more detail (Blobel and Dobberstein, 1975). The essential elements of their hypothesis are that secretory proteins are initially synthesized with an additional segment of amino acids at their N-termini. As they emerge from the shielding of the ribosome they trigger ribosome attachment to the membrane of the rough endoplasmic reticulum and facilitate the translocation of the protein across the membrane into the lumen. While the protein is still nascent, this signal sequence is removed, presumably by a membrane associated activity. Since their proposal, many studies have been done that verified the model and extended it to integral membrane proteins (For reviews see Zimmerman et al., 1980; Lodish, 1981; Docherty and Steiner, 1982; Sabatini et al., 1982). Membrane proteins simply must have some sort of "stop transfer" sequence to prevent the C-terminus from passing through the membrane (Sabatini et al., 1982).

The sequences of many N-terminal signal sequences have been analyzed and compared to reveal their general characteristics (Sabatini et al., 1982; Lodish, 1981; Docherty and Steiner, 1982; Zimmerman et al., 1980). Their lengths vary from a minimum of 15 to a maximum of about 30 residues, of which at least 11 in a row are hydrophobic or uncharged. Often, but not always, they contain one to three basic amino acids at their N-termini which may facilitate binding to the membranes. In addition, most but not

all, are cleaved after a small neutral amino acid (Gly, Ala, Ser, Cys). Other than these constraints, the sequences show very little pattern.

Many different laboratories have described hydrophobic N-terminal signal sequences associated with a variety of secretory and membrane proteins, the vast majority of which are proteolytically cleaved during, or very shortly after, translocation (Sabatini et al., 1982; Lodish, 1981). The few examples of proteins with signal sequences that are not cleaved also exhibit atypical topologies. For instance, the membrane proteins cytochrome P-450 (Bar-Nun et al., 1980), influenza neuraminidase (Blok et al., 1982), and sucrase-isomaltase (Hauri et al., 1982) have unusually long N-terminal hydrophobic sequences which probably also serve as stop transfer signals, and therefore act as membrane anchors. The erythrocyte plasma membrane protein band 3 (Sabban et al., 1981) and ovalbumin (Lingappa et al., 1979) contain uncleaved internal signal sequences.

A combination of RNA sequence data (Rice and Strauss, 1981) and N-terminal protein sequence data (Bell et al., 1978; 1982) reveal that both of the Sindbis virus proteins (E1, PE2) that are inserted into the endoplasmic reticulum have hydrophobic sequences near their N-termini. Both of these sequences presumably function as signal sequences, although they are both somewhat atypical ones. Shortly after the synthesis and release of the capsid protein into the cytoplasm (Clegg, 1975; Wirth et al., 1977), the N-terminus of nascent PE2 is inserted into the membrane of the rough endoplasmic reticulum, presumably via its signal sequence (Wirth et al., 1977; Bonatti et al., 1979). In a cell free system, insertion into microsomal membranes must begin before about 100 amino acids are synthesized or insertion never occurs, implying that the important signal for

insertion is at or near the N-terminus (Garoff et al., 1978). N-terminal sequencing of PE2 reveals that the first 19 amino acids of PE2 are hybrophobic or uncharged, as would be expected for a signal sequence (Bell et al., 1982). This sequence is atypical, as it is not cleaved from the protein until about 30 min later, and then only as part of a larger piece (Bonatti and Blobel, 1979; Welch and Sefton, 1979). As such, it is the only membrane protein of "typical topology" (a C-terminal membrane anchor, with the bulk of the protein extruding into the lumen) that we know of that does not have its signal sequence rapidly cleaved. In addition, it contains a potential glycosylation site at position 14. This site probably does contain an oligosaccharide chain, although this glycosylation does not seem to be the reason that the sequence is not cleaved (Bell et al., 1982).

The translation of the 26S RNA is completed by the synthesis and insertion of E1 into the membrane, the details of which is not well understood. In the vicinity of the two cleavage sites (the C-terminus of PE2 and the N-terminus of E1) there is sufficient hydrobic sequence to span the membrane up to four times. PE2 contains, in addition to its membrane anchor (34-61 amino acids from the C-terminus), another hydrophobic region of 23 amino acids ending six residues from its C-terminus. The 55 amino acid long 6K protein is entirely hydrophobic and is long enough to potentially span the membrane more than once. Since the order of the above two cleavages has not been determined, and up to four membrane crossings can be visualized, a variety of models have been postulated (see Bonatti et al., 1979; Rice and Strauss, 1981; Sabatini et al., 1982), but none verified. In Semliki Forest virus the 6K protein has been shown to function as a signal sequence, at least under some circumstances, as a temperature sensitive

mutant that is unable to insert PE2 into the membrane is able to properly insert E1 (Hashimoto et al., 1981).

Sindbis virus thus provides two atypical examples of membrane protein insertion. Any general models for insertion of membrane proteins will have to account for these and other exceptions.

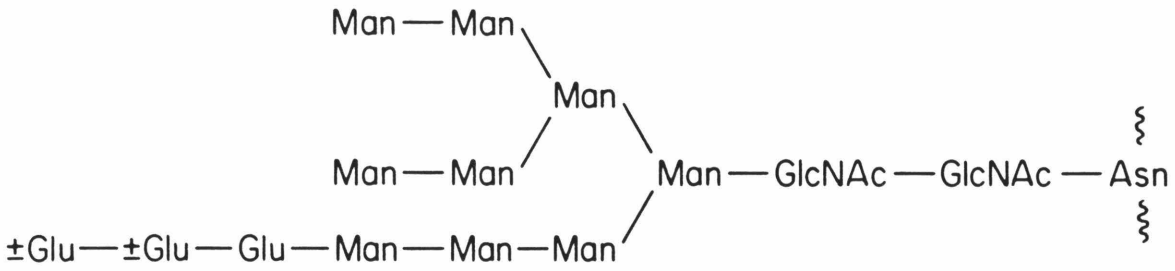
Glycosylation

Many membrane and secreted proteins contain N-glycosidically linked oligosaccharide chains (for review see Lennarz, 1980). These chains are attached to asparagines within the sequence Asn-X-Ser/Thr, where X is any other amino acid (Marshall, 1974). The attachment involves the en bloc transfer of a core oligosaccharide of the structure $Glc_{1-3}Man_{8-12}GlcNAc_2$ (see Fig. 1 for a typical example) to the asparagine via a dolichol intermediate (Struck and Lennarz, 1980). This transfer occurs in the rough endoplasmic reticulum, probably while the protein chain is nascent. As the protein passes from the rough endoplasmic reticulum through the Golgi apparatus, these core oligosaccharides are processed to their final forms (Schachter and Roseman, 1980). There are two major types of asparagine linked oligosaccharides, called complex (or Type A) and simple (or Type B), chains. Examples of typical structures for these are shown in Fig. 1, although many variations of both types have been described (see Cummings and Kornfeld, 1982; Kornfeld and Kornfeld, 1980).

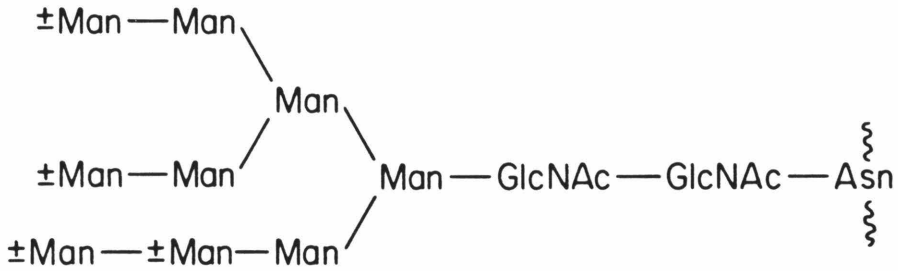
An example of the processing scheme for an asparagine linked oligosaccharide chain is shown in Fig. 2. After the attachment of the core oligosaccharide, and while still in the rough endoplasmic reticulum, it is trimmed by removal of the glucose and some of the mannose residues. In the case of simple chains, this is the end of processing, but for complex

FIG. 1. Typical asparagine linked oligosaccharide structures. From Cummings and Kornfeld, 1982; Kornfeld and Kornfeld, 1980. A: Core oligosccaharide. B: Simple type oligosaccharide. C: Complex type oligosaccharide. Man: Mannose. GlcNAc: N-Acetylglucosamine. Gal: Galactose. NANA: Sialic acid. Fuc: Fucose. Asn: Asparagine. Glu: Glucose.

A: CORE OLIGOSACCHARIDE



B: SIMPLE TYPE OLIGOSACCHARIDE



C: COMPLEX TYPE OLIGOSACCHARIDE

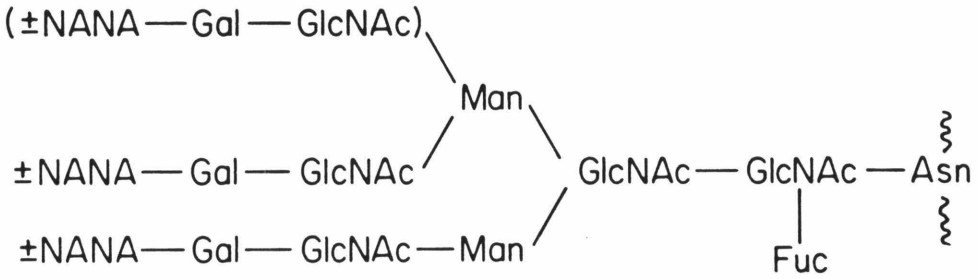
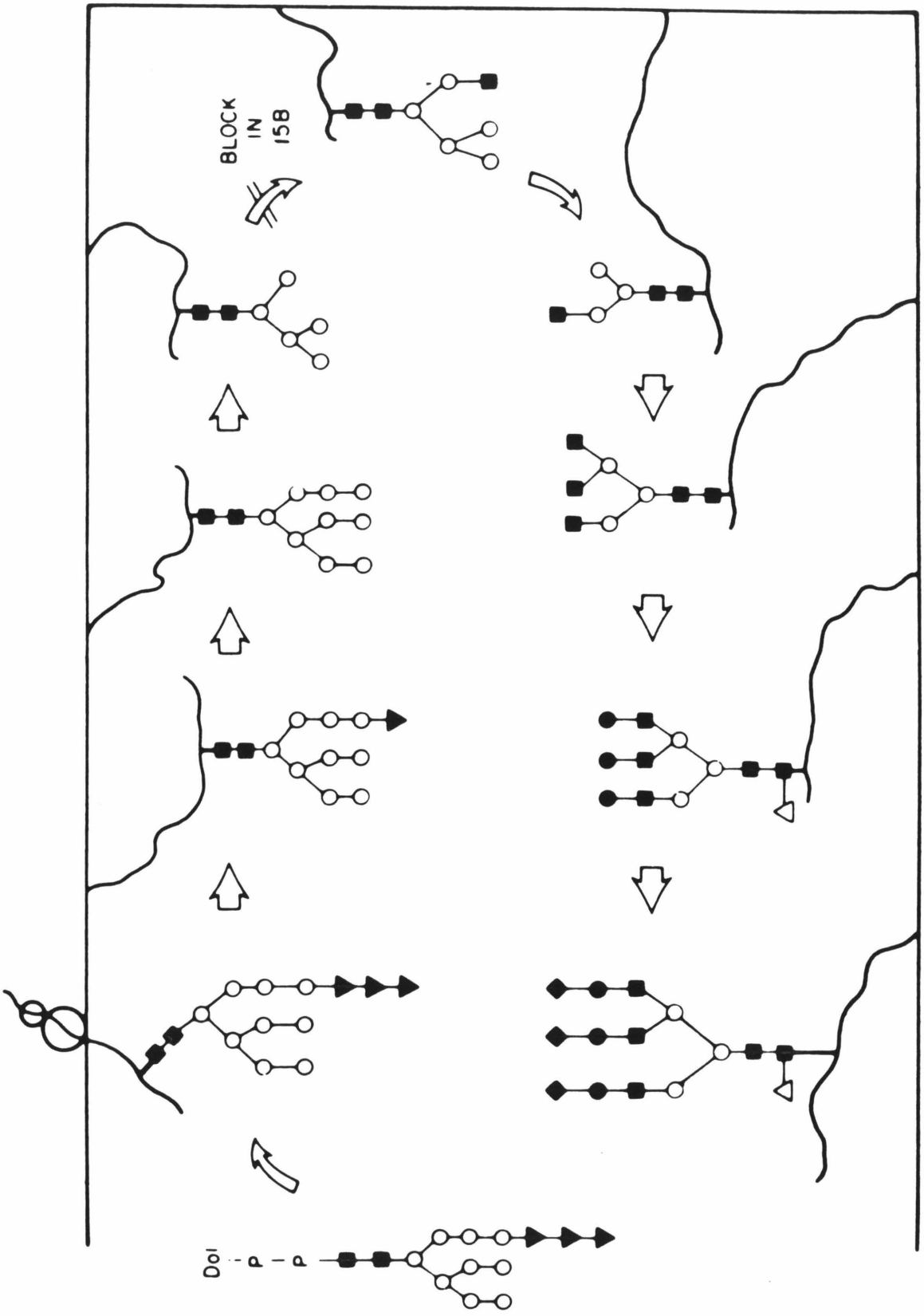


FIG. 2. Proposed sequence for the synthesis of complex-type oligosaccharides (Kornfeld et al., 1978). The symbols are: (Dol): Dolichol, the lipid carrier of the oligosaccharide. (■): N-acetylglucosamine. (○): mannose. (▲): glucose. (●): galactose. (◆): sialic acid. (△): fucose.


BLOCK
IN
158
Dol-P-P

chains, more trimming occurs, and glucosamine, galactose, fucose, and sialic acid residues are added stepwise in the Golgi apparatus (Schachter and Roseman, 1980).

The complete protein sequences of E1, E2, and E3 as deduced from the nucleotide sequence of the 26S mRNA (Rice and Strauss, 1981) reveal two potential glycosylation sites of the Asn-X-Ser/Thr type in both E1 and E2, and one in E3. Studies of the oligosaccharides of these proteins suggest that all of these potential sites are used. The oligosaccharides of Sindbis virus E3 have not been extensively studied, but E3 is glycosylated, as it incorporates radioactive mannose (Welch and Sefton, 1979). Pesonen (1979) has shown that in Semliki Forest virus, E3 contains a complex type oligosaccharide chain. E1 and E2 both contain about two oligosaccharide chains per molecule (Sefton and Keegstra, 1974), one each of the simple type and the complex type (Robbins et al., 1977). The structure of these chains are $Man_{5-7}GlcNAc_2$ and $NANA_{0-2}Gal_2Man_3Fuc_{0-1}GlcNAc_2$, respectively (Burke and Keegstra, 1979; Hakimi, et al., 1981). These structures are analogous to those shown in Fig. 1, except that in Sindbis virus the complex type chain does not contain the third branch.

When Sindbis virus (Keegstra et al., 1975; Burke and Keegstra, 1976, 1979; Weitzman et al., 1979), or other viruses including vesicular stomatitis virus (Etchison and Holland, 1974; Etchison et al., 1977) and retroviruses (Warren et al., 1972; Lai and Duesberg, 1972; Sefton, 1976), are grown in several different cell lines only minor differences in their glycosylation patterns are seen. These changes mostly consist of differences in the number of sialic acid residues (Warren et al., 1972; Keegstra et al., 1975; Burke and Keegstra, 1979) or fucose residues

(Etchison and Holland, 1974) rather than major changes in oligosaccharide structure. In contrast, different proteins within a given host cell type each exhibit their own characteristic glycosylation pattern (Sefton, 1976; Weitzman et al., 1979). These results suggest that the protein itself, as opposed to the host cell, contains the information that determines its glycosylation pattern. On the other hand, the host cell must contain the proper glycosyltransferases or the glycosylation pattern of the protein will be affected. For instance, Sindbis virus grown in lectin resistant cell lines, which lack a glycosyltransferase (Schlesinger et al., 1976), or in insect cells, which lack a sialyltransferase (Stollar et al., 1976), contain proteins with unusual oligosaccharide patterns that reflect the host cell's defect. One exception to this is the report that Sindbis E1 in BHK cells contains very little simple type oligosaccharide (Burke and Keegstra, 1976). It is not clear whether this is due to an underglycosylation of one of the sites or the replacement of the simple chain with an additional complex chain.

Robbins and his coworkers (Robbins et al., 1977; Krag and Robbins, 1977) have shown that Sindbis virus proteins are initially glycosylated via a lipid linked intermediate. This transfer is completed while the glycoproteins are still being translated (Sefton, 1977). The completion of glycosylation is not until late in the maturation of E1 and E2. PE2 and intracellular E1 both contain short uncompleted oligosaccharide chains, deficient in galactose and fucose residues (Bonatti and Cancedda, 1982; Hakimi and Atkinson, 1982).

The precise role of glycosylation is not well understood. Presumably it can have profound effects on conformation, which could in turn affect a

variety of properties of the protein, including solubility, enzymatic activities, or protein-protein interactions (Kornfeld and Kornfeld, 1980). In the case of Sindbis virus glycoproteins, tunicamycin, an inhibitor of asparagine linked glycosylation, prevents their migration to the cell surface, possibly due to aggregation (Gibson et al., 1979; Leavitt et al., 1977). In at least one case, the use of phosphomannosyl residues to direct lysosomal proteins to the lysosome (Hasilik and Neufeld, 1980; Kaplan et al., 1977), a specific glycosylation pattern is important for targeting a class of proteins to their destination.

Proprotein-Like Cleavages

In addition to the removal of their signal sequences, many proteins are further proteolytically processed late in infection (see Lodish, 1981; Zimmerman, 1980; Docherty and Steiner, 1982). These proproteins range from peptide hormones (Potts et al., 1980; Steiner et al., 1980; Mains and Eipper, 1980) to some viral membrane proteins (Min Jou et al., 1980; Klenk et al., 1981). In many cases the cleavages are thought to function as a means of activating some protein activity late in maturation or as a method for facilitating their proper folding (Docherty and Steiner, 1982).

Most of these cleavages occur in the Golgi apparatus (Habener et al., 1977; Eipper et al., 1976; Lodish, 1981), but some have been shown to occur either in secretory granules (Gainer et al., 1977) or following secretion (Bornstein and Sage, 1980). Many of these proteins are cleaved by a similar cleavage mechanism. This involves a cleavage after two to three basic residues (lys or arg) via a trypsin-like activity, followed by the removal of these basic residues by a carboxypeptidase B-like activity (Kemmler et al., 1971; Lodish, 1981; Docherty and Steiner, 1982; Klenk et al.,

1981). In the case of the several viral proteins that have been studied recently the consensus sequence at the cleavage point seems to be Arg-X-Lys/Arg-Arg (Shinnick et al., 1981; Schwartz et al., 1983; Bosch et al., 1981; Garoff et al., 1980; Rice and Strauss, 1981; Dalgarno et al., 1983). So far all examples of proproteins that are cleaved at pairs of basic residues (and therefore presumably by this mechanism) are cleaved intracellularly, whereas those that are cleaved outside the cell are cleaved by other mechanisms (Lodish, 1981; Docherty and Steiner, 1982).

In Sindbis virus the cleavage of PE2 to E2 and E3 is in many ways suggestive of such a proprotein cleavage. It is one of the last events to occur in E2 maturation, 30-60 min after synthesis (Jones et al., 1974; Bracha and Schlesinger, 1976). The precise location of this cleavage is unclear. In Semliki Forest virus (Green et al., 1981) E2 is not detected intracellularly, but PE2 is not detected at the plasma membrane, and numerous studies have reported conflicting results as to whether the cleavage is at the plasma membrane or intracellular. Antibody studies which inhibit PE2 cleavage (Bracha and Schlesinger, 1976; Jones et al., 1977; Ziemiecki et al., 1980) suggest that the cleavage occurs at the plasma membrane, whereas iodination studies (Sefton et al., 1973; Smith and Brown, 1977) and careful analysis of the carbohydrates of PE2 and E2 (Bonatti and Cancedda, 1982; Hakimi and Atkinson, 1982) suggest that the cleavage occurs intracellularly. Circumstantial evidence from the deduced amino acid sequence via cDNA sequencing of the 26S mRNA of both Sindbis virus (Rice and Strauss, 1981) and Semliki Forest virus (Garoff et al., 1980) implies that the cleavage may be intracellular. Both viruses contain pairs of basic amino acids at the junction between E3 and E2 which are

presumably cleaved by a mechanism like that of other intracellular pro-protein cleavages.

The function of this cleavage is not known, but it is presumed that it plays some important role in virus budding (Jones et al., 1974; Keranen and Kaariainen, 1975), possibly via a change in the conformation of E2 in the membrane to prime it for incorporation into virions (Brown, 1980). Cross-linking studies show that the interaction between PE2 and E1 is different than between E2 and E1 (Rice and Strauss, 1982).

Other Modifications During Maturation

In addition to the above modifications of the Sindbis glycoproteins, there are reports of several other types of alterations, including the attachment of fatty acids (Schmidt et al., 1979; Schmidt, 1982), sulfation (Pinter and Compans, 1975), and phosphorylation (Tan and Sokol, 1974; Waite et al., 1974). These are beyond the scope of this review, so they will not be discussed further.

Scope of the Thesis

In this thesis I investigate several aspects of Sindbis virus glycoprotein maturation in order to obtain more information concerning the maturation of membrane glycoproteins in general. Chapter 1 deals with the maturation of E3. E3 is very useful for approaching several questions from a previously unexamined angle. Since it is physically attached to E2 as the precursor protein PE2 until very late in maturation, it can be used to follow the maturation of E2. Comparisons between the appearance of E3 and E2 (in virions) in the culture fluid also provide a convenient method to study the maturation of virions. In the second chapter I describe the isolation and characterization of several hybridomas producing monoclonal

antibodies specific for Sindbis virus proteins. These potentially will prove useful for the study of various aspects of Sindbis virus infection, including the association of E1 and E2 during maturation, as one of the hybridomas is able to coprecipitate E1 and E2. Finally, a study of the glycosylation of E1 and E2 in BHK and chick cells compares the patterns of glycosylation at individual glycosylation sites in the two cell systems.

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CHAPTER 2

Biochemical studies of the maturation of the small Sindbis virus glycoprotein E3

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ABSTRACT

A small glycoprotein (E3) was purified from the culture fluid of Sindbis virus infected primary chick embryo fibroblasts. Tryptic peptide mapping and pulse-chase studies verified that this protein was produced as a by-product of the cleavage of the precursor protein PE2 to produce the envelope glycoprotein E2. A 2600-fold enrichment was achieved via a purification scheme involving differential ethanol precipitation, gel filtration, ion exchange chromatography, and affinity chromatography using a lentil lectin column. Amino acid composition analysis, N-terminal microsequencing, and labeling studies yielded information about the fine structure of E3 and its relationship to E2 and virion maturation. The N-terminal sequence of E3 is identical to that of PE2, including the result that 90% of the molecules appear to be blocked. The first 16 amino acids are predominantly hydrophobic or uncharged and presumably serve as the signal sequence for the insertion of PE2 into the membrane of the endoplasmic reticulum, but this sequence is unusual in that it is not immediately cleaved from PE2 and is glycosylated at the asparagine at position 14. The C-terminal two residues of E3, Lys-Arg, are removed during or shortly after cleavage from PE2. Labeling studies imply that although the PE2 → E2 + E3 cleavage is necessary for virion budding, these two events are not closely coupled. E3 is cleaved and released into the culture fluid under conditions where virions do not bud, and the kinetics of the appearance of E3 in the culture fluid and E2 in virions are quite dissimilar. The maturation of E3 is discussed as it relates to the processing of cellular membrane or secretory glycoproteins.

INTRODUCTION

Sindbis virus (an alphavirus) is a simple, enveloped, RNA containing virus which contains only three structural proteins, the two envelope glycoproteins, E1 and E2, and a nucleocapsid protein, C (for reviews see Strauss and Strauss, 1977; Simons and Warren, 1983). During infection the predominant virus specific RNA is a 26S (1.6×10^6 dalton) mRNA which encodes these three proteins (Simmons and Strauss, 1974a, 1974b). The 26S RNA has a single translation initiation site, and these proteins are translated as a single polyprotein (Cancedda et al., 1975) and processed by post-translational cleavages. This polyprotein is never produced as such, however, except as an abortive by-product in some temperature-sensitive mutants (Schlesinger and Schlesinger, 1973). Instead, the cleavages occur while it is still nascent (Strauss and Strauss, 1977) resulting in the sequential release of the proteins in the order C/PE2/6K/E1, where PE2 is a precursor to E2 and the 6K protein is a small nonstructural protein (Rice and Strauss, 1981).

Shortly after the capsid protein has been cleaved from the nascent polyprotein, the N-terminus of the nascent PE2 attaches and inserts into the rough endoplasmic reticulum (Wirth et al., 1977; Bonatti et al., 1979) and the protein is glycosylated (Sefton, 1977). In vitro the membrane insertion must begin before the first 100 amino acids of PE2 are translated or insertion does not occur (Garoff et al., 1978). Thus, the N-terminus of PE2 presumably serves as a signal sequence as proposed by Blobel and Dobberstein (1975). The translation of the 26S RNA is completed by the synthesis and insertion into the rough endoplasmic reticulum membrane of E1, possibly via the use of the 6K protein as a signal sequence

(Hashimoto et al., 1981), but the details of these last events are unclear (see Bonatti et al., 1979; Rice and Strauss, 1981; Sabatini et al., 1982 for several possible models).

PE2 is cleaved to produce E2 as the final proteolytic processing step during its maturation (Schlesinger and Schlesinger, 1972). This occurs about 30 minutes after translation and is one of the last events before the budding of the virion (Bracha and Schlesinger, 1976). Some investigators have suggested that this cleavage may play some important role in virus budding (Jones et al., 1974; Keranen and Kaariainen, 1975). The small N-terminal peptide, E3, has been found in the culture fluid of Sindbis infected cells (Welch and Sefton, 1979), whereas E3 of Semliki Forest virus remains associated with the virion as a third structural glycoprotein (Garoff et al., 1974). We are particularly interested in E3 because of its similarities to the "pre" and "pro" portions of many membrane and secretory proteins.

Since the signal hypothesis was proposed (Blobel and Dobberstein, 1975), many laboratories have described hydrophobic N-terminal leader (or "pre") sequences which function in the translocation of membrane and secretory protein across the membrane of the endoplasmic reticulum, the vast majority of which are proteolytically removed during, or very shortly after, translation (for reviews see Sabatini et al., 1982; Lodish, 1981; Zimmerman et al., 1980). The few cases studied where the signal sequence is not cleaved also exhibit topologies atypical of the norm. The secretory protein ovalbumin (Lingappa et al., 1979) and the erythrocyte plasma membrane protein band 3 (Sabban et al., 1981) both contain internal signal sequences. Membrane proteins such as cytochrome P-450 (Bar-Nun et al., 1980),

influenza neuraminidase (Blok et al., 1982), and sucrase-isomaltase (Hauri et al., 1982) contain unusually long hydrophobic sequences at their N-termini which may serve as "stop transfer" signals, and therefore act as membrane anchors as well as signal sequences. PE2 in Sindbis virus (and the closely related Semliki Forest virus) is an example in which the presumptive signal sequence is not cleaved, even though the topology is "normal", with the signal sequence at its N-terminus and the bulk of the protein, including the N-terminus, extruded into the lumen of the endoplasmic reticulum (Wirth et al., 1977; Bonatti and Blobel, 1979).

The kinetics of the cleavage of E3 from PE2 is suggestive of the cleavage of proproteins. Many membrane and secreted proteins, from peptide hormones (Potts et al., 1980; Steiner et al., 1980; Mains and Eipper, 1980), to some viral membrane proteins (Min-Jou et al., 1980; Klenk et al., 1981), contain sequences which are cleaved off late in maturation (see Lodish, 1981; Zimmerman, 1980). These cleavages usually occur in the Golgi apparatus (Habener et al., 1977; Eipper et al., 1976; Lodish, 1981), but examples have been found which occur in secretory granules (Gainer et al., 1977) or after secretion (Bornstein and Sage, 1980). A common sequence has been found at the cleavage points of most of the examples that are cleaved intracellularly, consisting of two to three basic amino acids (Lys, Arg) which are cleaved by trypsin-like activity followed by a carboxypeptidase B-like activity (Lodish, 1981; Klenk et al., 1981), but those cleaved after secretion do not seem to follow this pattern (Bornstein and Sage, 1980; Lodish, 1981).

In this study we have purified sufficient quantities of E3 to enable biochemical studies and have done labeling studies in order to investigate

the role of E3 in viral membrane protein and virion maturation.

MATERIALS AND METHODS

Growth and purification of radiolabeled virus. Radiolabeled [^{14}C]-leucine or [^{3}H]-leucine stocks of the HR (large plaque) strain of Sindbis virus (Burge and Pfefferkorn, 1966) were grown in monolayers of primary chicken embryo fibroblasts and purified as previously described (Pierce et al., 1974).

Preparation of radiolabeled and unlabeled E3. Primary chick embryo fibroblasts were prepared and infected with Sindbis virus as described (Pierce et al., 1974). A variety of radiolabeled preparations of E3 ([^{3}H]- or [^{14}C]-labeled amino acids, [^{3}H]-glucosamine [^{3}H]-mannose, or [^{14}C]-galactose) were prepared by one of the following methods. (i) Cells were labeled 3-12 hr post-infection in low salt medium (Pierce et al., 1974) containing 0.3-1.0% dialysed fetal calf serum and 1/10 the concentration of the appropriate amino acid. At 12 hr post-infection the culture fluid was collected and frozen. (ii) Cells were labeled 3-12 hr post-infection in Eagle's Minimal Essential Medium (Eagle, 1959) containing 0.3-1.0% dialysed fetal calf serum and 1/10 the concentration of the appropriate amino acid. At 12 hr post-infection the culture fluid was collected and precipitated with 8% final concentration polyethylene glycol as described previously (Pierce et al., 1974). The supernatant, which contains all of the E3, was frozen.

As needed, the radiolabeled preparations were thawed and purified through the first two steps of the purification scheme described below. Unlabeled E3 was prepared as in method (ii) above. A total of 400-420 roller bottles of PEG supernatant was used for the final preparation, and

four roller bottles of [^{3}H]-leucine labeled (1.25 mCi/roller bottle) E3 was added for the purpose of monitoring E3 through the purification scheme outlined below.

Purification of E3. E3 was purified from PEG supernatants by the following method:

(i) Differential ethanol precipitation (2X-4X ethanol precipitation): The medium was precipitated with 1.75 volumes of 100% ethanol, stored at -20°C overnight, and centrifuged at 13,200 x g (0°C) for 30 min. The supernatant was decanted, brought to 4.5 volumes of ethanol, and left overnight at -80°C. It was centrifuged again at 13,200 x g (0°C) for 30 min, the supernatant carefully aspirated off, and the pellet saved. Multiple rounds of the centrifugation were done in the same 250 ml centrifuge bottles, allowing the pellets to accumulate in the bottom.

(ii) Gel filtration. The pellet was resuspended in 100 mM NaCl, 50 mM Tris buffer, 0.01% NaN_3, 60 µg/ml phenylmethylsulfonylfluoride, pH 7.3 containing 1% sodium dodecyl sulfate (SDS) and 1% β-mercaptoethanol and loaded onto an Ultrogel AcA-34 column equilibrated in the same buffer but containing 0.1% SDS and 0.1% β-mercaptoethanol. For the purification of the large unlabeled preparation a 44 cm x 5.4 cm (100 ml void volume) column was run at 1 ml/min and 8 ml fractions collected. E3, which migrated with an R_f of 0.62, was pooled and precipitated with 5 volumes of 100% ethanol.

(iii) Ion exchange chromatography. The E3 pellet from step (ii) was resuspended in 20 mM Tris buffer, 0.5% Triton X-100, pH 7.2 and loaded onto a 33 cm x 5 cm (100 ml void volume) Sephadex DE-52 column at 2 ml/min. The column was then washed with a salt gradient (from 20 mM Tris Buffer,

no NaCl to 50 mM Tris Buffer, 450 mM NaCl, both in 0.5% Triton X-100, 0.01% NaN_3, 20 µg/ml phenylmethylsulfonylfluoride, pH 7.2) and 6.7 ml fractions were collected. E3 eluted at 175 mM salt and was pooled and precipitated with 5 volumes of ethanol.

(iv) Lens culinaris affinity column. The E3 pellet was resuspended in 100 mM NaCl, 50 mM Tris buffer, pH 7.3, containing 0.5% Triton X-100, 0.1% NaN_3, 0.1 mM $MnCl_2$, 20 µg/ml phenylmethylsulfonylfluoride and loaded onto a 10 ml (1 cm x 10 cm) column of lentil (Lens culinaris) lectin which had been purified and coupled to Sepharose 4B as described (Howard et al., 1971; Hayman and Crumpton, 1972). The sample was loaded onto the column at 4°C in a stepwise fashion. Portions of the sample were run into, but not through, the column and left in the column for 3 hr or more. This was repeated until all of the sample was loaded, and the column was then washed for 10 hr at 0.23 ml/min. Care must be taken at these steps, as much of E3 passes through the column if loaded too rapidly, and E3 slowly leaches off of the column if washed too extensively. E3 was eluted (and 4 ml fractions collected) with the same buffer containing 100 mM α-methylmannopyranoside. Unbound E3 was loaded onto a second column, eluted as before, and the two fractions pooled.

(v) Final ethanol precipitation. The pooled E3 fractions were precipitated with 4 volumes of ethanol, left at -80°C overnight, and centrifuged at 95,000 x g (4°C) for 6 hr. About 50% of the radioactivity remained in the ethanol supernatant, so 4 more volumes of ethanol were added. This was left at -80°C overnight and centrifuged as before, with

greater than 95% of the radioactivity precipitating. The samples were resuspended in 0.1% SDS.

SDS-polyacrylamide gel electrophoresis. The gels used contained 20% acrylamide, 0.067% bis acrylamide, and the buffer system of Laemmli (1970), except that the concentration of Tris buffer was halved. The samples were electrophoresed into 1.5 mm slab gels, which were either analyzed by fluorography according to Bonner and Laskey (1974), or stained with Coomassie blue and then fluorographed. Alternatively, the samples were electrophoresed into 6 mm x 10-20 cm tube gels, which were sliced into 1 mm fractions with a Mickle gel slicer and counted by liquid scintillation. Backgrounds and channel overlaps were corrected by a computer program. Small (0.5 mm thick by 10 cm long) slab gels of 15% acrylamide were stained with silver nitrate by the method of Merril et al. (1981). Photographic film (Kodak X-Omat) was prefogged (Laskey and Mills, 1976) and exposed at -80°C. The protein standards used were ovatransferrin (MW = 77K), bovine serum albumin (MW = 66K), ovalbumin (MW = 45K), chymotrypsinogen (MW = 25K), myoglobin (MW = 17K), and cytochrome c (MW = 12.3K).

Iodination of E3. A small quantity (<1 nanomole) of purified E3 was precipitated with 100 µg yeast RNA as carrier and 4 volumes of ethanol. This was resuspended in 0.5 M phosphate buffer (pH 7.4) and labeled with ^{125}I using chloramine T by the method of Erlich et al. (1978). The iodinated preparation was mixed with ^{3}H-leucine labeled E3 and digested with ribonuclease to remove the RNA carrier. This was run on a 20% acrylamide (0.067% bis acrylamide) tube gel (6 mm x 14 cm), sliced into 1 mm pieces, and counted by liquid scintillation.

Amino acid analysis. Duplicate samples of 0.5 nanomoles of purified

E3 were hydrolyzed with 6 N HCl at 110°C for either 12, 24, 46, or 95 hr. To correct for degradation during hydrolysis, values of some amino acids were extrapolated back to zero time of hydrolysis, assuming first order kinetics. Others, to correct for resistance to hydrolysis, were extrapolated to complete hydrolysis (usually approximately equivalent to the 46 or 95 hr time points). Cysteine and tryptophan are degraded during hydrolysis and were not quantitated. Whale skeletal myoglobin (MW 17,800) was used as a standard. All hydrolysates were analyzed on a Durrum D-500 MKII amino acid analyzer.

Protein sequenation. A 35 µl aliquot (10.2 nanomole) of the chemically pure E3 sample was diluted into 0.5 ml of 100% trifluoroacetic acid and loaded onto a non-commercial spinning cup sequenator, the construction and operation of which have been described (Hunkapiller and Hood, 1980). The mixture of phenylthiohydantoin amino acid derivatives released at each cycle were analyzed by reverse phase high performance liquid chromatography (Johnson et al., 1979).

Labeling experiments. 60 mm petri plates of primary chick cells were infected as described previously (Pierce et al., 1974), except where noted. Before labeling, the cells were washed 2-3 times with medium containing dialyzed fetal calf serum and deficient in the amino acid to be used in labeling. The harvested culture fluids or monolayers were analyzed on 20% polyacrylamide slab or tube gels (Laemmli, 1970).

RESULTS

Identification of E3 during Sindbis Virus Infection

When the culture fluid and cell lysate from Sindbis infected cells were examined on a polyacrylamide slab gel (Fig. 1) a small protein

appeared only in the culture fluid of infected cells (lanes 1, 3). This protein bound to an agarose-Lens culinaris column (data not shown) and incorporated radiolabeled mannose, galactose, and glucosamine (lanes 11-13), indicating that it was a glycoprotein with a complex-type oligosaccharide residue attached. Tryptic digests of this protein compared to those of E2 or PE2 (Fig. 2) showed that it was a by-product of the cleavage of PE2 to E2. All but one of the E3 peaks line up with PE2 peaks (Fig. 2A), whereas none of them correspond to E2 peaks. The one E3 peak which does not line up with PE2 is probably the carbohydrate containing peak, since PE2 contains predominantly short, unfinished oligosaccharides (Sefton and Keegstra, 1974) relative to the mature envelope protein. In addition, pulse chase experiments (Fig. 3) showed that E3 appeared in the culture fluid with about the same kinetics as the appearance of E2 and the disappearance of PE2 (30-60 min after labeling). The combination of these three experiments suggest strongly that this is a protein analogous to E3 in Semliki Forest virus.

On polyacrylamide gels E3 appears as a major band with two or more minor bands (Fig. 1), the relative intensities of which vary from one preparation to another. These multiple bands, which can also be seen on isoelectric focusing gels (Fig. 4) are probably caused by heterogeneity in E3, presumably in the oligosaccharides. Upon digestion with neuraminidase some, but not all, of the heterogeneity is lost (data not shown). This may be due to either incomplete digestion of sialic acid residues, or additional heterogeneity in E3.

Purification of E3. In order to obtain E3 of the necessary chemical purity for careful biochemical analyses, including amino acid compositions

FIG. 1. Distribution of viral proteins during Sindbis virus infection. Infected (I) and mock infected (M) chick cells grown in 60 mm petri plates were labeled with 200 µCi [^{3}H]-leucine from 3 to 12 hr post-infection. Virus was grown using one of two alternative protocols. Lanes 1-2, 7-8: Virus was grown in normal ionic strength (116 mM NaCl) medium (N). The culture fluid was harvested (lanes 1 and 2) and the cell monolayer lysed with 1% SDS (lanes 7 and 8). Lanes 3-6, 9-10: Virus was grown in low ionic strength medium (69 mM NaCl). The low salt culture fluid (L) was harvested (lanes 3 and 4), the cells were incubated with high ionic strength (216 mM NaCl) medium (H) for 30 min (lanes 5 and 6), and the cell monolayer was lysed (L/H) with 1% SDS (lanes 9 and 10). All fractions collected were analyzed by gel electrophoresis and fluorography. Lanes 11-13: Chick cells were labeled with [^{3}H]-mannose, [^{14}C]-galactase, or [^{3}H]-glucosamine between 3 and 12 hr post-infection in medium of normal ionic strength and the culture fluid harvested and analyzed as above.

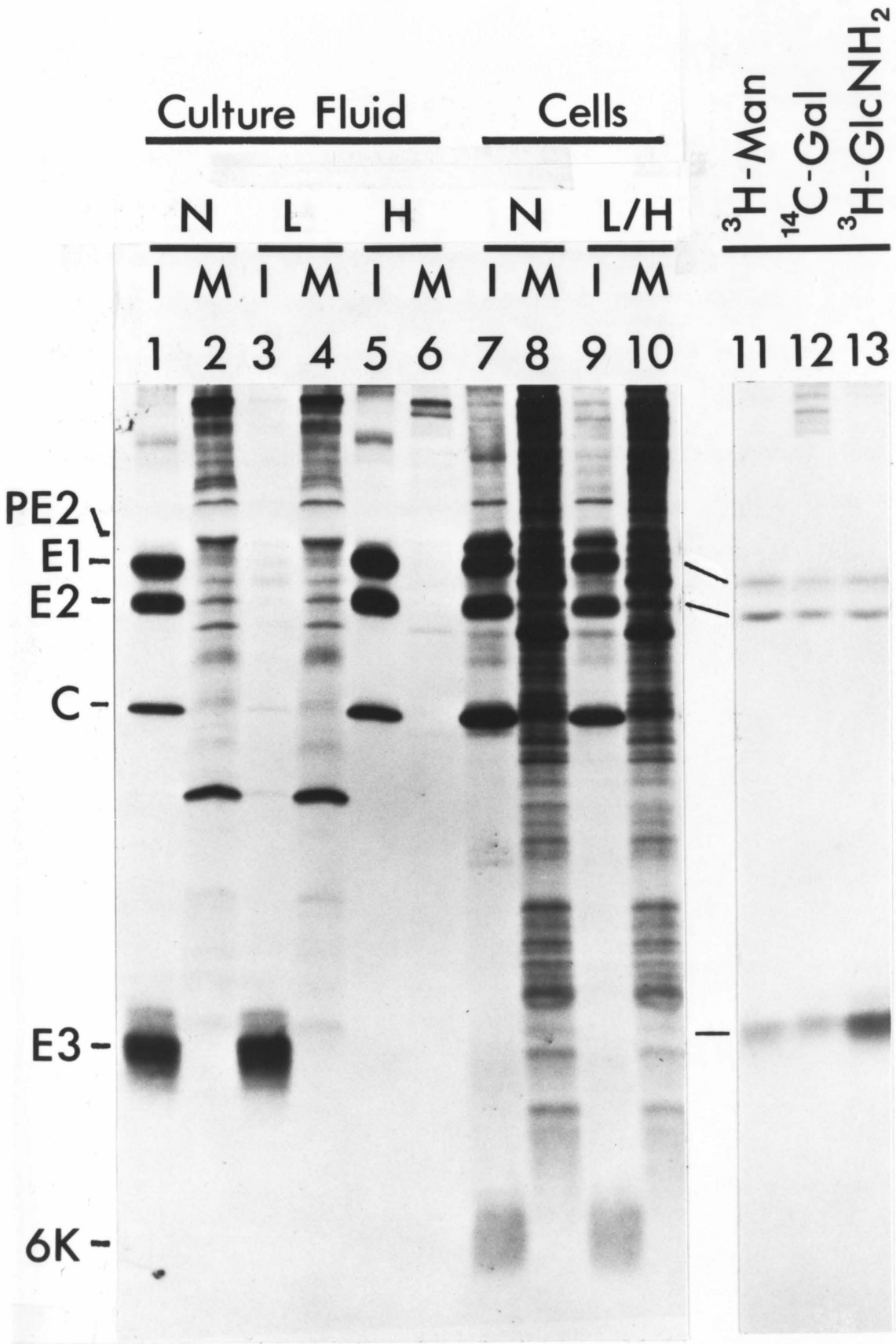
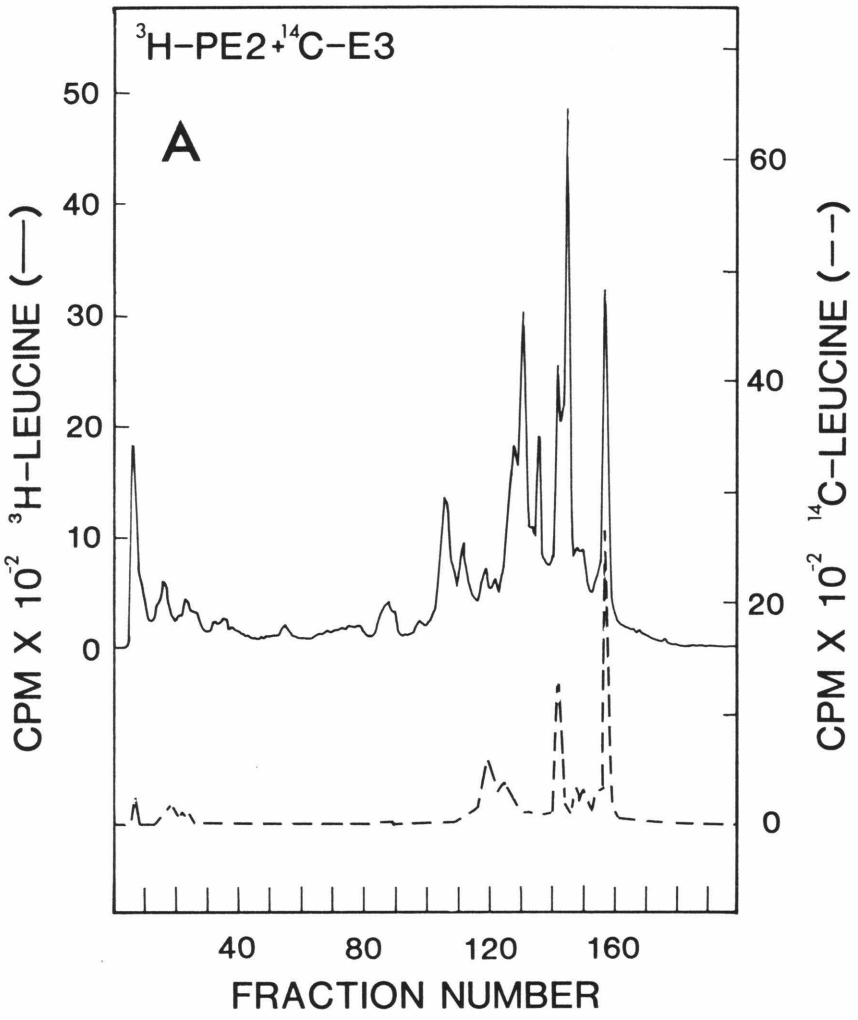

Culture Fluid
Cells
N
L
H
N
L/H
I M I M I M I M I M
1 2 3 4 5 6 7 8 9 10 11 12 13
^{3}H-Man
^{14}C-Gal
^{3}H-GlcNH$_2$
PE2
E1
E2
C
E3
6K

FIG. 2. High pressure liquid chromatograph peptide maps of E3 versus E2 or PE2. Radiochemically pure [^{14}C]-leucine E3 was purified as described in Materials and Methods. [^{3}H]-leucine labeled PE2 or E2, from an infected cell lysate and purified virions respectively, were purified by preparative gel electrophoresis as previously described (Rice et al., 1982). The samples were oxidized with performic acid (Hirs, 1967) and, after lyophilization, concentrated by ethanol preparation. Samples to be compared were mixed, digested exhaustively with trypsin (TPCK treated, Worthington), and the tryptic peptides were separated on a Dupont 830 high-pressure liquid chromatograph using a 25 cm Zorbax-C18 column (McMillan et al., 1978; Rice and Strauss, 1982). A: E3 (---) compared to PE2 (——). B: E3 (---) compared to E2 (——).


^{3}H-PE2+^{14}C-E3
A
CPM X 10^{-2} ^{3}H-LEUCINE (—)
CPM X 10^{-2} ^{14}C-LEUCINE (- -)
50
40
30
20
10
0
60
40
20
0
40
80
120
160
FRACTION NUMBER

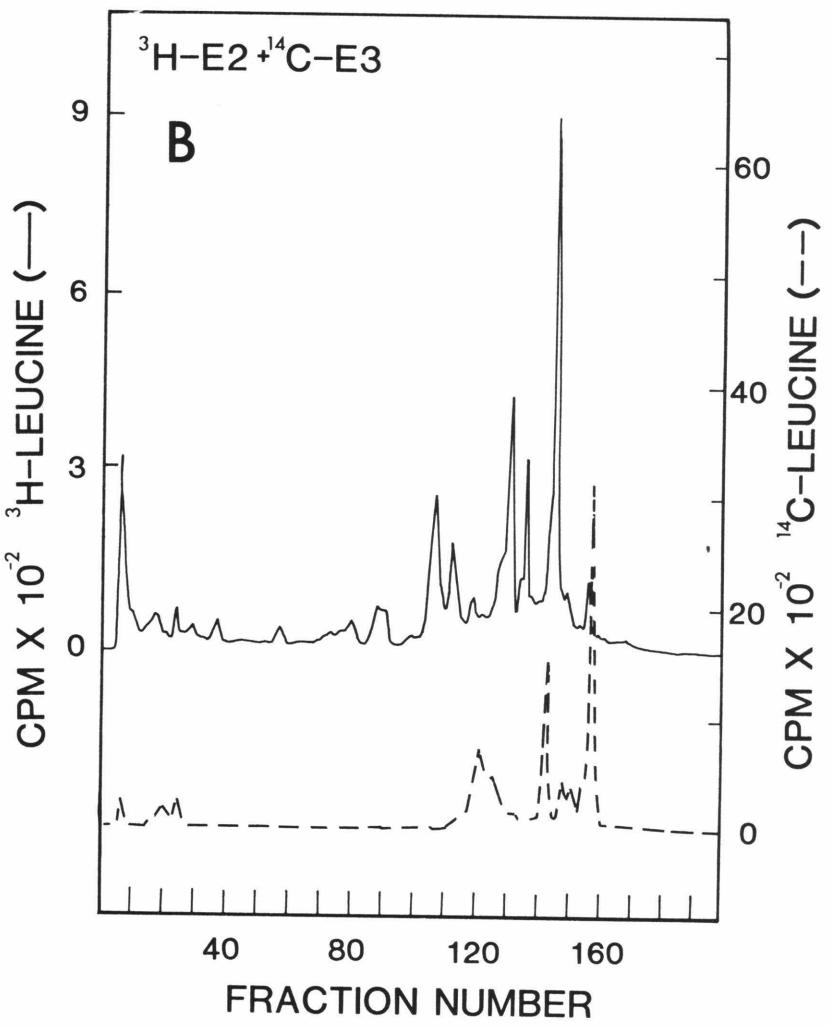

^{3}H-E2+^{14}C-E3
B
CPM X 10^{-2} ^{3}H-LEUCINE (—)
CPM X 10^{-2} ^{14}C-LEUCINE (- -)
9
6
3
0
60
40
20
0
40
80
120
160
FRACTION NUMBER

FIG. 3. Pulse-chase of viral proteins during infection. At 4 hr post infection 60 mm petri plates of Sindbis infected chick cells were washed twice with medium lacking methionine and incubated for 10 min in the presence of 10 µCi/ml [^{35}S]-methionine. After 10 min one plate was put onto ice and the rest were washed two times with media containing twice the normal concentration of methionine. Two ml of this medium was added to the plates and at 20, 30, 60, and 90 min after the initial addition of label, an additional plate was put on ice. The culture fluids were removed and the cell monolayers lysed with 1 ml of 1% SDS. Both the culture fluids (A) and lysed monolayers (B) were precipitated and analyzed by gel electrophoresis and autoradiography. Lane 1: [^{35}S]-labeled purified virus. Lane 2: 10 min pulse. Lanes 3 + 4: 10 min chase. Lanes 5 + 6: 20 min chase. Lanes 7 + 8: 50 min chase. Lanes 9 + 10: 80 min chase.

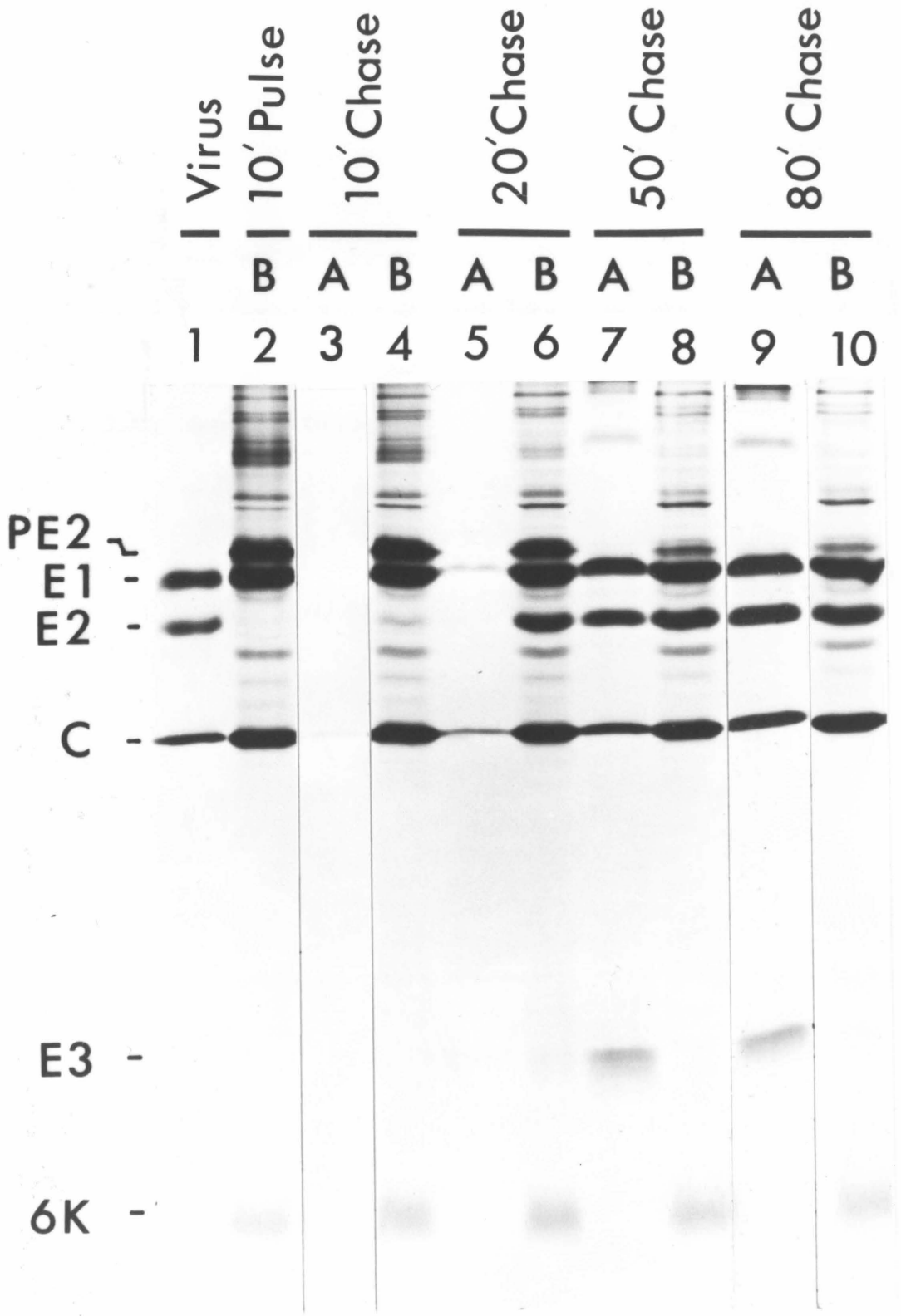
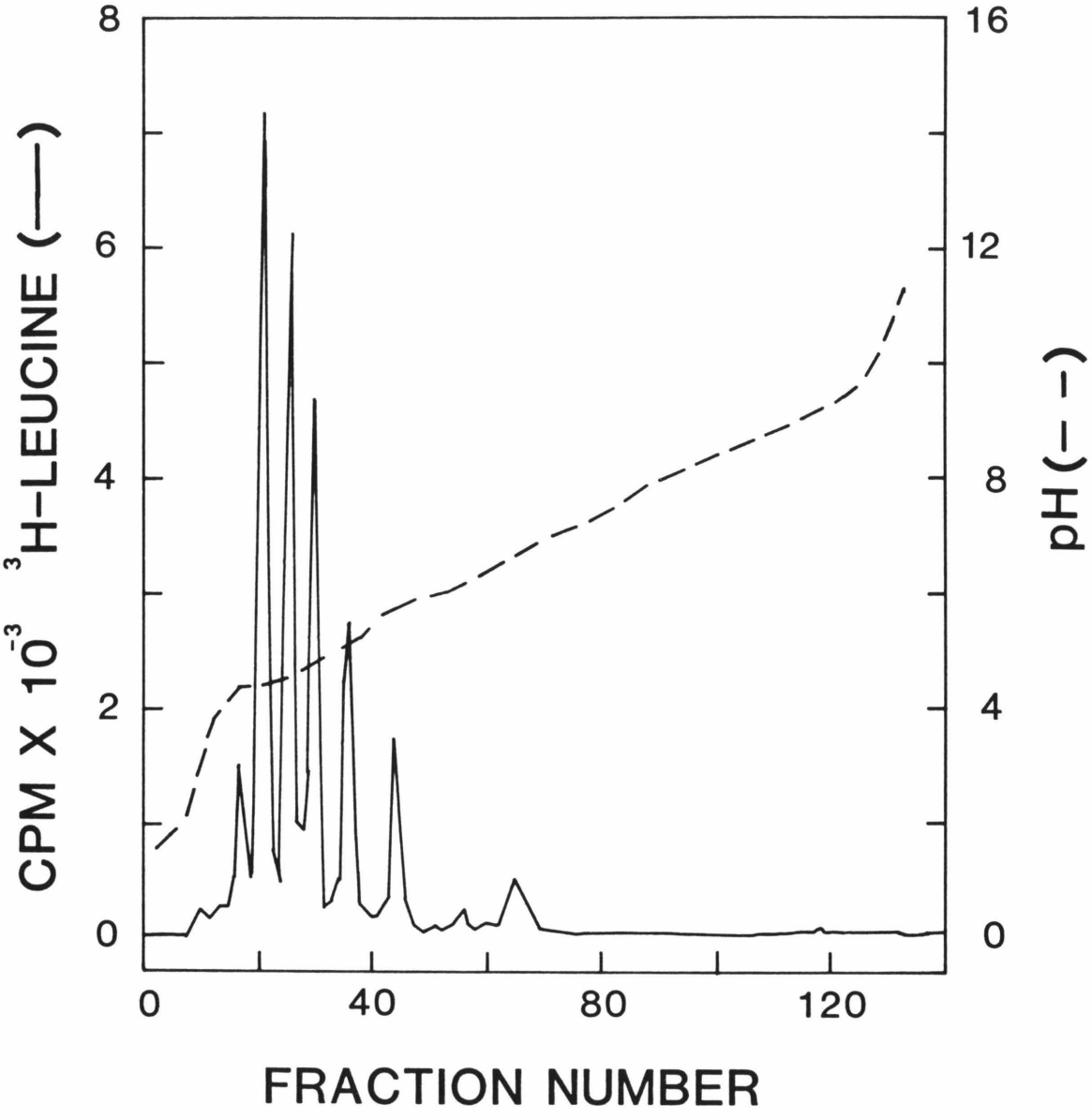

Virus
10′ Pulse
10′ Chase
20′ Chase
50′ Chase
80′ Chase
B
A
B
A
B
A
B
A
B
1
2
3
4
5
6
7
8
9
10
PE2
E1
E2
C
E3
6K

FIG. 4. Nonequilibrium isoelectric focusing gel of E3. A modification of the method of O'Farrell (1975) was used. E3 labeled with [^{3}H]-leucine was electrophoresed for 1500 volt hours into a 14 cm x 4 mm 4.25% (0.11% bis) acrylamide tube gel containing an ampholine mix of 4.95% pH 3-10 (Biorad), 0.34% pH 4-6 (Biorad), 0.34% pH 5-7 (Biorad), and 0.89% pH 9-11 (LKB), plus 9.2 M urea and 2% Triton X-100. 1.0 mm slices were cut and counted by liquid scintillation. A control gel was cut into 5 mm sections, immersed in 0.5 ml H_2O and the pH of the pieces measured. (—) radiolabeled E3. (---) pH measurements of blank gel.


8
6
4
2
0
16
12
8
4
0
0
40
80
120
CPM X 10^-3 ^3H-LEUCINE (——)
pH (– –)
FRACTION NUMBER

and N-terminal sequencing, a purification scheme was devised using a combination of several typical protein separation steps (column chromatography, ion exchange chromatography, and lentil lectin affinity chromatography) and the differential precipitation of E3 with ethanol (summarized in Table 1 and Fig. 5). Virus in the culture fluid is first precipitated with polyethylene glycol. Then two volumes of ethanol are added to the supernatant which causes the vast majority (99.6%) of the serum protein to precipitate whereas E3 remains in the supernatant. When the amount of ethanol is now raised to four volumes E3 quantitatively precipitates. At this stage E3 is radiochemically pure enough for many purposes and the 250-fold purification achieved greatly facilitates the subsequent column chromatography steps used to achieve chemically pure material.

When E3 reaches a certain degree of purity (after the lentil lectin step), it becomes more difficult to precipitate and ethanol concentrations of greater than 80% are required to effectively precipitate E3. In one instance, differential ethanol precipitation worked as an excellent final purification step, as virtually all the remaining contaminants precipitated with 80% ethanol (Fig. 6, lanes 7 and 9) and pure E3 was precipitated by 90% ethanol (lanes 8 and 10), but the variability in the amount of E3 that precipitates at 80% ethanol limits the use of this procedure as a purification step until further modifications are made.

Technical problems make it difficult to quantitate and follow E3 during the above purification. E3 does not stain well with Coomassie blue (Fig. 6, lane 8). The small amount of stained material seen probably corresponds to a minor contaminant in the pure E3 fraction. E3 does stain

Table 1

Description and Quantitation of E3 Purification Scheme

Step	Location of E3	Protein (mg)[a]	% of[b] starting E3	E3 (mg)[c]	Purification[d] factor
1. Harvest culture fluid at 12 hr post infection	Culture fluid	27000	ND[e]	ND[f]	1
2. Precipitate with 8% PEG, 0.4 M NaCl	Supernatant	27600	ND	ND	1
3. Precipitate supernatant with 1.75 volumes of ethanol	Supernatant	ND	ND	ND	ND
4. Precipitate supernatant with 4.5 volumes of ethanol	Pellet	106	100%	ND	255
5. Gel filtration (Ultrogel AcA-34)	R_f = 0.62	62.1	94%	ND	409
6. Ion exchange chromatography (Sephadex DE-52)	175 mM NaCl	20.0	87%	ND	1175
7. Lentil lectin chromatography (Elute with 0.1 M α-methyl-mannopyranoside)	Elution peak	5.87	45%	3.21	2070
8. Precipitate with 4 volumes of ethanol	Supernatant[g]	ND	ND	ND	ND
9. Precipitate with 8 volumes of ethanol	Pellet	4.03	38%	2.76	2580

Table 1 (continued)

[a]Determined by Lowry assay (Lowry et al., 1951).

[b]Determined by recovery of label through the steps, assuming all the label after the differential ethanol precipitation is in E3.

[c]Determined by fitting the data obtained from amino acid compositions to the complete sequence as obtained from the RNA sequence (Rice and Strauss, 1981).

[d]Enrichment of labeled E3 relative to total protein.

[e]Could not determine the amount of E3 in first two steps due to excess of free label. Other experiments have shown that essentially 100% of E3 is recovered through these steps.

[f]ND = not done.

[g]A variable amount of E3 precipitates at this step.

FIG. 5. Purification of E3. A: Gel filtration of E3 (after differential ethanol precipitation) using Ultrogel AcA-34. Excluded (V_o) and included (V_i) volumes as determined by blue dextran and phenol red respectively are marked by arrows. The E3 peak which was pooled is indicated with a horizontal bar. B: Ion exchange chromatography of E3 using Sephadex DE-52. A 50 µl aliquot of every fifth fraction of the load and wash (fraction 1-75) and 10 µl aliquot of every fraction of the elution (fraction 101-160) were counted. The conductivity of several standard NaCl solutions are shown on the right. The pooled peak is indicated by the horizontal bar. (——) [^{3}H]-leucine labeled preparation. (---) Conductivity measurements of a gel run in parallel with the gel containing sample. C: Lentil lectin affinity chromatography. The arrow indicates the additon of 0.1 M α-methylmannopyranoside. The fractions pooled are indicated by a horizontal bar. D: A 20% polyacrylamide gel of iodinated E3 preparation to determine its purity. (---) Radiochemically pure [^{3}H]-leucine labeled E3. (——) Iodinated E3 preparation. Arrow indicates the start of the separating gel.

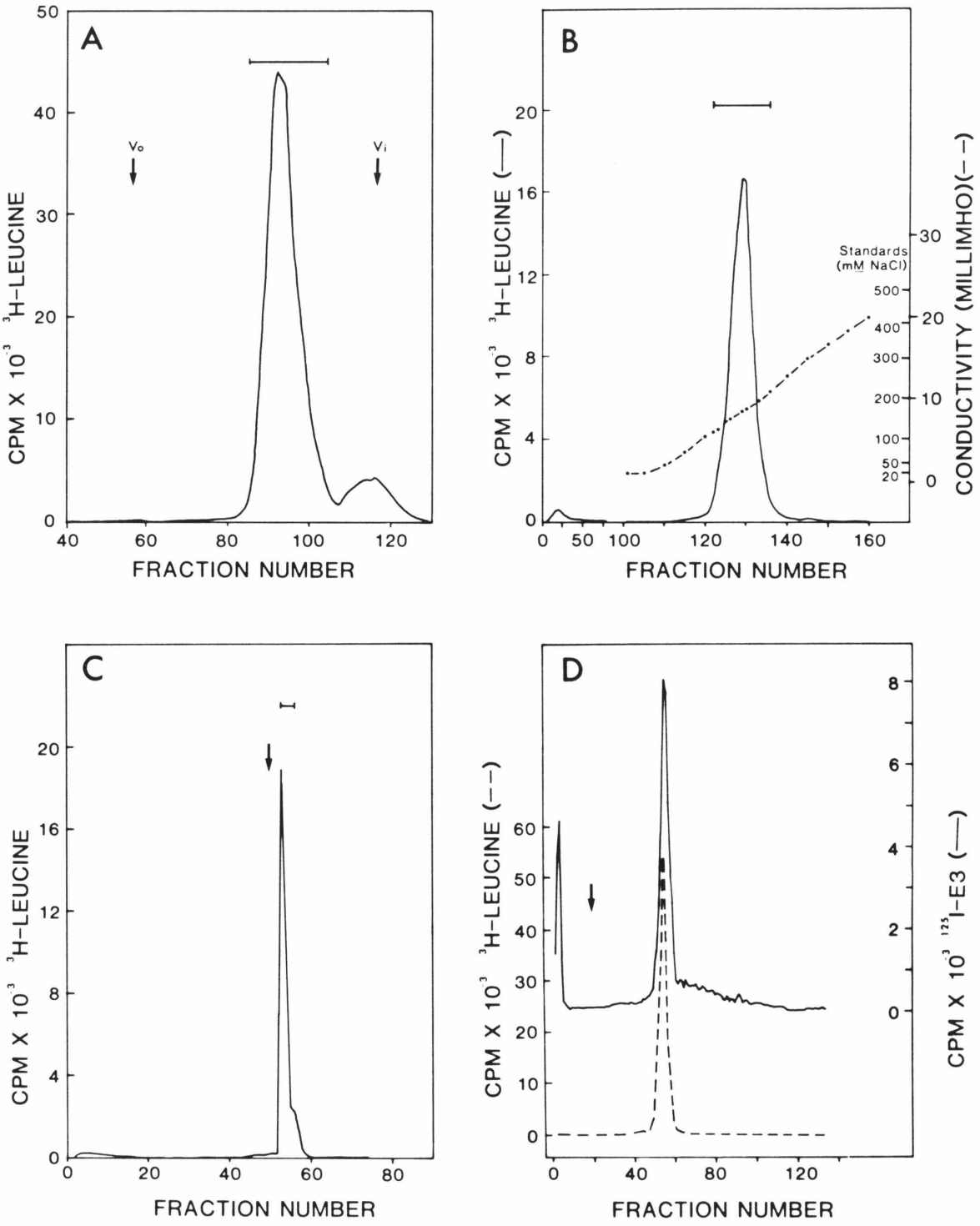
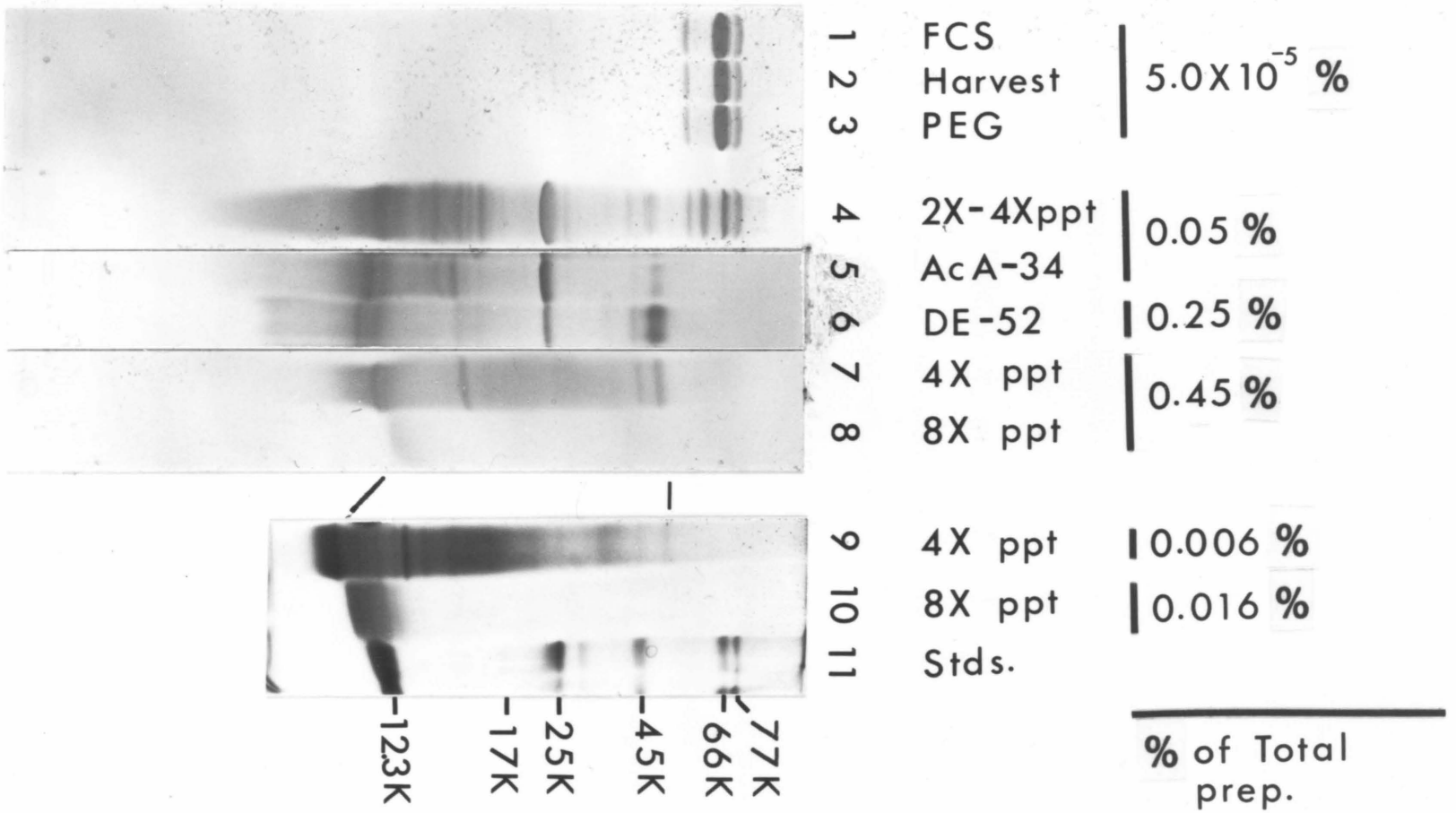

A
B
C
D
CPM X 10⁻³ ³H-LEUCINE
FRACTION NUMBER
Vo
Vi
CPM X 10⁻³ ³H-LEUCINE (—)
CONDUCTIVITY (MILLIMHO)(– –)
Standards (mM NaCl)
CPM X 10⁻³ ³H-LEUCINE (– –)
CPM X 10³ ¹²⁵I-E3 (—)

FIG. 6. Purity of E3 through the purification procedures. Aliquots of the preparation were saved during purification and analyzed by polyacrylamide gel electrophoresis. The percentage above each lane indicates the percentage of the total preparation loaded in the lane, and the headings indicates the purification step through which the sample has proceeded. Lane 1: Aliquot of fetal calf serum as an indicator of its contribution to the starting material. Lane 2: Culture fluid harvested from infected cells. Lane 3: Supernatant after PEG precipitation. Lane 4: Four volume pellet from 2X-4X differential ethanol precipitation. Lane 5: Pooled peak from gel filtration (Ultrogel AcA-34). Lane 6: Pooled peak from ion exchange column (Sephadex DE-52). Lanes 7 + 9: Four volume ethanol precipitate of pooled lentil lectin column peaks. Lanes 8 + 10: Eight volume ethanol precipitate of supernatant from 4 volume precipitation. Lane 11: Standards. Ovatransferrin (77K), bovine serum albumin (66K), ovalbumin (45K), chymotrypsinogen (25K), myoglobin (17K), cytochrome c (12.3K). Lanes 1-8: Coomassie blue stained 20% polyacrylamide gel. Lanes 9-11: Silver stained 15% polyacrylamide gel.


1 2 3 4 5 6 7 8 9 10 11
FCS
Harvest
PEG
5.0X10⁻⁵ %
2X-4Xppt
AcA-34
0.05 %
DE-52
0.25 %
4X ppt
8X ppt
0.45 %
4X ppt
0.006 %
8X ppt
0.016 %
Stds.
123K
77K
66K
45K
25K
17K
% of Total prep.

well with the silver staining method, however (Fig. 6, lane 10). Other methods of staining protein in gels, such as Schiffs staining and fluorescein labeled Conconavalin A staining did not succeed due to diffusion of E3 out of the gels during the extensive washing required by these procedures, and glutaraldehyde fixation failed to stabilize E3 in gels. Thus, unless the gel was dried down immediately after running the multiple bands diffused together to form a broad smear.

To verify the purity of the final E3 preparation an aliquot was radioactively labeled with ^{125}I and compared to [^{3}H]-leucine labeled E3 (Fig. 5D). Assuming that all of the contaminants are iodinated to the same degree as E3, this gel shows that the E3 preparation was greater than 90% pure. Silver staining also confirmed this estimate (Fig. 6, lane 10). The only stainable substances in the lane are E3 and contaminants which overlap with the lower molecular weight side bands of E3. It was very clear on the original silver stained gel that these were two different components, as they stained different colors. As a comparison, Fig. 6, lane 9 contains the proteins which precipitated with 80% ethanol.

We obtained 369 nmole (2.76 mg) of pure E3 from 420 roller bottles of chick cells (27 g protein, mostly serum proteins). This was 38% overall recovery of starting E3 and a 2580-fold enrichment (Table 1). These numbers are based on the assumption that all of the E3 is recovered through the polyethylene glycol precipitation and the differential ethanol precipitations, since the large excess of free label at these stages precluded monitoring the loss of radiolabeled E3. These assumptions were verified by mixing experiments using a small quantity of radiochemically pure E3 as a tracer (data not shown).

Amino Acid Composition

The results from amino acid compositions of E3 and comparisons with the theoretical values as deduced from the sequence of the 26S mRNA (Rice and Strauss, 1981) are shown in Table 2. The fit to the theoretical values is fairly good but there are some exceptions, which when used in conjunction with the RNA sequence (Fig. 8), yield information about the structure of E3. The most interesting difference is that the experimental amino acid composition almost completely lacks lysine, whereas the RNA sequence shows a lysine at position 63 (out of 64) in the sequence ($-Gly_{60}-Arg_{61}-Ser_{62}-Lys_{63}-Arg_{64}-COOH$). Thus, it is likely that at least the carboxy terminal $-Lys_{63}-Arg_{64}-COOH$ is not present in mature E3. The composition shows 5 instead of the theoretical 6 arginine residues, which is consistent with this hypothesis. The presence of the serine at position 62 is less clear. The composition shows an underrepresentation of serine by 1.7 residues, but serine and threonine (underrepresented by 1.1 residues) are prone to degradation during hydrolysis. We cannot definitely conclude whether or not this serine (or the arginine at position 61) is present or, if so, to what extent.

Amino Terminal Sequence

The primary amino acid sequence of the N-terminal 16 residues of E3 (Fig. 7) are compared with the amino acid sequence deduced from the sequence of 26S RNA (Rice and Strauss, 1981) and the amino terminal sequence of PE2 (Bell *et al.*, 1982) in Fig. 8. The sequences are identical, verifying that E3 forms the N-terminus of PE2 and that the processing of PE2 to E2 and E3 leaves the N-terminus of E3 unaltered. We were unable to detect residues 10 and 14, and residues 1 and 16 were only

Table 2

Amino Acid Composition of E3

	From analyzer[a]		From RNA sequence[c]	
	mole %	residues[b]		Δ[d]
ASP	> 11.71	6.8	< 3	
ASN			4	
THR	5.05	2.9	4	-1
SER	7.34	4.3	6	-2
GLU	> 7.81	4.5	< 4	
GLN			0	
PRO	8.33	4.8	5	
GLY	5.69	3.3	3	
ALA	12.10	7.0	6	+1
CYS	ND[e]	ND	4	
VAL	5.95	3.4	3	
MET	1.40	0.8	1	
ILE	3.88	2.2	2	
LEU	15.36	8.9	8	+1
TYR	2.76	1.6	2	
PHE	1.88	1.1	1	
HIS	2.17	1.3	1	
LYS	0.22	0.1	1	-1
ARG	8.36	4.9	6	-1
TRP	ND	ND	0	
TOTAL			64	
MOL. WT.			7475[f]	

Table 2 (continued)

[a]Results from amino acid analysis.

[b]Best fit using RNA sequence data and assuming that one Lys and one Arg are missing (see text).

[c]Amino acid composition deduced from the RNA sequence (Rice and Strauss, 1981) assuming all residues between the C-terminus of capsid protein and the N-terminus of E2 are present.

[d]Second column minus third column rounded to the nearest integer.

[e]Not determined.

[f]Not including carbohydrate content.

FIG. 7. Yields of amino acid phenylthiohydantoin (PTH)-derivatives from the spinning cup sequenator analysis of 10.2 nanomoles of E3. Aliquots of each cycle were analyzed by high pressure liquid chromatography, peaks were quantitated by comparison with a standard mixture of PTH-amino acids, and the yields were normalized to an injection of 100% of the sample. PTH-cysteine was not determined.

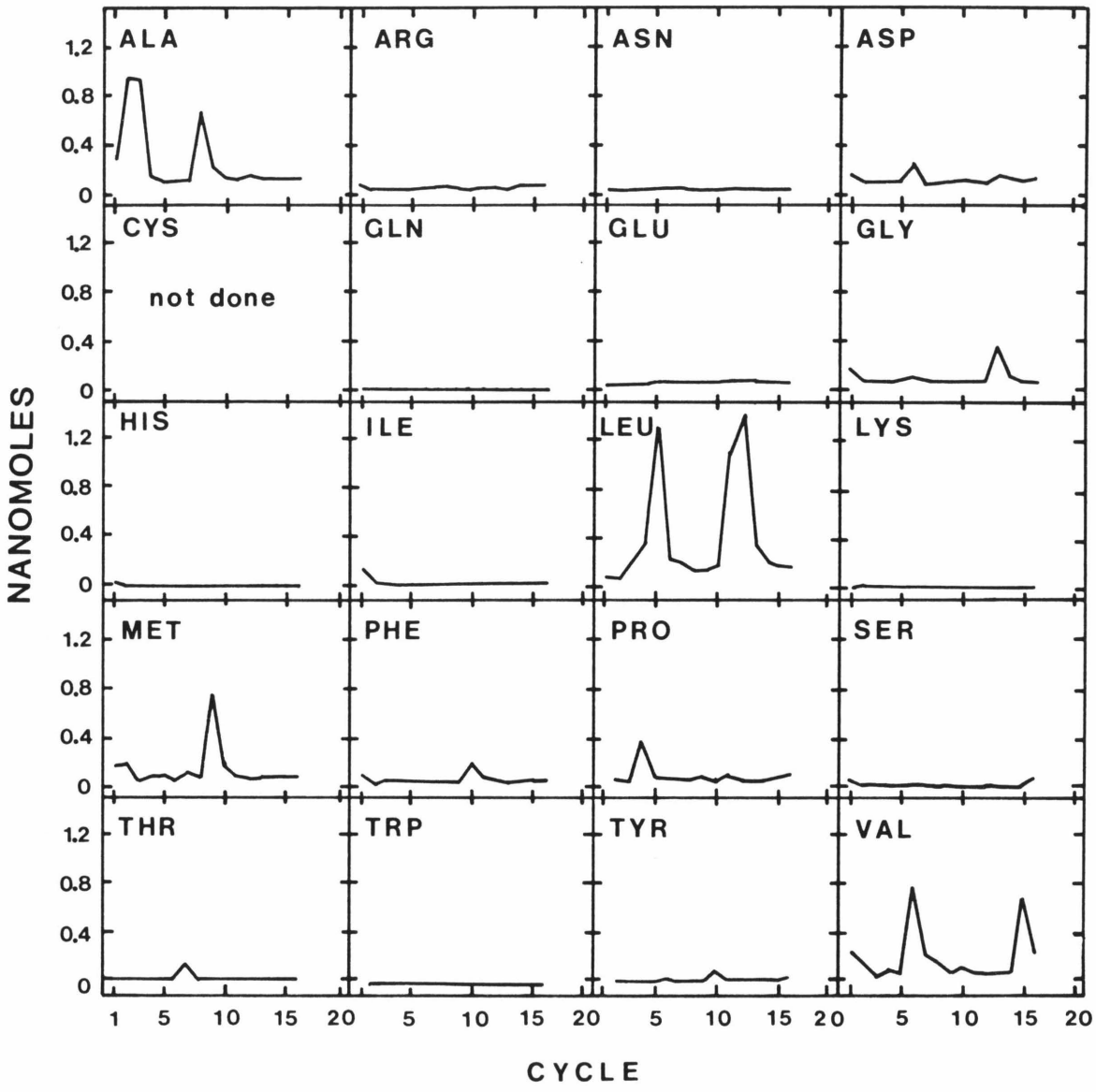
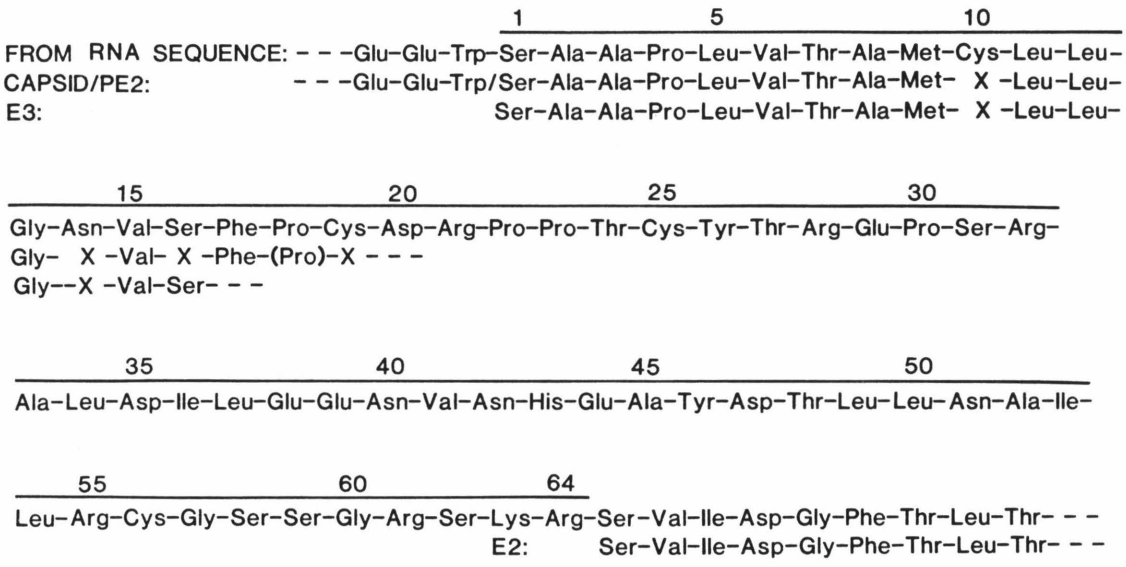

ALA
ARG
ASN
ASP
CYS
not done
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
NANOMOLES
0
0.4
0.8
1.2
1
5
10
15
20
CYCLE

FIG. 8. The N-terminal sequence of E3, as determined by Edman degradation and compared with other available sequence data. The symbol "X" indicates that no residue could be identified. The RNA sequence was determined by Rice and Strauss (1981), and the capsid, PE2, and E2 protein sequences were determined by Boege *et al.* (1980), Bell *et al.* (1982), and J. R. Bell *et al.* (1978), respectively. The potential 64 amino acid E3 sequence is marked by the solid overline.


1 5 10
FROM RNA SEQUENCE: - - -Glu-Glu-Trp-Ser-Ala-Ala-Pro-Leu-Val-Thr-Ala-Met-Cys-Leu-Leu-
CAPSID/PE2: - - -Glu-Glu-Trp/Ser-Ala-Ala-Pro-Leu-Val-Thr-Ala-Met- X -Leu-Leu-
E3: Ser-Ala-Ala-Pro-Leu-Val-Thr-Ala-Met- X -Leu-Leu-
15 20 25 30
Gly-Asn-Val-Ser-Phe-Pro-Cys-Asp-Arg-Pro-Pro-Thr-Cys-Tyr-Thr-Arg-Glu-Pro-Ser-Arg-
Gly- X -Val- X -Phe-(Pro)-X - - -
Gly--X -Val-Ser- - -
35 40 45 50
Ala-Leu-Asp-Ile-Leu-Glu-Glu-Asn-Val-Asn-His-Glu-Ala-Tyr-Asp-Thr-Leu-Leu-Asn-Ala-Ile-
55 60 64
Leu-Arg-Cys-Gly-Ser-Ser-Gly-Arg-Ser-Lys-Arg-Ser-Val-Ile-Asp-Gly-Phe-Thr-Leu-Thr- - -
E2: Ser-Val-Ile-Asp-Gly-Phe-Thr-Leu-Thr- - -

identified as serine by small amounts of characteristic breakdown products. The RNA data show that residues 1 and 16 are indeed serines and residue 10 is cysteine, whose phenylthiohydantoin derivatives are extensively degraded during sequencing, but residue 14 is an asparagine, which should have been detected. From RNA sequence analysis (Fig. 8) the asparagine at this position is the only potential glycosylation site of the Asn-X-Ser/Thr type (Marshall, 1974), and this asparagine is thus presumably glycosylated. The lack of sequence at position 14 confirms this view, and identical results were obtained for PE2 (Bell et al., 1982).

It was found that the yields of the phenylthiohydantoin derivatives were only about 10% of the expected values. This was not unexpected, as it has been shown that 80-90% of purified PE2 is unsequenceable due to N-terminal acetylation (Bell et al., 1982; Bell and Strauss, 1981). The similarities in sequenceable yields indicate that the N-terminus of E3 is probably not further altered after its cleavage from PE2.

Lysine Content of E3

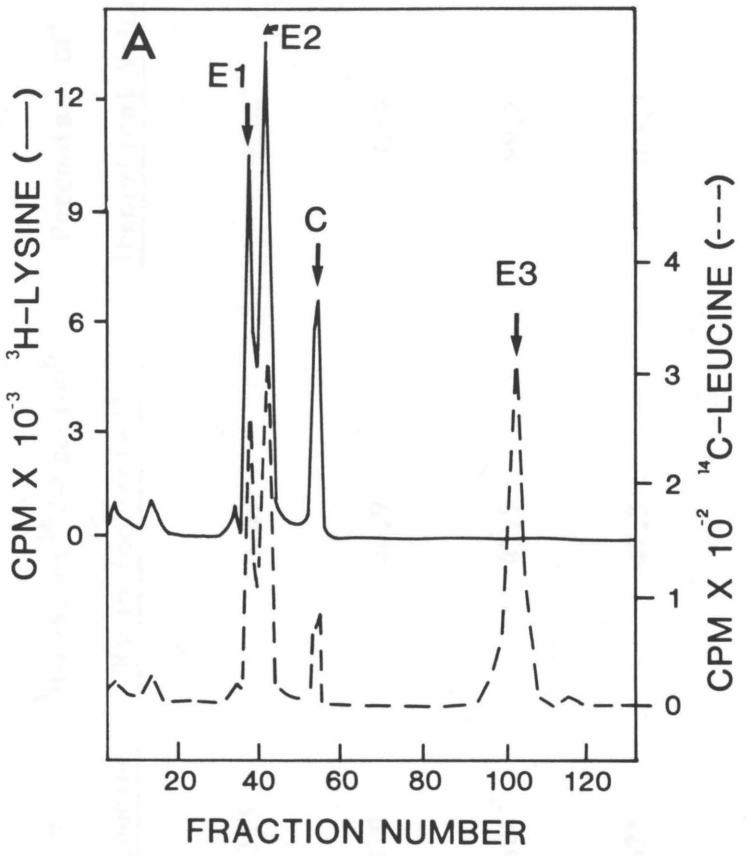
Because of the similarity of the potential cleavage site at the C-terminus of E3 to the cleavage sites of many proproteins that are cleaved intracellularly (Lodish, 1981), and the suggestive amino acid composition data, we further investigated the lysine content of E3. A pulse chase experiment was performed whereby cells were labeled with [^{3}H]-lysine or [^{14}C]-leucine for 30 min and chased with unlabeled medium for 30 min to investigate the percentage of E3 that contained lysine at short times after synthesis. The radiolabeled proteins that were released into the culture fluid as virions or E3 during the 30 min chase (virtually no label appeared in the culture fluid during the 30 min pulse, data not shown) were analyzed

on polyacrylamide gels for the incorporation of [^{3}H]-lysine and [^{14}C]-leucine (Fig. 9) and carefully quantitated. By using the theoretical ratios (from the RNA sequence) of the number of lysines and leucines in each of the proteins and assuming that the E1, E2, and capsid proteins isolated were intact, the percentages of E3 molecules that contain lysine can be calculated (Table 3). A maximum of 1% of the E3 molecules contained lysine, which is probably an overestimation in light of the background noise levels of the experiment and the fact that the small lysine peak which contributed most of the counts did not actually line up with the E3 peak (Fig. 9B). In addition, mock infected cells which were labeled with [^{3}H]-lysine produced a background in the vicinity of the E3 peak sufficient to obscure the detection of E3 containing less than about 2% lysine (data not shown). Thus, virtually all of the E3 molecules have had at least the two C-terminal amino acids removed during or shortly following the cleavage to remove E2 (see Fig. 8).

Distribution of Viral Specific Proteins during Infection

A careful analysis of the distributions of viral proteins during infection using two different growth conditions yielded information concerning the relationship between PE2 cleavage and virion budding. Virus was grown either in medium of normal ionic strength, or by the low salt-high salt reversal technique (Pierce et al., 1974). Under the latter conditions envelope proteins and nucleocapsids accumulate in the cells, but the virions do not bud until the addition of medium of high ionic strength, after which a rapid burst of budding occurs (Waite and Pfefferkorn, 1970; J. W. Bell et al., 1978). Aliquots of the different culture fluids and cell lysates were analyzed on polyacrylamide slab gels (Fig. 1). The viral

FIG. 9. Lysine content of E3. At 5.5 hr post-infection 60 mm petri plates of chick cells were labeled with 250 µCi of [^{3}H]-lysine or 12 µCi of [^{14}C]-leucine for 30 min in medium lacking the corresponding unlabeled amino acid followed by a 30 min chase with unlabeled medium containing twice the normal concentration of lysine and leucine. The harvested culture fluid was immediately brought to 200 µg/ml phenylmethylsulfonyl-fluoride and 1% SDS and put on ice in order to prevent degradation. Aliquots from [^{14}C]-leucine and [^{3}H]-lysine labeled plates were mixed and processed as follows: A: Culture fluid was precipitated with 4 volumes of ethanol to precipitate all protein present. B: Culture fluid was precipitated by 2X-4X differential ethanol precipitation as described in text to precipitate only E3. Both preparations were analyzed by gel electrophoresis on 6 mm X 20 cm tube gels, sliced into 1 mm slices and counted by liquid scintillation. The top of the gels are to the left and the bottom 7 cm of the gels were not sliced. (——) [^{3}H]-lysine. (---) [^{14}C)-leucine.


A
E1
E2
C
E3
CPM X 10^{-3} ^{3}H-LYSINE (—)
CPM X 10^{-2} ^{14}C-LEUCINE (- - -)
0
3
6
9
12
0
1
2
3
4
20
40
60
80
100
120
FRACTION NUMBER

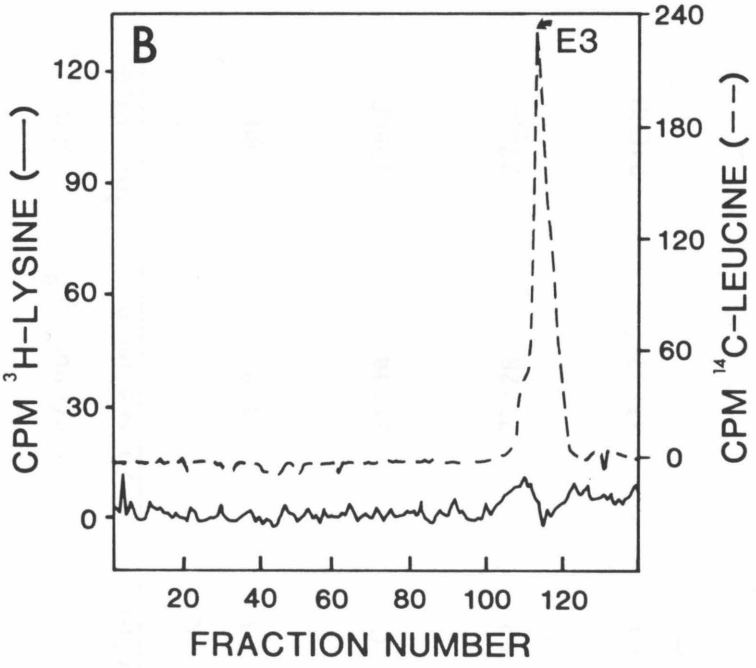

B
E3
CPM ^{3}H-LYSINE (—)
CPM ^{14}C-LEUCINE (- - -)
0
30
60
90
120
0
60
120
180
240
20
40
60
80
100
120
FRACTION NUMBER

Table 3

Lysine Content of E3

Protein	Lysine/Leucine[a] in sequence	CPM ^{3}H-Lysine	CPM ^{14}C-Leucine	^{3}H-Lysine:^{14}C-Leucine[b] Ratio (corrected)	Percentage of[c] Theoretical Value
E3	1/8	80	1313	0.49	1.06
C	25/14	13400	170	44.9	97.7
E2	27/28	27400	624	45.5	99.2
E1	23/27	21000	521	47.3	103.1

Table 3 (continued)

[a]From RNA sequence (Rice and Strauss, 1981).

[b] $\frac{^{3}\text{H-Lysine cpm}}{^{14}\text{C-Leucine cpm}} \times \frac{1}{\text{Lysine/Leucine Ratio}}$

[c]100% = average of corrected ^{3}H-Lys:^{14}C-Leu ratio (previous column) from C, E1, and E2.

protein bands from polyacrylamide tube gels were quantitated and normalized using the known amino acid compositions from the RNA sequence (Rice and Strauss, 1981) and the results tabulated in Table 4.

We found that E3 was released into the culture fluid, even under the low ionic strength conditions where virions (as monitored by E1, E2 and C) are not released. In addition, under either growth conditions there is not a 1:1 correspondence between release of E3 and virions, but rather a 2-3 fold excess of E3 released. Much of this excess can be explained by the large amount of E1 and E2 which remains associated with the cell and presumably the rest is due to turnover of these proteins before they mature into virions. Large quantities (30-60%) of E1 and E2 do not mature into virions. In addition, Table 4 confirms the results of others that E1, E2, and capsid exist at approximately a 1:1:1 ratio in virions (Rice et al., 1982; Schlesinger et al., 1972), but that an excess of capsid is made within infected cells (Cancedda and Schlesinger, 1974).

Comparison of the Kinetics of Release of E3 and Viral Structural Proteins

Additional information concerning the mechanism of viral budding can be obtained by comparing the kinetics of release of E3 and the structural proteins (as virions) into the culture fluid. To do this, a pulse chase experiment using a very long pulse was used. [^{3}H]-leucine was added to infected cells at 3 hr, before large quantities of structural proteins are being made, and left on the cells for 4 hr in order to label virtually all viral structural proteins present in the cells. At 7 hr, after the plateau of virion production has been reached (Strauss and Strauss, 1977), the label was removed and the medium changed and saved at 0.5-1 hr intervals, in order to follow the decay in the amounts of labeled viral protein being

Table 4

Distribution of Sindbis Virus Proteins during Infection[a]

	Low Salt-High Salt Reversal				Regular Infection		
Protein	Low Salt Harvest	High Salt Harvest	Cell Lysate	Total	Culture Fluid Harvest	Cell Lysate	Total
C	1.1	57.0	111.0	169.1	37.8	229.9	267.5
PE2	0.4	0.0	7.2	7.7	0.0	20.0	20.0
E3	136.9	4.5	10.0	151.5	149.9	17.3	167.1
E2	1.6	66.5	31.9	100.0	41.3	58.7	100.0
6K	0.0	0.0	57.4	57.4	0.0	70.1	70.1
E1	4.9	65.9	29.1	100.0	49.2	69.0	118.8

Table 4 (continued)

[a]Chick cells were infected, labeled and processed as described in Figure 1. Aliquots were run on 20 cm x 6 mm polyacrylamide tube gels, and one mm slices counted by liquid scintillation. The numbers are the relative quantities of viral proteins produced during infection. All quantities are normalized to total E2 produced equal to 100.0% (underlined)

released (Fig. 10, lanes 1-6, 12). The radioactivity in each of the viral proteins released into the culture fluid was carefully quantitated and their "specific activities" calculated by dividing by the number of plaque forming units released (Fig. 11). This correction yields a true specific activity for the structural proteins (quantity of labeled protein per virion), but not for E3 due to the lack of a 1:1 correspondence between release of E3 and structural protein (in virions). It does, however, allow for the correction for different durations of the chase harvests and for the drop in the number of healthy cells late in infection. The amounts of E1 and E2 could not be quantitated separately due to the inability to separate them on the tube gels used, but as can be seen from Fig. 10, the kinetics of their chase were essentially identical.

The key result of this experiment is that although E2 and E3 are physically connected until late in their maturation, the kinetics of their release into the culture fluid are very different. All but 7.4% of the E3 labeled during the 4 hr pulse has been released from the cells before the chase has even started, whereas 40% of the labeled envelope protein released from the cells throughout the experiment is released during the chase. Eighty-seven percent of the labeled E3 released throughout the 4 hr chase appears in the culture fluid within the first hr, but only 44% of the labeled envelope protein appears during this same period. Even after 4 hr of chase, a large percentage of the total labeled envelope protein (45%) is still in the cell. Some of this material may be a deadend pathway and not available for release, but clearly some is still being released and is available for incorporation into virus between 3 and 4 hr into the chase. Fig. 11 shows the drop in the specific activity of the viral proteins

FIG. 10. The kinetics of the release of E3 and structural proteins during Sindbis virus infection. Lanes 1-6, 12: A 60 mm petri plate of infected cells was labeled from 3-7 hr post-infection with 50 µCi/ml [^{3}H]-leucine in medium containing 1/10 the normal concentration of leucine in order to uniformly label all viral proteins in the cells. At 7, 7.5 , 8, 9, 10, and 11 hr post-infection the culture fluid was harvested, the cells washed, and new culture fluid containing twice the normal concentration of leucine added. At 11 hr post-infection the cells were lysed with 1% SDS. Lanes 7-11, 13: A 60 mm plate was processed as above, except no radioactivity was added at 3 hr and 12.5 µCi/ml [^{3}H]-leucine was added each time new medium (containing 1/10 the normal concentration of leucine) was added to the plates during the chase. All samples were precipitated and analyzed by gel electrophoresis. P: pulse. L: lysate. $^1/_2$, 1, 2, 3, 4: the number of hours after the "pulse" was stopped. A longer exposure of the E3 portion of the gel is shown below the full gel.

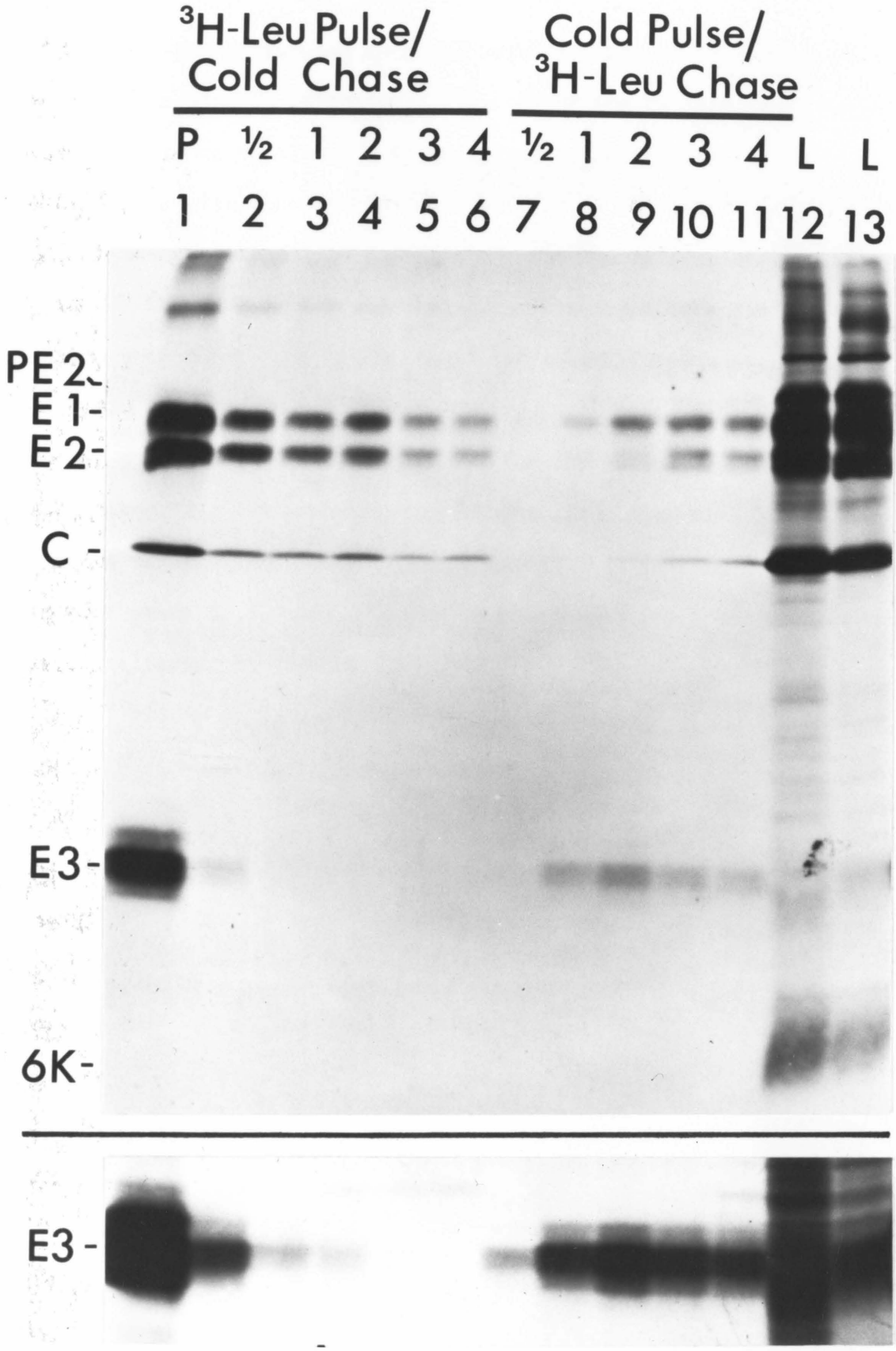
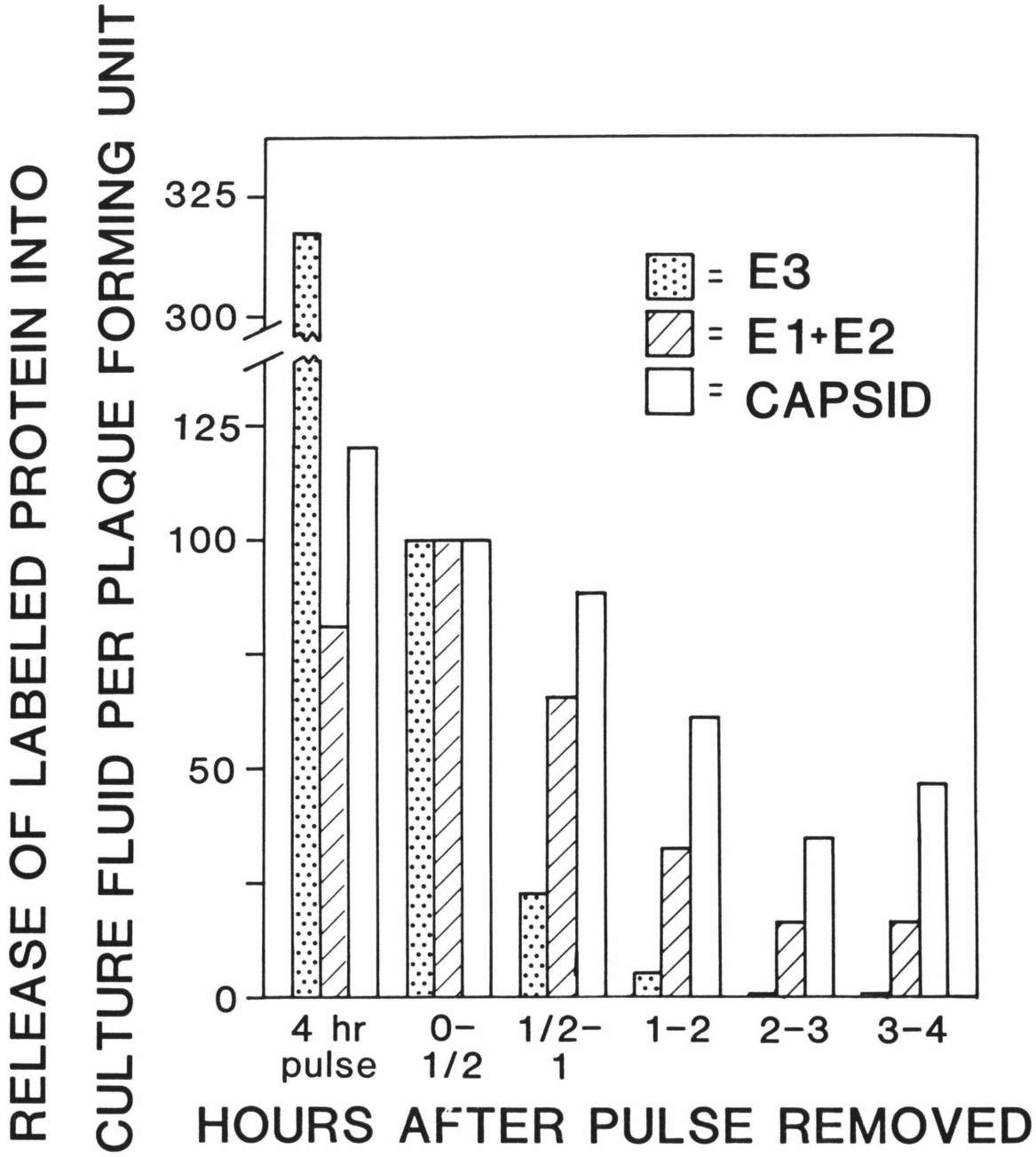

^{3}H-Leu Pulse/
Cold Chase
Cold Pulse/
^{3}H-Leu Chase
P ½ 1 2 3 4 ½ 1 2 3 4 L L
1 2 3 4 5 6 7 8 9 10 11 12 13
PE 2-
E 1-
E 2-
C -
E3-
6K-
E3 -

FIG. 11. Patterns of release into the culture fluid of E3 and structural proteins during Sindbis infection. Aliquots of the culture fluid harvests described in Figure 10 (lanes 1-6) were used to quantitate the radioactivity in the viral proteins using 6 mm x 20 cm polyacrylamide tube gels and liquid scintillation. In order to correct the release of labeled proteins for the number of viable cells, especially late in infection, the quantities were normalized to the number of plaque forming units in the samples using a standard plaque assay (Strauss et al., 1976). All the quantities are further normalized to 100 for the period 0-0.5 hr after the label was removed. The amounts of E3 at the 3 and 4 hr time points were the same as or less than the background noise level in that region of the gel (pooled peaks of 586 and 277 CPM, respectively) and therefore are possibly artifacts.


RELEASE OF LABELED PROTEIN INTO
CULTURE FLUID PER PLAQUE FORMING UNIT
325
300
125
100
50
0
= E3
= E1+E2
= CAPSID
4 hr pulse
0-1/2
1/2-1
1-2
2-3
3-4
HOURS AFTER PULSE REMOVED

throughout the experiment, normalized to 100 for the period of the first 0.5 hr of the chase. As noted above, E3 shows a rapid release, with most of E3 released after a transit time of 0.5-1 hr to the cell surface. E1 and E2, on the other hand, show a slower stochastic release, presumably because the newly synthesized unlabeled protein must compete with the large pool of premade labeled protein during the chase. The drop in the specific activity of labeled capsid protein released is much slower still and the amount left inside the cells after the chase is larger (86%). This implies an even larger pool of capsid protein in the cell which is consistent with the observation that although E1 and E2 are made in a 1:1 ratio, capsid is made in excess (Cancedda and Schlesinger, 1974).

The complementary experiment where only the chase contained [^{3}H]-leucine gave comparable results (Fig. 10). The E3 specific activity quickly increased such that virtually all of the E3 being released after 0.5 to 1 hr was labeled, whereas the specific activity of the structural protein continued to increase throughout the chase (quantitation not shown). In addition, a double label version of this experiment using a [^{3}H]-leucine pulse and a [^{14}C]-isoleucine chase (data not shown) confirmed that the level of release of E3 as well as virions stayed approximately constant throughout the chase period.

DISCUSSION

In this study, we have identified, purified, and analyzed a small Sindbis specific glycoprotein which is found in the culture fluid of infected cells. From our data (including pulse chase experiments, tryptic peptide mapping, and N-terminal sequencing) this protein has been shown to be produced from the cleavage of PE2 to E2, analogous to Semliki Forest

virus E3, even though it is not associated with the virions, as is E3 in Semliki Forest virus (Garoff et al., 1974). Similar studies have been done independently by Welch and Sefton (1979). Additional studies, including labeling experiments with Sindbis virus infected cells have yielded information concerning the maturation of virions and the viral envelope glycoproteins.

PE2, which is inserted into and translocated across the membrane while still nascent, presumably contains an N-terminal signal sequence which is not cleaved (Wirth et al., 1977; Bonatti et al., 1979; Bonatti and Blobel, 1979). Since E3 is located at the N-terminal end of PE2 in both Sindbis and Semliki Forest virus (Rice and Strauss, 1981; Garoff et al., 1980), the signal sequence should be at its N-terminus also, assuming that is is not further processed after cleavage from PE2. The N-terminal sequence reported here and previously (Rice and Strauss, 1981) confirms that at least the first 16 amino acids are uncharged, as expected of a signal sequence.

Although it seems likely that this is the signal sequence, in some ways it is atypical. The most striking difference is the fact that it is not cleaved off during translocation, although it seems to contain several potential cleavage sites similar to those reported for other signal sequences (Lodish, 1981; Inouye and Halegoua, 1980; Zimmerman et al., 1980), after the Gly_{13}, the Ser_{16}, or the Cys_{18}. So far E3 (PE2) is the only protein with a signal sequence of "typical topology" that we know of that is not cleaved. The other examples of proteins with uncleaved signal sequences have atypical topologies such as internal signal sequences or N-terminal membrane anchors.

There is also a potential glycosylation site of the Asn-X-Ser/Thr type (Marshall, 1974) at position 14, within the presumed signal sequence. Since this is the only potential glycosylation site in E3 (Rice and Strauss, 1981), and E3 is glycosylated, it is presumably glycosylated at this site. The failure to detect a phenylthiohydantoin derivative at position 14 during N-terminal sequenation supports this view. While glycosylation should not affect its function as a signal sequence, since glycosylation occurs after translocation (Lodish, 1981), this very hydrophilic sugar on the hydrophobic signal sequence should profoundly affect the conformation and folding, and therefore the properties, of E3. It is tempting to hypothesize that the glycosylation in the signal sequence may be the reason that the signal sequence is not cleaved from PE2 while nascent. However, Bell et al. (1982) showed that when glycosylation of PE2 was inhibited by tunicamycin, the putative signal sequence is still not cleaved.

The N-terminus of E3 is found to be identical to that of PE2 (Bell et al., 1982; Bonatti and Blobel, 1979), indicating that there is no further proteolytic processing of E3's N-terminus during or after its cleavage from PE2 (Fig. 8). This similarity extends to the finding that 90% of both PE2 (Bell et al., 1982) and E3 are unsequenceable. Since labeling studies in PE2 showed that this was due to acetylation of the N-terminal serine (Bell and Strauss, 1981), and PE2 and E3 share the same N-terminus, the unsequenceable E3 is also probably acetylated. These results suggest that the N-terminal acetylation does not perform any major function after translocation. Neither acetylated nor nonacetylated forms are apparently used or degraded preferentially, as this would skew the relative

amounts of these two species. N-terminal acetylation, which is common among structural proteins, is found to occur on the cytoplasmic side of the membranes (Jornvall, 1975), and therefore the acetylation of PE2 probably occurs prior to insertion into the endoplasmic reticulum (see Bell and Strauss (1981) for a more detailed discussion).

In addition to its probable function as a signal sequence, E3 has many of the characteristics of the "pro" portion of a proprotein (see Zimmerman et al., 1980). It is cleaved from PE2 very late in maturation, as are most proproteins. This cleavage is similar to a common cleavage among proproteins, involving the cleavage at two or more basic residues (Lys, Arg) via a trypsin-like activity followed by a carboxypeptidase B-like activity. The absence of the C-terminal Lys-Arg of E3 that we observed via amino acid compositions and lysine labeling experiments imply that E3 is cleaved by such a mechanism. All of the known examples of this type of cleavage occur intracellularly (Docherty and Steiner, 1982; Zimmerman et al., 1980). This observation argues circumstantially that the cleavage of PE2 to E2 and E3 occurs intracellularly. There are numerous other reports in the literature that offer conflicting data as to whether the cleavage occurs intracellularly (for instance, Smith and Brown, 1977; Bonatti and Cancedda, 1982; Hakimi and Atkinson, 1982) or at the plasma membrane (for instance Jones et al., 1977; Ziemiecki et al., 1980). In Semliki Forest virus Green et al. (1981) could not detect any intracellular E2, but also could not detect any PE2 at the plasma membrane. More sensitive techniques will have to be devised in order to resolve this conflict adequately.

Our data argue against the model sometimes proposed that the cleavage of PE2 is the event that triggers virus budding. When the low salt-high salt reversal method is used for virus growth, 90% of the E3 released is released into low salt medium, whereas greater than 95% of the virus does not bud until the addition of medium of high ionic strength. Although we cannot absolutely eliminate the possibility that it is the abnormal conditions of the low ionic strength medium that uncouples any cause-effect relationship, we feel that it is unlikely. Even in the virus infection under regular salt conditions, by 12 hr after infection a large fraction of the E2 made (60%) is still associated with the cells, although virtually all of the E3 has been released. The kinetics of the appearance of the structural proteins (as virions) and the release of E3 in the culture fluid supports our view. Although E2 and E3 are physically attached, and therefore follow the same maturation route until very late in their maturation, the kinetics of their appearances in the culture fluid are very different (Fig. 11). Labeled E3 is chased into the culture fluid much more quickly than E2. Whereas essentially all the labeled E3 has been chased into the culture fluid by 2 hr, a significant amount of E2 is still being released at the latest time that we examined.

Our results are consistent with the following model. PE2 is synthesized in the endoplasmic reticulum and processed to the point where E3 is cleaved with deterministic kinetics, proceeding through the various steps in maturation in well-defined intervals, with most of the newly made E2 (or PE2) reaching the plasma membrane within 1 hr of synthesis. E3 also follows these kinetics, as it is released into the culture fluid very rapidly upon cleavage from PE2. In contrast, once E2 reaches the cell

surface its release into virions follows stochastic kinetics, with newly made E1 and E2 competing with a large pool of preexisting envelope proteins for incorporation into virions. The chase of labeled capsid protein is even slower, consistent with our results and those of others (Cancedda and Schlesinger, 1974) that there is excess capsid protein made relative to envelope protein.

Johnson et al. (1981) have reported a different result using a combination of pulse-chase experiments and fluorescent photobleaching recovery studies. They find that Sindbis virus structural proteins are quickly chased into virions and that they may already be complexed with nucleocapsids before their appearance on the cell surface. We have not been able to combine the two sets of data into a consistent model but suggest one variable which may account for our different results. Whereas they used the wild type virus stock, we used a strain (HR) which was selected long ago for resistance of the virion to 60°C (Burge and Pfefferkorn, 1966). These two strains, with different properties, and thus changes within the structural proteins, may have proteins with different tendencies for aggregation which could profoundly affect the kinetics of virion maturation. This would also be consistent with the fact that they find extensive aggregation of structural proteins at the plasma membranes, whereas Rice and Strauss (1982) find, using crosslinking studies on the HR strain, a maximum of 6 structural proteins (3 sets of E1:E2 dimers) complexed to any large degree. Further studies will need to be done to resolve these differences.

Since the specific activities of all of the viral proteins can be easily calculated from their sequences (Rice and Strauss, 1981),

quantitative relationships can be readily calculated. There is a 1:1:1 relationship between the three structural proteins in virions as has been reported earlier (Rice et al., 1982; Schlesinger et al., 1972). If the total protein in all fractions of the infection is summed, there is an excess of capsid protein (see also Cancedda and Schlesinger, 1974), even relative to E3, which should be a good indicator of the total glycoprotein made. This excess could be due to one of two reasons. Either the ribosomes are abortively dissociating after capsid translation, or some percentage of the nascent PE2 is not attaching to and translocating across the membrane due to a limiting number of translocation sites (Lingappa et al., 1978) and is subsequently degraded. The excess of total E3 relative to E1 and E2 seen is probably due to recycling of viral glycoproteins from the plasma membrane, since endocytosis and membrane recycling are common events on plasma membranes.

These studies on the small nonstructural viral protein, E3, have allowed us to look at the maturation of Sindbis virus proteins and virions from a new perspective. As a result we have obtained new information and confirmed other data concerning their maturation. We feel that further studies along these lines could yield more information concerning the Sindbis virus infection.

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CHAPTER 3

Isolation and characterization of hybridomas producing monoclonal antibodies specific for Sindbis virus structural proteins

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ABSTRACT

Hybridomas were selected by the fusion of BALB/c MOPC21 NSI/1 myeloma cells with spleen cells from mice inoculated with either Triton X-100 treated Sindbis virions or with membrane preparations from cells infected with ts-23, a Sindbis virus temperature sensitive mutant that fails to cleave the precursor protein PE2 at the nonpermissive temperature. Ten stable hybridomas were obtained, seven producing antibodies specific for the envelope glycoprotein E1 and three producing antibodies specific for the capsid protein, and the immunoglobulin subclasses of these antibodies were determined. The seven E1-specific monoclonal antibodies were divided into two classes, which reacted with different antigenic domains of E1, as determined by a binding competition assay. The two classes of antibodies differed in several tested properties. One class, referred to as the E1(a) class and including five of the clones, reacted with a protein preparation (E1 + E2 solubilized in Triton X-100) that the second class, called E1(b) did not. In addition, E1(a) antibodies precipitated intact virus to a much greater degree than E1(b) antibodies. Two of the five E1(a) monoclonal antibodies inhibited viral infectivity, and one of these precipitated E2 along with E1 in Triton-treated virus preparations. These properties are discussed with regard to the known relationships between the viral structural proteins.

INTRODUCTION

The three viral structural proteins that are produced during Sindbis virus infection (two envelope glycoproteins, E1 and E2, and a nucleocapsid protein, C) mature via a complex pathway from a common polyprotein precursor, involving several proteolytic cleavages (Rice and Strauss, 1981). The two glycoproteins follow a maturation scheme very similar to that of cellular plasma membrane glycoproteins and secreted proteins, involving cotranslational insertion into the endoplasmic reticulum and transport through the Golgi apparatus to the plasma membrane (Green et al., 1981), during which they are modified by the addition of carbohydrates (Sefton and Keegstra, 1974) and fatty acids (Schmidt and Schlesinger, 1980). In addition, the precursor to one of these glycoproteins (PE2) has an N-terminal peptide (E3) removed very late in its maturation to produce E2 (Schlesinger and Schlesinger, 1972; Welch and Sefton, 1979). Finally, the three structural proteins (E1, E2, and C), plus the viral RNA, are assembled into virions which bud from the infected cells (Brown, 1980).

These processes have been studied extensively, using a variety of approaches (for reviews, see Strauss and Strauss, 1977; Simons and Warren, 1983). Specific probes, such as probes for a particular precursor form of a protein (i.e., PE2) or probes for a particular conformation of a protein (i.e., E2 when complexed to E1) would be very useful for further study of these processes. The new techniques for producing hybridomas which secrete large quantities of a single antibody should be very useful for generating these probes. Since this procedure produces antibodies specific for a single antigenic determinant, antibodies to a number of these specificities may be obtainable given the proper immunization and selection scheme.

Several laboratories have reported the generation of hybridomas which produce antibodies specific for Sindbis structural proteins (Roehrig et al., 1980, 1982; Chanas et al., 1982) and have used them to study in detail the contribution of particular viral proteins in several virus-mediated events, such as infection of susceptible host cells, and hemagglutination and hemolysis of erythrocytes. Monoclonal antibodies have also been tremendously useful in studying the function of antigenic determinants of a number of other viruses, including murine leukemia virus, (Stone and Nowinski, 1980; Oroszlan and Nowinski, 1980), mouse mammary tumor virus (Massey et al., 1980), influenza virus (Laver et al., 1979), and reovirus (Lee et al., 1981).

We report here the isolation and characterization of 10 hybridomas producing monoclonal antibodies specific for the structural proteins of Sindbis virus, including one which may be useful in investigating the interactions between E1 and E2 (or PE2) during maturation.

MATERIALS AND METHODS

Antisera and materials. Rabbit antisera specific to mouse immunoglobulin and immunoglobulin subclasses were obtained from Bionetics Laboratories, and Protein A from Staphylococcus aureus was obtained from Pharmacia. The preparation of rabbit antisera specific to Sindbis virus and individual Sindbis virus proteins have been described previously (Birdwell and Strauss, 1974; Rice and Strauss, 1982). Dinitrophenol coupled to bovine serum albumin (DNP-BSA) plus a hybridoma producing antibodies specific to this antigen were kind gifts of Dr. Jack Richards.

Cells, virus, and laboratory animals. Sindbis virus (HR large plaque strain, Burge and Pfefferkorn, 1966) was grown in monolayers of primary

chick embryo fibroblasts and purified as described previously (Bell et al., 1979). The growth of the Sindbis virus temperature sensitive mutant ts-23 to obtain virus infected membrane preparations was done in either chick or BALB/c 3T3 cells (kindly provided by C. F. Fox, UCLA). Plaque assays to quantitate infectious particles were done in chick cells (Strauss, et al., 1976).

The myeloma cell line BALB/c MOPC21 NSI/1, which produces kappa light chains but no heavy chain, was kindly provided by C. Milstein (Molecular Research Council, Cambridge), and grown in RPMI-1640 medium containing 1 mM pyruvate and 15% fetal calf serum. Antigens were inoculated into BALB/c mice and thymocyte feeder layers were obtained from three week old BALB/c or CD_2 F1 mice.

Preparation of ts-23 infected cell membranes. Chick cells or BALB/c 3T3 cells were infected with Sindbis virus ts-23, a mutant which fails to cleave PE2 to E2 at the nonpermissive temperature (Bracha and Schlesinger, 1976), at a multiplicity of 20 plaque forming units/cell (37°C). Ninety minutes post-infection the infecting medium was removed, 50 ml of minimal essential media (Eagle, 1959) containing 3% fetal calf serum was added, and the cells shifted to 40°C. At 6.5 hr post-infection the cells were washed twice with cold phosphate buffered saline (PBS, pH 7.2) and scraped into cold PBS. The cells were pelleted at 160 x g for 5 min and resuspended in swelling buffer (12.5 mM triethylamine, 12.5 mM KCl, pH 7.8). After 5-10 min in swelling buffer, the cells were homogenized with 60 strokes in a Dounce homogenizer and the buffer brought to 20 mM EDTA. The homogenate was brought to 60% sucrose (w/w), transferred to cellulose nitrate centrifuge tubes, overlaid with layers of 50% (w/w) and 25% (w/w) sucrose in TK

buffer (50 mM triethylamine, 50 mM KCl, pH 7.8) and centrifuged at 158,000 x g for 16 hr (4°C). Over 90% of the PE2 containing membranes are recoverable at the 25-50% sucrose interface, which was collected, diluted three-fold into TK buffer, and given 15 more strokes in a Dounce homogenizer. A 1 ml preparation containing the membranes from 10^7 cells has an absorbance of 2.2 at 280 nm. Mock infected membranes were purified by an identical method.

Iodination. Iodinations were done by the Chloramine T method of Erlich et al. (1978).

Purification of IgG from clone 6-8G. IgG was purified from the culture fluid of clone 6-8G using a protein A-sepharose column as described in Rice and Strauss (1982).

Immunoassays. Immune precipitation using Staphylococcus aureus (Cowan I strain) was as described in Rice and Strauss (1982). The solid phase immunobinding assay (plate binding assay) was as described in Nowinski et al. (1979). A secondary antibody (rabbit anti-mouse immunoglobulin) was routinely used unless otherwise noted. We also found it to be important to use fresh antigen for each assay. Variations of this assay for specific experiments are described in the figure legends.

Selection of hybridomas. The method used was essentially that of Nowinski et al. (1979) with some modifications. A brief description of this method, including the specifics of our selection protocols, is summarized below and in Table 1. Spleen cells from BALB/c mice inoculated with the antigens were fused with NSI/1 cells using polyethylene glycol and plated at about 10^5 cells/well in 96 well microtiter plates. They were grown in HAT selective medium (complete RPMI-1640 plus 1.0×10^{-4} M

hypoxanthine, 4.0×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine) for about 15 days before using plate binding assays to detect the growth of clones producing antibodies specific for the antigens tested. The fusions using Sindbis virus inoculated mouse cells were tested on plate binding assays using triton solubilized Sindbis virus as the antigen. The fusions using mice inoculated with membranes from ts-23 infected cells were tested on three parallel plate binding assays using either triton solubilized Sindbis virus, membranes from ts-23 infected cells, or membranes from mock infected cells as antigens. The cells in positive wells were dispersed, mixed with feeder cells (thymocytes from three week old mice) and plated at dilutions of 5-50 hybridoma cells per well. These were fed every 3-4 days with HT medium (HAT medium lacking the aminopterin) and tested again by plate binding assay after 12-15 days. Two to four wells were selected by a combination of their reactivity to the antigen and the observation in the microscope of a single healthy clone of cells and "minicloned" as before. This process was repeated (2-4 times) until all the wells showed up as uniformly positive by plate binding assay. Stable clones were grown up and injected into the intraperitoneal cavities of CD_2 F1 mice, and ascites fluids were drained from these mice periodically until they died. Dilutions of either these ascites fluids or the culture fluid from the hybridomas in culture were used in the assays described in this paper.

RESULTS

Hybridoma selection. We did a total of 5 different fusions, generating about 3000 initial microtiter wells, 10-15% of which contained viable hybridomas. Initial screening of these for reactivity to Sindbis protein produced 88 preliminary positive wells, ten of which eventually produced stable hybridomas specific for Sindbis virus proteins. A summary of the 5 fusions appears in Table 1.

Three different immunization and selection strategies were used to produce a variety of Sindbis specific hybridomas. Two fusions were done using mice immunized with Sindbis virions solubilized with Triton X-100, a nonionic detergent which should leave much of the native tertiary structure of the proteins unaltered. These were followed by three attempts to generate monoclonal antibodies specific for the portion of the nonstructural protein PE2 that is not present in E2. These fusions used mice immunized with membrane preparations from cells infected with ts-23, a temperature sensitive mutant that causes PE2 to accumulate in cells at the nonpermissive temperature (Bracha and Schlesinger, 1976). In each case three mice were used, each immunized with a slightly different antigen preparation, either membranes solubilized in Triton X-100, membranes solubilized in SDS, or membranes not treated with detergents. The spleen cells from the three mice were combined before fusion. For the last inoculation we used ts-23 infected BALB/c 3T3 cells (which should be antigenically identical to the inoculated mice) rather than chick cells as antigens in order to decrease the background of hybridomas reactive with host cell specific proteins. The cells resulting from these fusions were assayed in parallel for reactivity to Triton solubilized virus, ts-23

TABLE 1

Number of mice inoculated	Antigen Used	Boost/Fusion[a] (days after primary inoculation)	Quantity of antigen per mouse (inoculum/boost)	Fusion Ratio (spleen cells/ NSI/1 cells)	Stable Positive Clones Obtained	Plate Numbers[b]
1	Sindbis Virus in 0.02% Triton X-100	22/26	100 µg/50 µg	4:1	0	NA[c]
5	Sindbis Virus in 0.02% Triton X-100	8/11	50 µg/25 µg	5:1 and 16:1[d]	4	1-11
3	ts-23 infected[e] chick cell membranes	7/10	2.2 A_{280}/2.2 A_{280}	5:1	1	12-16
3	ts-23 infected[e] chick cell membranes	8/11	2.2 A_{280}/2.2 A_{280}	4:1	3	17-22
3	ts-23 infected[e] BALB/c membranes	19/23	2.1 A_{280}/2.1 A_{280}	4:1	2	23-28
15	Totals	NA[f]	NA	NA	10	NA

Table 1 (continued)

[a]Primary inoculations were done by subcutaneous, intraperitoneal, and intradermal injections. Boosts were done by either intraperitoneal or subcutaneous injections.

[b]The first portion of the stable clones' names refers to the original plate number, and the second portion refers to the microtiter well on that plate (i.e., clone 6-8G was obtained from well 8G on plate 6).

[c]Two microtiter plates. Not given consecutive numbers due to lack of positive clones.

[d]Two separate fusions were done with these cells. The 16:1 ratio was done using slightly overgrown ($2\text{-}4 \times 10^6$ cells/ml) NS-I/1 cells.

[e]Each of the three mice used was injected with one of the following three ts-23 preparations:

1) As described in Materials and Methods
2) Above, solubilized with 1.0% Triton X-100
3) Above, solubilized with 1.0% SDS

Spleen cells from the three mice were mixed together before fusion.

[f]Not applicable.

infected membranes, and mock infected membranes. The hope was that the culture fluid from clones producing antibodies to the nonstructural portion of PE2 would react with the ts-23 membranes, but not with the virus protein or mock infected membranes. Although we obtained 6 stable clones specific for structural proteins and numerous clones that reacted with both the infected and uninfected membranes, none were obtained to the nonstructural domain of PE2.

Immunoglobulin subclass determinations of stable hybridomas. The subclasses of the immunoglobulin heavy chains were determined with a plate binding assay using rabbit antisera specific for different mouse subclasses as the secondary antigens (Fig. 1). Since S. aureus protein A reacts only with IgG, and to different degrees with different subclasses of IgG (Ey et al., 1978), the binding of iodinated protein A to wells without a secondary antibody varies greatly according to the subclass of the monoclonal antibody. By the use of a secondary antibody that can bind protein A, the wells that are able to bind the secondary antibody (those with bound monoclonal antibodies of the proper subclass) will label more heavily with [^{125}I]. Therefore, each monoclonal antibody should show positive for only one subclass plus the rabbit antisera specific for whole mouse immunoglobulin. If the monoclonal antibody is one which itself reacts with protein A, then there will also be a background of [^{125}I] in the rest of the wells (see Fig. 1, 6-8G and 14-4C).

We were able to unambiguously identify the subclass of the antibodies produced by nine of the ten clones (Table 2). We found 3 IgM's, 6 IgG's of various subclasses (2 IgG1, 2 IgG2a, 1 IgG2b and 1 IgG3), and 1 IgG of unknown subclass. This last clone (28-6F) reacted equally with all of the

FIG. 1. Monoclonal antibody subclass determinations. Thousand-fold dilutions of ascites fluid from each stable hybridoma (plus NS-1 cells as a control) were tested on plate binding assays using rabbit antisera specific for the different mouse immunoglobulin subclasses (IgA, IgM, IgG1, IgG2a, IgG2b, IgG3) as the secondary antibody. Two separate experiments are shown. The left six lanes refer to the subclasses listed to the left, and right seven lanes refer to the subclasses listed to the right. No secondary antibody was used in the top row. α mouse Ig refers to Rabbit antisera to total mouse immunoglobulins. The nomenclature of the hybridomas is described in a footnote of Table 1.

2° Ab
CLONE
-7-2C
-22-12C
-6-8G
-14-4C
-28-6F
-25-10F
-25-10F
-17-10A
-17-3C
-6-9E
-2-6H
-αDNP-BSA
-NSI/1
2° Ab
None
αIgA
αIgM
αIgG1
αIgG2a
αIgG2b
αIgG3
αMouseIg
-None
-αIgG2a
-αIgA
-αIgM
-αIgG1
-αMouseIg
-αIgG2b
-αIgG3

TABLE 2

Characteristics of Stable Hybridomas

Clone No.[b]	Antigenic Specificity[a]: Viral Protein	Antigenic Specificity[a]: SDS/Triton X-100[c]	Immunoglobulin Subclass[d]	Relative ability to precipitate intact virus[e]	Competition with 6-8G[f]
2-6H	C[g]	--	IgM	0.2%	-
6-8G	E1	SDS=Triton	IgG2a	43%	++
6-9E	E1	SDS>>Triton	IgM	0.6%	±
7-2C	E1	SDS>Triton	IgG1	14%	++
14-4C	E1	SDS>Triton	IgG2a	19%	++
17-3C	E1(+E2)	SDS>Triton	IgM	93%	++
17-10A	C	--	IgG2b	1.1%	-
22-12C	E1	SDS>Triton	IgG1	14%	+++
25-10F	E1	--	IgG3	0%	-
28-6F	(C)	Triton>SDS	IgG	0%	-
α DNP-BSA	DNP-BSA[h]	--	(IgM)	0%	-
NSI/1	none	--	none	0%	-
α Sindbis	E1,E2,C	Triton>SDS	ND[i]	100%	ND

TABLE 2 (continued)

[a]Determined from a combination of a plate binding assay (Fig. 1) and immune precipitations followed by polyacrylamide gel electrophoresis (Fig. 2).

[b]Refers to plate and well number of initial plating following fusion.

[c]Comparison of reactivity to SDS and Triton X-100 solubilized viral protein. From Fig. 2.

[d]From Fig. 3.

[e]Immunoprecipitation using S. aureus. [^{35}S]-methionine labeled intact virus in PBS plus 1 mg/ml bovine serum albumin was reacted with 1/100 dilutions of ascites fluids followed by rabbit anti-mouse immunoglobulin as a secondary antibody. These were precipitated using S. aureus, washed, and counted by liquid scintillation. The results are given as a percentage of the label precipitated by a 1/50 dilution of the anti-Sindbis polyclonal antibody (Birdwell and Strauss, 1974). The amount of label precipitated by anti-DNP-BSA antibodies was subtracted from all values to correct for non-specific precipitation.

[f]From Fig. 5.

[g]Information in parentheses is from data that is suggestive but not firmly established.

[h]Stable hybridoma (obtained from Dr. Jack Richards) which is specific for dinitrophenol coupled to bovine serum albumin.

[i]Not Done.

wells of the plate binding, including the one that had no secondary antibody added, indicating that it was an IgG, but obscuring the identity of its subclass. It should be noted that we found that the degree to which iodinated protein A reacted directly with the antibodies of various IgG subclasses varied in different repeats of the assay. At various times all but one of the IgG producing clones (22-12C) reacted positively on plate binding assays without any secondary antibody. The slight reactions of clones 7-2C and 22-12C to the IgG2a wells is probably caused by a small amount of contaminating IgG1 specific antisera in the IgG2a antisera preparation.

Representative samples of clones containing each of the subclasses were labeled with [^{35}S]-methionine as described in the figure legend, immunoprecipitated with S. aureus, and analyzed on polyacrylamide gels (Fig. 2). To the sensitivity that this technique allows, each of the clones investigated secretes only one class of heavy chain and one class of light chain, but NSI/1 does not secrete any immunoglobulin. The migration of their heavy chains correspond to that expected for IgG and IgM as determined by the plate binding assay. The aberrant migration of the light chain of the iodinated clone 6-8G is presumably an artifact of the iodination.

Antigenic specificity of clones. Two types of experiments were used to determine the antigenic specificities of the antibodies produced by the stable hybridomas: either a plate binding assay using different Sindbis specific antigens (Fig. 3), or an immune precipitation of [^{35}S]-methionine labeled virus followed by polyacrylamide gel electrophoresis (Fig. 4). The antigenic specificities, as determined from these experiments, are

FIG. 2. Polyacrylamide gel analysis of selected monoclonal antibodies. Hybridomas representative of the different subclasses found were labeled with [^{35}S]-methionine. Cells were centrifuged as 160 x g for 5 min and resuspended to 1x10^{6} cells/ml in minimal essential medium (Eagle, 1959) lacking methionine and containing 15% dialyzed fetal calf serum. [^{35}S]-methionine was added to 50 µCi/ml and the cells incubated at 37°C for 4 hr. The cells were removed by centrifugation at 650 x g for 10 min and the culture fluid saved. Aliquots of the culture fluids were immunoprecipitated using rabbit anti-mouse immunoglobulin and S. aureus. The immunoprecipitation pellet was resuspended in Laemmli sample buffer, electrophoresed on 10% polyacrylamide slab gels (Laemmli, 1970), and analyzed by autoradiography. Pig IgG and rabbit IgM were used as size standards. Lefthand six lanes: Hybridomas labeled with [^{35}S]-methionine. Righthand lane: [^{125}I]-labeled clone 6-8G IgG (labeled by the chloramine-T method).

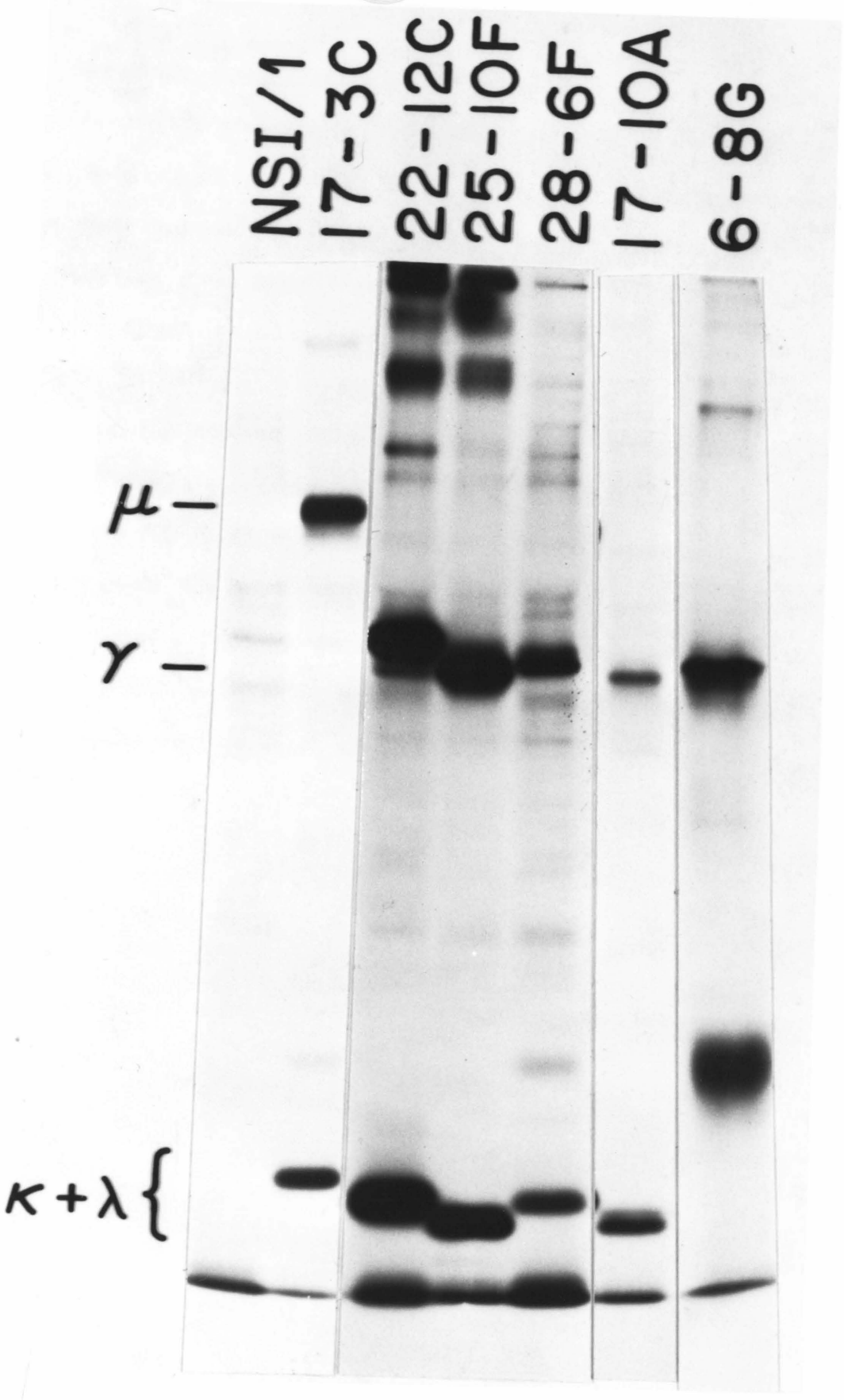

NSI/1
17-3C
22-12C
25-10F
28-6F
17-10A
6-8G
μ
γ
κ+λ

FIG. 3. Antigenic specificities of the monoclonal antibodies isolated. Culture fluids from stable hybridomas were tested on plate binding assays using either virus, E1+E2, or E1 as antigens. The antigens used were prepared as described by Bell et al. (1979). Antigen preparations used: Row A: Purified virus solubilized in 0.05% Triton X-100 in PBS (2-4 µg protein/well final concentration). Row B: E1+E2 in 0.5% Triton X-100, 200 mM NaCl, 50 mM Tris, 1 mM EDTA, pH 7.4. Diluted 30-fold into PBS (0.5 µg protein/well final concentration). Row C: E1 in 0.05% Triton X-100, 50 mM sodium succinate, 300 mM $CaCl_2$, 1 mM DTT, pH 5.5. Diluted five-fold into PBS (0.25 µg protein/well final concentration). Antibody preparation used: Rα E1, Rα E2, Rα E3: Monospecific antisera to viral structural proteins produced in rabbits (Rice and Strauss, 1982), diluted to 1/5000 in PBS. 6-8G, 6-9E, etc.: Culture fluids from hybridomas. 24-4F represents a clone which we subsequently lost.

SV A–
E1 + E2 B–
E1 C–
–RαE1
–RαE2
–RαC
–6-8G
–6-9E
–7-2C
–14-4C
–17-3C
–17-10A
–22-12C
–24-4F
–25-10F
2-6H

FIG. 4. Sindbis specific proteins immunoprecipitated by ascites fluids. [^{35}S]-methionine labeled virions were solubilized in either 0.5% Triton X-100 (A) or 0.5% SDS (B) and immunoprecipitated with the ascites fluids of stable hybridoma clones grown in mice. Aliquots of ascites fluids, polyclonal antisera, or preimmune sera were diluted into TNA (200 mM NaCl, 50 mM Tris, 1 mM EDTA, pH 7.4, 0.5% Triton X-100, 1 mg/ml BSA) as follows. α SV: Sindbis virus specific polyclonal antiserum (Birdwell, 1974) was diluted to 1/50 in TNA. α E1, α E2, α C: Polyclonal antisera monospecific for viral structural proteins (Rice and Strauss, 1982) were diluted into TNA (E1, 1/25 dilution; E2, 1/30 dilution; C, 1/10 dilution). Preimmune: Preimmune rabbit serum was diluted to 1/10 in TNA. 2S-10F, 17-10A, etc.: Ascites fluids were diluted to 1/100 in TNA. Ten µl aliquots of the diluted antisera were mixed with 10 µl of either Triton X-100 or SDS solubilized virus and incubated for 10 hr at 4°C. Ten µl of a 1/10 dilution of rabbit anti-mouse immunoglobulin was added for 1 hr and 50 µl of 10% S. aureus was added. After incubation for 8 hr at 4°C, the preparations were layered over 1 ml of 30% sucrose in TNA and the precipitates pelleted. The supernatants were removed and the precipitates dissolved in Laemmli sample buffer (1% SDS, 1% β-mercaptoethanol, 10% glycerol, 2% bromphenol blue, 0.05 M Tris, pH 6.8), electrophoresed on 10% polyacrylamide slab gels by the method of Laemmli (1970) except that one half the concentration of Trisma was used in the gel buffers, and analyzed by autoradiography. A: Virus diluted into TNA. B: Virus, brought to 0.5% SDS, heated to 56°C for 5 min, and diluted 10-fold with TNA.

αSV
αE1
αE2
αC
Preimmune
25-10F
17-10A
6-9E
2-6H
αBSA-DNP
NSI/1
6-8G
7-2G
14-4C
22-12C
28-6F
17-3C
A B
E1
E2
C

summarized in Table 2. The three antigens used for the plate binding assay were Triton X-100 solubilized virus, an E1 plus E2 mixture produced by pelleting intact nucleocapsids out of Triton solubilized virus, and E1 purified from the E1/E2 mixture by chromatography on a glass wool column (Bell et al., 1979). We decided to use these antigens because Triton X-100 is a relatively gentle nonionic detergent that should not extensively denature the antigens, although the exposure of the separated E1 to dithiothreitol and low pH (5.5) during purification may denature this preparation further. In addition, in several cases the antigens used for inoculation and selection were Triton X-100 treated.

Antibodies from seven of the nine clones tested on the plate binding assay show specificity for E1, and can be divided into two classes, reacting with at least two different antigenic specificities on E1. Antibodies from five of the clones (6-8G, 7-2C, 14-4C, 17-3C, 22-12C) reacted with all three of the antigen preparations tested, whereas those from the other two (6-9E, 25-10F) react with solubilized virus and pure E1, but not with the E1/E2 mixture. These two classes of antibodies, and the clones that secrete them, will be referred to as E1(a) and E1(b), respectively. Thus, whereas the antigenic site(s) that the antibodies from E1(a) clones react with are exposed in all the preparations, the site(s) that the antibodies from E1(b) clones react with is exposed in Triton X-100 solubilized virus, but is somehow hidden after removal of nucleocapsids. After treatment with a reducing agent and removal of E2, the E1(b) specific antigenic site is reexposed. This implies that there may be some interaction between all three viral structural proteins, even in the presence of nonionic detergents. The remaining two antibodies tested

(2-6H, 17-10A) are probably specific for capsid proteins. Antibodies from clone 2-6H are only very weakly reactive and it is thus hard to be certain of its specificity. Antibodies from clone 17-10A reacts slightly with the E1/E2 mix, but the monospecific polyclonal anticapsid antisera (lane 3) does also, possibly due to a low level of residual nucleocapsid proteins in the preparation.

Sindbis virus proteins treated with either Triton X-100 or SDS were immunoprecipitated with ascites fluid from the 10 stable clones and analysed by gel electrophoresis (Fig. 4). The ascites fluids from the five E1(a) specific clones precipitated both Triton and SDS treated E1. The ascites fluid from the two E1(b) specific clones were ineffective at immunoprecipitating E1 with S. aureus. Ascites fluid from clone 6-9E weakly precipitated only SDS solubilized E1, and that from clone 25-10F did not precipitate any detectible amounts of E1. One of the E1(a) clones (17-3C) exhibited the interesting property that its ascites fluid also precipitated Triton solubilized E2 to some degree.

Antibodies from the three remaining clones (2-6H, 17-10A, 28-6F) are probably capsid specific, but the large amount of nonspecific precipitation of capsid protein during the immunoprecipitations interferes with the interpretation. Ascites fluids from clones 2-6H and 17-10A do not precipitate enough capsid to be visible above the background level, but ascites fluid from clone 28-6H, which was not tested on the plate binding assay, precipitated a significantly greater amount of [^{35}S]-methionine label from Triton X-100 treated virus (data not shown) than the negative controls (ascites fluid from the DNP-BSA specific hybridoma or NSI-1 cells). Careful examination of the gel pattern of the Triton solubilized protein

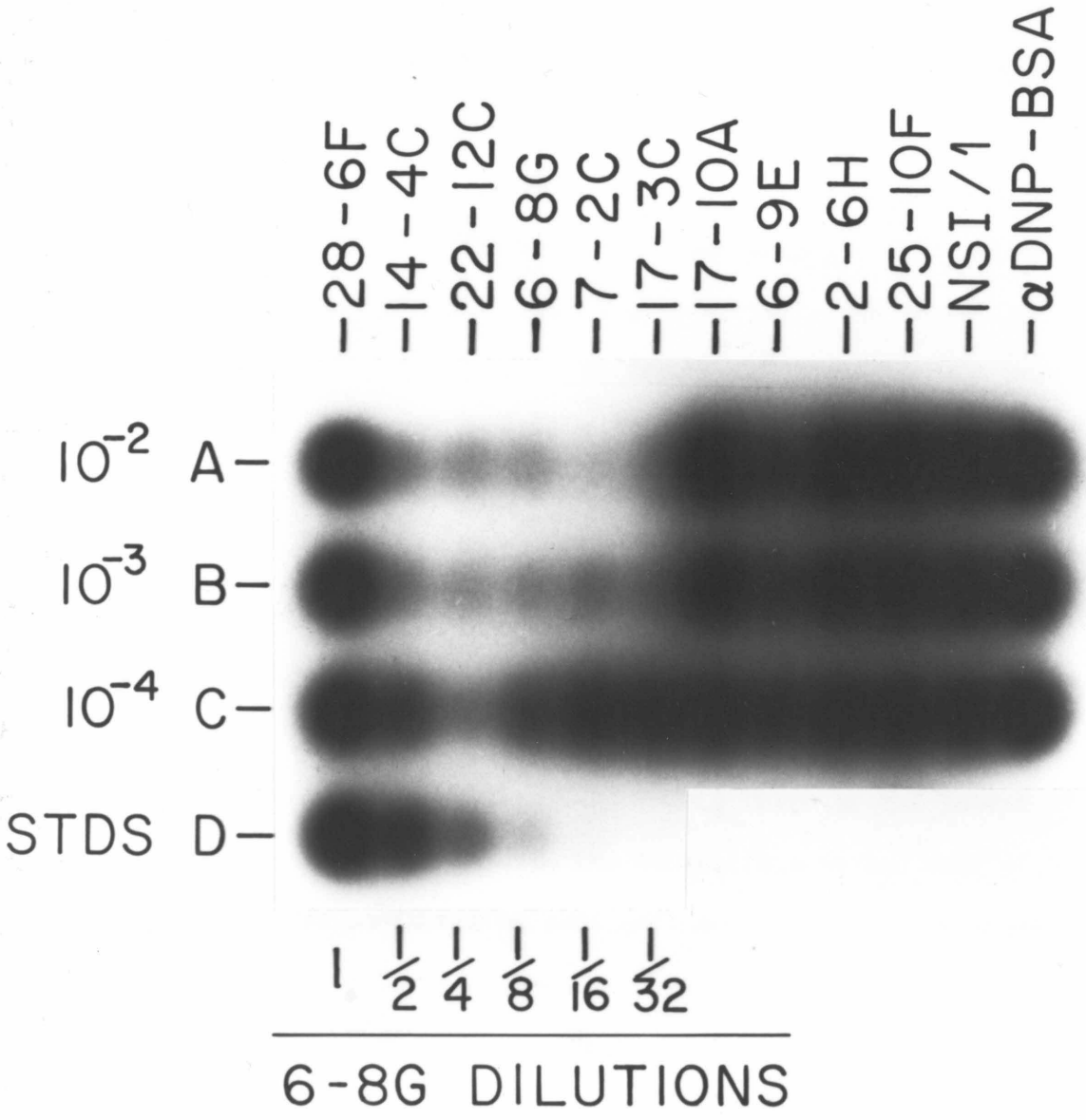
precipitated by 28-6F reveals a capsid band that is more intense than the controls (Fig. 4), identifying 28-6F as probably capsid specific.

The ability of the ascites fluids to precipitate whole virus was also tested (Table 2). Ascites fluids from the five E1(a) specific clones precipitated significant quantities of virus but ascites fluids from the two E1(b) specific clones and the three capsid specific clones fail to precipitate more than about 1% of the virus.

Competition of binding to Sindbis virus proteins. A variation of the plate binding assay was used to test the ability of the ascites fluids from the 10 clones to interfere with the ability of [^{125}I]-labeled 6-8G IgG to bind to viral proteins (Fig. 5). Other investigators (i.e., Stone and Nowinski, 1980) have found using monoclonal antibodies that the individual antigenic determinants can be grouped into a limited number of regions of antigenicity by competition studies such as these. These groupings based on competition experiments correlate well with groupings made based on other properties of the antibodies. We found that the E1 specific hybridomas could be grouped into two such groups by the experiment described in Fig. 5. Ascites fluids from the five E1(a) clones effectively competed with [^{125}I]-labeled 6-8G IgG, whereas that from the two E1(b) clones competed poorly (6-9E) or not at all. Ascites fluid from the three capsid specific clones and the controls (α-DNP-BSA, NSI/1) as was expected, did not compete.

The competitions at the higher concentrations of antibodies show a plateau effect presumably due to the saturation of all of the available antigenic sites at these dilutions. Likewise, the ineffective competition by ascites fluid from clone 6-9E is probably not due to a low concentration

FIG. 5. Competition between monoclonal antibodies for binding to Sindbis virus proteins. A variation of the plate binding assay was used. Ascites fluids diluted 10^2, 10^3, and 10^4-fold into PBS were bound to Triton solubilized virus in microtiter wells by the standard method. Following the ascites binding and washes, 50 µl of [^{125}I]-labeled clone 6-8G IgG diluted 10^4-fold into PBS was added to the microtiter wells. The plate was incubated 2 hr at 37°C, washed 10 times with PBS containing 1% bovine serum albumin, and dried. The plate was analyzed by autoradiography as described previously (Nowinski et al., 1979). DNP-BSA specific ascites fluid, NSI/1 ascites fluid, and buffer lacking any primary antibody were used as controls. Row A, B and C contain 10^2, 10^3, and 10^4 dilutions of ascites fluids respectively. Row D shows the binding of sequential two-fold dilutions of [^{125}I]-labeled 6-8G IgG when no ascites fluid was used to compete with it.


-28-6F
-14-4C
-22-12C
-6-8G
-7-2C
-17-3C
-17-10A
-6-9E
-2-6H
-25-10F
-NSI/1
-αDNP-BSA
10^{-2} A-
10^{-3} B-
10^{-4} C-
STDS D-
1 1/2 1/4 1/8 1/16 1/32
6-8G DILUTIONS

of antibodies, since it competes to the same degree even when diluted 10-fold. It is presumably due either to a low avidity for its antigenic site, or to the location of its binding site sufficiently far from that of the 6-8G antibodies such that it only marginally sterically hinders the binding of 6-8G IgG.

Neutralization of Sindbis virus infectivity. By exposing virions to a series of dilutions of hybridoma ascites fluids or polyclonal antisera and then immediately assaying the surviving infectivity, we investigated the ability of the antibodies from the different clones to inhibit infectivity. Our results (Table 3) indicated that none of our clones were very effective in inhibiting infectivity when compared with rabbit antisera specific for Sindbis. Ascites fluids from two of the clones (6-8G, 17-3C) showed clear, although inefficient inhibition of infectivity, but that from most of the other clones tested gave ambiguous results. A miscalculation of the total plaque forming units in the starting material prevented us from getting a clear answer from this experiment. Even at the lowest concentration of virus, the plaques on the control plates and most of the test plates were either confluent or semiconfluent. For a clear answer, this experiment will have to be redone.

A similar effect to that seen in the competition tests can be seen here with ascites fluids from clones 6-8G and 17-3C. The similar degree of inhibition by 10-fold different dilutions of antibody indicate that the limiting factor in the infectivity inhibition may be due to the avidity of the antibody itself, rather than a dilution effect. In contrast, the effect of dilution on the rabbit antisera to Sindbis virus is quite large (Table 3).

TABLE 3

Neutralization of Sindbis Virus infectivity by antisera[a]

Antisera Used	Antisera Dilution	PFU/plate[b]
Control[c]	NA[d]	Semiconfluent[e]
None	NA	Confluent[f]
Rabbit preimmune	1/10	Confluent
Rabbit α Sindbis[g]	1/10, 1/100, 1/1000	22, 79, 254
Rabbit α E1 IgG[h]	1/10	Confluent
Rabbit α E2 IgG[h]	1/10	153
6-8G Ascites	1/10, 1/100	305, 314
17-3C Ascites	1/10, 1/100	296, 316
2-6H Ascites	1/10	Semiconfluent
6-9E Ascites	1/10	Semiconfluent
17-10A Ascites	1/10	Semiconfluent
14-4C Ascites	1/10, 1/100	Semiconfluent, Semiconfluent
22-12C Ascites	1/10, 1/100	Semiconfluent, Semiconfluent
7-2C Ascites	1/10, 1/100	Confluent, Confluent

TABLE 3 (continued)

[a]Sindbis virus (10^6 plaque forming units in 200 µl) was mixed with 200 µl aliquots of dilutions of the various antisera and incubated for 1 hr at 37°C. The mixture was brought to 4 ml and the remaining infectivity immediately determined by plaque assay (Strauss and Strauss, 1976). In addition, aliquots of the virus were incubated with no antisera or preimmune antisera from rabbits. All dilutions were made into PBS containing 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 1% fetal calf serum.

[b]Plaque forming units per plate of a $4x10^3$-fold dilution of the starting concentration of the virus. A miscalculation of the titer of starting virus made it impossible to calculate the relative rates of inactivation.

[c]Aliquot (200 µl) of virus diluted to 4 ml and kept on ice during the 1 hr incubation.

[d]Not applicable

[e]Semiconfluent: too many plaques to count (greater than 400 plaques), but portions of the cell lawn were uninfected, giving the lawn a mottled look.

[f]Confluent: all cells in lawn were lysed.

[g]See Birdwell and Strauss, 1974.

[h]See Rice and Strauss, 1982.

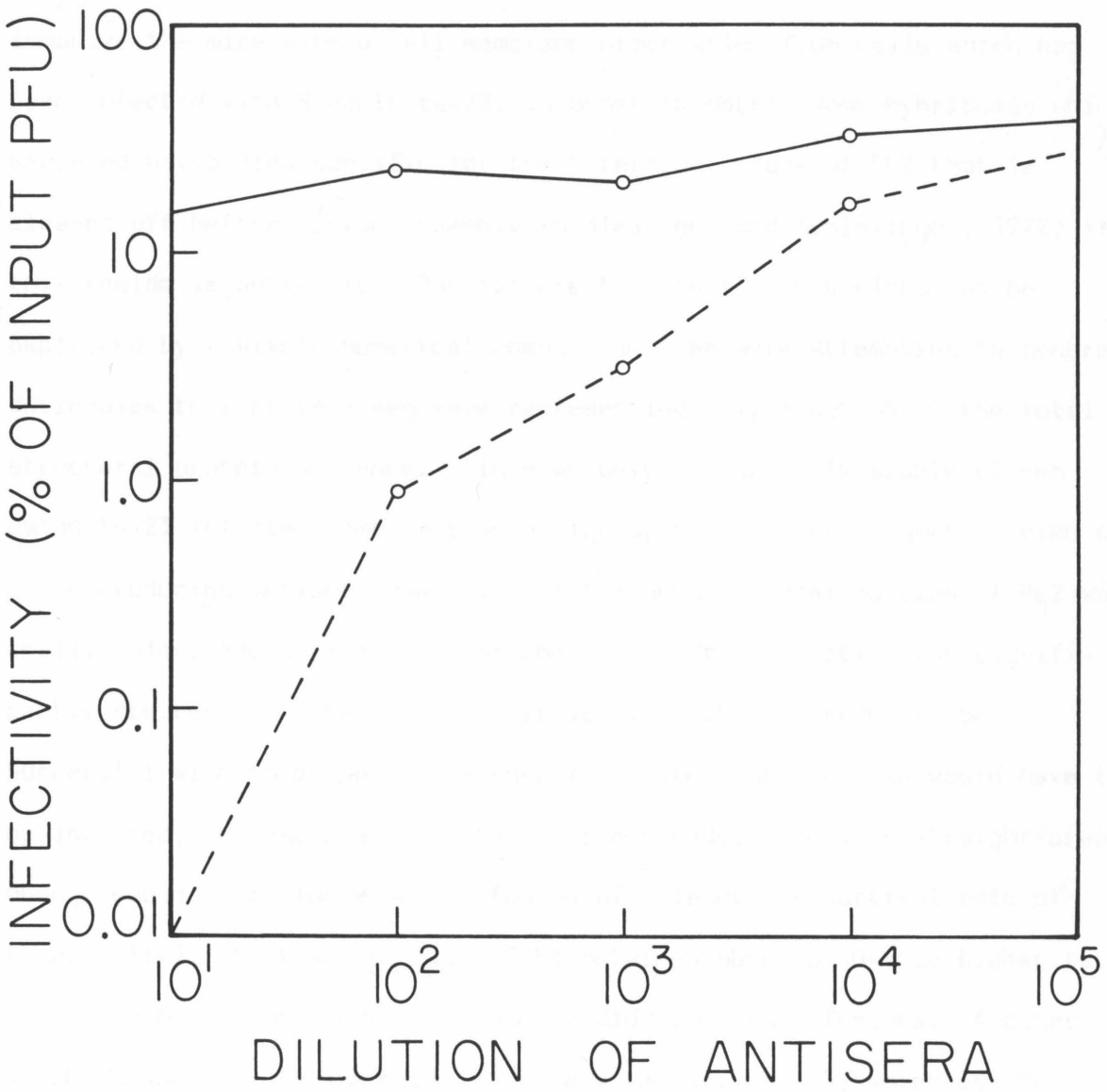
The inhibition of infectivity by ascites fluid from clone 17-3C as compared with rabbit antisera to Sindbis virions was more thoroughly investigated using multiple dilutions of antibodies and lower quantities of starting virus ($3x10^3$ PFU vs. $1x10^6$ PFU). Fig. 6 shows the results of this experiment. Although the rabbit antisera shows a strong correlation between concentration and infectivity, the ability of ascites fluid from clone 17-3C to inhibit infectivity is only slightly affected by concentration within the range tested (varying only from 15% to 37% residual virus infectivity over a 10,000-fold difference in antibody concentration). Thus, as seen before, the limitations of the ascites fluids from the hybridomas may be due to the avidity of the antibodies or its inherent ability to inhibit infectivity, not concentration effects. All of the concentrations tested seem to essentially saturate the antigenic sites.

We cannot rule out the possibility that a nonspecific inhibition of infectivity is caused by some component of clone 17-3C and clone 6-8G ascites fluids that is not present in the other ascites fluids, but we feel that this is unlikely.

DISCUSSION

One of our aims was to generate hybridomas specific for various Sindbis virus proteins to help compare antigenic determinants of the different proteins with various viral characteristics. In addition, by using preparations containing the nonstructural protein PE2 as an antigen, we hoped to produce monoclonal antibodies specific for a nonstructural protein to use a tool for studying viral protein maturation. Although we were unsuccessful in generating PE2 specific antibodies, we obtained 10

FIG. 6. Comparison of the inhibition of infectivity by 17-3C ascites fluid and Sindbis specific rabbit antisera. Sindbis virus ($3x10^3$ plaque forming units) in 200 µl of PBS containing 0.9 m$\underline{M}$ $CaCl_2$, 0.5 m$\underline{M}$ $MgCl_2$, and 1% fetal calf serum was mixed with 200 µl of the antisera diluted 10^1, 10^2, 10^3, or 10^4-fold in the same buffer. They were incubated for 1 hr at 37°C, immediately diluted to 2 ml in the same buffer, and the infectivity of the preparation assayed by plaque assay (Strauss and Strauss, 1976). (——): Relative infectivity after treatment with clone 17-3C ascites fluid. (---): Relative infectivity after treatment with Sindbis specific rabbit antisera.


INFECTIVITY (% OF INPUT PFU)
100
10
1.0
0.1
0.01
10^1
10^2
10^3
10^4
10^5
DILUTION OF ANTISERA

hybridomas producing antibodies specific for the structural proteins, among them several very interesting ones.

The strategy we used to look for PE2 specific hybridomas was to immunize the mice with a cell membrane preparation from cells which had been infected with Sindbis ts-23, in order to obtain some hybridomas which secreted antibodies specific for the N-terminal piece of PE2 that is cleaved off before virion assembly (Schlesinger and Schlesinger, 1972) if this region is antigenic. Our failure to obtain such a clone can be explained by a simple numerical comparison. We were attempting to generate antibodies to a protein sequence representing only about 6% of the total structural protein sequence. Since we only isolated six stable clones using ts-23 infected membranes as antigens, the chances of getting even one clone producing antibody specific for the nonstructural portion of PE2 were small, unless the antigenicity of the nonstructural portion was significantly greater than the average. It is clear that in order to be successful with this approach either many more stable clones would have to be isolated, or some change in the approach made. The most straightforward change would be to increase our fusion efficiency (or survival rate of fused cells). Nowinski et al. (1979) obtained about a 10-fold higher rate of viable hybridoma production than we did from their fusions. Another approach would be to immunize the mice with purified E3, which is the N-terminal piece of PE2 (Rice and Strauss, 1981), and use ts-23 infected membranes for the plate binding assay. We tried this once, but lost the cells due to contamination (data not shown). A lack of more purified E3 prevented a repeat of this experiment, but since that time we have purified sufficient quantities (2 mg) to be able to repeat this approach.

The seven clones producing E1 specific antibodies that were isolated can be easily separated into two classes, termed E1(a) and E1(b), that exhibit markedly different properties and bind to two different domains of E1. The five E1(a) clones (6-8G, 7-2C, 14-4C, 17-3C, 22-12C) produced antibodies that reacted with all three antigen preparations used in the plate binding test described in Fig. 3, were relatively effective at precipitating intact virions by immunoprecipitation and effectively competed with the binding of iodinated clone 6-8G IgG to viral proteins. Conversely, the two E1(b) clones (6-9E, 25-10F) produced antibodies that did not react with the Triton X-100 E1/E2 mixture used as an antigen on the plate binding assays described in Fig. 3, were very ineffective at precipitating intact virions, and did not compete effectively with clone 6-8G IgG for binding to viral proteins. Antibodies from E1(a) clones also were more effective than those from E1(b) clones at immunoprecipitating Triton X-100 or SDS solubilized viral proteins (Fig. 4), but this difference is less striking.

The competition test using iodinated clone 6-8G IgG (Fig. 5) allowed us to demonstrate that the antibodies from the E1(a) and E1(b) groups bind to at least two different regions of antigenicity on E1. The assumption is that if the binding sites of two antibodies are sufficiently close to each other, the binding of one antibody will sterically hinder the binding of the other, whereas if the binding sites are separated, the antibodies will have little, if any, effect on each other. Stone and Nowinski (1980) found with monoclonal antibodies directed against murine leukemia virus proteins that each protein could be divided into a fairly limited number of these regions of antigenicity (2 for gp70 and 2 for p15(E)), and that these

groupings corresponded very closely with the ability of the antibodies to affect other properties of the protein. Our groupings based on the competition experiment also matched up well with the other differences in properties that we found. We should note that antibodies from the two clones in the E1(b) group do not necessarily react with the same antigenic domain in E1. Although they share similar (but not identical) properties, we have not tested whether they compete with each other for binding to E1.

The reactivity pattern of E1(b) antibodies, especially in Fig. 3, is very hard to explain. We expected the conformation of E1 in the Triton X-100 solubilized virus and the Triton X-100 solubilized E1/E2 mixture to be identical, since the only difference was that the cores had been centrifuged out of the latter. We propose two possible explanations for this difference. First, it is possible that even in Triton solubilized virus there is some interaction between the viral capsid and envelope proteins such that the removal of capsid causes a change in conformation of E1 or E2 to hide an antigenic site of E1. It has been previously shown (Rice and Strauss, 1982) that at least E1 and E2 form stable associations in Triton. Further purification of E1 could then reexpose that site, due to either the removal of E2 or the treatment with low pH (5.5) and a reducing agent which would remove any disulfide bonds. Although the ease by which these proteins can be separated in solution by pelleting the capsids argues against this explanation, it is possible that this interaction is weak, and only when reacting with a common surface (the plate) are these interactions stabilized. A more trivial explanation would be that an artifact was introduced into the E1/E2 preparation. Even if this is the case, the

difference between the two antigenic classes is presumably real, as the two groups react differently with the E1/E2 preparation, whatever its condition.

Antibodies from clone 17-3C exhibits a very interesting, and potentially quite useful, property. Although its primary antigenicity is for E1(a), it coprecipitates some E2 in immunoprecipitation experiments with Triton solubilized, but not SDS solubilized (Fig. 4) virus. The most likely explanation for this phenomenon is that this antibody is able to bind to E1 without disrupting the dimeric E1/E2 complex. The separation of E1 from E2 by the other monoclonal antibodies, or by polyclonal antibodies, is presumably due to a competitive disruption of the E1/E2 interaction. These antibodies could prove very useful for studying the interaction between E1 and E2 (or PE2) during the course of viral glycoprotein maturation, assuming this cross reactivity is also present in E1/E2 complexes in infected cell membranes.

Surprisingly, most of the E1 antibodies produced are more reactive to SDS solubilized E1 than to Triton X-100 solubilized E1, even though some of the clones (7-2C, 6-9E) were derived from mice immunized with Triton solubilized virus, and all the clones were selected using Triton solubilized antigens. This is possibly the case because SDS solubilized E1 is a better antigen than Triton solubilized E1, both in raising an immune response and reacting with antibodies. Clone 6-8G is the only clone which does not exhibit this tendency, its antibodies reacting about equally well with both preparations of antigens.

When ascites fluids from the E1 specific clones were tested for their ability to inhibit virus infectivity that from only two of the clones (6-8G

and 17-3C) inhibited viral infectivity to any significant degree. Even these, at the higher antibody concentrations, inhibited infectivity 100-1000-fold less effectively than Sindbis specific polyclonal antisera. Any inhibition was surprising, as previous results using monospecific polyclonal antisera indicated that only E2 specific polyclonal antisera was able to inhibit infectivity (see Table 3 and Dalrymple et al., 1976). On the other hand, a recent report by Chanas et al. (1982) describes two hybridomas producing antibodies specific for Sindbis E1, one of which is able to inhibit viral infectivity. This inhibition may be an indirect effect caused by steric hindrance of the interaction of E2 with the cell membrane when the antibodies are complexed with E1. If only a limited number of exposed E2 molecules are needed for infectivity, and only a subset of the sites in the E1(a) domain will sterically hinder the accessibility of E2, then rabbit antisera to E1, by its polyclonal nature, will not effectively inhibit infectivity whereas some monoclonal antibodies will. In polyclonal antisera a significant fraction of the E1 molecules will have an antibody bound to their E1(a) domain that is ineffective at inhibiting infectivity, and this fraction of the molecules will allow infectivity. With a monoclonal antibody, if it binds to a site which will sterically hinder E2, all of the sites on the virus will be filled, and infectivity will be affected. Since the antibody is only indirectly blocking a neighboring protein, the steric effect may not be complete, leading to a residual low level of infectivity even when all of the sites are saturated, which is the result we observed.

In several instances the monoclonal antibodies produced only weak effects when tested. In at least two of these instances (the competition

for binding between antibodies from clones 6-9E and 6-8G, and the inhibition of infectivity by antibodies from clones 17-3C and 6-8G) the effect is probably due to inherent properties (such as avidity) of the antibody-antigen complex rather than a dilution effect. In both cases, the ability of the antibody to produce the observed effect varied only slightly over a 100-1000-fold variation in concentration, implying that all of the antigenic sites were essentially saturated at all the dilutions.

The antibodies from the three capsid specific hybridomas have not been extensively characterized. They were negative for inhibition of viral infectivity, competition with clone 6-8G IgG for binding to viral proteins, and precipitation of whole virus, as would be expected for a protein buried within the virion.

The results presented above show the ability of monoclonal antibodies to elucidate and dissect the effects of individual antigenic determinants on viral properties, which are often masked when polyclonal antisera is used. Such probes may also prove useful in the future for investigating the events during viral maturation.

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CHAPTER 4

Comparison of the specificity and extent of glycosylation of Sindbis virus proteins produced in hamster and chicken cells

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ABSTRACT

The tryptic glycopeptides of the Sindbis virus envelope glycoproteins E1 and E2 grown in BHK and chick cells were purified by gel filtration followed by high pressure liquid chromatography. Each of the purified glycoproteins was analyzed by N-terminal sequencing to identify from which of the potential glycosylation sites it was derived. The identity of the type of oligosaccharide chain attached to each glycopeptide was determined from gel filtration analysis of the pronase digested glycopeptides, and the relative incorporation of radiolabeled galactose, mannose, and glucosamine into each glycopeptide was used to confirm these determinations. We found that the glycosylation pattern for the two proteins were essentially identical in the two hosts. The E2 glycosylation sites at Asn_{196} and Asn_{398} contained exclusively complex type and simple type oligosaccharide chains respectively. In E1, the glycosylation site at Asn_{135} contained only complex type chains, but the site at Asn_{245} contained a mixture of simple (15-25%) and complex (75-85%) type chains. These results are compared to previous results and a prediction as to the relative importance of the different glycosylation sites to the function of the proteins is made.

INTRODUCTION

The two Sindbis virus membrane glycoproteins (E1 and E2) are glycosylated in a manner similar to that of other membrane and secreted glycoproteins containing asparagine linked oligosaccharides (for review see Lennarz, 1980). A core oligosaccharide ($Glc_{1-3}Man_{8-12}GlcNAc_2$) is transferred in one step from a lipid linked intermediate to the nascent polypeptide chain (Sefton, 1977; Robbins et al., 1977). Some of the core mannose residues are trimmed while still in the endoplasmic reticulum and N-acetylglucosamine, galactose, fucose, and sialic acid are added in the Golgi apparatus (Robbins et al., 1977; Schachter and Roseman, 1980). The two classes of oligosaccharide chains that do or do not contain these terminal sugars are called complex type and simple type, respectively (Kornfeld and Kornfeld, 1980).

These two Sindbis virus proteins each contain two potential glycosylation sites of the Asn-X-Ser/Thr type (Rice and Strauss, 1981). Several studies indicate that each protein contains oligosaccharide chains of both the simple ($Man_{5-7}GlcNAc_2$) and complex ($NANA_{0-2}Gal_2Man_3Fuc_1GlcNAc_4$) types in approximately a 1:1 ratio (Sefton and Keegstra, 1974; Burke and Keegstra, 1979; Hakimi et al., 1981). The predominant complex and simple oligosaccharide chains of E2 have been tentatively assigned to position 196 and 398, respectively (Rice and Strauss, 1981), but those of E1 have not been localized.

When a variety of enveloped viruses, including Sindbis virus (Keegstra et al., 1975; Burke and Keegstra, 1976, 1979; Weitzman et al., 1979), vesicular stomatitis virus (Etchison and Holland, 1974; Etchison et al., 1977), or retroviruses (Warren et al., 1972; Lai and

Duesberg, 1972; Sefton, 1976), are grown in several different cell lines only minor differences in their glycosylation patterns are detectable. These changes are largely due to differences in the number of sialic acid residues (Warren et al., 1972; Keegstra et al., 1975; Burke and Keegstra, 1979) or fucose residues (Etchison and Holland, 1974) rather than major changes in oligosaccharide structure. In contrast, different proteins within a given type of host cell each exhibit their own characteristic glycosylation pattern (Sefton, 1976; Weitzman et al., 1979). These results suggest that the protein itself, rather than the host cell, contains the information that determines its glycosylation pattern. If the host cell lacks the proper oligosaccharide processing capability however, the protein's glycosylation pattern will be altered. For instance, Sindbis virus grown in lectin resistant cell lines, which lack a N-acetylglucosaminyltransferase (Schlesinger et al., 1976), or in insect cells, which lack a sialyltransferase (Stollar et al., 1976), contain proteins with unusual oligosaccharide patterns that reflect the host cell's defect.

Sindbis virus E1 offers a possible exception to the above rule. Burke and Keegstra (1976) have reported that when E1 is grown in BHK cells, but not chick cells, it is deficient in simple oligosaccharide chains. In contrast, Hakimi and Atkinson (1980) have reported that E1 grown in chick cells is also deficient in simple oligosaccharide chains, although to a lesser extent. We would like to resolve this difference. In this paper we investigate the extent of glycosylation and the nature of the oligosaccharide chains added to the different glycosylation sites in E1 and E2. We compare the results obtained following infection of two host cells, BHK and chick.

MATERIALS AND METHODS

Growth and Purification of Virus

Virus was purified by polyethylene glycol precipitation, velocity sedimentation, and isopycnic density banding (Bell et al., 1979). Roller bottles of either BHK cells or primary chick embryo fibroblasts were infected with the HR strain of Sindbis virus (Burge and Pfefferkorn, 1966) at 20 plaque forming units per cell by the method of Pierce et al. (1974) except that at 1.5 hr post-infection Eagles minimal essential medium (Eagle, 1959) containing 3% dialyzed fetal calf serum was added. Bottles of infected cells were labeled with various radioactive sugars from 3 hr to 15 hr post-infection. The virus used to produce the BHK grown glycopeptides was grown in 28 roller bottles of BHK cells. Two of these roller bottles were labeled with 125 µCi [^{3}H]-glucosamine and two with 20 µCi [^{14}C]-galactose. That used to produce the chick cell grown E1 glycopeptides was a mixture of 30-40 roller bottles of previously polyethylene glycol precipitated and frozen virus and 5 roller bottles of labeled virus (1 roller bottle containing 160 µCi [^{3}H]-mannose, 2 roller bottles containing 150 µCi [^{3}H]-glucosamine, and 2 roller bottles containing 35 µCi [^{14}C]-galactose). Finally, that used to produce chick cell grown E2 glycopeptides was a mixture of 30-40 roller bottles of the frozen virus and 4 roller bottles labeled with a combination of labels (470 µCi [^{3}H]-mannose, 175 µCi [^{3}H]-glucosamine and 375 µCi [^{14}C]-glucosamine.

Purification of Envelope Proteins and Their Glycopeptides

Virions purified by isopycnic density centrifugation were solubilized with Triton X-100 and the nucleocapsids removed by centrifugation. The two glycoproteins were then separated from one another via a glass wool column

as described by Bell et al. (1979). After ethanol precipitation the purified proteins were resuspended in 8 M urea, 0.5% SDS, 0.2 M Tris buffer, pH 8.0 and reduced and acetylated by sequential treatment with 20 mM dithiothreitol for 60 min at 37°C, 50 mM ICH_2CONH_2 for 30 min at 25°C, and 30 mM dithiothreitol for 15 min at 37°C (all steps in a N_2 atmosphere). After two rounds of ethanol precipitation to remove the urea the samples were exhaustively digested with TPCK treated trypsin (Worthington). The peptides from the trypsin digests were separated on Biogel-P10 columns followed by reverse phase high pressure liquid chromatography (HPLC) columns as described in Figs. 1 and 2. The pooled HPLC peaks were dialyzed at 4°C against 100 mM NH_2HCO_3 and lyophilized. Aliquots of these samples were analyzed by either pronase digestion or N-terminal sequencing to determine the type of oligosaccharide attached to the peptide and the site of attachment.

In two cases the pooled peaks contained a mixture of two peptides. In these cases a Concanavalin A-Sepharose column (250 µl bed volume) was used to effectively separate the two peptides (Cummings and Kornfeld, 1982; Kornfeld et al., 1981). The two peaks from these columns were dialyzed and lyophilized as before and used for N-terminal sequencing and pronase digestion.

Analysis of Proteins and Glycopeptides by Pronase Digestion

Aliquots of the purified glycopeptides or labeled standards (chick cell grown E1 or E2, or a BHK cell grown E1 plus E2 mixture) were mixed with 200 µg unlabeled virus and resuspended in 300 µl 100 mM Tris buffer, pH 8.0, 10 mM $CaCl_2$. The samples were digested for 48 hr at 60°C, with 25 µl aliquots of 10 mg/ml pronase (predigested for 2 hr at 37°C) added to

each sample at 0, 12, 24, and 36 hr. The sample was layered onto a Biogel P-4 column (1cm x 120cm) equilibrated in 100 mM Tris buffer, pH 8.0, 0.02% NaN_3, containing 200 µg/ml bovine serum albumin (trypsin digested) as a carrier. Ten drop (0.4 ml) fractions were collected and counted by liquid scintillation. The patterns of radioactivity were compared with the standards, which were either mixed with the sample before digestion or digested in parallel and analyzed on the column immediately before or after the samples.

N-terminal Sequencing of Purified Glycopeptides

Aliquots of the purified glycopeptides were subjected to automatic Edman degradation on a non-commercial gas phase sequencer (Hewick et al., 1981). The phenylthiohydantoin amino acid derivatives were analyzed by a modification of the reversed phase high pressure liquid chromatographic procedure previously described (Johnson et al., 1979), quantitated by comparison to a standard mixture, and the yields normalized to an injection of 100% of the sample.

RESULTS

Labeling Studies on BHK and Chick Cell Grown E1 and E2

Fig. 1 shows the pronase digestion patterns of the simple and complex type oligosaccharides for E1 and E2 grown in either BHK or chick cells. Pronase digestion followed by column chromatography on Biogel P-6 is often used to analyze the glycosylation of Sindbis virus glycoproteins. The glycoproteins show a characteristic pattern including three galactose containing peaks that represent complex-type oligosaccharide chains containing zero, one, and two sialic acid residues, and a broad peak that does

not contain galactose that represents the heterogeneous simple-type chains found in Sindbis virus (Burke and Keegstra, 1976, 1979) A Biogel P-4 column was used to analyze these patterns, rather than a P-6 column as used by others, because in our hands the P-4 column gave significantly better resolution than the P-6 column. Good separation of the simple and complex peaks was obtained except for a small galactose labeled peak in some of our chick cell preparations (Fig. 1, panels B and D) which migrated at the leading (left hand) edge of the simple peak. This was not one of the three standard complex peaks, but a previously uncharacterized peak. The pronase patterns from other laboratories (Hakimi and Atkinson, 1980; Burke and Keegstra, 1976) also exhibit this small peak, but its analysis has been neglected. Because the emphasis of this paper is not oligosaccharide structure, we will not further characterize it.

Next, radioactively labeled E1 or E2 from BHK and chick cells were mixed, digested with pronase, and analyzed on P-4 columns (Fig. 2). The patterns are very similar (except for the peak in chick cell E2 as mentioned above). As reported previously (Burke and Keegstra, 1976), the glycoproteins from BHK cells contain a larger percentage than chick cells of the large complex type oligosaccharides containing two sialic acid residues.

Since the compositions of the simple and complex oligosaccharide chains of E1 and E2 are known (Burke and Keegstra, 1979; Hakimi et al. 1981), we were able to make estimates of the relative amounts of each in E1 and E2 by quantitation of the radioactivity in Figs. 1 and 2 (see Table 1). The results indicate that although there is some under-representation of simple oligosaccharide chains in E1, it is not the

FIG. 1. Identification of the simple type and complex type oligosaccharide chains of E1 and E2. Mixes of [^{14}C]-galactose and [^{3}H]-mannose or [^{3}H]-glucosamine E1 or E2 were digested with pronase and analyzed on Biogel P-4 columns as described in Materials and Methods. Panel A: BHK cell grown E1. [^{14}C]-galactose plus [^{3}H]-glucosamine labeled proteins. Panel B: Chick cell grown E1. [^{14}C]-galactose plus [^{3}H]-mannose labeled proteins. Panel C: BHK cell grown E2. [^{14}C]-galactose and [^{3}H]-glucosamine labeled proteins. Panel D: Chick cell grown E2. [^{14}C]-galactose and [^{3}H]-mannose labeled proteins. (▬▬): ([^{14}C]-galactose label. (———): [^{3}H]-mannose or [^{3}H]-glucosamine label. The three vertical lines near the top of each panel indicate the three standard complex oligosaccharide peaks, and the horizontal bar indicates the broad simple oligosaccharide peak. Vertical lines under this horizontal bar mark the major peaks within the simple peak. The unusually small complex peak within the simple pattern is not marked.

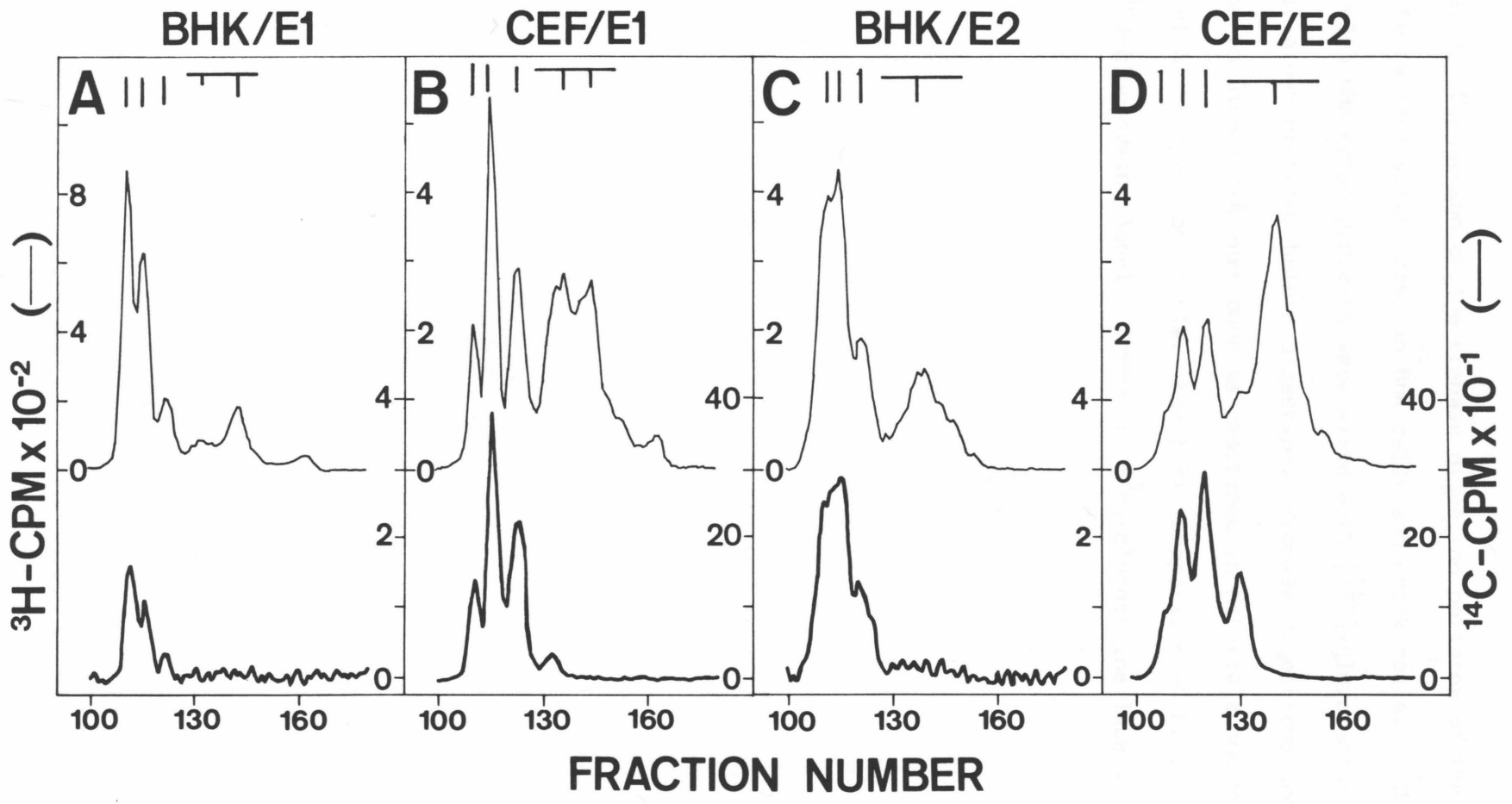

BHK/E1
CEF/E1
BHK/E2
CEF/E2
A
B
C
D
^{3}H-CPM x 10^{-2} (—)
^{14}C-CPM x 10^{-1} (—)
FRACTION NUMBER
100
130
160

FIG. 2. Comparison of the pronase digestion patterns of the envelope proteins from virus grown in BHK cells and chick cells. [^{3}H]-glucosamine labeled BHK grown proteins were mixed with [^{14}C]-glucosamine labeled chick cell grown proteins before digestion. Pronase digestions and gel filtration on Biogel P-4 were done as described in Materials and Methods. Panel A: Comparison of E1s. Panel B: Comparison of E2s. (——): [^{14}C]-glucosamine label. (━━━): [^{3}H]-glucosamine label.

A
E1
B
E2
CPM x 10-2 3H-GLUCOSAMINE (——)
CPM x 10-2 14C-GLUCOSAMINE (——)
FRACTION NUMBERS
100
140
180
0
1
2
3

Table 1

Molar Ratios of Simple and Complex Type Oligosaccharide[a] Chains in E1 and E2

Host Cell/Protein	Figure	Sugar Labeled	Molar Ratio (Simple/Complex)
BHK/E1	2A	Glucosamine	0.63
BHK/E1	1A	Glucosamine	0.71
CEF/E1	2A	Glucosamine	0.98
CEF/E1	1B	Mannose	0.72
BHK/E2	2B	Glucosamine	0.69
BHK/E2	1C	Glucosamine	0.87
CEF/E2	2B	Glucosamine	1.00-1.28[b]
CEF/E2	1D	Mannose	0.71-0.81[b]

[a]Calculated from the pronase digest patterns in Figs. 1 and 2. The radioactivity in the simple and complex peaks were summed and corrected for the number of the labeled sugars in the known predominant oligosaccharide structures. Complex (E1+E2): $NANA_{0-2}Gal_2Man_3Fuc_1GlcNAc_4$. Simple (E1): $Man_5GlcNAc_2$. Simple (E2): $Man_7GlcNAc_2$ (Burke and Keegstra, 1979; Hakimi et al., 1981).

[b]Chick cell grown E1 contained enough of the small galactose labeled oligosaccharide comigrating with the simple peak to complicate the quantitation. The numbers given are obtained by ignoring it totally, or including it in the simple peak, respectively.

dramatic result seen by Burke and Keegstra (1976), and it varies somewhat from preparation to preparation. The results also indicate that there may be some underrepresentation of simple oligosaccharide chains in E2, which also varies with the preparation.

Purification of E1 and E2 Glycopeptides Grown in BHK and Chick Cells

Radiochemically pure individual tryptic glycopeptides of E1 and E2 were obtained using a purification scheme involving Biogel P-10 column chromatography followed by reversed phase high pressure liquid chromatography. This purification scheme generally was sufficient to obtain preparations containing only one labeled glycopeptide plus at most one to two unglycosylated tryptic peptides. Both the Biogel P-10 columns (Fig. 3) and the HPLCs (Fig. 4) were necessary for clean separation. The P-10 column by itself gives insufficient separation of tryptic peptides. In addition, many of the nonglycosylated tryptic peptides migrate in the same regions of the column (data not shown). Although the HPLC gives sharp peaks, in many cases peptides eluting in different regions of the P-10 column elute from the HPLC at identical fraction numbers, and they often turned out to be different peptides (i.e., BHK/E2/2A and BHK/E2/4B_2, see Table 3 for nomenclature description). In two instances (BHK/E2/3A and BHK/E2/4B) the resulting samples from this purification were still a mix of two glycopeptides. In these cases, a concanavalin A-Sepharose column was used to cleanly separate the two peptides (data not shown). The recovery from each time the samples were manipulated was 80-90%. Although this was quite good, the extensive manipulations of the sample during purification meant that the final recovery was a small percentage of the starting material (10-20%).

FIG. 3. Gel filtration (Biogel P-10) separation of tryptic glycopeptides. Trypsin digested envelope proteins (E1 or E2) grown in either BHK cells or chick cells were passed through a 1 cm x 120 cm Biogel P-10 column equilibrated in 100 mM NH_4HCO_3. Aliquots of the 20 drop (800 µl) fractions were counted by liquid scintillation and peaks were pooled. Horizontal bars mark the fractions pooled, and the numbers above the bars correspond to a portion of the full peptide name as defined in Table 3. The numbers down the left portion of each panel represent CPM x 10^{-2}. The arrows marked V_o and V_i represent the excluded and included volumes of the column as monitored by blue dextran and phenol red, respectively. Panel A: BHK cell grown E1 labeled with [^{3}H]-glucosamine and [^{14}C]-galactose. Panel B: Chick cell grown E1 labeled with [^{3}H]-mannose, [^{3}H]-glucosamine, and [^{14}C]-galactose. Panel C: BHK cell grown E2 labeled with [^{3}H]-glucosamine and [^{14}C]-galactose. Panel D: Chick cell grown E2 labeled with [^{3}H]-mannose, [^{3}H]-glucosamine, and [^{14}C]-glucosamine.

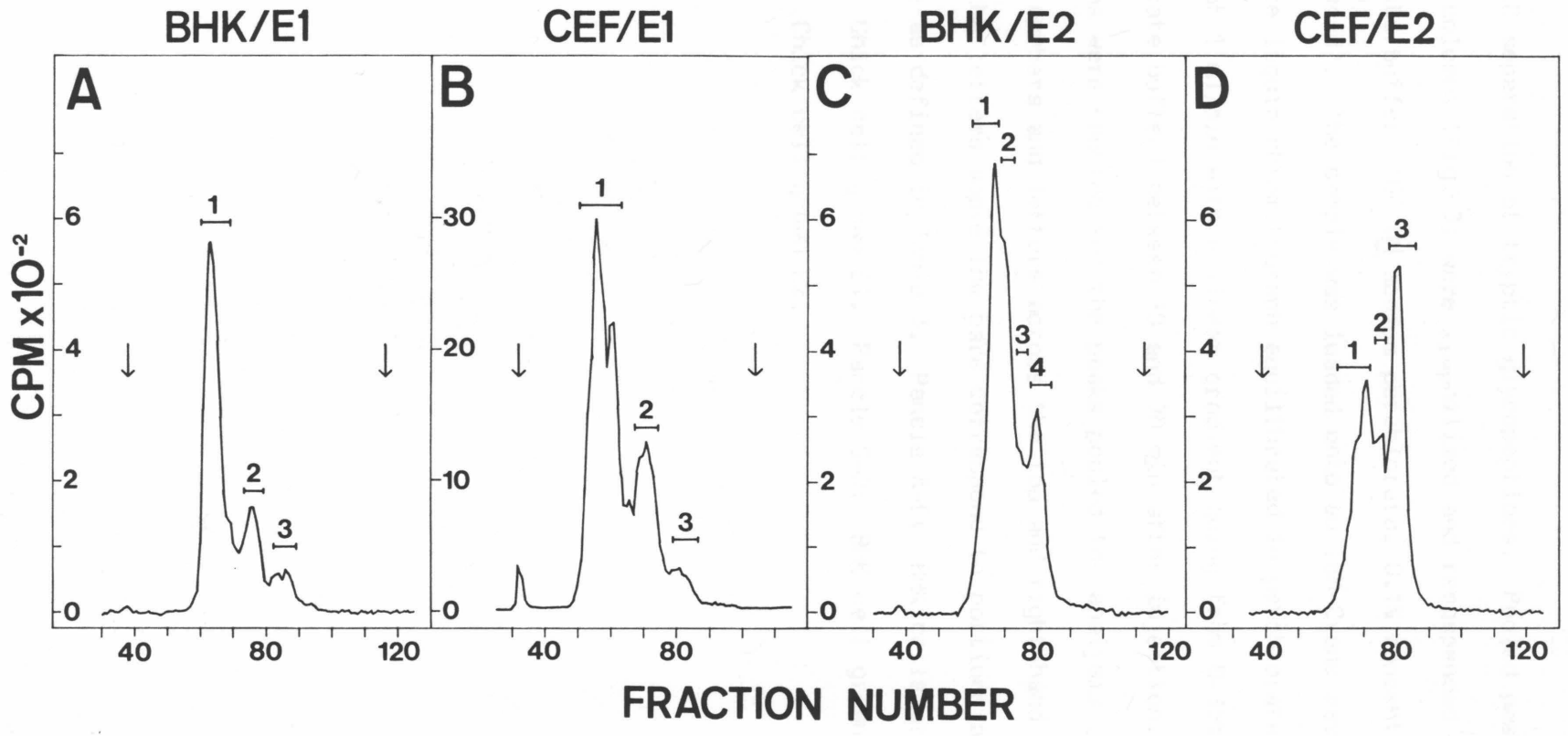
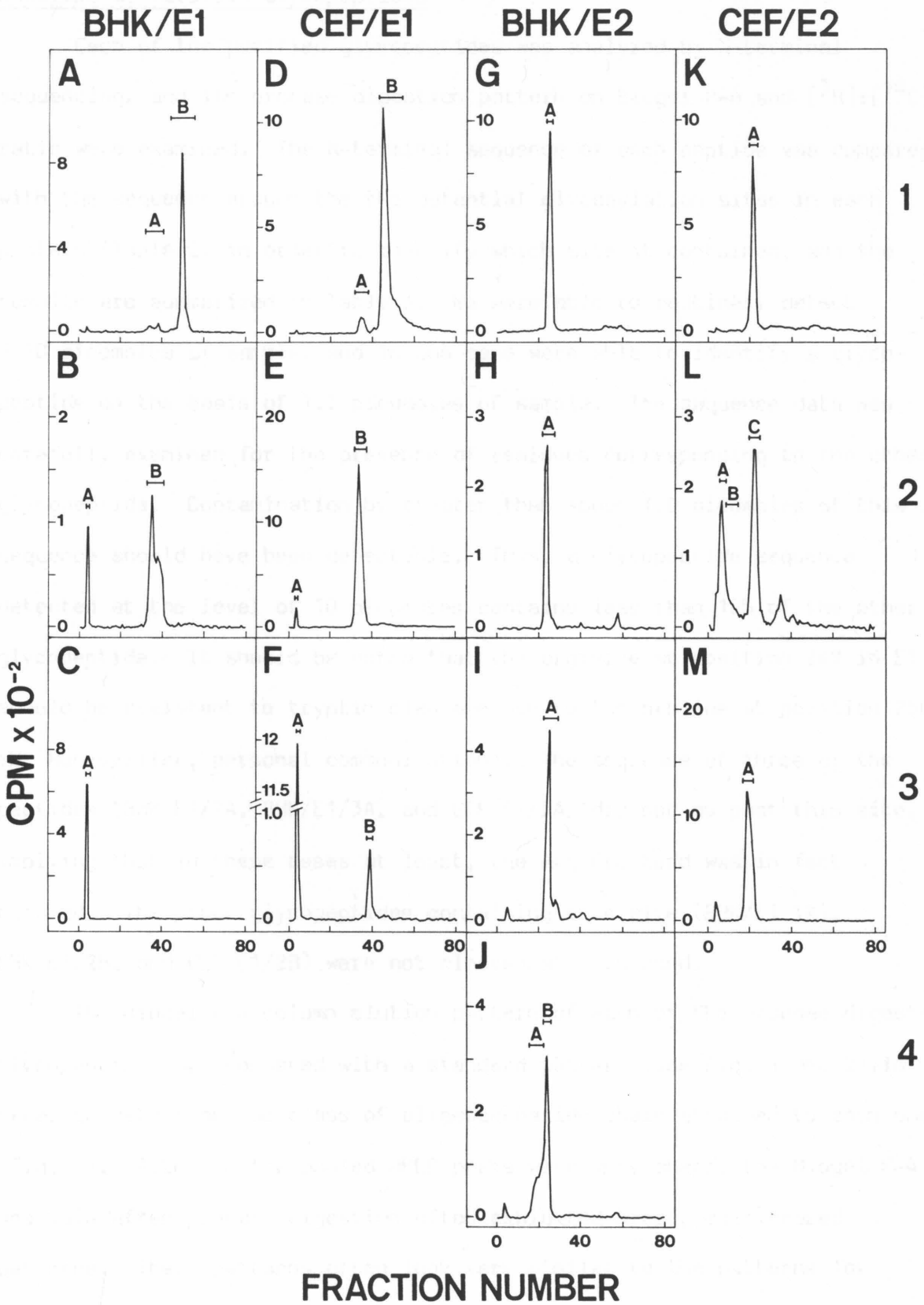

BHK/E1
CEF/E1
BHK/E2
CEF/E2
A
B
C
D
CPM x10⁻²
FRACTION NUMBER
1
2
3
4
0
2
4
6
10
20
30
40
80
120

FIG. 4. HPLC separation of tryptic glycopeptides. Pooled peaks from Biogel P-10 columns (Fig. 3) were lyophilized and resuspended in 75-100 µl of perchlorate buffer (100 mM sodium perchlorate, 0.1% concentrated phosphoric acid). The sample was loaded onto an IBM-Cyano reverse phase high pressure liquid chromatograph equilibrated in perchlorate buffer and eluted off at 1 ml/min with a linear gradient going from 0-35% acetonitrile (in perchlorate buffer) between 10 and 70 min after injection. Aliquots of the fractions were counted and the peaks pooled for analysis (horizontal bars). The numbers and letters across the top and right-hand sides of the figure and the letters above the bars correspond to portions of the full peptide name as defined in Table 3. Panels A-C: BHK cells grown E1. Panels D-F: Chick cell grown E1. Panels G-J: BHK cell grown E2. Panels K-M: Chick cell grown E2.


BHK/E1
CEF/E1
BHK/E2
CEF/E2
A
B
C
D
E
F
G
H
I
J
K
L
M
1
2
3
4
CPM x 10⁻²
FRACTION NUMBER

Analysis of Purified Glycopeptides

Each of the purified glycopeptides was analyzed by N-terminal sequencing, and its pronase digestion pattern on Biogel P-4 and $[^{3}H]:[^{14}C]$ ratio were examined. The N-terminal sequence of each peptide was compared with the sequence around the two potential glycosylation sites in each protein (Table 2) in order to identify which site it contained, and the results are summarized in Table 3. We were able to routinely detect 5-10 picomoles of sample, and in one case were able to identify a glycopeptide on the basis of 1.7 picomoles of sample. The sequence data was carefully examined for the presence of residues corresponding to the other glycopeptide. Contamination by greater than about 1.0 picomoles of this sequence should have been detectable. Thus, a glycopeptide sequence detected at the level of 10 picomoles contains less than 10% of the other glycopeptide. It should be noted that the arginine at position 249 in E1 should be resistant to tryptic cleavage due to the proline at position 250 (M. Hunkapiller, personal communication). The sequence of three of the peptides (BHK/E1/2A, BHK/E1/3A, and CEF/E1/3A) did not go past this site, implying that in these cases at least, the Arg-Pro bond was in fact cleaved. The other glycopeptides containing this site (BHK/E1/1A), BHK/E1/2B, and CEF/E1/2B) were not cleaved at this bond.

The Biogel P-4 column elution pattern of each of the pronase digested glycopeptides was compared with a standard pattern (see Fig. 1 and 2) in order to determine the class of oligosaccharide chain attached to each one (Fig. 5). Although the pooled HPLC peaks were very sharp, the Biogel P-4 analysis after pronase digestion often exhibited broad, multipeaked patterns. These patterns often look very similar to the patterns for

TABLE 2

Sequence of Tryptic Peptides Containing the Potential Glycosylation Sites of Sindbis Virus E1 and E2[a]

Protein	Site	Sequence[b]
E1	1	...GLR/IVYG*NTTSFDVYVNGVTPGTSK/DL... (135)
E1	2	...WK/*NNSGR/PLQETAPFGCK/IAV... (245)
E2	1	...SGK/*NITYECK/CGD... (196)
E2	2	...TVR/*NFTVDR/DGL (398)

[a]From Rice and Strauss (1981).

[b]The single letter amino acid code is used: A=ala, C=cys, D=asp, E=glu, F=phe, G=gly, H=his, I=ile, K=lys, L=leu, M=met, N=asn, P=pro, Q=gln, R=arg, S=ser, T=thr, V=val, W=trp, Y=tyr. The first amino acid of the peptide containing the potential glycosylation site is numbered relative to the N-terminus of the protein. *=potential glycosylation site. /=potential tryptic cleavage site around the glycosylation site. The potential cleavage site after position 249 in E1 is partially resistant to cleavage due to the proline that follows it.

TABLE 3

Summary of Analysis of Purified Glycopeptides

Peptide [a]	Type of Carbohydrate: Pronase Digest[b]	Type of Carbohydrate: Ratio of Labels[c]	Type of Carbohydrate: Con A Column[d]	Glycosylation Site[e]	Quantity Detected[f] (pmoles)	Total[g] (nmoles)
BHK/E1/1A	C	C	-	2	$7(N_{246})$	0.16
BHK/E1/1B	C	C	-	1	$62(I_{135})$	3.59
BHK/E1/2A	*	C	-	2[h]	$8(N_{246}/G_{248})$[i]	0.39
BHK/E1/2B	S	S	-	2	$11(G_{248})$	1.16
BHK/E1/3A	S	S	-	2[h]	$9(G_{248})$	0.78
CEF/E1/1A	Lost	C	-	(2)[j]	-	(0.22)[k]
CEF/E1/1B	C	C	-	1	$20(V_{136})$	4.02
CEF/E1/2A	*	C	-	(2)[j]	0	(0.05)[k]
CEF/E1/2B	S	S	-	2	$13(N_{246})$	1.22
CEF/E1/3A	S	S	-	2[h]	$8.3(N_{246})$	0.48
CEF/E1/3B	*	S	-	-	-	-
BHK/E2/1A	C	C	-	1	$90(I_{197})$	4.92
BHK/E2/2A	C	C	-	1	$14(E_{200})$	2.52
BHK/E2/$3A_1$	C	C	C	1	$16(I_{197})$	1.23
BHK/E2/$3A_2$	S	S	S	2	$12(F_{399})$	0.92
BHK/E2/4A	S	S	-	2	$65(F_{399})$	1.72
BHK/E2/$4B_1$	C	C	C	1	$40(I_{197})$	1.01
BHK/E2/$4B_2$	S	S	S	2	$118(F_{399})$	2.97
CEF/E2/1A	C	C	-	1	$30(I_{197})$	5.70
CEF/E2/2A	C	C	-			0.37
CEF/E2/2B	C	C	-	1	$4(E_{200})$	
CEF/E2/2C	C	C	-	1	$1.7(E_{200})$	0.13
CEF/E2/3A	S	S	-	2	$38(F_{399})$	8.57

Table 3 (continued)

[a]The peptide name identifies the source and purification history of the peptide. The first and second parts identify the host cell and protein, respectively. The third portion identifies the purification history of the protein. The number refers to the pooled peak from the Biogel P-10 column used to separate tryptic peptides, and the letter refers to the pooled peak from the HPLC used to further separate these Biogel P-10 peaks. When a Concanavalin A column was needed to further separate peptides (BHK/E2 only) a subscript 1 or 2 was used to identify the peak eluting with α-methylglucoside (complex carbohydrates) or α-methylmannopyranoside (simple carbohydrates) respectively. This identification system is used throughout this paper.

[b]Pronase digests of the tryptic peptides (Fig. 3) were compared with the known typical patterns for simple or complex type oligosaccharides. In the case where the patterns were atypical and could not be assigned, an asterisk (*) is used.

[c]Aliquots of the final preparations of each peptide were counted by liquid scintillation and the $[^{14}C]:[^{3}H]$ ratios were calculated. Using our knowledge of the radioactive sugar labels used and the known structures of simple and complex oligosaccharide chains (Burke and Keegstra, 1979; Hakimi et al., 1981) the identity of the type of oligosaccharide was determined.

[d]In two cases a Concanavalin A column was needed to separate two species from a single HPLC peak. The resulting peaks were assigned as simple or complex both by their $[^{3}H]:[^{14}C]$ ratio and by the previously reported ability of α-methylglucoside and α-methylmannopyranoside to elute complex and simple type oligosaccharides, respectively (Cummings and Kornfeld, 1982).

Table 3 (continued)

[e]The site of glycosylation was determined by comparing the amino acid sequencing results with the complete protein sequence as determined by Rice and Strauss (1981). The sequences in the vicinity of the potential glycosylation sites in E1 and E2 are shown in Table 2.

[f]Quantitation was determined by measuring peak heights of phenylthiohydantoin amino acid derivatives of selected residues and comparing them with a known quantity of a standard mix of phenylthiohydantoin derivatives. The residue used for the quantitation is shown in parentheses. The quantitation is accurate only to about a factor of two.

[g]Total quantity of the glycopeptides extrapolated back to starting material (pure E1 or E2). Thus is done by correcting the quantity of sequence obtained (previous column) for the percentage of the sample loaded and correcting for a repetitive yield of 94%. Then this quantity was corrected for the loss of radioactivity through each of the purification steps used. Differential losses at steps where more than one peptide were together cannot be corrected for.

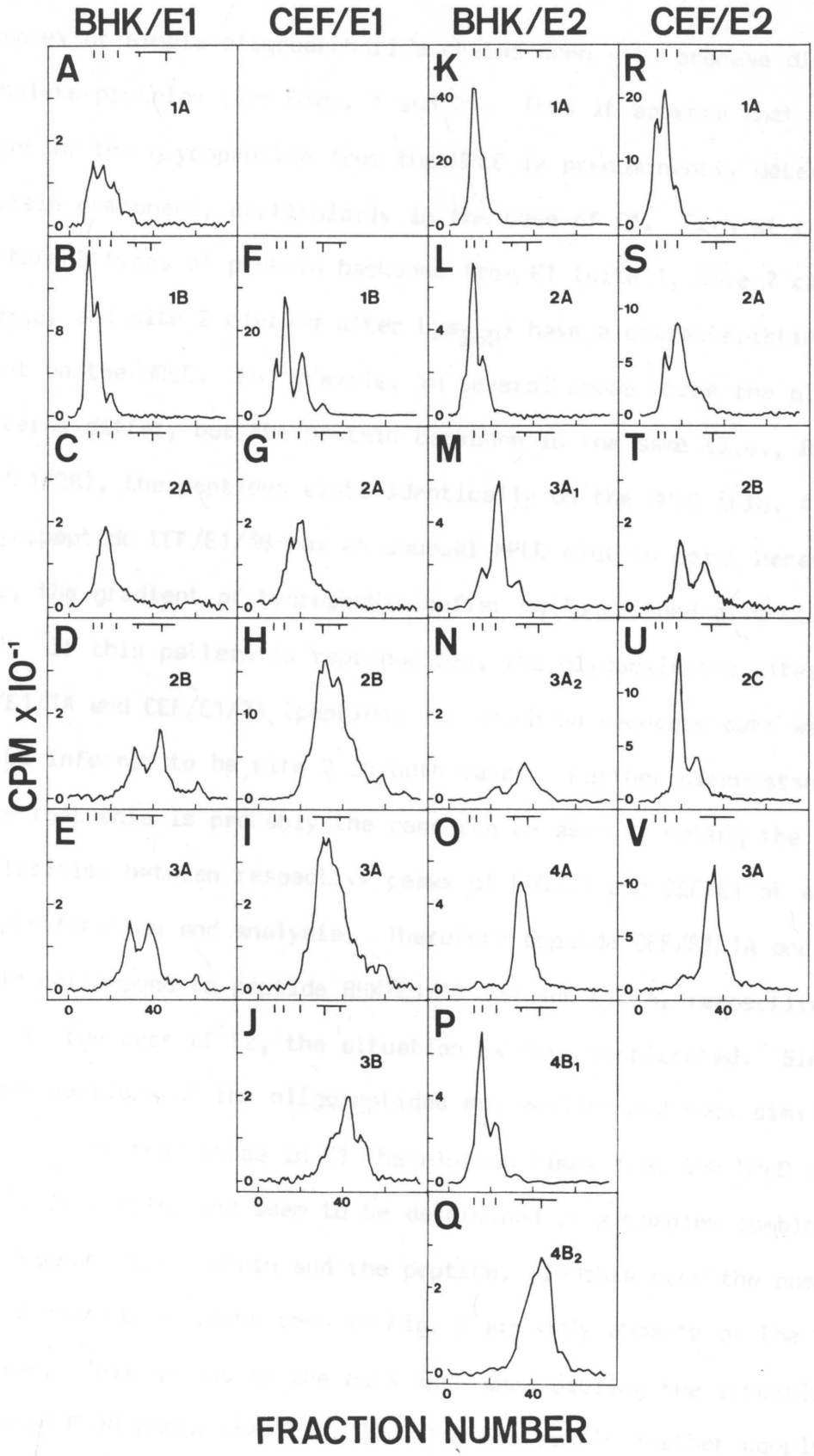
[h]Sequenceable only through the Arg_{249}, implying an Arg-Pro cleavage.

[i]Average of the two residues shown.

[j]No sequence data was available for these. The glycosylation site was inferred from their elution position from the HPLC column (see text).

[k]These quantitations were determined by comparing the radioactivity in these samples to that in CEF/E1/1B. The assumption is that each of the complex chains should label approximately equally.

FIG. 5. Pronase digestion patterns of purified glycopeptides. Samples were digested and chromatographed as described in Materials and Methods, and the nomenclature for the glycopeptides is that described in Table 3. The positions of peaks from the standard patterns are indicated in the upper portion of each panel. The three vertical lines represent the three complex oligosaccharide peaks, and the horizontal bar represents the broad simple oligosaccharide peak typically seen. Vertical lines under this horizontal bar mark the major (and in some cases the minor) peaks within the simple peak pattern (see Figs. 1 and 2 for examples). Panels A-E: BHK cell grown E1. Panels F-J: Chick cell grown E1. Panels K-Q: BHK cell grown E2. Panels R-V: Chick cell grown E2.


BHK/E1
CEF/E1
BHK/E2
CEF/E2
A
1A
B
1B
F
1B
K
1A
R
1A
L
2A
S
2A
C
2A
G
2A
M
3A1
T
2B
D
2B
H
2B
N
3A2
U
2C
E
3A
I
3A
O
4A
V
3A
J
3B
P
4B1
Q
4B2
CPM x 10-1
FRACTION NUMBER

complex or simple oligosaccharide chains seen with pronase digests of the complete proteins (see Figs. 1 and 2). Thus it appears that the elution point of the glycopeptide from the HPLC is predominantly determined by the protein component, particularly in the case of E1. Each of the three different types of protein backbone from E1 (site 1, site 2 cleaved after Arg_{249}, and site 2 cleaved after Lys_{260}) have a characteristic elution point on the HPLC. For example, in several cases where the glycosylation patterns differ, but the protein backbone is the same (i.e., BHK/E1/1A and BHK/E1/2B), the peptides elute identically on the HPLC (Fig. 4) (glycopeptide CEF/E1/3B has an unusual HPLC elution point because in this case, the gradient of hydrophobic buffer malfunctioned slightly during the run). If this pattern is reproducible, the glycosylation sites for CEF/E1/1A and CEF/E1/2A (peptides for which no sequence data was obtained) can be inferred to be site 2 in both cases. Further circumstantial evidence that this is probably the case can be seen by noting the close similarities between respective peaks of BHK/E1 and CEF/E1 at all steps of the purification and analysis. Therefore peptide CEF/E1/1A and CEF/E1/2A should correspond to peptide BHK/E1/1A and BHK/E1/2A, respectively.

In the case of E2, the situation is more complicated. Since the protein portions of the oligopeptides are smaller and more similar to each other in size than those in E1 the elution times from the HPLC are all relatively similar and seem to be determined by a complex combination of the oligosaccharide chain and the peptide. In this case the complex oligosaccharide patterns seen in Fig. 5 are only subsets of the complete patterns. This is due to the cuts made when pooling the incompletely separated P-10 peaks (see Fig. 3). The pattern is further complicated by

the small fourth complex peak, which appears in several of the complex patterns (i.e., CEF/E2/2A-2C and BHK/E2/$3A_1$). The pronase pattern of the isolated peptides also enable us to detect this peptide in the BHK cell grown E2 (BHK/E2/$3A_1$), although to a much lesser extent.

We also found several peptides with oligosaccharide chains that did not fit the standard pattern. An oligosaccharide chain that was labeled with galactose but did not comigrate with any of the standard complex peaks was found in both chick and BHK cell E1 (CEF/E1/2A and BHK/E1/2A). In addition an unusual small simple oligosaccharide chain was found in chick cell E1 (CEF/E1/3B). In all three cases, these unusual oligosaccharides were minor portions of the total peptides and will not be further discussed.

Finally, the ratios of [^{3}H]:[^{14}C] were used to independently identify the peptides as simple or complex (summarized in Table 3). In all of the cases, these results were consistent with the results determined by pronase digest patterns. In the case of the three atypical pronase digest patterns seen, these ratios were used to determine that the two glycopeptides that were approximately the size of typical complex chains (BHK/E1/2A and CEF/E1/2A) had a complex chain as they contain [^{14}C]-galactose, whereas the small glycopeptide (CEF/E1/3B) had a simple chain as it did not contain [^{14}C]-galactose.

In E2, all of the complex type oligosaccharide chains are attached to site 1 (Asn_{196}) and all of the simple type chains are attached to site 2 (Asn_{398}) (Table 3). The glycosylation pattern of E1 is more variable. Site 1 (Asn_{135}) contains only complex type chains, but site 2 (Asn_{245}) contains a mix of complex and simple chains. In BHK cells, 10-15% of the

complex carbohydrate is attached to site 2 (20-25% of the total oligosaccharide attached at that site). For E1 from chick cells we find that about 6% of the complex carbohydrate is attached to site 2 (about 15% of the total oligosaccharide attached to that site). Thus the two host cells glycosylate the glycoproteins in a nearly identical fashion within the limitations of our quantitation.

Table 4 summarizes the ratios of nanomoles of site 2 to that of site 1. The error in these quantities are too large to determine whether there are significant differences in the glycosylation of site 1 and site 2 in the different cases. If there is any trend, it is that E1 in both cell systems is somewhat underglycosylated.

DISCUSSION

The Sindbis virus envelope glycoproteins seem to be glycosylated in a quite specific way. The predominant species of complex and simple oligosaccharide chains in E2 have been assigned to the first (Asn_{196}) and second (Asn_{398}) glycosylation sites respectively (Rice and Strauss, 1981; see also Burke and Keegstra, 1979). We have confirmed and extended this result using a method that should detect even minor variations in the patterns, and have not detected any exceptions. In the case of E1, conflicting reports about its glycosylation have appeared. Burke and Keegstra (1976) reported that E1 grown in BHK cells, but not chick cells, is drastically underrepresented in simple oligosaccharides, whereas Hakimi and Atkinson (1980) reported that E1 grown in chick cells is also deficient in simple chains, although to a more moderate degree. We have found that the glycosylation patterns in both host cells are essentially identical, and that in both cases simple chains are underrepresented. In both cases site 1

Table 4

Ratio of Nanomoles of Glycosylated Site 2 to Glycosylated Site 1[a] in Starting Material

Protein	Ratio (Site 2:Site 1)
BHK/E1	0.69
CEF/E1	0.42 (0.49)[b]
BHK/E2	0.58
CEF/E2	1.38

[a]The number of nanomoles of site 1 or site 2 for a given protein (Table 3, last column) are summed and the ratios of site 1:site 2 determined.

[b]The numbers in parentheses include the quantitation arrived at by indirect means (see Table 3, footnotes j and k and text).

(Asn_{135}) contains only complex chains, whereas site 2 (Asn_{245}) contains predominantly simple chains, but also a small proportion (15-25%) of complex chains. Therefore, the presence of complex oligosaccharides at site 2 accounts for at least part of the underrepresentation of simple chains in E1. The quantitation suggests that site 2 may also be underglycosylated.

It is interesting to note that in the closely related Semliki Forest virus (Garoff et al., 1980) and Ross River virus (Dalgarno et al., 1983) the second E1 glycosylation site is missing. The single glycosylation site in each of these viruses (Asn_{141} in both Semliki Forest virus and Ross River virus) contains a complex chain (Matilla et al., 1976; Pesonen, 1979; Dalgarno et al., 1983) and is found at almost the identical position as the first site in Sindbis virus (Asn_{135}), which also contains a complex chain. On the other hand, the E2 in both viruses contains two glycosylation sites (Asn_{200} and Asn_{262} in both Semliki Forest virus and Ross River virus), as does Sindbis virus. At least for Semliki Forest virus, E2 contains both complex and simple chains, although it is not known where these chains are attached (Matilla et al., 1976; Pesonen, 1979). The position of the first, but not the second, of these glycosylation sites is closely conserved across these three viral systems.

We would predict from our data, and that of others in Sindbis virus, Semliki Forest virus, and Ross River virus, that the glycosylation at the first site in both E1 and E2 is quite important for proper protein maturation or function, as their locations and glycosylation patterns are conserved across a wide range of hosts and viruses. In contrast, glycosylation at the second site in E1 should be of little or no importance, as the

glycosylation pattern seems to be nonspecific and highly variable and the site itself is missing in closely related viruses. The case of the second glycosylation site in E2 is less clear. Although the pattern of glycosylation in E2, and therefore probably also the pattern at the second site in E2, is conserved throughout a wide range of hosts (Burke and Keegstra, 1976, 1979; Keegstra et al., 1975; Weitzman et al., 1979; Hakimi and Atkinson, 1980), its precise position in the molecule is not crucial, as can be seen by its divergent positions in Sindbis virus, Semliki Forest virus and Ross River virus.

The testing of this prediction must wait for a more detailed understanding of the precise function of the two envelope glycoproteins in the viral infection cycle, and the identification of the importance of particular protein domains to their function.

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"It is easier to stay out than to get out."

Mark Twain