VARIATION IN ALFALFA MOSAIC VIRUS WITH SPECIAL REFERENCE TO ITS IMMUNOCHEMICAL PROPERTIES

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To:

My Parents

Table of contents

Summary

1 - Alfalfa mosaic virus (AMV) was isolated from lucerne (Medicago sativa) plants with a variety of disease symptoms in each of 13 sites in South Australia indicating that the virus is widespread in the state. Host range and symptomatology of the collected field isolates were determined on a limited number of host plant species and shown to be extremely variable. Pathogenicity of twelve single local lesion AMV isolates was studied in detail by mechanical inoculation to 22 different plant host species under two different environmental conditions. These experiments showed that each isolate was biologically distinct and that the host range and symptomatology of each isolate was affected by the environmental condition. Differences were observed in host range and symptomatology between some of the field isolates with their corresponding single local lesion isolates indicating that there was heterogeneity in the viral populations of the AMV field isolates. The host range and variability of the twelve single local lesion AMV isolates precluded their grouping into strains of the virus.

2 - Five biologically most distinct single local lesion isolates of AMV (H4, N20, S30, S40 and W1) were selected for further study and their biochemical and biophysical properties were compared. It was shown that the isolates have different physical stabilities and particle length ratios. Their coat proteins had similar molecular weights but different numbers of disulphide bonds as revealed by polyacrylamide gel-electrophoresis under reduced and non-reduced conditions. The analysis of total RNA of each isolate by agarose gel electrophoresis showed that each has at least four RNA components designated RNAs 1-4 in order of decreasing molecular weight. The size of each segment was indistinguishable between the isolates, but their relative amounts varied. Additional RNA segments found in preparations of some AMV isolates were shown to have AMV sequences by northern blot analysis. Nucleotide sequence analysis of all RNA segments of these isolates by northern and dot-blot hybridization showed that despite their biological and physicochemical differences all five isolates have a high degree of homology.

3 - Despite the high degree of sequence homology between the coat protein genes of the five AMV isolates, their capsids had various requirements for stability as it was not

possible to prepare soluble coat protein preparations of all the isolates by any of the previously published methods. Consequently, a method for the preparation of soluble coat protein of all AMV isolates was developed (Appendix 1). However, protein solubility was retained only in the presence of 0.1 M $CaCl_2$. If the salt concentration was reduced below 0.1 M, protein from some of the AMV isolates precipitated. Proteins prepared by this method were shown to be immunoreactive and to activate the infectivity of the AMV genome. However, during prolonged exposure to buffer containing 0.1 M $CaCl_2$, AMV coat protein undergoes slow proteolysis thereby loosing its ability to activate the AMV genome but not its immunoreactivity.

4 - Polyclonal antisera were raised in rabbits against preparations of coat proteins from two of the AMV isolates and against native as well as glutaraldehyde-fixed virus particles of all five isolates. Antisera were also raised in chickens against native and glutaraldehyde-fixed virus particles of two of the AMV isolates. All antisera raised in rabbits were titrated against different antigenic forms of AMV (native and glutaraldehyde-fixed as well as coat protein subunits) in gel-immunodiffusion tests. Antibodies recognised isolated coat protein in some of the antisera raised against native or glutaraldehyde-fixed AMV and in all antisera raised against isolated coat proteins by gel-immunodiffusion, indirect ELISA and western immunoblotting.

Comparisons of immunogenicity of native and glutaraldehyde-fixed preparations of all five AMV isolates showed that glutaraldehyde-fixation enhanced its immunogenicity. It was also found that glutaraldehyde-fixation renders AMV more efficient as a test antigen in gel-immunodiffusion tests when compared to native virus or isolated coat protein preparations, irrespective of the type of immunogen used for antiserum production. The binding of glutaraldehyde-fixed virus to antibodies was shown to be specific as antibodies to non-related viruses were not able to recognise glutaraldehyde-fixed AMV particles. The better reactivity of glutaraldehyde-fixed AMV in this test was demonstrated to be correlated with its enhanced stability. In contrast to gel-immunodiffusion tests, the fixed AMV was the least reactive test antigen in indirect ELISA compared to native or isolated coat protein preparations, irrespective of the type of antibodies used.

5 - The poor antigenic reactivity of glutaraldehyde-fixed AMV in indirect ELISA was investigated using ^{35}S-labelled AMV (Appendix **2**). It was observed that glutaraldehyde-fixed virus bound very poorly to microtitre plates in 10 mM phosphate buffer, pH 7.0, when compared to that of native virus. However, the glutaraldehyde-fixed virus bound better than native virus to microtitre plates precoated with anti-AMV sera, irrespective of the type of immunogen used for raising the antisera. Furthermore, the adsorption of glutaraldehyde-fixed AMV to microtitre wells could be enhanced by raising pH or increasing ionic strength of coating buffer.

The adsorption properties of proteins from native virus preparations of the different AMV isolates to the microtitre well was studied using ^{35}S-labelled virus. When 10 mM phosphate buffer, pH 7.0, (in which all AMV particles remained intact) was used as coating buffer, different isolates had different ability to be adsorbed to the plates. The serological comparison between AMV isolates using this buffer gave indications of wide differences between AMV isolates. When virus particles were applied in conventional coating buffer, (carbonate buffer, pH 9.6), all isolates had similar binding abilities. However, in this buffer, no intact AMV particles were detected and all isolates were serologically similar. These data demonstrate that the results of indirect ELISA were directly correlated with the binding ability of the isolates and this test is not a reliable method for studying serological relationships among AMV.

6 - The serological comparison of biologically distinct AMV isolates was investigated by three different tests; gel-immunodiffusion, two formats of ELISA and western immunoblotting. The comparison was made at the level of isolated coat protein, native and glutaraldehyde-fixed particles using their corresponding polyclonal antisera. It was shown that the biologically distinct isolates of AMV were serologically very closely related but not identical. However, the degree of relationships depended on the type of antisera and test antigens used. The presence of isolate-specific epitopes were best revealed in gel-immunodiffusion tests when using antisera against isolated coat protein or native virus particles and glutaraldehyde-fixed or native virus particles as test antigens. Under these experimental conditions it was possible to obtain isolate-specific or group specific

polyclonal antisera to some of the isolates. Western immunoblotting was not able to differentiate any of the AMV isolates.

7 - Mouse monoclonal antibodies (McAb) were produced to a mixture of native virus preparations of five AMV isolates and screened by three formats of indirect ELISA to select a maximum diversity of McAbs. Seven McAbs were able to differentiate between biologically diverse AMV isolates. They reacted with unique isolate-specific epitopes on the coat protein subunits, native virus or glutaraldehyde-fixed virus particles in indirect ELISA. A number of McAbs were also obtained which had been directed against common antigenic determinants of all the AMV isolates. Two McAbs were selected which precipitated either native or glutaraldehyde-fixed virus, respectively, in gel-immunodiffusion tests. A heterospecific McAb was obtained which precipitated native virus preparations of cucumber mosaic virus (CMV) but not AMV. This McAb differentiated glutaraldehyde-fixed virus preparation of CMV from its corresponding native virus particle by the formation of a pronounced spur in immunodiffusion tests.

8 - Both polyclonal and McAbs revealed the presence of three types of antigenic determinants (**cryptotopes**, **metatopes** and **neotopes**) on different antigenic conformations of AMV. Evidence is presented to show that isolate-specific epitopes are located on the surfaces of intact AMV particles, and are those antigenic determinants which are exposed on the surface of the isolated coat protein.

Statement

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and, to the best of my knowledge and belief, does not contain material previously published or written by another person, except where due reference is made in the text. I consent to the thesis being made available for photocopying and loan if accepted for the award of the degree.

Signed

M.R. Hajimorad

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Chapter 1

General Introduction

Alfalfa mosaic virus (AMV) has a host range of more than 430 plant species in some 51 dicotyledonous families. The virus is transmitted mechanically and by a number of different aphid species in a non-persistant manner. It can also be transmitted through the seeds of some of its hosts (Hull, 1969; Jaspars and Bos, 1980). AMV is responsible for a number of serious diseases of crop plants (Gibbs, 1962; Frosheiser, 1969; Crill et al., 1970; Tu and Holmes, 1980; Knorr et al., 1983; Bailiss and Ollennu, 1986; Ohki et al., 1986; Hiruki and Miczynski, 1987; Dall et al., 1989). It is one of the most thoroughly studied plant viruses at the molecular level and has been the subjects of many reviews (Van Vloten-Doting and Jaspars, 1977; Van Regenmortel and Pink, 1981; Jaspars, 1985; Francki et al., 1985).

AMV has a tripartite genome consisting of three single-stranded RNAs (RNAs 1, 2, and 3) of positive sense with molecular weights of about 1.1, 0.8, 0.7 x 10^6, respectively. The partial transcript of the 3' end of RNA3 with a molecular weight of about 0.3 x 10^6 encoding the coat protein gene (RNA4) is also encapsidated (Jaspars and Bos, 1980). The coat protein has at least three functions in the multiplication of the virus, activation of the viral genome (Bol et al., 1971; Smit et al., 1981), encapsidation of the newly formed viral RNAs and the programming of the viral RNA polymerase for plus-strand synthesis (Nassuth and Bol, 1983; Houwing and Jaspars, 1987). The 3' terminal 142 residues of all four RNAs are conserved after which there is no significant sequence homology, except for RNA3 which contains the complete sequences of RNA4 at its 3' end (Pink and Pink, 1979; Gunn and Symons, 1980; Cornelissen et al., 1983 a,b; Barker et al., 1983; Ravelonandro et al., 1984). Each of the four RNAs is encapsidated separately with a single species of coat protein of molecular weight about 24,300 (Kruseman et al., 1971; Krall et al., 1972; Krall et al., 1976; Collot et al., 1976; Van Beynum et al., 1977) to form quasi-spherical particles about 18 nm in diameter, plus a series of bacilliform particles of the same width but differing in length depending on the RNA encapsidated (Hull et al., 1969). The main

nucleoproteins are designated as bottom component (B), middle component (M), top component b (Tb) and top component a (Top a), encapsidating RNAs 1, 2, 3 and 4, respectively (Hull et al., 1969). A combination of B, M, and Tb nucleoproteins is infectious (Van Vloten-Doting et al., 1970). Although most AMV isolates contain these four nucleoproteins, but up to 13 minor additional nucleoproteins have been detected in preparations of some isolates (Bol and Kaashoek, 1974). Bacilliform particles longer than component B have been found in some isolates of AMV, but the encapsidated RNA was shown to be no longer than RNA3 (Hull, 1970; Heijtink and Jaspars, 1974).

The complete nucleotide sequences of all four RNAs from AMV strain 425 (Brederode et al., 1980; Cornelissen et al., 1983 a, b; Barker et al., 1983) and RNA3 from isolate S (Ravelonandro et al., 1984) have been determined. From these data, as well as from *in vitro* translation experiments (Mohier and Hirth, 1975; Van Tol and Van Vloten-Doting, 1979; Van Vloten-Doting and Neelemam, 1982), and analysis of proteins in infected alfalfa, tobacco and cowpea cells (Samac et al., 1983; Berna et al., 1986), it is evident that the AMV genome encodes for at least three unrelated non-structural proteins (Joshi et al., 1984; Berna et al., 1986; Van Pelt-Heerschap et al., 1987). However, their exact functions are still unknown. The observation that RNAs 1 and 2 can replicate independently of RNA3, and the results of studies of virus mutants with changes in RNAs 1 and 2 indicate that the gene products of RNAs 1 and 2 are a part of the viral replicase complex (Nassuth et al., 1981; Nassuth and Bol, 1983; Nassuth et al., 1983; Sarachu et al., 1985). RNA3 is a dicistronic messenger from which a protein of molecular weight 3.2 x 10^3 (P_3) is translated which has been detected serologically *in vivo* (Godefroy et al., 1986; Stussi-Garaue et al., 1987). This protein is considered to be responsible for movement of the virus from cell to cell (Godefroy et al., 1986; Huismen et al., 1986). Polyclonal antisera raised against the *in vitro* translation product of RNA3 reacted with P_3 of AMV, but not with the corresponding proteins from tobacco streak virus (TSV), cucumber mosaic virus (CMV) and brome mosaic virus (BMV) (Van Tol and Van Vloten-Doting, 1981).

AMV has many similarities to the Ilarviruses. Although the morphology of particles in these two groups is different, AMV-like bacilliform particles have been seen in

preparations of some Ilarviruses (Basit and Francki, 1970) and an AMV spontaneous mutant has been shown to contain Ilarvirus-like particles (Roosien and Van Vloten-Doting, 1983). Another similarity between viruses in the two groups is that for infectivity, inocula must contain either RNA4 or coat protein in addition to the three genomic RNAs (Van Vloten-Doting, 1975; Gonsalves and Garnsey, 1975b; Jaspars, 1985). Coat protein of both groups contains substantial quantities of zinc and it is believed that zinc-finger motif plays a role in genome activation (Sehnke et al., 1989). Remarkably, the protein of each virus can cross-activate the genome of the other (Gonsalves and Garnsey, 1975a) despite a lack of nucleotide sequence homology of their coat protein genes (Barker et.al., 1983; Ravelonandro et al., 1984; Cornelessine et al., 1984), serological relationships (Gonsalves and Garnsey, 1975a), or tryptic finger-print patterns (Van Vloten-Doting and Jaspars, 1977). There is about 24% sequence homology between the 3'-terminal 140 non-coding residues of the AMV and TSV RNAs 3 and even less with those of RNAs 2 (Koper-Zwarthoff and Bol, 1980). However, in spite of the sequence differences, the RNAs possess similar secondary structures (Koper-Zwarthoff and Bol, 1980). For AMV it has been shown that the integrity of AMV coat protein is essential for activating the genome (Bol et al., 1974; Zuidema et al., 1983b) and that the mixture of RNAs and protein is not infectious unless each of the genomic RNA has bound a few coat protein molecules (Smit and Jaspars, 1980; Smit et al., 1981).

Another similarity between the Ilarviruses and AMV is that, the RNAs of both are able to withdraw protein subunits from intact virus particles, indicating that their RNAs contain specific sites with high affinity for their coat proteins (Van Vloten-Doting and Jaspars, 1972; Houwing and Jaspars, 1978; Zuidema and Jaspars, 1985). It has been proposed that the binding of protein to the 3' end of the AMV RNA makes it recognisable by the replicase (Houwing and Jaspars, 1978; Stoker et al., 1980; Zuidema et al., 1983a) and that the location of the major coat protein binding sites are located near the 3' ends of the genomic RNAs of AMV and TSV in both heterologous and homologous RNA-coat protein combinations (Koper-Zwarthoff and Bol, 1980; Zuidema et al., 1983a; Zuidema et al., 1984; Zuidema and Jaspars, 1984; Zuidema and Jaspars, 1985).

The genome organization of AMV and the Ilarviruses resemble the tripartite genomes of the Cucumoviruses and Bromoviruses which together share a number of important features (Jaspars, 1974; Van Vloten-Doting, 1976; Van Vloten-Doting and Jaspars, 1977 ; Lane, 1979, 1981; Jaspars and Bos, 1980; Kaper and Waterhouse, 1981; Fulton, 1981, 1983). The evolutionary relationship of these viruses has been established on the basis of their nucleotide sequences, but different segments of the genome exhibit different evolutionary rates (Koper-Zwarthoff and Bol, 1980; Murthy, 1983; Rezaian et al., 1984; Cornelissen and Bol, 1984; Fauquet, et al., 1986; Davis and Symons, 1988). Many attempts have been made to classify these viruses into a group. Van Vloten-Doting and Jaspars, (1977) divided them into either isocapsidic (Bromo and Cucumoviruses) or heterocapsidic (AMV and Ilarviruses) viruses. The International Committee on Taxonomy of Viruses (ICTV) recognizes them as four distinct groups (Matthews, 1982) but it has been suggested they all be included in a family for which the name Tricornaviridae has been suggested (Van Vloten-Doting et al., 1981).

The ICTV lists three members in the Bromovirus group, brome mosaic virus (BMV), broad bean mottle virus (BBMV), cowpea chlorotic mottle virus (CCMV) (Lane, 1979) ; three members in the Cucumovirus group, cucumber mosaic virus (CMV), tomato aspermy virus (TAV) and peanut stunt virus (PSV); and 11 distinct viruses in the Ilarvirus group (Fulton, 1983). The presence of numerous natural variants of AMV with different pathogenicity have been reported (Bancroft et al., 1960; Crill et al., 1971; Paliwal , 1982; Walter and Kuszala, 1985; Hiruki and Miczynski, 1987). However, despite the presence of such biological variability, the group has remained monotypic with AMV as its sole member (Matthews, 1982).

Viruses with RNA genomes have high mutation rates because of the lack of proof-reading enzymes (Holland et al., 1982). The constant generation of base changes must result in the generation of virus variants (Van Vloten-Doting and Bol, 1988). Most viruses have numerous variants which in the literature have been referred to as isolates, strains or serotypes. These terms are often ill-defined and hence can be confusing. In this thesis AMV variants will be referred by the more or less non-committal term, isolates. However, the status of the isolates studied in this thesis will be evaluated in Chapter 8. In addition to

base changes (mutation), variants of multi-partite viruses can also arise by exchanges of RNA species between related viruses (pseudorecombinants). Pseudorecombinants have been experimentally constructed and showed to have the properties of both parents (Bancroft, 1972; Bancroft and Lane, 1973; Majorana and Paul, 1969; Dingjan-Versteegh et al., 1972, 1974a; Habili and Francki, 1974a; Hartmann et al., 1976; Mossop and Francki, 1977; Hanada and Tochihara, 1980; Rao and Francki, 1982; Edwards et al., 1983; Lakshman and Gonsalves, 1985; Allison et al., 1988). In nature, pseudorecombination between compatible virus isolates may contribute to natural variation and help viruses to overcome host resistance (Van Vloten-Doting and Jaspars, 1977). However, this has never been demonstrated to occur *in vivo*. Also, with the accumulation of data (Bujarski and Kaesberg, 1986; Robinson et al., 1987), it seems that recombination can occur between RNA viruses which may be responsible for the appearance of new variants.

The existence of numerous virus variants creates problems in their identifications. It has been shown that a single mutation in the coat protein gene of tobacco mosaic virus (TMV) can affect both its stability (Siegel et al., 1962) and serological specificity (Von Sengbusch and Wittman, 1965). However, most single amino acid mutations in the coat protein do not alter the antigenicity (Wang and Knight, 1967; Van Regenmortel, 1967). It has also been postulated that the amino acid sequences of the coat protein of TMV may influence symptom types in tobacco (Von Sengbush and Wittman, 1965). Recently, it has been shown that the coat protein gene sequences of TMV can be responsible for changes in host responses (Saito et al., 1987; Knorr and Dawson, 1988; Dawson, et al., 1988). It seems that this is not a common feature of all viruses, as in tomato golden mosaic virus the coat protein is not required for symptom development (Gardiner et al., 1988). However, it is believed that the extent of changes in the coat protein is paralleled by the variations elsewhere in the viral genome as serologically related viruses share most of their other properties (Van Regenmortel, 1982). Consequently, virologists have considered serological properties as one of the most valuable criteria for virus classification and identification (Hamilton et al., 1981; Francki, 1983) despite the reservation that the coat protein cistron represents only a small percentage of the total coding capacity of the viral genome.

There have been intensive serological studies on some viruses from each of the groups in the tricornaviridae and considerable reliance for classification within the groups has been placed on serological tests (Habili and Francki, 1975; Devergne et al., 1981; Rybicki and Von Wechmar, 1981; Rao et al.,1982; Uyeda and Mink, 1982), but little attempt has been made to study serological differences between AMV variants (Rybicki and Von Wechmar, 1985). However, an attempt to classify isolates of AMV has been made using the biological characteristics such as host range and symptomatology (Crill et al., 1971). As has been suggested, the host range and symptom expression are of little value for the characterization of viruses because there are numerous examples of similar symptoms produced on the same plant species by obviously unrelated viruses (Francki and Hatta, 1980; Francki, 1983; Symons, 1984). Nevertheless, symptomatology remains as a useful tool for separation of variants of a virus.

AMV isolates not only differ in their host ranges and pathogenicity, but also in other aspects, such as their *in vivo* behaviour (Hull et al., 1970; Dingjan-Versteegh et al., 1974b), amino acid composition of their coat proteins (Krall, 1975), leader sequences of their RNAs 4 (Swinkles and Bol, 1980), 5'-end structures of their genomic RNAs (Ravelonandro et al , 1983; Langereis et al., 1986), their particle length ratios (Van Vloten-Doting et al., 1968; Schwenk et al.,1971) and their *in vitro* translation products (Forster et al.,1985). However, despite these differences, experiments using RNA competition hybridization assays failed to reveal significant differences in their nucleotide sequence homology (Bol et al., 1975).

It has been generally assumed that isolates of AMV with widely different pathogenicities and geographical origin are serologically indistinguishable (Bancroft et al., 1960; Tomaru et al., 1968; Paliwal, 1982; Walter and Kuszala, 1985; Hiruki and Miczynski, 1987). The primary structures of the coat protein of three AMV isolates (425, S and VRU) have been determined by direct protein sequencing (Krall et al., 1976; Collot et al., 1976; Castel et al., 1979) and also have been deduced from the nucleotide sequences of their coat protein genes (Brederode et al., 1980; Barker et al., 1983; Revelonandro et al., 1984) which show that the AMV coat protein is strongly conserved. However, it was shown that two domains on AMV coat protein are susceptible to substitutions, one near the

middle (amino acids 71,82,103) and one close to the C-terminus (amino acids 184-211). As the antibodies to viruses are directed mostly against conformational determinants (Sela et al., 1967), it is possible that the coat proteins of these isolates are antigenically differentiable due to the amino acid substitutions, but this has never been studied. Most of the comparative serological studies between AMV isolates have been done using polyclonal antisera raised against native AMV particles and using the same preparations as test antigens (Bancroft et al., 1960; Silber and Heggestad, 1965; Tomaru et al., 1968; Paliwal, 1982; Walter and Kuszala, 1985). Isolated protein subunits possess antigenic determinants which are not present on polymerised particles (Jerne, 1960), and hence such determinants have probably not been compared between different AMV isolates.

The antigenic structure of AMV has been shown to be complex and similar to that of BMV which is composed of three types of conformational determinants, **neotopes** (epitopes created after polymerization of monomers, Van Regenmortel, 1966; Von Wechmar and Van Regenmortel, 1968), **cryptotopes** (epitopes of a monomer that are no longer exposed after polymerization, Jerne, 1960; Van Regenmortel, 1966) and **metatopes** (epitopes common to polymerized and depolymerized protein, Moed and Veldstra, 1968; Von Wechmar and Van Regenmortel, 1968). In studying the serological interrelationships between the members of the bromovirus group, it was shown that the relationship varied depending on the type of antigenic determinants compared. Their related antigenic determinants were more easily recognisable on swollen virus particles or free coat protein subunits than in compact intact virus particles (Rybicki and Von Wechmar, 1981). It has been shown that dissociated coat proteins of potyviruses appear to be more closely serologically related than their assembled counterparts (Shepard et al., 1974). However, the N-terminal regions of their coat proteins possess the virus specific epitopes which are exposed on the surfaces of their particles. Nevertheless, apparently normal virus particles can consist of protein subunits devoid of terminal peptides which are responsible for virus specific epitopes (Shukla et al., 1988). The possibility of the existence of some differential epitopes between AMV variants on different antigenic conformation has not been investigated. As AMV is not a stable virus (Hull, 1969), and because most of the comparative serology on AMV variants has been studied with native particles as

immunogens and test antigens, it is possible that some of the antigenic determinants, such as cryptotopes or neotopes have not been compared. Indeed, there has been some serological evidence for differences between AMV variants revealed by the formation of precipitin "spurs" in Ouchterlony tests (Van Vloten-Doting et al., 1968; Roosien and Van Vloten-Doting, 1983). In contrast to antisera used by others, these workers used virus preparations heated for 1hr at 30°C as immunogen (Van Vloten-Doting et al., 1968).

Since the introduction of the double antibody sandwich enzyme linked-immunosorbent assay (DAS-ELISA) in plant virology by Clark and Adams, (1977), this technique has been widely used not only for diagnostic purposes (Lister, 1978; Bar-Joseph et al., 1979; Chu and Francki, 1982; Lommel et al., 1982; Moran et al., 1985; Van der Vlugt et al., 1988), but also for comparative serological studies which have revealed serological differences between related viruses (Koenig, 1978; Barbara et al., 1978; Lister and Rochow, 1979; Bar-Joseph and Salomon, 1980; Rybicki and von Wechmar, 1981; Devergne et al, 1981; Rao et al.,1982). Although this technique has been used for the diagnosis of AMV (Marco and Cohen, 1979; Leath and Barneth, 1981; Knorr et al., 1983; Hampton and Weber, 1983; Mclaughlin et al., 1984; Ohki et al.,1986; Hiruki and Miczyncki,1987; Miczyncki and Hiruki, 1987; Pesic and Hiruki, 1988; Dall et al., 1989), it has not been used for comparisons between biologically distinct AMV isolates (Rybicki and Von Wechmar, 1985).

The immunogenicity of AMV has been classified as moderate (Bancroft et al., 1960), however, the maximum reciprocal titres of antisera prepared against a native virus preparation has not exceeded 128 (Forster et al., 1985; Paliwal, 1982) or 256 (Pesic et al., 1988; Avegalis and Katis, 1989) when tested in gel-immunodiffusion tests. It has been shown that the immunogenicity of some poorly immunogenic viruses can be enhanced by fixation using formaldehyde (Von Wechmar and Van Regenmortel, 1968; Francki and Habili, 1972; Rybicki and Von Wechmar, 1981). However, it has been claimed that this treatment can change the antigenicity of the viruses (Rybicki and Von Wechmar, 1981; Rybicki and Coyne, 1983; Musil and Richter, 1983). The effect of fixation on AMV has not been studied before. Such a study should reveal the possibility of increasing the

immunogenicity of the virus and furthermore, fixation may provide a conformationally stable antigen for comparative serological studies.

The difficulty of differentiating AMV variants with polyclonal antibodies could be due to the presence of higher amounts of antibodies to common epitopes shared between isolates while masking the activity of the differentiating antibodies present in lower concentration. In polyclonal antisera, it is impossible to control the precise composition and relative concentrations of antibodies to the various viral epitopes (Nowinski et al., 1983). As a result of the pioneering studies of Kohler and Milstein (1975), it is now possible to obtain immortal cloned cell lines (hybridomas) capable of continuously and reproducibly producing unique monoclonal antibodies (McAbs). Hybridomas are generated by hybridization of antigen triggered B cells (antibody-producing lymphocyte cells) with murine myeloma cells (malignant cells). The hybridoma acquired from its lymphocyte parent, the ability to produce a specific antibody and from its myeloma cell parent, the ability to be cultured indefinitely.

McAbs offer the following advantages over conventional polyclonal antibodies: (a) an unlimited quantity of antibody can be produced from a small quantity of antigen; (b) pure antibodies specific for a single antigenic determinant can be obtained, even when impure antigens are used as immunogen; (c) hybridomas can be preserved by freezing in liquid nitrogen, thereby assuring a supply of antibody whenever required; (d) highly specific McAbs may reveal serological relationships between microorganisms or antigens that were previously unrecognized with polyclonal sera; (e) the use of McAbs eliminates the quantitative and qualitative variability in specific antibody content found in different batches of polyclonal antisera (Halk and De Boer, 1985). The main limitations on the use of McAbs are that they are expensive to produce, are sometimes too specific for the task in hand, and may be assay-specific (Van Regenmortel, 1986a; Martin, 1987).

Although the first reports of McAbs production against plant viruses appeared in 1982 (Al Moudallal et al., 1982; Dietzgen and Sander, 1982; Briand et al., 1982), in the following two years McAbs were produced against more than 30 plant viruses (Van Regenmortel, 1986a); a number which has now been exceeded by far. The use of McAbs in the field of plant virology has been the subject of recent review articles (Sander and

Dietzgen, 1984; Van Regenmortel 1984, 1986a; Halk and De Boer, 1985). McAbs produced against plant viruses have been used for detection and diagnosis of viral infections (Gugerli and Fries, 1983; Hsu and Lawson, 1985; Diaco et al., 1985; Huss et al., 1986; Vela et al., 1986; Rose and Hubbard, 1986; Dekker et al., 1987; Rajeshwari et al., 1987; Rose et al., 1987; Sherwood et al., 1987; Dore et al.,1987a; Bahrani et al., 1988; Torrance, et al., 1988; Culvar and Sherwood, 1988; Grassi et al., 1988; Sherwood et al., 1989), neutralization of viral infectivity (Dietzgen, 1986a; Dietzgen et al., 1987; Aebig et al., 1987), ultrastructural location of a viral non-structural protein (Mackenzie and Tremaine, 1988), analysis and mapping of epitopes (Altschuh et al., 1985; Dougherty et al., 1985; Tremaine et al., 1986; Dore et al., 1987b, 1988), purification of viral antigens (Diaco et al., 1986a), analysis of viral structural proteins (Dietzgen and Francki, 1988), characterization of viral antigens (Al Moudallal et al., 1982; Morrow et al., 1984; Dietzgen, 1986a; Mackenzie and Tremaine, 1986; Koenig and Torrance, 1986; Sober et al.,1988) and for production of anti-idiotypic antibodies (Hu et al, 1988).

As each McAb is specific for a single antigenic determinant, analysis of many virus strains may be made on that basis. Hence, McAbs have been used in studying strain differences and virus relationships in different plant taxonomic groups including Sobemoviruses (Tremaine, et al., 1985), Potato virus X (PVX) (Torrance et al., 1986), Nepoviruses (Huss et al., 1987), Luteovirus (Hsu et al., 1984; Martin and Stace-Smith, 1984; Diaco et al., 1986b; Massalaski and Harrison, 1987; Hewish et al., 1987; Forde, 1989), Tobamoviruses (Briand et al., 1982; Dietzgen, 1986a), Phytoreoviruses (Kitagawa, et al., 1987), Potyviruses (Hill et al., 1984; Dougherty et al., 1985), Geminiviruses (Thomas et al., 1986; Dekker et al., 1988), Dianthoviruses (Hiruki et al.,1984a,b; Hiruki and Figueiredo, 1985), beet necrotic yellow vein virus (Torrance et al., 1988), Cucumoviruses (Porta et al., 1989) and Ilarviruses (Halk et al., 1984). These studies have revealed some serological differences between isolates previously undetectable by polyclonal antibodies.

Attempts to produce McAbs against AMV was initiated by Halk, (1983) and Halk et al., (1984). They generated stable hybridoma cell lines secreting specific antibodies against one isolate of AMV but the ability to differentiate AMV isolates was not reported.

However, they were able to reveal some aspects of the antigenic structure of AMV (Halk, 1986).

The scope of this thesis

Before starting this project, the general accepted view was that AMV variants were not serologically easily distinguishable (Jaspars and Bol, 1980) and only two serologically distinguishable field isolates (isolates 425 and YSMV) and one mutant derived from isolate 425 were known (Van Vloten-doting et al., 1968; Roosien and Van Vloten-Doting, 1983). This project was undertaken to examine the antigenic properties of field isolates of AMV with diverse biological, biophysical and biochemical properties. With this goal in mind the following approach was followed:

1 - A survey was conducted in lucerne stands around South Australia to collect AMV field isolates.

2- The collected isolates were screened on the basis of their host ranges and symptomatology.

3 - Selected isolates were further characterised biophysically and biochemically.

4 - The immunogenicity of AMV was enhanced by glutaraldehyde-fixation and polyclonal antisera were produced against different antigenic forms of AMV.

5 - A panel of monoclonal antibodies were raised against AMV.

6 - Attempts were made to differentiate the selected AMV isolates by using both polyclonal and monoclonal antibodies by various serological methods.

Chapter 2

General Materials and Methods

I Materials

A. Virus isolates

The virus isolates of AMV studied in this thesis were collected from lucerne (Medicago sativa) stands at different sites around South Australia as shown on Fig.3 -1. Native or glutaraldehyde-fixed virus and isolated coat protein preparations of the other viruses, used in some of the serological experiments were obtained either from the departmental collection or kindly supplied by Miss W. S. Wahyuni and Messrs S. T. Sackey and B. Chen (department of Plant Pathology, University of Adelaide).

B. Materials used for Polyacrylamide-gel electrophoresis (PAGE)

Stock solutions of acrylamide from BDH Chemicals, England and BIS (N,N' -Methylene-bis-acrylamide) from Bio-Rad Laboratories, U.S.A. were prepared and then stored in the dark at 4^0C. Ammonium persulphate (electrophoretic grade) from Bio-Rad Laboratories was stored in an air tight container over $CaCl_2$. N,N,N'-N'-Tetramethyl ethylenediamine (TEMED) was obtained from Sigma. Silver nitrate and formaldehyde solution (formalin) were obtained from Ajax chemicals, Australia.

C. Protein used as markers for polyacrylamide gel electrophoresis

Proteins markers (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and α-lactalbumin) were obtained as a kit from Pharmacia (Sweden). Prestained SDS molecular weight markers [α_2 -macroglobulin (human plasma), ß-galactosidase (E.coli), fructose-6-phosphate kinase (rabbit muscle), pyruvate kinase (chicken muscle), fumarase (porcine heart), lactic dehydrogenase (rabbit muscle), triosephosphate isomerase (rabbit muscle)] were all obtained from Sigma, Chemical Co., U.S.A.

D. Materials used for cDNA synthesis and molecular hybridization analysis

The chemicals and biochemicals which were used specifically for this purpose are listed in Table 2 - 1.

Table 2-1 : Chemicals and biochemicals specifically used for molecular hybridization.

Chemicals	Sources
DL-Dithiothreitol (Cleland's reagent)	Sigma Chemical Co.,U.S.A.
Sephadex G-50	Pharmacia Sweden
^{32}P dATP	Bresatec, Australia
dTTP	Boehringer Mannheim, West Germany
dGTP	Boehringer Mannheim, West Germany
dCTP	Boehringer Mannheim, West Germany
Ficoll 400	Pharmacia, Sweden
Reverse transcriptase	Bethesda Research Laboratories, U.S.A.
Salmon sperm DNA (primer)	Sigma Chemical Co., U.S.A.
Triethylamine	Ajax Chemicals, Australia
Ribonucleic acid (Torula yeast)	Sigma Chemical Co.,U.S.A.
X- ray film	Fuji X-ray, Japan

E. Materials used specifically for hybridoma technology

All washable instruments were soaked overnight in 1% of Linbro 7xTM (Flow Laboratories, Australia) in tap water, drained, washed with a soft brush, rinsed several times in distilled water (DW) and further sterilized by autoclaving at 121^{o}C for 15 min. All

liquid solutions were filtered through an appropriate sterile filter (Millipore, USA), collected in a sterilized container and stored at 4°C. The myeloma or hybridoma cells were grown in either tissue culture flasks or tissue culture plates (24 or 96 wells) from Nunc (Sweden) and kept in a humid atmosphere of 5% CO_2 at 37°C by using an incubator from Forma Scientific Co., U.S.A. The counting and the viability of the cells was done with an inverting microscope (Olympus or Zeiss, West Germany) using a counting grid from Assistant, West Germany. For preservation of the hybridoma or myeloma cells, cryotubes from Nunc (Sweden) were used. Groups of 4-6 cryotubes were inserted into a cryoflex from Nunc (Sweden) and were kept in a liquid nitrogen container at 4°C. The media used for growing the cells were sterilized by filtration, using a master flex pump (Model 7014 - 20 from Laboratory Supply, Coler -Palmer, Australia) for passing the media through three layers of Millipore filter (type AP), Millipore filter (type HA, 0.45μm), and finally through Millipak filter (0.22μm) from Millipore U.S.A. All other chemicals and biochemicals used are as listed in Table 2-2.

F. Materials used for serological techniques

Gel-immunodiffusion tests were done in disposable petri-dishes (Johas, Australia). Microtitre ELISA plates were purchased from Nunc (Sweden). Other chemicals and biochemicals used are as listed in table 2-3.

G. Buffers and solutions

Freeze dried RNA preparations were suspended in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.3). TAE (Loening, 1967) was used as electrophoresis buffer for preparative or analytical gel electrophoresis of RNA and contained 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 8.1 (adjusted with glacial acetic acid).

H. Miscellaneous materials

Chemicals and biochemicals used in this study are listed in Table 2-4. Other chemicals and biochemicals which were used but are not mentioned, were all analytical reagent grade.

Table 2-2: Chemicals and biochemicals used specifically in hybridoma technology.

Chemicals	Sources
Aminopterin	Flow Laboratories, U.S.A.
8-azaguanine	Sigma Chemical Co., U.S.A.
Barbitone (Na-salt)	BDH Chemicals, England
Dimethyl ether	Ajax Chemicals, Australia
Dimethyl sulphoxide	Ajax Chemicals, Australia
Foetal calf serum (FCS)	Flow Laboratories, U.S.A.
Hypoxanthine and thymidine (50x)	Flow Laboratories, U.S.A.
L-glutamine	Sigma Chemical Co., U.S.A.
Penicilline G	Sigma Chemical Co., U.S.A.
Pristane	Sigma Chemical Co., U.S.A.
Polyethylene glycol (4000 MW)	Merck Co., West Germany
RPMI-1640	Flow Laboratories, U.S.A.
Sephacryl S-300	Pharmacia, Sweden
Silicon dioxide powder (Silica, Fume)	Sigma Chemical Co., U.S.A.
Streptomycin	Sigma Chemical Co., U.S.A

I. Instruments

Ultracentrifugation was done either in a Beckman L8-70, L2-65, L-50, or TL-100 refrigerated centrifuge. Low and medium speed centrifugation was done in either a Sorvall RC-2B or Sorvall RC-3 centrifuge. Analytical centrifugation was done in a Beckman Model E analytical ultracentrifuge equipped with an An-D rotor. Sucrose density-gradients were fractionated with an ISCO Model 640 density gradient fractionator coupled to an ISCO Model UA-5 absorbance monitor. The colour reaction in each ELISA well was measured by its absorbance at 405 nm using a Bio-Rad Model 1550 EIA reader. Electrophoresis of proteins in polyacrylamide gels was done in a Mini Protein^{TM} II Dual Slab Cell apparatus or a vertical

Table 2-3: Chemicals and biochemicals used for serological studies.

Chemicals	Sources
Agarose (Typell)	Sigma Chemical Co., U.S.A.
Alkaline phosphatase Type Vll -S	Sigma Chemical Co., U.S.A.
Alkaline phosphatase conjugated rabbit anti - mouse (IgG) and (IgM)	Sigma Chemical Co., U.S.A.
Alkaline phosphatase conjugated streptavidin	Zymad Laboratory, U.S.A.
Bacto - agar	Difco Laboratories, U.S.A.
Bovine serum albumin (BSA)	Sigma Chemical Co., U.S.A.
5-Bromo-4-chloro-3-indolyl phosphate(BCIP)	Sigma Chemical Co., U.S.A.
Diethanolamine	Ajax Chemical Co., Australia
Diethylaminoethyl cellulose (DE22)	Whatman Biochemicals Ltd , England
Goat anti - mouse sub-class specific antibodies	Sigma Chemical Co., U.S.A.
Glutaraldehyde	Sigma Chemical Co., U.S.A
Glycine	BDH Chemicals, England
Heparin	Sigma Chemical Co., U.S.A.
Mouse IgG	Sigma Chemical Co., U.S.A.
N,N dimethylformamide	Sigma Chemical Co., U.S.A.
NHS - LC - Biotin	Pierce Chemical Co., U.S.A.
Nitroblue tetrazoline , Grade lll	Sigma Chemical Co., U.S.A.
Phenazine methosulphate	Sigma Chemical Co., U.S.A.
P - nitrophenyl phosphate	Sigma Chemical Co., U.S.A.
Polyvinylpyrolidone (4000 MW)	Sigma Chemical Co., U.S.A.
Sodium Azide	Sigma Chemical Co., U.S.A.
Tween 20	Sigma Chemical Co., U.S.A.

slab gel apparatus (Model SE 600), from Hoefer Scientific Instruments, U.S.A. and the electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose membranes was done by either a Mini Trans-Blot or Transblot TM cell from Bio-Rad Laboratories U.S.A. Polyacrylamide gels were dried in a Bio-Rad slab drier Model 443. The RNA

Table 2-4: Miscellaneous chemicals and biochemicals.

Chemicals	Sources
Adjuvants (Freund's complete and incomplete)	Sigma Chemical Co., U.S.A.
BCA and BCA protein assay reagent	Pierce Chemical Co., U.S.A.
Bromophenol blue	BDH Chemicals, England
Coomassie brilliant blue	ANAX England
Chloroform	BDH Chemicals, Australia
2,5 - Diphenyloxazole (PPO)	Koch - Light Laboratories Ltd., England
Dimethyl POPOP	Ajax Chemicals Ltd., Australia
Ethidium bromide	Sigma Chemical Co., U.S.A.
Hypoclearing agent	Kodak Pty LTD, Australia
Dialysis membranes	Union Carbide Corporation, U.S.A.
Ethylenediaminetetra-acetic acid (EDTA)	Ajax Chemicals Ltd., Australia
8 - Hydroxyquinoline	May and Baker Ltd., England
Ilford rapid fixer	Ilford Pty Ltd , Australia
L-ascorbic-acid	Sigma Chemicals Co., U.S.A.
n-butyl alcohol	May and Baker, Australia
Nitrocellulose membrane (0.2μm)	Schleicher and Schuell , Germany
Phenol	BDH Limited, Poole , England
Polyethylene glycol (PEG 6000)	ACE Chemical Co., Australia
Sigmacote	Sigma Chemical Co., U.S.A.
Sodium dodecyl sulphate (SDS)	BDH Chemicals Ltd., England
Soluene 350	Packard , Australia
^{35}Sulphate	Amersham , England
Toluidine blue	BDH Chemicals, England
Tris (hydroxymethyl) aminomethane (Tris)	Sigma chemical Co., U.S.A.

fractionation by tube gel electrophoresis was done in an apparatus which was made in our laboratory from plastic containers, inserted with 12 plexiglass tubes (15cm x 0.5cm). RNA electrophoresis for analytical purpose was done in a horizontal slab gel apparatus from Bethesda Research Laboratories, U.S.A. Absorption spectra were recorded with a Beckman DU-8B spectophotometer and scintillation spectrophotometry was done in a Packard Tricarb liquid scintillating spectrometer. For freeze-drying, a Dynavac freeze drier unit and for dot-blot a Minifold filtration apparatus from Schleicher and Schuell, Keene, N.H. were used.

II Methods

A. Collection of leaf samples from the field and isolation of AMV

Leaf samples were collected from lucerne plants showing abnormal growth symptoms at several sites in South Australia. Leaf material was ground in 10 mM phosphate buffer, pH 7.0, using a pestle and mortar and the extracts were inoculated mechanically to Nicotiana clevelandii previously dusted with carborundum powder. All the plants which developed mosaic symptoms within three weeks were shown to be infected with AMV when tested by immunodiffusion with antiserum to the Q strain of AMV (provided kindly by Dr. R.I.B. Francki). The host range and symptom induction by each isolate was tested on Phaseolus vulgaris (cv. Hawkesbury Wonder), Lycopersicon esculentum (cv. Rutgers), Cucumis sativus (cv. Supermarket), Vicia faba (cv. Aqua Dulce) and Chenopodium amaranticolor.

B. Biological purification of selected field isolates of AMV by local lesion passage

Twelve of the collected field isolates which caused different symptoms in the above four host species were selected for further study. Each isolate was passaged five times from single local lesions on a suitable host plant. Each isolate was then stored either by direct drying of the infected leaves over $CaCl_2$ and maintaining the dried material at 4^0C in sealed tubes containing $CaCl_2$, by freeze-drying partially purified virus preparations in tubes and storing the tubes at 4^0C or direct freezing of the leaves in liquid nitrogen and

immediate storage at -70°C. Each isolate was also maintained in N. clevelandii in the glasshouse. Single local lesion transfer of nine AMV field isolates (H1, H2, H3, H4, H5, LA1, NI, N3, N20) was passaged on P. vulgaris , two (H4 , S30) on Vigna. unguiculata and one (S40) on V. faba.

C. Host range and symptomatology

The twelve AMV isolates were mechanically inoculated to a range of plant species and cultivars. One series of tests was done in the glasshouse (16-25°C) and another series in a growth chamber held at 25°C with continuous illumination (10,000 lux). In the two series of tests each isolate was inoculated to 10-15 plants of each species at two or three different times and the plants were observed for symptoms over 3 weeks. Inoculated and younger leaves not showing symptoms were tested for the presence of AMV by inoculation to either P. vulgaris or V. unguiculata. With Capsicum frutescens, it is difficult to recover viruses by mechanical inoculation (Marco and Cohen, 1979) and hence symptomless leaves were assayed by the double antibody sandwich enzyme-linked immuno-sorbent assay (DAS-ELISA).

D. Aphid transmission

Wingless Myzus persicae bred on chinese cabbage (Brassica chinensis cv. Pak Choy), were fasted for 90 min and then allowed to probe for 5 min on AMV infected N. clevelandii. Groups of five aphids were then placed on each seedling of Medicago sativa (cv. Hunter River). After 16-24 hr, the plants were sprayed with Metasystox to kill the aphids.

E. Plant inoculation and virus propagation

Plants were grown and maintained in an insect-proof glasshouse (except where otherwise stated) with natural illumination and an average temperature of 25°C. N. clevelandii was used for virus propagation. Plants dusted with carborundum (500 mesh) and inoculated mechanically by rubbing extracts of infected tissue in 10 mM phosphate buffer, pH 7.0. The excess inoculum was washed off with tap water.

F. Infectivity assays

Infectivity of AMV-RNAs and added coat proteins were assayed on half-leaves of French bean plants (P. vulgaris cv. Hawkesbury wonder) which had been previously kept in the dark for 48 hr. Mixtures of RNA and coat protein in 10 mM Tris buffer, pH 8.3 containing 1 mM EDTA were incubated for 30 min at room temperature before inoculation.

G. Virus purification

AMV was purified at 4°C from N. clevelandii plants that had been inoculated for 10-14 days. Leaves with prominent symptoms were ground in 0.1 M phosphate buffer, pH 7.0, containing 0.1 M ascorbic acid and 20 mM EDTA (1g leaf material/ml buffer). The slurry was emulsified with a 1:1 (v/v) mixture of chloroform and n-butanol (1 ml/gm leaf tissue) for 1 min (Van Vloten-Doting and Jaspars, 1972). The emulsion was centrifuged at 4,000g for 10 min and the buffer layer was recovered. After addition of polyethylene glycol (PEG 6000 MW) to a final concentration of 1.5% (w/v), the mixture was stirred for 1 hr and then centrifuged for 3 hr at 65,000g. (Addition of PEG 6000 was found to increase the efficiency of virus resuspension from the pellets after centrifugation). The pelleted virus was resuspended in 10 mM phosphate buffer, pH 7.0, containing 2% Triton X-100. After a further three cycles of differential centrifugation (10 min at 12,000g to clarify and 3 hr at 65,000g to sediment the virus), the partially purified virus was resuspended in 10 mM phosphate buffer, pH 7.0.

To purify the virus further, preparations were centrifuged in 6-30% (w/v) sucrose density-gradient columns in 10 mM phosphate buffer, pH 7.0, for 3.5 hr at 24,500 rpm in a Beckman SW 28 rotor. Fractions containing the virus were recovered with an ISCO fractionator and dialysed overnight against 10 mM phosphate buffer, pH 7.0. The virus was concentrated by centrifugation for 3hr at 65,000g and resuspended in 10 mM phosphate buffer, pH 7.0.

H. Preparation of virus labelled with ^{35}S

This was done essentially as described by Francki (1968). N. clevelandii seedlings inoculated 4-5 days previously, were removed from the pots and their roots were washed

free of soil. Six to eight small plants were placed with their roots in 100 ml beakers and the roots were moistened with 0.5-1 ml of water containing 3-5 mCi ^{35}[S]-sulphate in aqueous solution, pH 6-8 (Amersham, code SJS.1). The plants were prevented from wilting by adding minimal amounts of water to the roots for about 12 hr and were then immersed in about 30ml of water and maintained at 25°C under artificial light for 4-5 days. The radioactive virus was isolated by the method described above except that it was sedimented by ultracentrifugation at either 400,000g for 30 min or 144,000g for 90 min. The virus was further purified using either sucrose-density gradient centrifugation as described above or centrifugation through a cushion layer of 10% sucrose in 10 mM phosphate buffer, pH 7.0, at 400,000g for 30 min. The purified virus was kept in PEN buffer (10 mM NaH_2PO_4, 1mM EDTA, pH 7.0, and 1mM sodium azide) (Van Vloten-Doting and Jaspars, 1972) at 4°C. The purity of cushion purified virus preparations was further tested by sucrose density-gradient centrifugation which showed that about 97% of the [^{35}S] was in the fractions containing virus. This was determined by monitoring absorption at 254 nm, assaying the fractions for radioactivity and DAS-ELISA.

I. Fractionation of AMV nucleoproteins

From partially purified virus preparations the top a nucleoprotein component was separated as described by Van Vloten-Doting and Jaspars, (1972). Virus preparations were mixed at 0°C with an equal volume of 10 mM NaH_2PO_4 containing 60 mM $MgSO_4$ adjusted to pH 7.0, with NaOH. The solution became turbid and after 2 hr was layered over 10% sucrose dissolved in 10 mM phosphate buffer, pH 7.0, containing 30 mM $MgSO_4$ and centrifuged for 30 min at 35,000 g. Under these conditions the nucleoprotein components containing RNAs 1, 2, and 3 were pelleted whereas most of the nucleoprotein containing RNA 4 remained in the supernatant. The pellets were resuspended in 10 mM phosphate buffer, pH 7.0, and dialysed against the same buffer overnight at 4°C. The supernatant which contained most of the top a nucleoprotein was also dialysed against 10 mM phosphate buffer, pH 7.0, pelleted by centrifugation for 90 min at 144,000g and dissolved in the same buffer.

J. The stabilisation of AMV capsids by glutaraldehyde

To the virus preparation of each virus isolate, glutaraldehyde was added to a final concentration of 0.25% and dialysed against 10 mM phosphate buffer, pH 7.0, containing 0.25% glutaraldehyde at 4°C overnight (Francki et al., 1980). To remove the free glutaraldehyde, the preparation was then dialysed against 10 mM phosphate buffer, pH 7.0, without glutaraldehyde for 24 hr at 4°C. The buffer was changed at least three times. The preparation was clarified by low speed centrifugation at 12,000g for 10 min and the concentration of virus was measured spectrophotometrically. The virus preparation was stored at 4°C until required.

K. Agarose gel electrophoresis of glutaraldehyde-fixed and native virus preparations

Agarose at 1.5% (w/v) in 10 mM phosphate buffer, pH 7.0, was sterilized by autoclaving at 121°C for 15 min . The electrophoresis was done in a horizontal slab gel apparatus in 10 mM phosphate buffer, pH 7.0. To each sample, bromophenol blue [0.1% (w/v) in 50% (v/v) glycerol] was added to a final concentration of 10% (v/v). Electrophoresis was done at 4°C for 2-3 hr. The gel was stained first with ethidium bromide and after visualization of the RNA bands, it was stained to detect protein with Coomassie blue stain (0.1 g Coomassie blue R dissolved in 1.0 ml methanol and made up with 25% (v/v) isopropanol/10% (v/v) acetic acid to the final volume of 200 ml) for 20 min and the gel was then destained in 10% (v/v) isopropanol/10% (v/v) acetic acid.

L. Determination of nucleoprotein particle ratios

1. Analytical centrifugation

Partially purified preparations of each AMV isolate in 10 mM phosphate buffer, pH 7.0, was used. Each virus preparation at a concentration of 4 mg/ml was subjected to centrifugation at 33,400 rpm in an An-D rotor of a Model E analytical ultracentrifuge equipped with Schlieren optics.

2. Sucrose density-gradient centrifugation

A partially purified virus preparation of each AMV isolate was layered on top of a linear density-gradient containing 5-30% (w/v) sucrose in 10 mM phosphate buffer, pH 7.0, (Van Vloten-Doting and Jaspars, 1972) and centrifuged at 35,000 rpm in a Beckman Spinco SW 41 rotor for 2 hr. The tubes were then monitored with an ISCO density-gradient absorbance reader coupled to a fractionator.

M. Stability of nucleoproteins of different AMV isolates

Partially purified virus preparations of each AMV isolate, propagated and purified under the same conditions, were exposed to various buffers and the preparations were incubated at different temperatures for specified lengths of time. The effects of these conditions on the stability of different isolates was assessed by sucrose density-gradient centrifugation using a linear gradient of 5-30% (w/v) sucrose in the same buffer in which the virus was incubated. Centrifugation was for 2 hr at 35,000 rpm in a Beckman SW 41 rotor and the content of each tube was monitored with an ISCO apparatus.

To test the effect of temperature on comparative stability of the isolates, a preparation of each virus isolate in 10 mM phosphate buffer, pH 7.0, was divided into aliquots. One aliquot was incubated at 4°C and the other at 25°C for 16 hr. To test the comparative stability of the isolates during storage, the preparation of each isolate in PEN buffer was stored at 4°C for a period of four months. For testing the effect of pH, 10 mM phosphate buffer adjusted with NaOH to pH 6.5 and 7.0 and carbonate buffer, pH 9.6, (Clark and Adams, 1977) were used. One volume of virus preparation of each isolate in 10 mM phosphate buffer, pH 7.0, was mixed with nine volumes of each of the test buffers and incubated at 25°C for 16 hr.

N. Isolation and characterisation of AMV coat protein

1. Isolation of coat protein

A modified method of Gonsalves and Garnsey, (1975a) as described in Appendix **1** was used. Highly or partially purified virus preparations were dialysed against 1 M $CaCl_2$ for 48 hr at 4°C. The precipitated RNA was removed by centrifugation at 12,000g for 10

min and the supernatant was subjected to centrifugation at 400,000g for 30 min to remove any intact or only partially degraded virus particles. The uppermost 3/4 of the supernatant was aspirated from the tubes and dialysed for 48 hr at 4°C against 0.1 M $CaCl_2$ and 10 mM sodium acetate, pH 6.0, with three changes of the buffer. The preparation was again centrifuged at 400,000 g for 30 min to remove insoluble material. Ultraviolet spectra of protein preparations from all five AMV isolates tested had 280/260 nm ratios above 1.45 and low absorbance above 300 nm indicating that the preparations contained soluble protein essentially free of RNA. However, reducing the $CaCl_2$ concentration below 0.1 M resulted in precipitation of the protein (see Appendix **1**).

Coat protein was also isolated by the method of Kelly and Kaesberg, (1962). An equal volume of 2M NaCl was mixed with a viral preparation and incubated at 45°C for 1 hr and after the centrifugation (12,000g for 10 min), the pellet was dissolved in 10 mM phosphate buffer, pH 7.0, containing 50 mM SDS and dialysed at 4°C against distilled water for 16 hr. The protein was precipitated by the addition of 0.66 volume of saturated ammonium sulphate solution. The precipitated protein after centrifugation at 12,000g for 10 min was resuspended in 10 mM phosphate buffer containing 50 mM SDS and dialysed against 10 mM phosphate buffer, pH 7.0 containing 5 mM SDS. Ultraviolet spectra of the protein preparation, after a final cycle of centrifugation, showed a ratio of 280/260 of 1.1. This protein was used for only one set of experiments, as it was only soluble in the presence of SDS, which is known to have a diminishing effect on antigen and antibody binding (Halfman et al.,1986).

2. SDS-polyacrylamide gel electrophoresis

The discontinuous buffer system of Laemmeli (1970) with 12% polyacrylamide resolving gel and 5% stacking gel was used. To prepare 20 ml of the 12% gel, 6 ml of stock acrylamide solution [30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide in double distilled water (DDW)], 5 ml of stock gel buffer solution, [18.17g Tris-base, 4 ml of 10% (w/v) SDS, DDW to 100 ml and 6N HCl for adjusting the pH to 8.8] and 7 ml of DDW were mixed and 10 µl of TEMED and 60 µl of a freshly prepared solution of 10% (w/v) ammonium persulphate was added. The mixture was added to the apparatus leaving 1 cm

from the top empty. A flat surface was ensured by overlaying with 1 ml water saturated n-butanol and the gel was allowed to polymerise. After polymerisation, the n-butanol layer was poured off and the gel washed with DDW. The stacking gel solution was prepared by mixing 1.15 ml acrylamide stock solution, 1.7 ml of stock gel buffer solution [6.06 g Tris-base, 4 ml of 10% SDS (w/v), DDW to 100 ml and pH adjusted to 6.8 with 6N HCl] and 3.85 ml of DDW. After addition of 10 µl of TEMED and 40 µl of 10% (w/v) ammonium persulphate the mixture was poured on top of the resolving gel and the combs were inserted. AMV preparations or protein preparations from which salts had been removed by microdialysis (Overall, 1987) against 50 mM Tris-HCl, pH 6.8, containing 0.5% (w/v) SDS, were heated at 95°C for 5 min in sample buffer (Laemmeli, 1970) containing 62.5 mM Tris-HCl, pH 6.8, 3 % (w/v) SDS, 5% (w/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.01% (w/v) bromophenol blue. For electrophoresis under non-reduced condition the samples were prepared in the same sample buffer but without the 2-mercaptoethanol and the samples were not heated.

3. Silver staining of the SDS-polyacrylamide gels for protein

This was done as described by Wray et al , (1981). Following the electrophoresis, the gel was soaked in 50% (v/v) methanol in DDW and left on a rocking platform overnight. This solution was changed at least three times. Silver nitrate (0.8 gm) dissolved in 4 ml of DDW was added dropwise to a 21 ml solution of 0.36% NaOH containing 1.4 ml of fresh 14.8 M ammonium hydroxide, and DDW was added to 100 ml. The methanol washed gel was quickly rinsed twice in glass-distilled water (1-2 min) and overlaid with the silver solution. The gel remained in this solution under constant agitation for 15 min, followed by two washes in DDW (5 min each). The gel was then developed in a solution prepared by mixing 2.5 ml of 1% (w/v) citric acid and 0.25 ml of 38% (w/v) formaldehyde in 500 ml of DDW with gentle agitation, washed with distilled water (DW) several times and incubated in Ilford rapid fix solution (1: 5 dilution with water). After the removal of background, the gel was rinsed several times with DW and incubated in hypoclearing agent (at the strength recommended by the manufacturer) for 30 min with agitation. After rinsing with distilled water, the gel was transferred to a solution of 10%

(v/v) methanol, 5% (v/v) glycerol in DDW and shaken for 2 hr. Finally it was photographed or dried.

O. Isolation of RNA

1. Preparation of RNase-free glassware and solutions

All solutions and heat resistant equipment used for ribonucleic acid handling were autoclaved at 121°C for 15 min. Heat sensitive equipment was washed with 0.2 M KOH dissolved in 90% (v/v) ethanol and rinsed thoroughly with autoclaved distilled water.

2. Isolation of total RNA from plant tissues

This was done as described by Langridge and Brooker, (1987). Leaf-tissue from N. clevelandii, (2g) was ground with a pestle and mortar in liquid nitrogen to a fine powder. The powder was then mixed in 40 ml extraction buffer [(50 mM Tris-HCl, pH 8.5, containing 10 mM $MgCl_2$, 30 mM KCl, 5 mM EDTA, 20% (w/v) sucrose to which 0.5% (w/v) iodoacetic acid was added just prior to extraction)]. The mixture was centrifuged to a speed of 4080g and stopped immediately. The supernatant was poured into a tube containing a 40 ml water-saturated phenol/chloroform (1:1 v/v) mixture to which 4 ml of 10% (w/v) SDS was added. After 1 min of vortexing it was subjected to centrifugation at 8000g for 5 min. The upper phase was recovered and re-extracted twice with 40 ml of the phenol/chloroform (1:1 v/v) mixture. Finally, the supernatant was recovered and mixed with 4 ml 3 M sodium acetate, pH 4.8, and precipitated with 80 ml of ethanol at -20°C overnight. The precipitate was recovered by centrifugation at 12,000g, washed twice with ethanol, freeze-dried, dissolved in TE buffer and stored at -20°C until required.

3. Isolation of viral RNA

RNA of each isolate was extracted as described by Peden and Symons (1973) either from the total or fractionated nucleoproteins. An equal volume of water saturated phenol containing 0.1% (w/v) 8-hydroxyquinoline and buffer containing 0.6 M sodium acetate, 0.6% (w/v) SDS, 20 mM EDTA, pH 7.6, was added to the virus preparation. The mixture was shaken continuously at 25°C for 1 hr and then the aqueous phase was separated by centrifugation at 3,000g for 10 min. To the buffer phase, one-half volume of

the water saturated phenol was added and the mixture was shaken at 25°C for a further 30 min and centrifuged as described above. The buffer phase was then precipitated with 2.5 volume of cold-redistilled ethanol at -20°C overnight. The precipitate was sedimented at 5,000g for 10 min. The pellet was washed twice in ethanol and freeze-dried. The precipitate was suspended in TE buffer and stored at -20°C until required.

4. Agarose gel electrophoresis of RNA

a. Preparative gel electrophoresis

This was done as described by Rao and Francki, (1982). RNA was extracted from nucleoprotein fractions enriched in M, B and top a components as described in section 9 of this chapter. Agarose (1.5%) in TAE was autoclaved and cast in 15x1 cm glass tubes to a depth of approximately 13 cm. Before pouring the gel into the tube, a piece of sterilized dialysis tubing was stretched tightly over the end of each tube, secured with a rubber band and autoclaved for 15 min at 121°C. After setting, the top of each gel was trimmed with a sterilized razor to produce a flat loading surface and to maintain a uniform gel length. The tubes were pre-run at 0.03 mA/tube for at least 30 min before loading, using 1x TAE as electrophoresis buffer. RNA samples were prepared in TE and after addition of bromophenol blue in 50% (v/v) glycerol to a final concentration of 10%, were heated at 60°C for 5 min . The samples were immediately cooled on ice and samples of 100 µg of RNA were loaded on each tube. The RNA preparations were allowed to enter the gel by initial electrophoresis at 0.03 mA/tube for at least 30 min and then at 12 mA/tube for 3 - 4 hr. To minimize the change in pH during electrophoresis, the buffer was exchanged at intervals between the reservoirs with a sterilized syringe manually. After electrophoresis, the gels were stained in toluidine blue 0 for 30-60 sec (0.05% (w/v) toluidine blue 0 prepared in 0.02M sodium acetate, pH 7.8) and destained by two changes of TAE and several changes of autoclaved DDW until the bands appeared. The bands were observed on a light box and the desired fractions were cut with a sterile scalpel blade and transferred to a tissue grinder tube. The RNA extraction buffer was added and the tubes were stored at -20°C overnight. After homogenization, an equal volume of phenol was added, shaken for 30 min and subjected to low speed centrifugation (3,000 g for 10 min). The phenol

extraction was repeated, and the RNA recovered by ethanol precipitation and was further washed twice. The isolated RNA components were freeze-dried, suspended in TE buffer and stored at -20°C until required.

b. Analytical gel electrophoresis

The electrophoresis was done in a horizontal gel apparatus under non-denaturing conditions (Francki et al., 1986) using 1.5% agarose. RNA samples were prepared as described above and preparations containing 1-2 µg of RNA loaded in each well. The electrophoresis was done at 100V for 2-3 hr. Before terminating the run, 10-20 µl of ethidium bromide [1% (w/v) in sterile double distilled water (SDDW)] was added to the electrophoresis buffer and after a further 30 min electrophoresis, the gel was observed by UV transillumination and was photographed with a Polaroid camera.

P. Nucleic acid hybridization analysis of viral RNAs

1. Synthesis and purification of ^{32}P-labelled DNA complementary (cDNA) to viral RNA

RNA from each virus isolate was reverse transcribed into cDNA by the random primer method of Taylor et al , (1976) using reverse transcriptase as detailed by Palukaitis and Symons, (1980, and references therein). Briefly, 2-3 µg of purified RNA was used as template in a reaction mixture containing 2-3 µl (1mg/ml) of primer, 5µl of buffered salt solution (200 mM Tris-HCl, pH 8.3, 700 mM KCl, 100 mM $MgCl_2$), 5 µl of 200 mM dithiothreitol, 3 µl of deoxynucleotide triphosphates (TTP, ATP, GTP, 8.3mM each), 2-3 µl reverse transcriptase, (200 units/µl), 2-3 µl (20-30 µCi) α-^{32}P labelled dCTP (1-1.5 µM), and SDDW was added to a total volume of 45 µl and finally 5 µl of 40 µM sodium pyrophosphate was added (care was taken that both enzyme and pyrophosphate were added simultaneously to the reaction). The mixture was vortexed briefly and incubated at 42°C for 1.5-2 hr. The reaction was stopped by the addition of 5µl of 5% (w/v) SDS, 5 µl of 0.4M EDTA, 15µl of 4M NaOH and 125 µl of SDDW. After gentle vortexing it was incubated overnight at room temperature.

The cDNA was purified under sterile conditions by Sephadex G-50 filtration. Autoclaved sephadex G-50 in 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA was packed into a 5ml pipette. The column was washed with a freshly prepared, sterile 0.1 M ammonium bicarbonate solution. The cDNA was loaded and eluted with the bicarbonate solution. A total of twenty fractions were collected, the first 1.0 ml and subsequently 0.5 ml samples. The fractions were assayed by Cerenkov counting. The cDNA peak fractions were retained and to each tube triethylamine was added to 10% (v/v). The fractions were pooled and freeze-dried. The dried cDNA was resuspended in 0.5-1.0 ml of SDDW and an aliquot used for determination of radioactivity by scintillation counting. The prepared cDNA was stored at -20^{0}C until required.

2. RNA blotting procedures

a. Dot - blots

Dot-blotting was done essentially as described by Palukaitis et al., (1985). Nitrocellulose membranes were soaked in SDDW and then in 20xSSC (20 xSSC = 175.3g NaCl, 93.3g Na_3-Citrate, 0.2 ml of 0.2 N HCl per litre). It was placed over two layers of Whatman 3 MM paper (pre-soaked in 20xSSC) on a Schleicher and Schuell " Minifold" filtration apparatus. Bromophenol blue dye was used in the sample to monitor the application of samples to the nitrocellulose sheet. After loading, the nitrocellulose membrane was removed and transferred between a pair of dry 3 MM Whatman paper sheet and baked in a vacuum oven at 85^{0}C for 2 hr and placed into a plastic bag.

b. Northern blots

This was done as described by Palukaitis et al. , (1983). After visualising the RNA gel in ultraviolet light, the gel was trimmed to the right size and placed between two sheets of pre-soaked (in SDDW and then in 20xSSC) nitrocellulose membranes with three sheets of pre-soaked (SDDW and 20xSSC) Whatman 3 MM chromatography paper above and below the gel. This sandwich was placed between a stack of paper towels and two glass sheets with a weight on top. After bidirectional blotting overnight the membrane was baked and processed as above for dot-blotting.

3. RNA-cDNA hybridization procedures

This was done essentially as described by Palukaitis et al., (1985) using Maule's (Maule et al., 1983) buffer [3x SSC, 0.08% (w/v) bovine serum albumin, 0.08% (w/v) Ficoll, 0.08% (w/v) polyvinyl-pyrrolidine (PVP), 1 mM EDTA and 250 µg/ml of phenol extracted yeast RNA] as pre-hybridization and hybridization buffers. For pre-hybridization, 5 ml of buffer/130 cm^2 of nitrocellulose sheet in a plastic bag was used, all air bubbles were removed and the plastic was sealed and immersed in a water bath with constant shaking for 24 hr at 42°C.

For hybridization, the cDNA was added to the plastic bag (50,000-200,000 cpm/ml of buffer) and after removal of air bubbles, the bag was resealed and hybridization was allowed to take place at 60-65°C in a water bath with constant shaking. After 24 hr the nitrocellulose membrane was washed six times for 5 min (2x at room temperature and 2x at 55°C) in 2xSSC containing 0.5% SDS and twice for 15 min with 0.1xSSC containing 0.5% SDS at 55°C (Palukaitis et al. 1983). The membrane was then placed between two sheets of Gladwrap, all excess liquid and bubbles were removed and the blots were exposed to X-ray film at -70°C, using an intensifying screen.

Q. Serological techniques

1. Serological assays

a. Gel immunodiffusion tests

The tests were done in 90 mm petri dishes containing 15 ml of 0.75% agar or agarose in 10 mM phosphate buffer, pH 7.6, with 0.02% sodium azide when native or glutaraldehyde-fixed virus preparations were used as test antigens. In experiments using the coat protein preparations as test antigens, the agarose gel was prepared in 10 mM sodium acetate, pH 6.0, containing 100 mM $CaCl_2$. Holes 3 mm in diameter were removed from the agar plates and each was filled with 15 µl of either antiserum or antigen. Native or glutaraldehyde-fixed virus was diluted in 10 mM phosphate buffer, pH 7.0, and coat protein preparations in 10 mM Na-acetate, pH 6.0, containing 100 mM $CaCl_2$. Antisera were always diluted in the same buffer in which the gel had been prepared.

b. Enzyme -inked immunosorbent assay (ELISA)

i. Variations of ELISA

Different variations of ELISA used in this thesis are summarised in Fig.2-1. ELISA formats 1 and 2 are based on the double antibody sandwich-enzyme linked immuno-sorbent assay (DAS-ELISA), (Clark and Adams,1977) and were used for serological studies involving glutaraldehyde-fixed AMV particles or dissociated coat protein as test antigens with polyclonal antibodies. ELISA formats 3, 4, 5 and 6 are based on indirect ELISA (Jaegle and Van Regenmortel, 1985). Formats 3, 4 and 5 were used for serological comparisons of AMV isolates using polyclonal antibodies raised in rabbits. Formats 6, 7 and 8 which are modified forms of indirect ELISA (Van Regenmortel and Buckard, 1980), were used for titration of mouse polyclonal antibodies, screening of hybridoma culture supernatants and determination of reactivity of monoclonal antibodies (McAbs). ELISA format 9 is an indirect ELISA and was used for subclass determination of McAbs (Hammerling and Hammerling, 1981) and for determining the immunoglobulin concentration in ammonium sulphate precipitated hybridoma supernatants (R.Fisher, private communication). ELISA formats 10, 11 and 12 are biotin-avidin systems (Zrein et al., 1986) which were used for comparisons of sensitivity of detecting viral antigens with polyclonal and McAb antibodies.

ii. Conjugation of γ-globulin with enzymes

(1) Conjugation with alkaline phosphatase

The conjugation was performed as described by Clark and Adams, (1977) and unless otherwise stated, the enzyme and γ-globulin were mixed in a ratio 5:2 (w/w), respectively. The enzyme solution was centrifuged at 12,000g for 10 min and the pellet dissolved directly in the γ-globulin. The mixture was dialysed against three changes of PBS at 4^0C for 16 hr, glutaraldehyde solution was added to a final concentration of 0.06%, and the mixture incubated for 4-6 hr at room temperature. Free glutaraldehyde was removed by dialysis against PBS at 4^0C overnight and bovine serum albumin and sodium

Fig. 2-1

Different formats of ELISA used in this thesis. The abbreviations are as follows :

AB^R = rabbit antibodies

AG^N = Native AMV

AB^M = Mouse antibodies

AG^F = Glutaraldehyde-fixed AMV

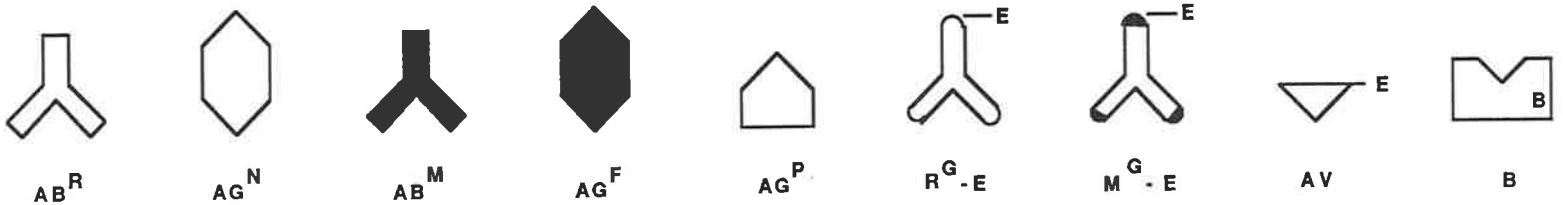
AG^P = Dissociated coat protein of AMV

R^G- E = Goat anti-rabbit conjugated with alkaline phosphatase

M^G-E = Goat anti-mouse conjugated with alkaline phosphatase

AV = Streptavidine conjugated with alkaline phosphatase

B= Biotin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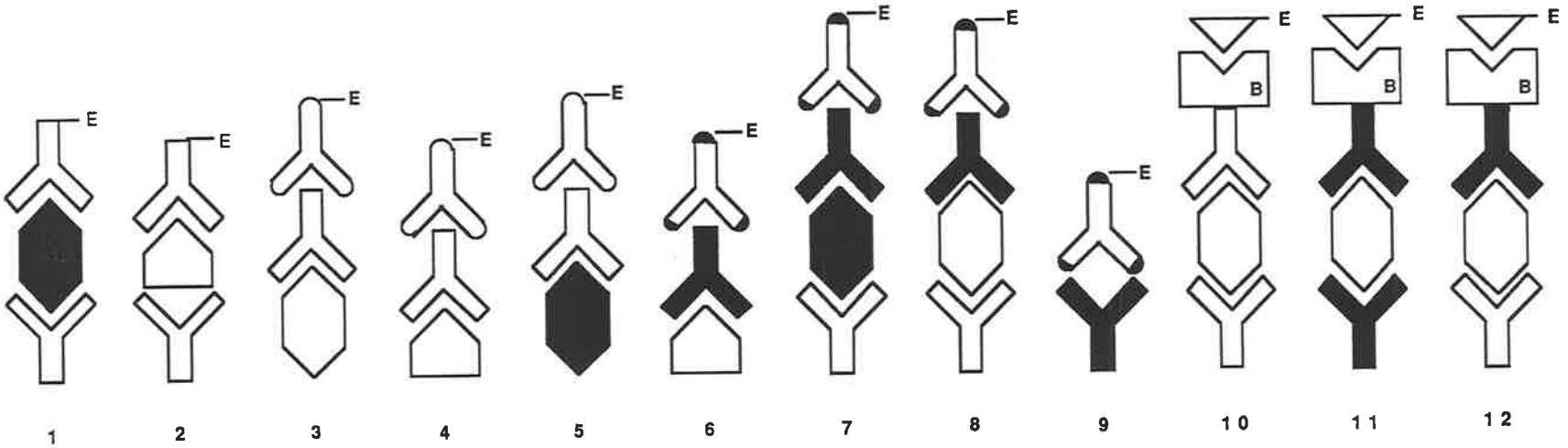
AB R
AG N
AB M
AG F
AG P
R G - E
M G - E
AV
B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azide were added to final concentrations of 5 mg/ml and 0.02% (w/v), respectively. The conjugated antibodies were stored at 4°C.

(2) Conjugation with biotin

This was done as described by Diaco et al., (1985) but with slight modification. Purified γ-globulin at a concentration greater than 1 mg/ml was dialysed overnight against 0.1 M $NaHCO_3$, pH 8.0, at 4°C. After dialysis, the concentration of the antibody was adjusted to 1 mg/ml. Immediately before use 1.65 mg of NHS-LC-biotin was dissolved in 1 ml of dimethyl sulphoxide from which about 50 µl (approximately 145 n mol) was added to the preparation of 1 ml antibody (1mg). After 1 hr incubation at 25°C it was dialysed at 4°C against 0.1 M $NaHCO_3$, pH 8.0, for 48 hr with four changes of fresh buffer. Finally, NaN_3 was added to a final concentration of 0.05% (w/v) and the conjugate was stored at 4°C. Alkaline phosphatase conjugated streptavidin was reconstituted as recommended by the manufacturer by dissolving the contents of one lyophylized bottle (1mg) in 2 ml of distilled water. After addition of 50% (v/v) glycerol it was stored at 4°C until required.

iii. Buffers , incubation times and conditions for various steps in ELISA

Buffers were prepared according to Clark and Adams, (1977). PBS-Tween, pH 9.6 (8.0 gm NaCl, 0.2 gm KH_2PO_4, 1.15 gm Na_2HPO_4, 0.2 gm KCl, 0.5 ml Tween-20 and DW to 1 litre) was used as washing buffer. For coating of antibody to the plate, carbonate buffer, pH 9.6 (1.59gm Na_2CO_3, 2.93gm $NaHCO_3$, 0.2gm NaN_3 per litre of DW) was used and the plates incubated at 25°C for 3 hr. Antibodies used in other steps were always diluted in sample buffer [PBS-Tween, pH 6.0 containing 2% (w/v) PVP, 0.02% (w/v) NaN_3] and the plates incubated for 16 h at 4°C or in a humid atmosphere of 5% CO_2 at 37°C when polyclonal or monoclonal antibodies were used (Dietzgen and Sander, 1982).

For coating of native or glutaraldehyde-fixed virus particles and dissociated coat protein preparations to the microtitre plates, 10 mM phosphate buffer, pH 7.0, or carbonate buffer, pH 9.6, and 10 mM Na-acetate, pH 6.0, containing 0.1 M $CaCl_2$ were used, respectively, and plates were incubated for 3 hr at 25°C. Preparations of native and

glutaraldehyde-fixed virus were diluted in 10 mM phosphate buffer, pH 7.0, containing 2% (w/v) PVP, 1% (w/v) BSA and 0.05% (v/v) Tween-20 and dissociated coat protein was diluted in 10 mM Na-acetate, pH 6.0, containing 0.1 M $CaCl_2$, 2% (w/v) PVP, 1% (w/v) BSA and 0.05% (v/v) Tween-20, when they were added to the antibody precoated plates. Antigens in the DAS-ELISA and biotin-avidin systems were incubated at 4°C overnight and in other formats of ELISA for 3 hr at 25°C. The alkaline phosphatase conjugated antibodies, biotinylated antibodies and the alkaline phosphatase conjugated streptavidin were diluted in conjugate buffer [(PBS -Tween, 2% (w/v) PVP, 0.5% (w/v) BSA, pH 7.4 , 0.02% (w/v) NaN_3)] and the plates were incubated at 25°C for 3, 2 and 2 hr, respectively. Substrates prepared in substrate buffer (97 ml diethanolamine, 0.02% (w/v) NaN_3, pH adjusted to 9.8 with HCl and DW to 1 litre). After coating the plates with either antigens or antibodies (except for DAS-ELISA) any remaining free sites were blocked by the addition of 0.1M NaCl containing 1% (w/v) BSA, followed by incubation at 25°C for 1 hr. After each step, the plates were washed three times for 3 min each time.

iv. Determination of the optimal parameters for ELISA

For the optimisation of ELISA formats 1 and 2 which are based on DAS-ELISA (Fig.2. 1) the parameters which gave an ELISA dilution response curve (Clark and Barbara, 1987) were chosen. To obtain such parameters, different concentrations of coating antibody were used to trap eight series of two-fold dilutions of homologous antigens . Enzyme conjugated antibodies diluted 1:1000 and 1:500 were used and after the addition of substrate (1mg/ml), the optical densities at 405 nm were measured. If the background after 2 hr hydrolysis exceeded optical densities of 0.05 the level of conjugated antibody was reduced.

The parameters of the ELISA formats 3, 4 and 5 (Fig.2-1) were adjusted as described by Jaegle and Van Regenmortel, (1985). Four different concentrations 0.125, 0.25, 0.5 and 1µg/ml of native or glutaraldehyde-fixed virus and 100, 50, 25 and 12.5 ng/ml of isolated coat protein preparations of the homologous isolates were adsorbed directly to the ELISA plates in the appropriate buffer. Each antiserum was diluted $1:10^3$, $1:5 \times 10^3$ and $1:10^4$ in sample buffer of Clark and Adams, (1977). For each dilution, a

series of eight two-fold dilutions were prepared in the same buffer and added to the microtitre wells. To each concentration of antigen, different dilutions of antiserum were added. Anti-globulin enzyme conjugate was always used as recommended by the manufacturer. After addition of substrate, which was always used at a concentration of 1mg/ml, the optical density was measured. The results of ELISA were evaluated and a dilution of antiserum which gave a rapidly growing optical density curve with no trace of a plateau (Jeagle and van Regenmortel, 1985) was chosen. If the minimum optical density at highest dilution of antiserum exceeded 0.05, then the concentration of test antigen was further optimised.

The concentrations of coating antibodies in ELISA formats 7 and 8 (Fig.2-1) were the same as for DAS-ELISA and only one concentration of antigen or antibodies was used through these tests and anti-mouse conjugated immunoglobulin was used as recommended by the manufacturer. In ELISA formats 10, 11 and 12 (Fig.2-1) the same concentrations of polyclonal antibodies as in DAS-ELISA were used . A suitable concentration of biotinylated antibody was obtained by evaluating different dilutions of these antibodies. The alkaline phosphatase conjugated streptavidin was used as recommended by the manufacturer.

v. Determination of binding efficiency of viral antigens to the ELISA plates

Native or glutaraldehyde-fixed [^{35}S] labelled virus preparations were used in these experiments. Samples of antigen with known concentration were applied either to antibody coated or uncoated ELISA wells which had been cut into single rows with a band saw. After the required incubation time, each well was washed three times (each 3 min) with washing buffer, and air dried. For each experiment a known concentration of labelled antigen was dispensed into ELISA wells and dried under an infra red lamp. Each well was excised with a hot scalpel, placed in a tube and filled with 200 µl of Soluene-350 (addition of Soluene-350 was found to increase the removal of adsorbed antigen from the microtitre well). The tubes were incubated for 2-3 hr at room temperature before adding 3 ml of scintillation liquid [4% (w/v) 2,5 diphenyloxazole (PPO) in toluene]. The specific radioactivity of each well was determined by scintillation spectrophotometry.

c. Western immunoblotting.

i. Electrophoretic transfer of protein from the gel to nitrocellulose

The transfer buffer was prepared as described by Towbin et al , (1979), except instead of methanol, ethanol was incorporated [192 mM glycine, 25 mM Trizma-base, 20% (v/v) ethanol in DW]. After electrophoresis, the polyacrylamide gels were trimmed to remove the stacking gel, marker dye and any other excess material. The trimmed gel was then immersed in cold transfer buffer for 10 min. Two pieces of Whatman 3MM and nitrocellulose membrane of the same size as the gel were cut , soaked in transfer buffer together with one pair of Scotch-brites. While everything was kept wet, the sandwich for electrotransfer was prepared as follows. One Scotch brite-pad was placed on a plastic support frame (cathode) and a piece of pre-soaked Whatman 3 MM filter paper was overlaid on it and gently rubbed with gloved hands. The gel was placed on top of the paper and rubbed gently to remove all air bubbles. Nitrocellulose was placed on the gel uniformly and rubbed with a circular motion after addition of buffer, to establish a weak electrostatic interaction. This was better achieved by rolling a 5 ml pipette over the nitrocellulose to remove excess buffer between the nitrocellulose and gel (R.G.Dietzgen, personal communication). The second piece of Whatman filter paper was placed on top and air bubbles were removed. Finally, another Scotch-brite pad was added and the sandwich was clamped tightly and carefully in order to avoid sliding of the assembly. The sandwich was placed slowly in a transblot chamber with a stir bar at the bottom of the tank. After addition of transfer buffer, the electroblotting was allowed to proceed at constant voltage of 100V for 70 min with a Mini Transblot or 4 hr with a Transblot TM Cell.

ii. Immunoblotting

After electroblotting, the nitrocellulose was removed from the assembly. The evaluation of successful transfer was made based on the presence of prestained marker protein on the nitrocellulose. It was quickly air dried and incubated in PBS for 16 hr at 37^{0}C to remove SDS and renature the proteins (Birk, et al , 1987). After a brief wash in rinse buffer [1 mM Tris-HCl, pH 7.4, 15 mM NaCl, 0.1 mM EDTA containing 0.01%

(v/v) Triton x-100], the residual binding sites on the nitrocellulose were blocked by incubation in rinse buffer containing 3 % (w/v) BSA for 1-2 hr at room temperature on a shaking platform. The blot was transferred into a plastic bag, dilution of antibody preparation in sample buffer [rinse buffer containing 1% (w/v) BSA, 0.02% (w/v) sodium azide, 5 units/ml heparin] was added, air bubbles removed, sealed and agitated on a rocking platform for 1-2 hr at room temperature. [Heparin was included in the immunoblot sample buffer to avoid charge related non-specific reactions; (Dietzgen and Francki, 1987)]. Following exhaustive washing with four changes of rinse buffer-tween, each for 15 min, the membrane was incubated for 1-2 hr at room temperature in alkaline phosphatase conjugated affinity-purified goat anti-rabbit IgG (H+L) or goat anti-mouse IgG (H+L) diluted 1:2,000 in sample buffer. After washing in four changes of TTBS [2.5 mM Tris-HCl, pH 7.5, 15 mM NaCl, 0.01% (v/v) Triton x-100, 0.01 mM $MgCl_2$ and 4 x 10^{-8} M $ZnCl_2$] on a rocking platform at room temperature, the membrane was overlaid with 0.1 ml/cm of the substrate solution of Ey and Ashman, (1986). The substrate solution was prepared as follows : For every 0.33 mg of nitroblue tetrazolium in 1 ml of solution A (0.1 M Tris-Cl, 25 mM diethanolamine, 0.1 M NaCl, 2 mM $MgCl_2$ and $1x10^{-6}$ M $ZnCl_2$, pH 9.55), 6.7 µl of solution B (2 mg/ml of phenazine methosulphate in DW) and 3.4µl of solution C (40 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate in anhydrous dimethylformamide) were mixed by rapid shaking. After addition of substrate, the blot was incubated in the dark at 37°C until sufficient colour developed. The blot was then rinsed thoroughly with distilled water and dried. It was photographed and stored in the dark.

2. Production of polyclonal antibodies

a. Production of polyclonal antibodies against native and glutaraldehyde-fixed virus preparations in rabbits

Antisera to five of the AMV isolates, selected because of their differences in host reactions, were prepared in rabbits injected either with highly purified native or glutaraldehyde-fixed virus preparations. Each rabbit was initially injected intravenously with 250µg AMV, in 10 mM phosphate buffer, pH 7.0, and thereafter six more times;

after 1 (250µg, intravenously), 3 (500µg, subcutaneously), 6 (500µg, subcutaneously), 8 (500µg, intramuscularly), 15 (1mg, intramuscularly) and 21 weeks (500µg, intravenously). Virus was emulsified with an equal volume of Freund's complete adjuvant for each subcutaneous or intramuscular injection. The rabbits were bled through the ear vein at regular intervals. The blood was clotted after incubation for 2-3 hr at 37°C and left overnight at 4°C. The sera were recovered as supernatants after centrifugation at 2,000g for 10 min. They were titrated by immunodiffusion in agar gels against preparations of 1.0 mg/ml of homologous native or fixed virus and their corresponding isolated coat proteins.

b. Production of polyclonal antibodies against native and glutaraldehyde-fixed virus preparations in chickens

Antisera were prepared against two AMV-isolates S30 and S40, using highly purified native or glutaraldehyde-fixed virus preparations as immunogen. Each chicken was initially injected intravenously with 250 µg of AMV in 10 mM phosphate buffer, pH 7.0, and thereafter six more times by the following routes; intravenously (250µg), subcutaneously (500µg), intramuscularly (500µg), intramuscularly (1mg), intravenously (500µg), intravenously (500µg) after 1, 3, 7, 11, 13 and 15 weeks, respectively, (chickens injected with S40 antigens) and 1, 3, 9, 13, 15 and 17 weeks, respectively, (chickens injected with S30 antigen). Virus was emulsified with an equal volume of Freund's complete adjuvant for each subcutaneous or intramuscular injection. The chickens were bled through the veins of the wings, using a syringe and the sera were tested by immunodiffusion in agar against preparations of 1 mg/ml of homologous native or fixed virus.

c. Production of polyclonal antibodies against isolated coat protein

Antisera were produced in rabbits subjected to periodic immunization with AMV proteins of the S40 and S30 isolates of AMV. Each rabbit was immunized by an initial intravenous injection of 250µg viral protein and a further four injections of 250µg, 500, 500µg and 2mg of protein 1, 3, 6, and 9 weeks thereafter. All injections were intravenous except the third which was administered intramuscularly with the protein emulsified in

Freund's complete adjuvant. Rabbits injected with protein preparation of AMV-S30 received an additional intravenous injection of 2mg protein 11 weeks after commencement of immunization. The animals were bled as described before and each antiserum was titrated by gel-diffusion against 1mg/ml of native or glutaraldehyde-fixed homologous virus preparations, or against 1mg/ml of viral protein.

d Production of strain specific or group specific polyclonal antibodies

Antisera specific to an individual strain or a group of isolates was prepared by cross-absorption in tubes as described by Rao, (1982). The amount of antigen necessary to exhaust an antiserum were determined by intragel cross-absorption using the homologous system. Approximately 500µl of heterologous viral antigen was added to 0.5 ml of antiserum and the mixture was incubated at 25°C overnight. After centrifugation at 12,000 g for 10 min the unreacted antibodies were recovered in the supernatant.

The intragel cross-absorption was done as described by Von Wechmar and Van Regenmortel, (1968). Antiserum wells were pre-charged with the absorbing antigens 24 hr before recharging with the antisera (Previously the quantity necessary to exhaust the homologous antibodies were determined for each antiserum by cross-absorption of antisera with different concentrations of homologous antigen).

3. Production and characterization of Monoclonal antibodies (McAbs)

a. Immunization

Three female (2 months old) BALB/c mice were immunized with 200µg of a mixture of equal amounts of highly purified native virus preparation of five AMV isolates(H4, N20, S30, S40, W1) in 10 mM phosphate buffer, pH 7.0, or with 200µg of a mixture of isolated coat proteins of the same AMV isolates in 10 mM Na-acetate, pH 6.0, containing 100 mM $CaCl_2$ as described in Table 2-5.

For all intraperitoneal injections, the antigen was always mixed with an equal volume of Freund's complete adjuvant. Intravenous injections were administered 3-4 days before sacrificing each mouse. Each mouse was bled at different intervals having been pre-warmed by exposure to an infra red lamp for a few minutes. The blood was collected from

Table 2-5 : Immunization schedules of three BALB/c mice for production of monoclonal antibodies.

Time (days)	Route of injection	Immunogen[a]	Mouse A	Mouse B	Mouse C
1	Intraperitoneal[b]	Native virus	+[c]	+	+
23	Intraperitoneal	Native virus	+	+	+
37	Intraperitoneal	Native virus	+	+	+
53	Intravenous	Native virus	+	-[d]	-
63	Intraperitoneal	Native virus	-	+	+
80	Intravenous	Native virus	-	+	-
105	Intraperitoneal	Coat protein	-	-	+
115	Intraperitoneal	Coat protein	-	-	+
125	Intravenous	Coat protein	-	-	+

[a] Immunogen was either 200 µg of equal amounts of highly purified native virus preparations of five AMV isolates (H4, N20, S30, S40 and W1) in 10 mM phosphate buffer, pH 7.0, or 200 µg of a mixture of equal amounts of coat protein of the same isolates in 10 mM Na-acetate, pH 6.0, containing 100 mM $CaCl_2$.

[b] For all intraperitoneal injections, the same volume of immunogen was mixed with complete Freund's adjuvant before injection.

[c] Indicates the administration of the injection.

[d] Indicates the injection was not administered.

the tail vein and antisera titrated by two formats of indirect ELISA (Fig. 2-1, formats 6 and 8) but using mixtures of native virus preparations or dissociated coat proteins from the five AMV isolates as test antigens.

b. Preparation and storage of stock solutions and growth Media

Commercially available HT solution (50 x, containing 5000µM hypoxanthine and 800 µM thymidine) was stored at -20°C. A solution containing 62.5 mg penicillin G and 0.1gm streptomycin per ml of SDDW was dispensed in 1 ml aliquots and stored at -20°C. Glutamine stock solution was prepared by dissolving 14.3gm of L-glutamine in 500 ml of SDDW and dispensed in aliquots of 10 ml and stored at -20°C. Foetal calf serum (FCS) was always filter sterilized and stored at -20°C. L-broth agar was prepared by dissolving

10 gm tryptone, 5gm yeast extract and 10 gm NaCl in 1 litre of SDDW, and the pH was adjusted to 7.0 with 4 N NaOH and, after addition of 15gm agar, it was sterilized by autoclaving.

RPMI-1640 medium was prepared by dissolving 10.44 gm of RPMI-1640 and 2 gm of sodium bicarbonate in 800 ml of DDW and the pH was adjusted to 7.2-7.3 with HCl. To this, 10 ml stock solution of glutamine (final concentration 2 mM) and 1ml stock solution of streptomycin and penicillin (final concentrations of 100μ units and 100μg/ml, respectively) were added and the total medium was made up to 1 litre with DDW. Finally, FCS was added to 10 or 15 % (v/v) as required. This will be referred to as the basic medium here after. For the fusion, selection and cloning, the medium containing 15% FCS was used but after selection of hybridomas the concentration of FCS was decreased to 10-12%. The medium was initially filtered through three layers of filter paper. Using a master flex pump (model 7014-20, Laboratory Supply, Coler-Palmer Australia), the medium was then filtered through a millipore filter (Cat. no. Ap20-04700 type AP), then a millipore filter type HA , (0.45 μm) and finally through a sterile filter (Millipak filter - 0.22 μm, Millipore U.S.A.). Complete sterilization was achieved by this practice as when the sterility of each preparation was checked by culturing on L-broth agar medium or by direct incubation of an aliquot at 37°C no sign of bacterial growth was observed.

For preparation of HT media, to 1 litre of basic medium, 20 ml of HT stock solution was aseptically added. For preparation of HAT medium, to 1 litre of HT medium, 4 ml of aminopterin was aseptically added. All these media were stored at 4°C.

c. Fusion

i. Preparation of fusogen (50% PEG solution)

This solution was prepared as described by Lane et al., (1984). One day before fusion, 2gm polyethylene glycol 4000 (Merck Co.) was melted directly in a test tube by holding the tube in a beaker containing boiling water. The melted PEG was dissolved in 2ml of warm (37°C) basic medium. After addition of 0.2 ml of dimethyl sulphoxide this solution was sterilized by passing through a sterile filter (Millex-Gs, 0.22μm, Millipore,

U.S.A.) using a glass type syringe and collected in a sterile container under aseptic conditions. It was then kept at 4°C until required.

ii. Preparation of peritoneal extraction solution and extraction of peritoneal exudate cells (PEC)

This was done as described by Fazekas et al., (1980). Sucrose (0.34M) and NaCl (0.9% w/v) were dissolved in DDW. Aliquots of 50 ml were taken in small flasks and sterilized by autoclaving. They were then kept at room temperature aseptically until required.

A non-immunized mouse served as a source of peritoneal cells. It was killed by either dimethyl ether inhalation or cervical dislocation and transferred to a plastic bag containing 70% ethanol for surface sterilization. All other steps were followed under aseptic conditions in an inoculation hood. The mouse was mounted on a piece of board and the abdomen area was washed and wiped with sterile gauze using 95% ethanol. The abdominal skin was removed with sterile surgical instruments and 5 ml of cold (4°C) peritoneal extraction solution was injected into the peritoneal cavity using a 5 cc syringe with a 18 gauge needle. The fluid was distributed around the cavity by gentle massaging of abdomen with alcohol washed fingertips. Then as much as possible of the fluid was withdrawn using another needle and syringe, avoiding contact with other organs. The extracted cell suspension was transferred into a test tube and the extraction was repeated once more. The pooled extracts were centrifuged at 200g, at 4°C for 5 min. The macrophages from one mouse were added to 100 ml of basic medium at 37°C and distributed, 1 ml/well of a 24 well cell culture plate (approx. 1 $x10^5$ PEC). The plates were equilibrated in an incubator in a humid atmosphere of 5% CO_2 at 37°C.

iii. Growth of Myeloma cells

A P3-X63-Ag 8.653 myeloma cell line (Kearney et al , 1979) which does not express any immunoglobulin was used throughout this work. A few weeks before fusion it was expanded in T-75 flasks in basic medium containing 0.13 mM 8-azaguanine and 5% (v/v) FCS. (To prepare 0.13 mM 8-azaguanine medium, the content of a bottle of lyophylized 8-azaguanine was reconstituted in 10 ml of basic medium and then diluted to

500 ml with the basic medium containing 5% (v/v) FCS. Incorporation of 8-azaguanine in the media was discontinued two passages before the fusion, and switched to basic medium with 15 % FCS (v/v) (no azaguanine). One day before fusion the myeloma cells were divided into two portions 1:1 with basic medium containing 15% FCS (v/v). The cells were in logarithmic growth by the fusion day (half-confluent with orange supernatant).

iv. Preparation of spleen cells for fusion

An immunized mouse with a titre of at least $1x10^4$ in indirect ELISA against viral antigens was bled and then killed by cervical dislocation. After surface sterilization and mounting on a dissecting board (as described before) the peritoneum was cut and stomach was lifted up in order to reveal the spleen. The spleen was removed and placed in 10 ml of cold ($4^{\circ}C$) basic medium containing 15% FCS (v/v). The fat and other tissues attached to the spleen were removed and the spleen was transferred into another petri-dish with the same medium. The spleen was injected (multiple sites) with cold basic medium using a 22 gauge needle (Halk et al , 1984). It was then gently massaged with a needle while the stroma was left intact and any cell clumps were pipetted vigorously. The cell suspension was transferred to a test tube, left for 5 min at room temperature, pipetted to a second test tube while all the debris was left behind. The cell suspension was centrifuged at 300g for 5 min. The pelleted cells were resuspended in 20 ml of basic medium containing 15% (v/v) FCS and the total number of lymphocytes was counted as follows : an equal volume of cell suspension was mixed with a 1% (w/v) trypan blue solution and 20 µl was distributed on a counting grid. After replacing the lamella, the number of live cells (bright colour) were counted in the square areas using an inverting microscope. The number of cells per ml of solution was calculated according to the following formula :

Number of counted cells x 2 x 10^4 = number of cells per ml, where 2 is dilution of cells with 1% (w/v) trypan blue solution.

v. Preparation of tumour cells for fusion

All myeloma flasks were checked for any signs of contamination and the status of the cells was assessed. Four flasks with cells in log phase of growth were chosen. The cells were gently suspended in flasks and then transferred into 50ml test tubes and

subjected to centrifugation at 200g for 5 min. The pelleted cells were resuspended in 10 ml basic media containing 15% (w/v) FCS and the total number of cells was estimated as described above.

vi. Fusion strategy

Fusion was performed as described by Lane et al., (1986). The spleen cells and the appropriate number of myeloma cells were mixed and centrifuged at 300g for 5 min. The pellet was resuspended in 10 ml of basic medium containing 15% (w/v) FCS and the suspensions were centrifuged at 300g for 15min. While the cells were being prepared the pH of the fusogen was adjusted to pH 7.0 with sterilized 1N NaOH or HCl solution until the mixture turned pink. The supernatant was aspirated off and the cells were mixed by tapping the tubes with a finger. Then, 1 ml of fusogen was added slowly over 45 seconds, while gently swirling the tube, and gradually diluted by dropwise addition of 2 ml of warm basic medium with 15% (v/v) FCS over 2 min with continuous swirling. Then 10 ml of basic medium at 37°C with 15% (v/v) FCS was added over 3 min with continuous stirring. Finally, the volume was brought up to 50 ml with basic medium and the tube was incubated at 37°C for at least 10 min followed by centrifugation at 300g for 5 min. The supernatant was aspirated off and the cells were mixed by a few taps with a finger. The cells were resuspended in 195 ml RPMI containing 15% (v/v) FCS and 2 x HAT and were plated out quickly, 1 ml in each well of the PEC plates. The plates were incubated as described before.

d. Maintenance of the plates until screening

Before changing the media (5-7 days after fusion), the plates were observed with a phase-contrast inverting microscope and the growth of hybridoma cells in the wells of each plate was mapped. Then half the medium in each well was aspirated off using a sterile Pasteur pipette attached to a vacuum line. Each pipette was used for only one row of wells of a plate and the plates were fed with 500 µl of basic medium containing HT. When the wells were half-confluent and the supernatant changed colour from pink to yellow (10-15 days post-fusion) samples of 1 ml of the medium was taken carefully with a pasteur pipette from each well and transferred to an Eppendorf tube. The tubes were marked with the

corresponding number of the plate, fusion, and the position of well on the plate. The wells were fed with 1ml of basic medium containing HT.

e. Screening procedures

Three formats of indirect ELISA (Fig. 2-1, formats 6, 7 and 8), each exposing a different antigenic conformation of each of the five AMV isolates, were used for screening of hybridoma supernatants. All buffers and incubations were done as described in section 16-4 of this chapter. On each ELISA plate, mouse polyclonal antisera and supernatants from growing myeloma cells were used as positive and negative controls, respectively.

f. Preservation of hybridoma cells

The supernatant from each of the desired hybridoma cells was aspirated off aseptically and 1 ml of freshly prepared freezing media [90% FCS (v/v), 10% dimethyl sulphoxide (v/v)] was added to each well and cells were gently suspended. The cell suspension was sucked up with a sterile pasture pipette and transferred into a cryotube. Each tube was marked with date, corresponding fusion number, and the location of the well on the plate. The tubes were placed in a small polyurethane-foam container and kept for a few min at -20°C and were then transferred to -70°C overnight. The tubes were later inserted in a cryoflex tube holder and transferred in a liquid nitrogen container.

g. Regeneration of hybridoma cells

The cryotubes were removed from liquid nitrogen and were thawed in a water bath at 37°C. Immediately after the melting of the last piece of ice, the tubes were transferred to an inoculation hood and decapped with forceps. The content of each cryotube was transferred into a 20 ml test tube and 2 ml of HT medium at 4°C was added dropwise to each tube over 10 min. After addition of each drop, the tube was shaken gently. The tubes were incubated at 37°C for 15 min and 5 ml of the medium was added to each tube, dropwise over 10 min and finally the volume of each tube was made up to 20 ml by the addition of the same medium. After centrifugation at 200g for 5 min the pelleted cells were suspended in basic medium containing HT with macrophages and distributed in the wells of tissue culture plates.

h. Limited dilution cloning

As soon as the results of the screening was obtained, the cloning was performed as follows :

The selected hybridoma cells were mixed in the same well using a Pasteur pipette. Two different procedures were used for limited dilution cloning :

i. Limited dilution cloning without counting the number of hybridoma cells

This was done essentially as described by Hammerling and Hammerling, (1981). The number of viable hybridoma cells in a well was estimated visually, and 2-5 µl of a resuspended hybridoma cell suspension was taken by a micropipette and diluted into 20 ml of complete medium containing $4x10^4$ ml of peritoneal exudate feeder cells. From this solution, 10 ml was plated out into 96 well tissue culture plates by using an 8 channel Titertek pipette delivering 100 µl of cell suspension per well. The remaining 10 ml was mixed with an equal volume of complete medium containing feeder cells and again 10 ml of suspension was plated out as above. The solution left was diluted again with an equal volume of the same media and all were plated out as before.

ii. Limited dilution cloning by counting the number of hybridoma cells

By counting an aliquot of the cell suspension, the number of cells was determined. The dilution of cells in the medium (as above) was prepared so that after plating, the number of cells per plate were four. Then, 4 ml of that preparation was taken and diluted to 20 ml using the same medium and plated out into 96 wells of a plate. Each treatment was always done in duplicate and 100 µl of the cell suspension was added per well.

After limited dilution cloning the plates were assessed within 7-10 days using an inverted microscope and the location of wells containing hybridomas were located and mapped. The wells in which cells appeared to have grown from a single cell were selected and transferred into 2-4 wells of a 24 well plate. When the supernatant changed colour from pink to yellow, it was collected and screened for antibody as described before.

i. Characterization of McAbs

i. Isotyping

The isotypes of McAbs were determined by two immunological methods as follows :

(1) Gel-immunodiffusion tests

The gel composition was the same as before. Ammonium sulphate concentrated supernatant (10-15 µl) was loaded in the central well and individual surrounding wells were filled with goat anti-mouse subclass-specific antibodies as described by Hammerling and Hammerling, (1981). The plates were incubated at 25°C and the results recorded 2-3 days later.

(2) Indirect ELISA

This was done essentially as described by Hammerling and Hammerling, (1981) and as has been shown in Fig. 2-1, (format 9). Ammonium sulphate concentrated supernatant was diluted in coating buffer applied directly to the ELISA plates and incubated for 2 hr at 25°C. The plates were blocked as before. Then 1 : 20,000 dilution of goat anti-mouse sub-class immunoglobulin in conjugate buffer containing heparin, was added. After 2hr incubation at 25°C, a preparation of 1: 2000 rabbit anti-goat antibodies in conjugate buffer containing heparin (5 units/ml) were added to the plates and incubated for another 2 hr at 25°C. Finally, substrate (1mg/ml) was added and hydrolysis allowed to proceed for 90 min at room temperature.

ii. Reactivity of McAbs

(1) Indirect ELISA

Three formats of indirect ELISA which exposed various antigenic conformation of each AMV strain (Fig. 2-1, formats 6, 7 and 8) were used to detect the reactivity of each monoclonal antibody. The conditions of ELISA were described in section 16-3 of this Chapter.

(2) Gel-immunodiffusion tests

This test was performed essentially as described for isotyping, except in some cases, 1-3% polyethylene glycol 6000 was added to the agarose to induce the precipitin reaction (Goding, 1986 ; Van Regenmortel et al., 1985).

j. Mass production of McAbs

i. *In vitro* production

After selection of a particular hybridoma clone, it was expanded initially in a 50 T flask using 3 ml pre-warmed (37°C) basic medium containing HT. To promote the growth of cells at early stages peritoneal cell exudates were also mixed with the medium. Selected hybridoma clones were mixed in the well and about 0.5 ml of suspension was taken and added to the flask. Depending on the growth of cells the volume of medium was increased gradually. As the cells became confluent in the flask, they ware divided into two parts and one part was transferred into a new bottle. To reduce the amount of non-immunoglobulin protein in the supernatant, the last feeding prior to harvesting was done with media without any FCS.

ii. *In vivo* production

Hybridoma cells expanded *in vitro* as described before were pelleted by centrifugation at 200g for 5 min and diluted in basic medium without FCS. The number of cells per ml was determined by counting. For each hybridoma, 3-4, male (2 months old), BALB/c mice were injected with 0.5 ml pristane intraperitoneally 10-14 days prior to the injection of hybridoma. Each mouse was injected intraperitoneally with $1x10^6$ hybridoma cells. Occasionally the harvested ascitic fluid containing cells was injected intraperitoneally to pristane primed mice for further ascites production.

4. Purification of γ-globulin

a. Preparation and purification of γ-globulin from polyclonal antisera

This was done according to Clark and Adams, (1977). One ml of antiserum was mixed with 9 ml of distilled water, 10ml of saturated ammonium sulphate solution was

added and the mixture kept on ice for 30-60 min. The precipitated γ-globulin was sedimented by centrifugation at 5,000g for 10 min, dissolved in 2 ml of 1/2 strength PBS and dialysed three times against the same buffer at 4°C. The γ-globulin was further purified by column chromatography through 2-3 cm packed DE 22 cellulose. The column was pre-equilibrated in half-strength PBS and the effluent collected in 1 ml fractions which were monitored spectrophotometrically at 280 nm. The first protein fractions with high absorbance were mixed and the concentration of purified γ-globulin was adjusted to approximately 1 mg / ml and stored in silicon treated tubes at - 20°C.

b. Purification of McAbs

i. Extraction and concentration of McAbs from culture supernatants

As the cells became confluent and the media changed colour, the supernatant was harvested. To do that, the content of each flask was poured into a test tube and subjected to centrifugation at 200g for 5 min. The supernatant was removed and the pelleted cells were resuspended in 3 ml of basic medium containing 15% FCS (v/v) and transferred back to the flask and expanded again. The secreted McAbs were concentrated at 4°C according to Jonak (1980). Saturated ammonium sulphate solution (pH adjusted to 7.0 with NaOH) was mixed with an equal volume of supernatant. The mixture was stirred gently and kept on ice for 1 hr, followed by low speed centrifugation at 5,100g for 10 min. The supernatant was discarded and the pellet was dissolved in as little as possible of buffer A (20 mM tris-HCl, pH 7.8, containing 40 mM NaCl) and then dialysed against buffer B (the same as buffer A, except it contained 20 mM NaCl) at 4°C. The dialysis was performed in a 2 litre cylinder and the buffer was changed at least twice at intervals of 4 hr. After the dialysis, the content of each bag was removed and subjected to centrifugation at 5,100g for 10 min. The pellet was discarded, the supernatant was retained and the concentration of protein was measured spectrophotometrically. It was diluted 1 : 10 and stored at -20°C for further purification by affinity chromatography.

ii. Extraction of ascites fluid

When the growth of tumour was satisfactory (10-14 days post injection) mice were prepared for extraction of ascites fluids as follows :

A maximum of 5 ml of sterilized physiological saline, 0.15 M NaCl (Coll, 1987) was injected into the peritoneal cavity and after a few min the mouse was anesthetized by ether inhalation. The peritoneal cavity was briefly massaged and then punctured by a 23g needle and the ascites fluid was removed dropwise through the needle by applying gentle pressure around the peritoneal cavity. After removal of ascites, the punctured area was rubbed with a sterilized pad using ethanol as disinfectant. Each mouse was tapped two or three times at 3-4 days intervals depending on the condition of the animal.

iii. Delipidization of ascites fluid prior to affinity chromatography

This was done essentially as described by Neoh et al., (1986). The ascities fluid was diluted 1 : 1 with MAPS2 binding buffer (Bio-Rad) or 10 mM phosphate buffer, pH 7.0, and clarified by centrifugation at 3,000g for 5 min. The pellet was discarded and 15mg silicon dioxide powder was added to every ml of the supernatant. The suspension was shaken gently at 25°C for 30 min, centrifuged for 5 min at 12,000g and the supernatant was stored at -20°C in aliquots.

iv. Purification of mouse IgG McAbs from culture supernatants and ascites fluid by affi-gel protein A

All steps were carried out at 4°C as follows : Affi-gel protein A agarose (5 ml) was obtained in a Kit (MAPS II Kit, Bio Rad). One ml of the gel was packed in a 1 x 10 cm column and equilibrated with 5 bed volumes of binding buffer (using either 1.5 M glycine, 3 M NaCl adjusted to pH 8.9 with 5 M NaOH or reconstituting 471 g of supplied powder in 1500 ml in SDDW and adjusting the pH to 9.0 with NaOH). To each buffer sodium azide to the final concentration of 0.05% (w/v) was added. Half a ml of ascites fluid or ammonium precipitated hybridoma supernatant was diluted 1 : 1 with binding buffer and applied to the column, followed by continuous recycling by a peristaltic pump for 1 hr. The retained proteins in the column were washed off with 15 bed volumes of binding

buffer. The IgG was eluted with 15 bed volumes of elution buffer (prepared by reconstituting 25 g of supplied powder in 1100 ml of water or using 0.1M glycine buffer containing 0.05% (w/v) sodium azide, pH 3.0 adjusted with HCl). The eluate was neutralized immediately, e.g. by collecting in a tube containing 1.6 ml of 1 M Tris-HCl, pH 9.0, or by adding 2M Tris-base. The eluted antibody preparations were concentrated against solid polyethylene glycol (PEG) followed by dialysis against 20mM Tris-HCl, pH 7.8, containing 20mM NaCl at 4°C (Dietzgen and Francki, 1988) and the antibody concentration was determined spectrophotometrically. The McAb preparations were stored at -20°C in aliquots in PBS, pH 7.0 (at concentrations below 1mg/ml, BSA was added to a final concentration of 1mg/ml).

After each cycle of column chromatography, the column was regenerated with regeneration buffer (provided by manufacturer), or was stored at 4°C in PBS, pH 9.0, containing 0.05% (w/v) sodium azide. Each column was used and regenerated up to 12 times.

v. Purification of IgM from mouse ascites fluid

This was done essentially as described by Neoh et al., (1986). After the ascites fluid was clarified and delipidized, it was dialysed against several changes of distilled water at 4°C which resulted in precipitation of the IgM. After resuspension in distilled water, it was removed and subjected to centrifugation for 10 min at 12,000g. The IgM was precipitated and dissolved in veronal-buffered saline (VBS), pH 7.2, (made up from 0.004 M Na-barbitone, 0.15M NaCl, 0.8mM Mg^{2+} and 0.3mM Ca^{2+}) (BDH, code no.10365, 2c). Then it was filtered through a 3-5 cm packed S-300 Sephacryl column, pre-equilibrated by at least 3 bed volumes of VBS buffer. The collected fractions were monitored with a spectrophotometer at 280 nm for the presence of protein. The early fractions containing IgM were further identified by their serological reactivity. The IgM antibodies at concentrations above 1 mg/ml were directly stored at -20°C in VBS containing 500 mM NaCl.

5. Determination of γ-globulin concentration

Concentration of purified γ-globulin was measured spectrophotometrically, (Clark and Adams, 1977). The concentrations of secreted γ-globulin in hybridoma supernatants was measured by a standard curve obtained from an indirect ELISA (Fig. 2-1, format 9) using various known concentrations of mouse IgG as standards. The plates were coated with two-fold dilutions of mouse IgG from 100 μg/ml to 0.3 μg/ml using PBS, pH 7.4, as coating buffer. Each ammonium sulphate precipitated preparation of McAbs was diluted 1 : 200 and 1 : 2000 and added to the same plate in duplicate. The plate was incubated for 2 hr at 25°C, any free sites on plates were saturated with blocking solution as before for 1 hr at 25°C, alkaline phosphatase conjugated rabbit anti-mouse (IgG) and (IgM) were added and after addition of substrate buffer, the optical density was measured at 405 nm. By plotting the optical density at 405 nm against the concentration of mouse IgG, a standard curve was obtained.

6. Storage of antisera and purified γ-globulin

Polyclonal antisera were stored in 50% (v/v) glycerol at -20°C. However, for continuous use, approximately 2-3 ml of antiserum was stored at 4°C in the presence of 0.02% (w/v) sodium azide (Rao, 1982). Purified γ-globulin from polyclonal antisera were stored in silicon treated vials at -20°C. Delipidized ascities, ammonium sulphate concentrated hybridoma supernatants, affinity purified IgG and column chromatography purified IgM were stored at -20°C. Concentration of purified IgG or IgM was always kept above 1 mg/ml during storage.

R. Spectrophotometry

Concentration of purified AMV, its RNA and protein were determined in a spectrophotometer using $E^{0.1\%}_{260nm}$ of 5 and 25 and $E^{0.1\%}_{280nm}$ of 0.7 respectively (Jaspars and Bos, 1980). However, the concentration of isolated protein was also measured by the Pierce BCA protein assay according to the manufacturer, using a known concentration of BSA as standard. By plotting the net (blank corrected) absorbance at 562 nm against protein concentration, a standard curve was obtained. Concentration of purified γ-globulin

was also determined spectrophotometrically, using $E^{0.1\%}_{280nm}$ of 1.4 (Clark and Adams, 1977).

Chapter 3

Incidence, and variation in the host ranges and symptomatology of AMV isolates from South Australia

I Introduction

As there were no biologically characterised AMV isolates readily available, a survey was conducted in lucerne stands around South Australia with the aim of collecting field variants of AMV and determining the incidence of this virus in this state. AMV was reported in Australia during the 1960's (Swenson and Venables, 1961; Behncken, 1966) but did not appear to be widespread until more recently. The virus is now commonly found in forage legumes and it has been suggested that this is a consequence of introduction into Australia of three aphid species (Therioaphis trifolli (MON.), Acyrthosiphon kondoi Shin. and A. pisum Harr.) and importation of seed of numerous lucerne (Medicago sativa) cultivars (Garran and Gibbs, 1982). Some of the imported seed was shown to be infected with AMV (Garran and Gibbs, 1982). The same three aphids were introduced into New Zealand and importations of lucerne seed there were also found to be carrying AMV (Forster et al., 1985). These introductions were correlated with a significant increase of AMV incidence in lucerne crops (Forster et al., 1985). Although incidence of this virus in the lucerne stands of South Australia has been increasing in recent years (R.I.B. Francki and J.W. Randles, unpublished data), no survey had been conducted for virus incidence or its variants.

In this chapter are presented some results showing the incidence of AMV in South Australia, together with the biological characterization of some of the isolates from lucerne at different geographical sites.

II Experimental

A. Incidence and distribution of AMV in lucerne

Leaf samples were collected from 170 plants showing abnormal growth (Table 3-1) at 13 sites in South Australia (Fig. 3-1). Of these, 125 had pronounced symptoms which

could be categorised into seven distinct types (Table 3-1). A leaf sample from each of the plants was tested for virus by mechanical inoculation to Nicotiana clevelandii and those test plants which developed disease symptoms were checked for the presence of AMV by immunodiffusion. The virus was detected serologically in all the N. clevelandii which developed mosaic symptoms (Table 3-1).

Table 3-1: Tests for AMV on lucerne samples collected from the field.

Description of symptoms	No. of samples with AMV / No. of samples tested
Mild or severe mosaic	8/51
Mosaic and leaf stunting	2/24
Mosaic and leaf rolling	0/6
Mosaic, chlorotic vein banding and spotting	16/29
Mosaic and leaf reddening	2/3
Mosaic, leaf narrowing and vein banding	0/6
Leaf chlorosis and vein banding	3/6
Symptomless	0/10
Symptoms not recorded[a]	12/35
Total	43/170

[a] Sampling was after grazing which made symptom description unreliable .

Data summarised in Fig.3-1 show that AMV is widely distributed in South Australian lucerne crops. The data in Table 3-1 also show that only 31 of the 125 lucerne plants with symptoms (25%) were infected with AMV indicating that field symptoms in this species are not a reliable indication of AMV infection.

B. Aphid transmission

A number of attempts were made to transmit several of the AMV isolates from N. clevelandii back to lucerne either by mechanical inoculation or with the aphid, Myzus persicae. Transmission was achieved only in two experiments, which are shown in Table 3-2. The successfully infected plants were maintained in a glasshouse for over 18 months. During this time the plants remained essentially symptomless, although some shoots

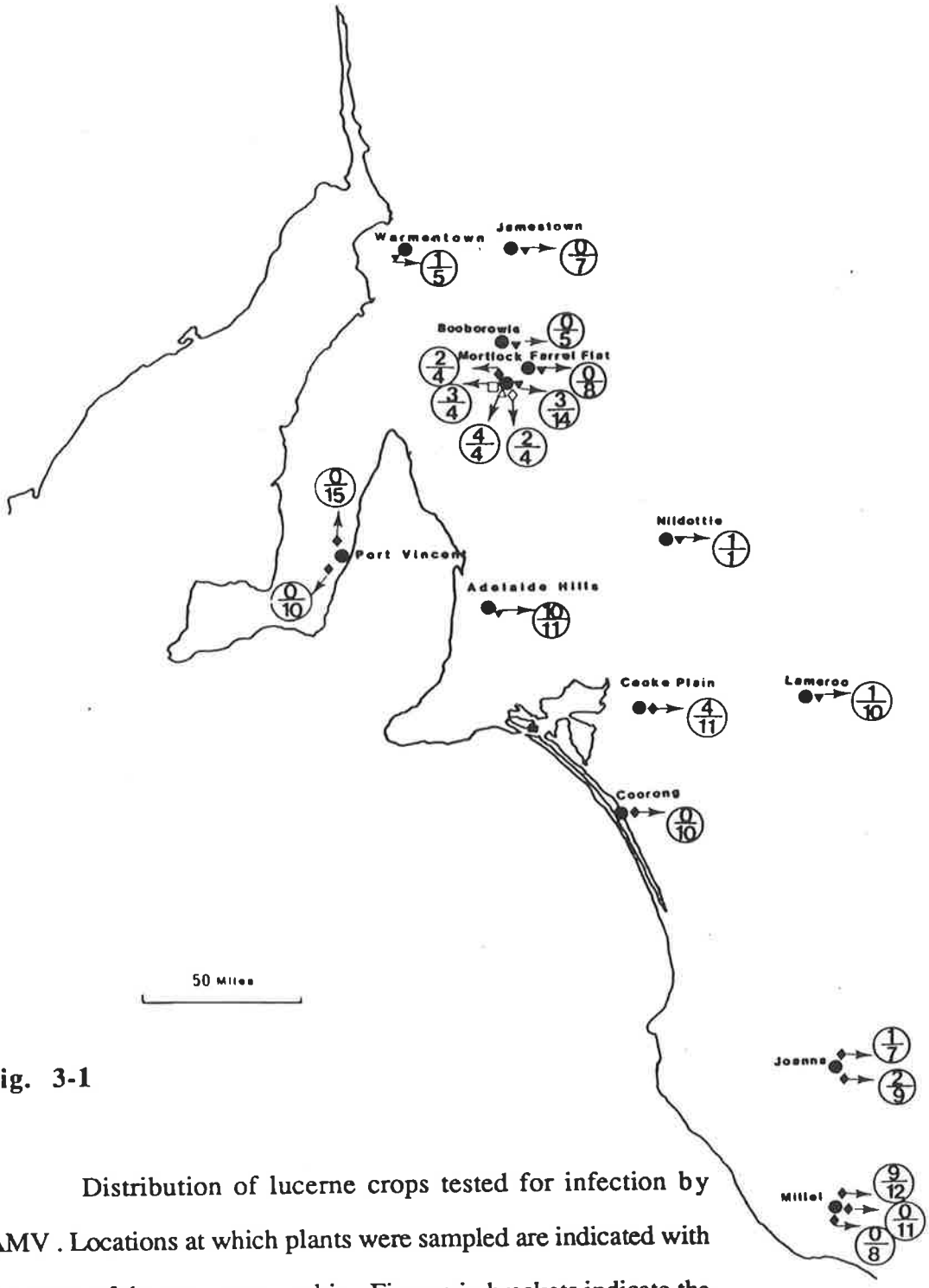


Fig. 3-1

Distribution of lucerne crops tested for infection by AMV . Locations at which plants were sampled are indicated with the name of the nearest township . Figures in brackets indicate the number of plant samples shown to contain AMV (numerator) and the total number of samples tested (denominator) . Different symbols near each site represent individual fields from which samples were taken .

developed transient mild mosaic or chlorotic flecks. However, AMV remained detectable by both DAS-ELISA or infectivity assay on Phaseolus vulgaris and Vigna unguiculata irrespective of whether any symptoms were evident or not.

Table 3-2: Transmission of AMV from Nicotiana clevelandii to lucerne plants (Medicago sativa cv. Hunter River) .

Virus isolates[a]	No. of plants infected / No. of plants inoculated[b] Tests with Myzus persicae	Tests by mechanical inoculation
H4	-[c]	0/30
H5	0/19	-
LA1	0/6	-
N20	0/24	0/30
S30	5/23	0/16
S40	0/7	-
W1	0/22	3/21

[a] Origin of isolates given in Table 3-3

[b] Plants tested for infection by DAS-ELISA, 5 wk after inoculation .

[c] Signifies not tested .

C. Biological variation of AMV isolates

To determine the variability of AMV, each of 41 field isolates was inoculated to P. vulgaris, Chenopodium amaranticolor, Cucumis sativus, Lycopersicon esculentum and Vicia faba from N. clevelandii. On the basis of symptoms produced on these hosts, the isolates could be differentiated into 18 distinct clusters (Table 3-3) similar to those described by Crill et al., (1971). Four of the clusters included eleven, seven, four and three isolates respectively; two other categories included two isolates each; and each of the remaining 12 isolates were distinguishable from all the others examined.

C. amaranticolor is often used to detect AMV and distinguish it from cucumber mosaic virus (CMV) which does not cause systemic symptoms in this host (Francki et al , 1979; Jaspars and Bos, 1980). However, three of the AMV isolates failed to infect C. amaranticolor and a further 10 isolates produced local lesions on the inoculated leaves but failed to infect the plants systemically (Table 3-3). Such an AMV isolate has also been

Table 3-3 : Reactions of 41 field isolates of AMV on five host plant species and their differentiation into '18 clusters' by the numerical system of Crill et al. , (1971).

Symptoms of AMV Field Isolates on :										Numerical classification of AMV Field Isolates										Name of AMV field Isolates	No. of isolates
P. vulgaris		L. esculentum		C. sativus		V. faba		C. amaranticolor													
I[a]	S[b]	I	S	I	S	I	S	I	S												
1	2	3	4	5	6	7	8	9	10												
+	+	+	+	+	+	+	+	+	+	1	2	3	4	5	6	7	8	9	10	H4 , S30 , NI , N12	4
+	0[c]	+	+	+	+	+	+	+	+	1	0	3	4	5	6	7	8	9	10	N20 , W1 , LA1	3
+	+	0	0	+	+	+	+	+	+	1	2	0	0	5	6	7	8	9	10	N17	1
+	0	0	0	+	+	+	+	+	+	1	0	0	0	5	6	7	8	9	10	H2,H1,H6,N9,N16,N21,N29,H6,S34,S33,S5	11
+	+	0	0	+	+	+	+	+	0	1	2	0	0	5	6	7	8	9	10	S22	1
+	+	0	0	+	+	+	0	+	0	1	2	0	0	5	6	7	0	9	10	N18	1
+	0	0	0	+	0	+	+	+	+	1	0	0	0	5	0	7	8	9	10	S39	1
+	+	0	0	0	0	+	+	+	+	1	2	0	0	0	0	7	8	9	10	S36	1
+	0	0	0	+	+	+	+	+	0	1	0	0	0	5	6	7	8	9	0	S31,S25,H8,N28,N22,S35,N23	7
+	0	0	0	+	+	+	0	+	+	1	0	0	0	5	6	7	0	9	10	N25, N24	2
+	0	0	0	0	0	+	+	+	+	1	0	0	0	0	0	7	8	9	10	H5	1
+	0	+	+	0	0	+	0	0	0	1	0	3	4	0	0	7	0	0	0	S40	1
+	0	0	0	+	+	+	+	0	0	1	0	0	0	5	6	7	8	0	0	NI	1
+	0	0	0	+	0	+	+	+	0	1	0	0	0	5	0	7	8	9	0	S15	1
+	0	0	0	0	0	+	0	+	+	1	0	0	0	0	0	7	0	9	10	H3	1
+	0	0	0	+	0	+	+	0	0	1	0	0	0	5	0	7	8	0	0	N19	1
+	0	0	0	0	0	+	+	+	0	1	0	0	0	0	0	7	8	9	0	H7, N26	2
+	0	0	0	0	0	+	0	+	0	1	0	0	0	0	0	7	0	9	0	N27	1

[a] Indicates symptoms on inoculated leaves . [b] Indicates symptoms on systemically infected leaves . [c] Indicates no infection . 1, 3 , 5 , 7, 9 indicate the positive recovery of virus from inoculated leaves and 2, 4, 6 , 8, 10 from systemic leaves of Phaseolus vulgaris, Lycopersicon esculentum cv. Rutgers, Cucumis sativus cv. Supermarket, Vicia faba cv. Aqua Dulce and Chenopodium amaranticolor, respectively.

reported recently from Canada by Hiruki and Miczynski, (1987). This indicates that C. amaranticolor is unreliable for AMV detection and differentiation from CMV.

Twelve of the virus isolates were used to study the variation of AMV in more detail. All these isolates were passaged through five single local lesion transfers and were then inoculated to 22 indicator plant species and cultivars. Results of these experiments are summarised in Table 3-4. The number of different plants infected by the isolates varied from 14 to 21. Also, whereas some of the isolates were able to infect as many as 21 hosts systemically, others infected as few as six (Table 3-5). Symptoms induced by the isolates on all the plants tested varied widely (Table 3-4), including those recommended as diagnostic indicators such as P. vulgaris, P. sativum, V. faba, V. unguiculata as well as C. amaranticolor (Jaspars and Bos, 1980) (Figs. 3-2, 3-3 and 3-4). This indicates that it would be difficult to unequivocally identify the virus isolates as AMV by inoculation to indicator plants alone. In my experience, symptoms induced in Nicotiana glutinosa (Fig. 3-2 B) are probably the most reliable for the tentative identification of AMV.

It was also observed that the symptoms produced by some of the single lesion isolates on certain hosts, differed from those induced by the field isolates from which they were derived. Examples of some of the most striking differences are summarised in Table 3-6 indicating that some of the field isolates must have been mixtures of AMV variants.

The host range studies on the twelve local lesion AMV isolates were repeated in a growth room at 25°C with continuous illumination of 10,000 lux. Many of the indicator plants reacted very similarly to those grown in the greenhouse; however, some were significantly different (Table 3-7). This indicates that environmental conditions have an important effect on the symptoms expressed by AMV on some indicator plants.

D. Attempts to classify AMV isolates into clusters

Crill et al , (1971) suggested a numerical classification of AMV isolates into what they considered to be strains. These workers inoculated each of their isolates to P. vulgaris , P. sativum , V. unguiculata and G. globosa and each isolate was assigned a numerical classification based on symptoms on the inoculated and systemically infected leaves.

Table 3-4 : Reactions of selected plant species and cultivars to AMV single local lesion isolates .

Host Plant	AMV Isolates[a]											
	H1	H2	H3	H4	H5	LA1	NI	N3	N20	S30	S40	W1
Phaseolus vulgaris cv . Hawkesbury wonder	N/-[b]	N/-	N/-	N/M,E	N/-	N/-	N/-+	N/-	N/-	+/M,E	N/-	N/-
cv .Bountiful	N/-	N/.+	N/-	C,N,/M,E	N/-	N/-	N/-	N/-	N/-	C,N/M,E	N/-	N/-
cv .Top Crop	N/-	N/- +	N/-	C,N/M,E	N/-	N/-	N/-	N/-	N/-	C,N,/M,D,E	N/-	N/-
Pisum sativum cv . Green Feast	S/S	S/S	-/-	S/M	-/-	S/S	S/S	S/S	S/M	S/S	-/-	S/(M)
Vicia faba cv . Aqua Dulce	N/+	N/M	N/-	N/K,M	N/-	N/K	+/M	N/(M),K	N/(M)K	N/(M),(K)	N/-	N/(K)
Vigna unguiculata cv. Black Eye	N/-	N/-	N/-	N/D,(K)	N/-	N/-	N/-	N/-	N/-	N/(M),K,D	N/-	N/-
Glycine max	+/M	+/M	-/-	+/M	-/-	+/M	+/+	+/M	+(M),(K)	+/(M),(K)	+/M	+/M
Solanaceae												
Nicotiana tobaccum cv. White Burley	C/M	C/+	C/.+	+/+	C/.+	+/+	C/M	+/M	M(M),B	C/M	C/M	C/M
cv . Xanthi n.c.	C/M	C/+	C/M	C/M	-+/.+	+/+	+/M	+/M	+/(M),L	+/M	C/M	C/M,L
Nicotiana clevelandii	N/(M)	C/(M)	C/M	C,N,(M)	N/(M)	+/M	+/M	C/(M)	M/(M)D,L	N/(M)	C/(M),L	N/(M)
Nicotiana glutinosa	+/M,K	+/K	+/M	+/M	+/K	+/K	+/M,K	C/M	M/(M),L	+/K	N/K	+/M
Nicotiana edwardsonii	N/M	+/M	+/M	N/M	C,N/(M)	+/M	+/M	+/M	N/(M),L	C/K	C,N/M	C,N/M
Petunia hybrida cv. Bobbydazzler	+/M	+/M	+/M	+/M	M/(M)	+/(M)	+/M	+/(M)	+/(M)	+/M	+/M	C/M
Physalis floridana	+/(M)	+/M	-+/.+	+/M	-+/.+	M/(M)	+/M	M/(M)	M/(M)D,E	M/M	+/M	M/(M)
Lycopersicon esculentum												
cv .Rutgers	-/-	-/-	-/-	+/M,D	-/-	+/(K)	+/K,D	-/-	-/-	+/(K)	-/-	+/(K)
cv . Gross Lisse	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/(K)	-/-	-/-	-/-	-/-
Capsicum frutescens cv. Giant Bell	+/M	+/M	-/-	+/M	-+/.+	+/(M),L	+/M	+/(M),B	+/(M),B	+/M	-/-	+/M,L
Sloanum melongena cv. Long Tom	+/M	+/M	-/-	+/M	+/M	+/+	+/M	+/M	+/(M)	+/M	-/-	+/M
Cucurbitaceae												
Cucumis sativus cv. supermarket	C/M	C/M	-/-	C/M	-/-	+/M	C/M	C/M	C/(M)K,D	C/M	-/-	C/M
Amarantaceae												
Gomphrena globosa	N/+	N/+	N/+	N/+	N/-	N/M	N/M	N/+	N/(M)	N/-	N/-	N/+
Chenopodiaceae												
Chenopodium amaranticolor	N/M	C,N/M	C,N/M	N/+	C/M	C,N/M	C,N/M	+/M	+/(M)L	N/M	N/-	C/M
Chenopodium quinoa	C,N(M)	C,N/M	C,N/M	C,N/-	+/M	+/M	+/M	C,N/M	C,N/(M)L	+/M	C/+	C,N/(M)
Total number of hosts infected	20/16	20/16	14/8	21/21	14/6	21/17	21/17	21/17	20/16	21/20	15/9	21/17

[a] Isolates H1 - H5 were collected from the same field in the Adelaide Hills , LA1 from Lameroo , NI from Nildottie , N3 and N20 from Mortlock , S30 and S40 from the same field in Millel and W1 from Warmenton.

[b] Symbols to the left of bars indicate symptoms on the inoculated leaves and to the right, on systemically infected leaves; + = symptomless infection (virus recoverd by back inoculation), - = immune to virus (virus not recoverd by back inoculation), -+ = results not constant from experiment to experiment, C = chlorotic local lesions, N= necrotic local lesions, M = mosaic (severe when in brackets), K = necrosis (plants died when in brackets), S = chlorosis, B = leaf narrowing, E = epinasty, D = stunting, L = leaf distortion or crinkling.

Table 3-5 : AMV isolates having a number of common hosts.

Virus isolates	S30	S40	H4	W1	N1	LA1	H5	H3	H2	HI	N3
N20	A(20)15[a]	B(15)9	A(20)16	C(20)16	N(20)15	D(20)16	E(14)6	C(14)8	F(19)1	G(20)16	C(20)16
S30		A(15)9	A(21)20	A(21)16	A(21)16	A(21)16	A(14)6	A(14)7	A(20)15	A(20)15	A(20)15
S40			H(15)9	B(15)9	I(15)9	C(15)9	K(12)4	B(13)6	I(15)9	I(15)9	C(15)9
H4				H(21)17	A(21)17	H(21)17	H(14)6	H(14)8	H(20)16	H(20)16	H(20)16
W1					J(21)17	D(21)17	C(14)6	J(14)8	C(20)16	C(20)16	C(20)16
NI						L(21)17	C(14)6	C(14)8	C(20)16	C(20)16	C(20)16
LA1							C(14)9	C(14)8	C(20)16	C(20)16	C(20)16
H5								E(13)5	I(14)6	I(14)6	C(14)6
H3									J(14)8	I(14)8	C(14)7
H2										M(20)16	C(20)16
HI											C(20)16

[a]The letters correspond to the host(s) listed below on which any pair of viruses can be readily differentiated (A=Vigna unguiculata, B=Chenopodium amaranticolor, C=Nicotiana tabacum cv. White burley, D=Nicotiana clevelandii, E=Gomphrena globosa, F=Nicotiana tobacum cv. Xanthi n. c., G=Nicotiana glutinosa, H=Phaseolus vulgaris cv. Hawkesbury wonder, I=Cucumis sativus cv. Supermarket, K=Chenopodium quinoa, J=Petunia hybrida, cv. Bobbydazzler, L=Capsicum frutescens cv. Giant Bell, M=Physalis floridana, N=Glycine max). The figures in parantheses represent the number of host species shared by any pair of isolates. A total of 22 plant species were inoculated. The figures on the right of the parantheses represents the number of host species producing systemic infection shared by any pair of AMV isolates.

Fig. 3-2

Symptoms produced by selected single local lesion AMV isolates on Lycopersicon esculentum cv. Rutgers (A), Nicotiana glutinosa (B), Chenopodium amaranticolor (C), Nicotiana clevelandii (D) under greenhouse conditions. Symptoms induced by the following AMV isolates:

a, S40

b, H4

c, S30

d, N20

e, W1

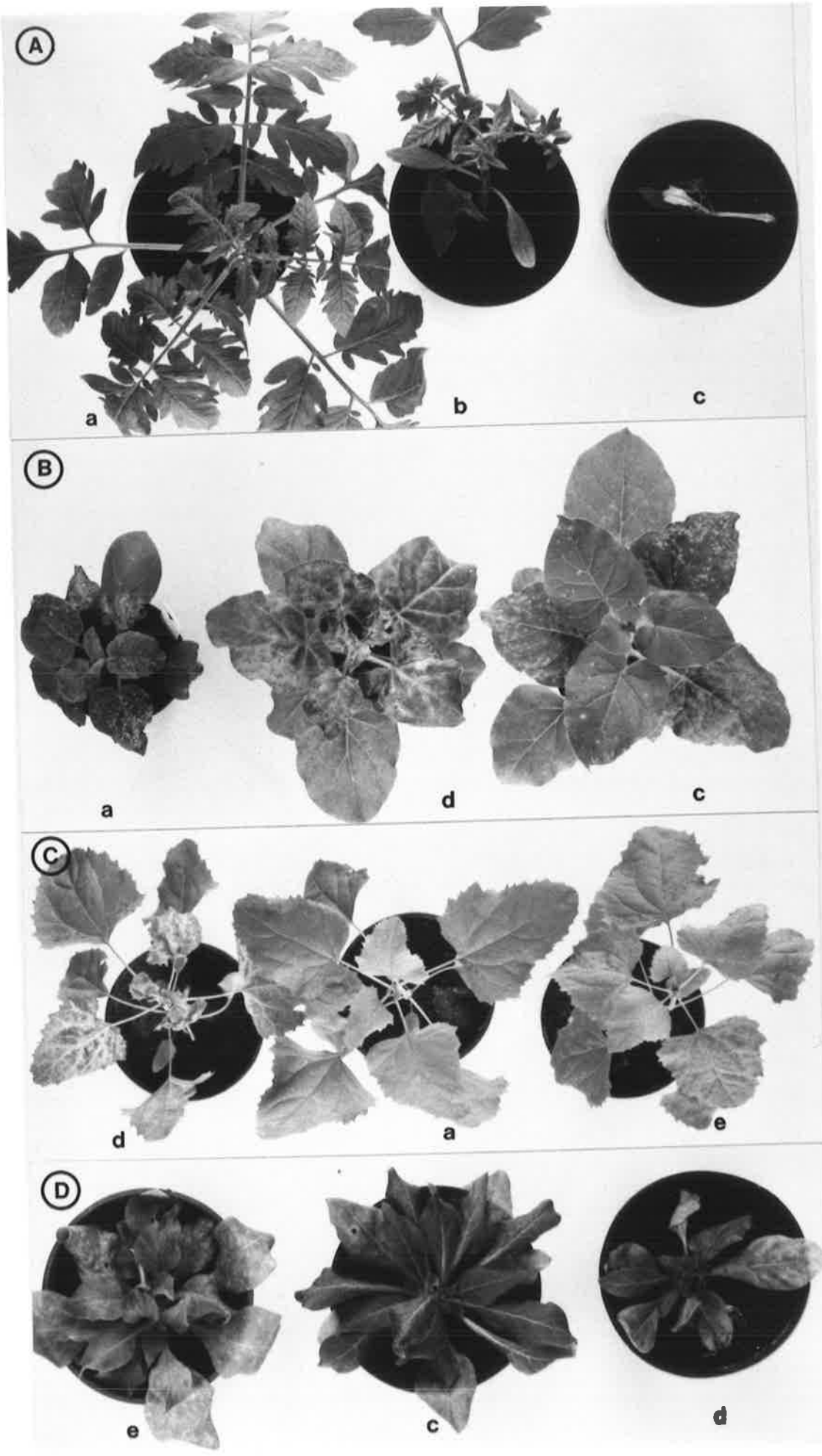

A
a
b
c
B
a
d
c
C
d
a
e
D
e
c
d

Fig. 3-3

Symptoms produced by selected single local lesion isolates of AMV on Glycine max (A), Solanum melongena cv. Long Tom (B), Chenopodium quinoa (C), and Capsicum frutescens cv. Giant Bell (D) under greenhouse conditions. Symptoms were induced by the following AMV isolates:

a, N20

b, S40

c, H5

d, S30

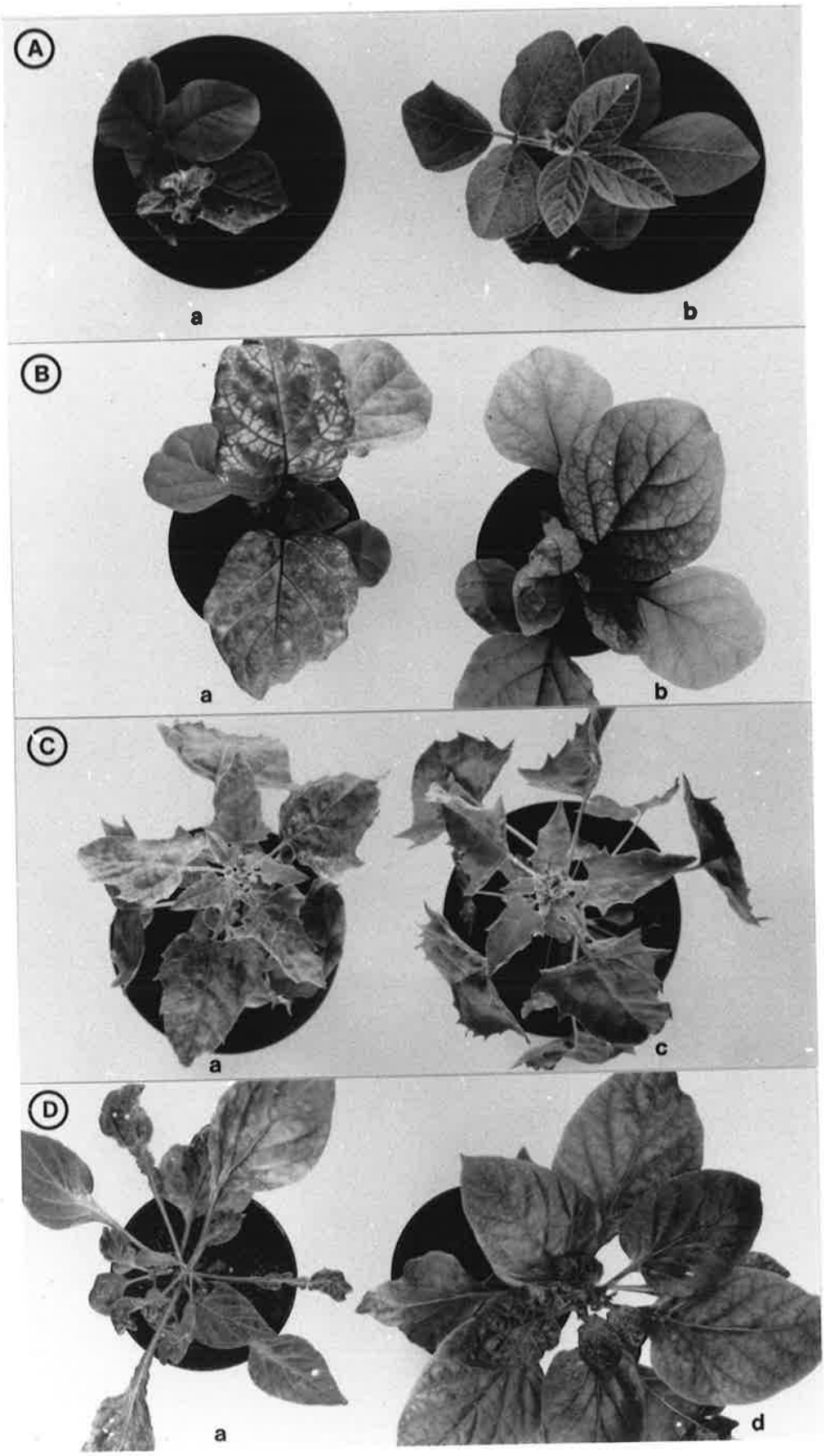

A
a
b
B
a
b
C
a
c
D
a
d

Fig. 3-4

Symptoms induced by single local lesion AMV isolates of H4, N20 and S30 on Physalis floridana (A), Nicotiana tabaccum cv. Xanthi n.c. (B), Cucumis sativus cv. Supermarket (C), Phaseolus vulgaris cv. Hawkesbury wonder (D), Gomphrena globosa (E), and Vicia faba cv. Aqua Dulce (F).

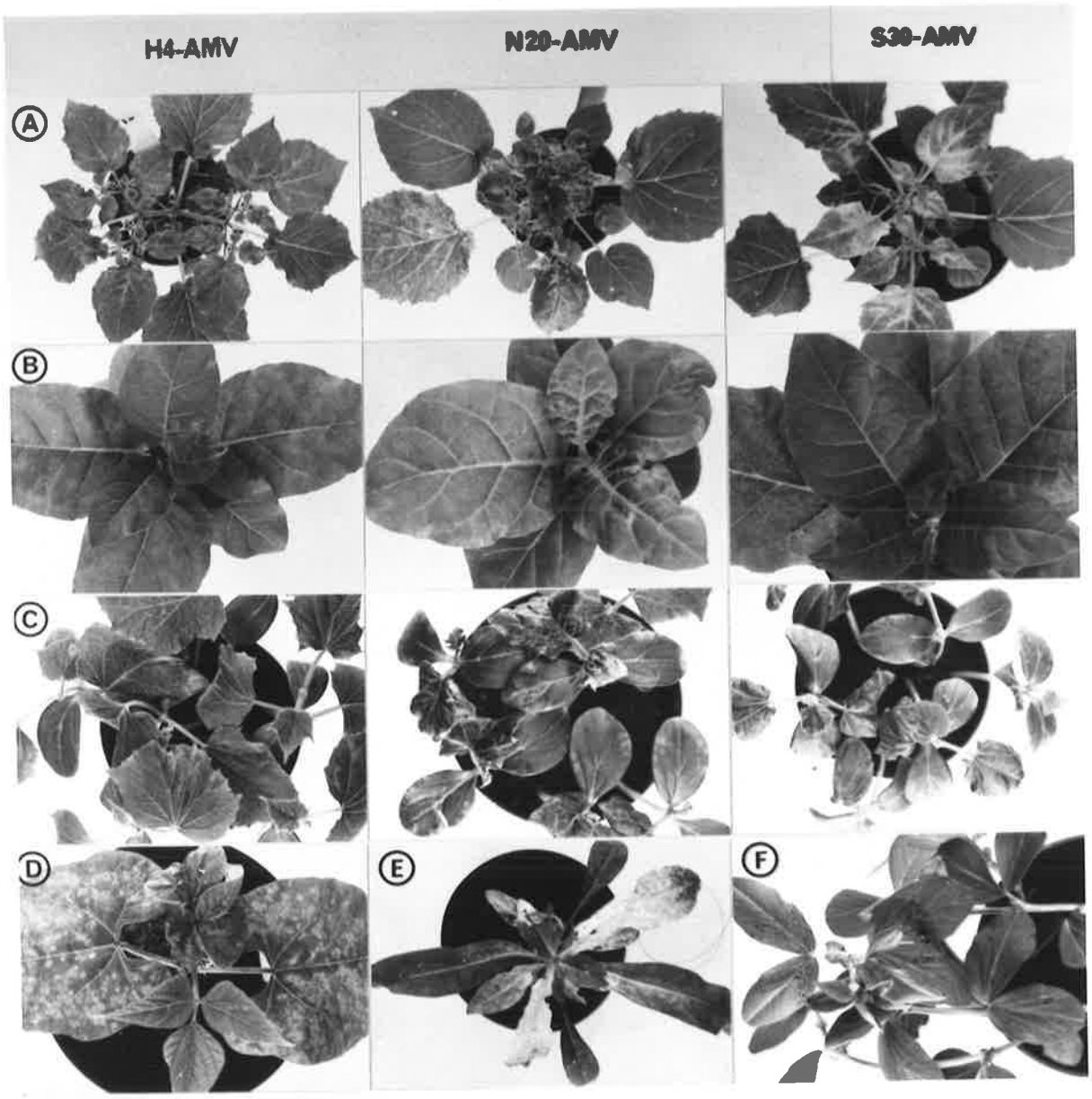

H4-AMV
N20-AMV
S30-AMV
A
B
C
D
E
F

Table 3-6: Differences in symptom production by AMV isolates before and after local lesion purification.

Host Plant	Virus isolate	Symptoms included
Lycopersicon esculentum (cv. Rutgers)	N20 Field	Top necrosis, Plants killed
	Purified[a]	No infection
Lycopersicum esculentum (cv. Rutgers)	S40 Field	Top necrosis, Plants Killed
	Purified	No infection
Vigna unguiculata	S30 Field	Necrotic lesions, Stem necrosis
	Purified	Necrotic lesions, Stem necrosis, Stunting and mosaic
Vigna unguiculata	NI Field	Necrotic lesions, top necrosis, Plants Killed
	Purified	Necrotic lesions, no systemic spread

[a] Isolates were purified biologically by five single lesion passages

When the twelve local lesion AMV isolates were classified on the basis of their reactions on the four host plants recommended by Crill et al , (1971), they fell into five groups (Fig 3-5 line A). However, when tomato (L. esculentum cv Rutgers) was substituted for peas (P. sativum) in the analysis, the isolates fell into five different groups (Fig. 3-5, line B). Moreover, when the tomato cultivar was changed from Rutgers to Gross Lisse, the groupings changed again (Fig. 3-5, line C). The groupings were also changed when C. amaranticolor (Fig. 3-5, line D) or C. quinoa (Fig 3-5 line E) were substituted for G. globosa. Of the twelve AMV isolates considered, only two clusters of three isolates, H1, H2 and N20 on the one hand and W1, LA1, and NI on the other,

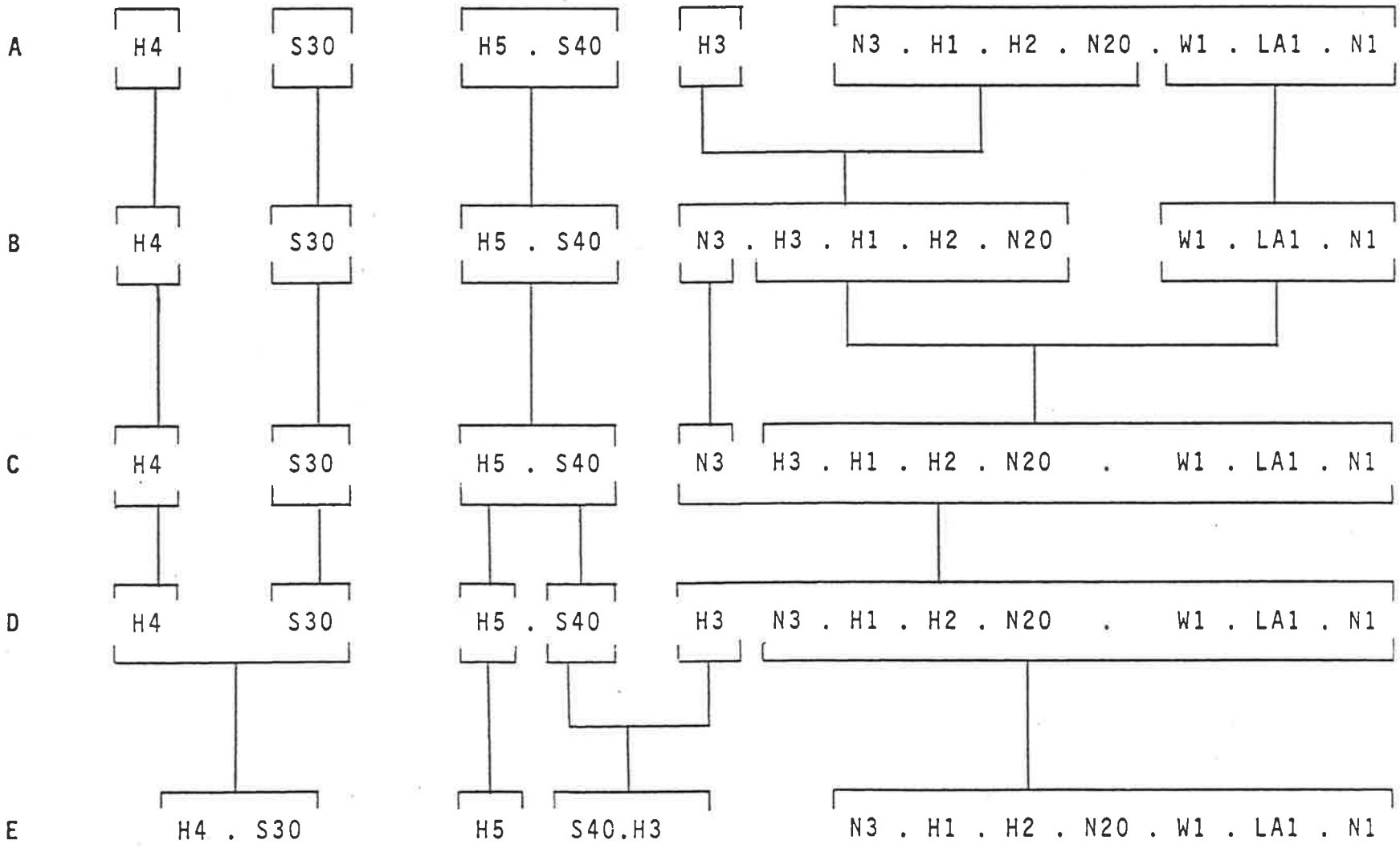
Table 3-7: Differences in reactions to infection by AMV in some host plants grown under different environmental conditions

Host Plant	Virus Isolate	Plant Reactions [a] Glasshouse [b]	Growth Chamber [c]
Cucumis sativus cv. Supermarket	H1	Chlorotic local lesions: mosaic	Chlorotic local lesions only
Glycine max	S40 & LA1	Latent local infection: mild mosaic	No infection
	NI	Latent local infection: Systemic infection	No infection
Gomphrena globosa	S30	Necrotic local lesions only	Necrotic local lesions: latent infection
	H2 & H3	Necrotic local lesions: latent infection	Necrotic local lesions only
Lycopersicon esculentum cv. Rutgers	LA	Latent local infection: necrosis	No infection
	H4	Latent local infection: mosaic and stunting	Latent local infection: necrosis
Nicotiana tabacum cv. White Burley	H3	Chlorotic lesions: Latent infection	No infection
Phaseolus Vulgaris cv. Top Crop	S30	Chlorotic and necrotic local lesions: epinasty, chlorosis and mosaic	Chlorotic and necrotic local lesions: latent infection
Pisum sativum cv. Greenfeast	S40	No infection	Latent local infection: mild mosaic
Vicia faba cv. Aqua Dulce	H1	Necrotic local lesions: latent infection	Necrotic local lesions: necrosis
Vigna unguiculata cv. Blackeye	S30	Necrotic local lesions: mosaic and partial necrosis	Necrotic lesions: necrosis
	H4	Necrotic local lesions: systemic necrosis	Necrotic lesions: latent systemic infection

[a] Reactions in inoculated : systemically infected leaves .
[b] At 16-25°C with natural light .
[c] At 25°C and continuous fluorescent illumination of 10,000 lux.

Fig. 3-5

Grouping of AMV isolates based on their reactions on host plants by the numerical classification method used by Crill et al. (1971). A-grouping based on reactions produced on Phaseolus vulgaris, Pisum sativum, Vigna unguiculata and Gomphrena globosa as recommended by Crill et al , (1971). Groupings after substituting data on P. sativum to those on Lycopersicon esculentum cv. Rutgers and Gross Lisse are shown in B and C, respectively; and from G. globosa to that on Chenopodium amaranticolor and C. quinoa in D and E, respectively.


A
H4
S30
H5 . S40
H3
N3 . H1 . H2 . N20 . W1 . LA1 . N1
B
H4
S30
H5 . S40
N3 . H3 . H1 . H2 . N20
W1 . LA1 . N1
C
H4
S30
H5 . S40
N3
H3 . H1 . H2 . N20 . W1 . LA1 . N1
D
H4
S30
H5 . S40
H3
N3 . H1 . H2 . N20 . W1 . LA1 . N1
E
H4 . S30
H5
S40.H3
N3 . H1 . H2 . N20 . W1 . LA1 . N1

always remained grouped together (Fig 3-5). However, the data presented in Table 3-5 show that all the isolates can be distinguished from each other and hence their assignment to any clusters based on host range would be arbitrary.

III Conclusion

The results presented here allow the following conclusions to be drawn:

1 - The occurrence of AMV in the lucerne stands surveyed indicates that the virus is widely distributed in South Australia.

2 - The collected isolates of AMV were biologically very variable.

3 - Field isolates of AMV may contain a mixture of variants.

4 - Environment had a significant effect on the symptoms produced by some AMV isolates.

5 - Symptoms of AMV on lucerne can be transient and virus can be readily detected in plants showing no symptoms.

6 - Field symptoms were not a reliable indication of AMV infection.

7 - AMV cannot be reliably identified by symptoms induced on any host plant species.

8 - Reaction of AMV isolates on C. amaranticolor is variable and can not be used for distinguishing AMV from CMV.

9 - The host range and variability of AMV precluded the meaningful grouping of isolates into "strains" of the virus.

Chapter 4

Comparative biochemical and biophysical properties of five biologically distinct isolates of AMV

I Introduction

It was shown in the previous chapter that the AMV isolates collected in South Australia were biologically very variable and readily distinguishable by their symptomatology. To determine if these isolates could also be differentiated by their biochemical and biophysical properties, the five most biologically distinct AMV isolates were selected and further characterised. In this chapter are described experiments on the comparative biochemical and biophysical properties of the H4, N20, S30, S40 and W1 AMV isolates.

II Experimental

A. Particle composition of different AMV isolates

Analysis of purified virus preparations of all five AMV isolates by analytical centrifugation showed that particle composition of each isolate is distinct (Fig. 4-1). As with previously described AMV preparations, (Van Vloten-Doting et al., 1968; Hull, 1969, Schwenk, et al., 1971) at least three components were observed and designated (B) bottom component, (M) middle component, (Tb) top b component. These components contained mainly B-RNA, M-RNA, and Tb-RNA,respectively (Bol et al , 1971; Bol and Lak-Kaasheek 1974).

Analysis of the virus preparations by sucrose density-gradient centrifugation also showed three distinct bands, corresponding to B, M and Tb components in each isolate (Figs 4-2 and 4.3, traces a) . The sedimentation properties of the preparations of four of the isolates (H4, S30, S40 and W1) were similar in Fig.4-1, 4-2 and 4-3. However, the particles of N20 showed some differences (compare Fig. 4-1b with panel B trace a in Figs. 4-2 and 4.3). This isolate was propagated in N.clevelandii and purified by the same method in both experiments. Hence, the observed difference in particle ratio cannot be a

Fig. 4-1

Schlieren patterns of virus preparations of the five AMV isolates grown and purified under similar conditons. Partial purified preparations of each AMV isolate at concentrations of 4 mg/ml in 10 mM phosphate buffer, pH 7.0, were used. All photographs taken 8 minutes after reaching a speed of 33,450 rpm. Sedimentation from right to left. Traces a and b are of AMV-S40 and N20, respectively, photographed at a bar angle of 50^0; and traces c, d and e are of AMV-S30, H4 and W1, respectively, photographed at a bar angle of 60^0.

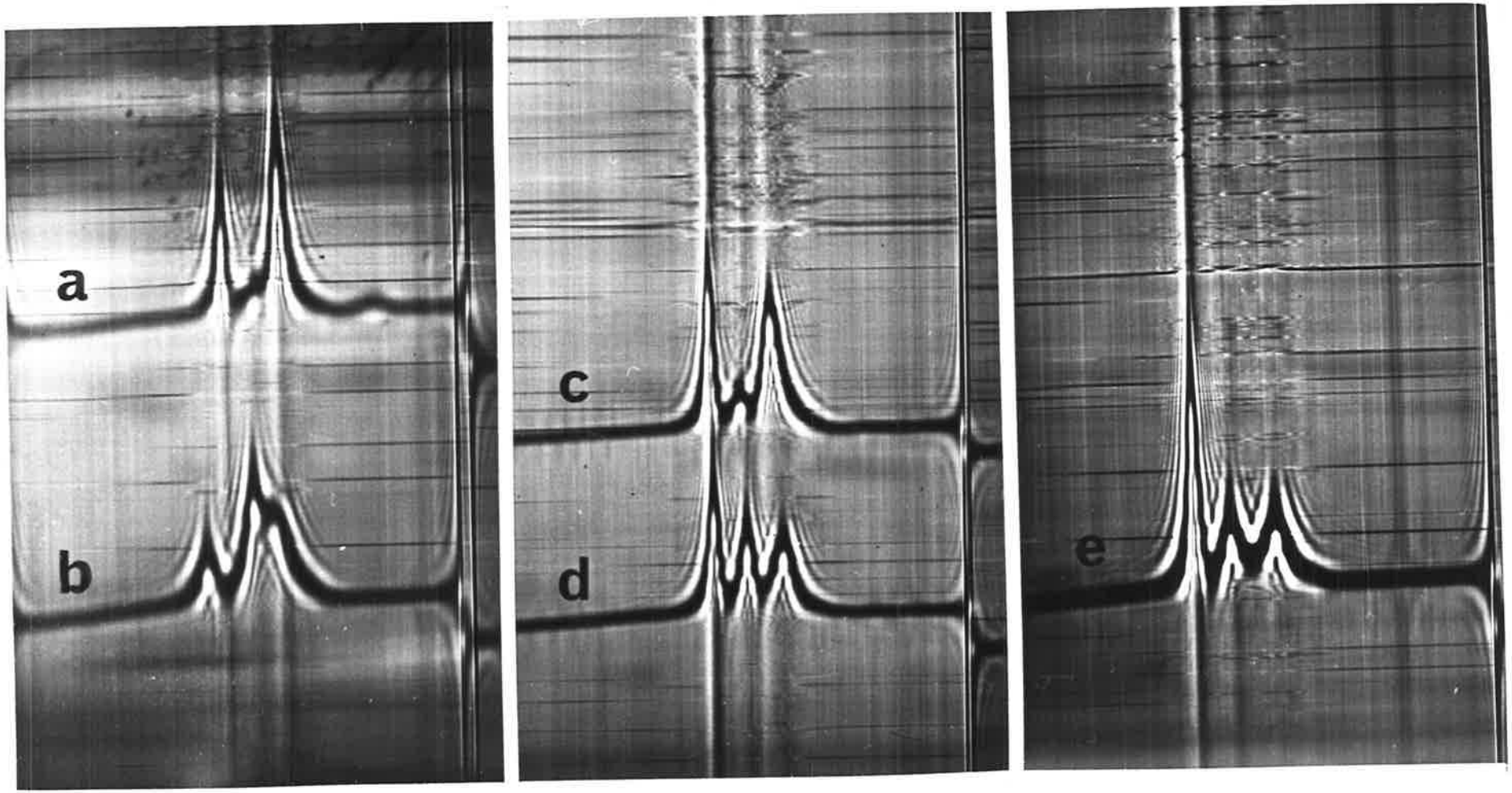

a
b
c
d
e

Fig. 4-2

Comparison of stability of native virus particles of AMV isolates H4 (panel A), N20 (Panel B), S30 (Panel C), S40 (Panel D) and W1 (Panel E) kept in 10 mM Phosphate buffer, pH 7.0, for 16 hr at 4°C (traces a) as controls, or in PEN buffer, pH 7.0, for 4 months at 4°C (traces c) or in 10 mM phosphate buffer, pH 6.5, for 16 hrs at 25°C (traces b). In each experiment, except for traces c, 50µl (100 µg) of freshly prepared native virus preparation in 10 mM phosphate buffer, pH 7.0, was mixed with 9 volumes of the appropriate buffer and incubated as required. In the experiment in which the virus was incubated in PEN buffer, (traces c), the final pellet from the last cycle of preparative centrifugation was dissolved in this buffer. Each treatment was subjected to centrifugation at 35,000 rpm for 2 hrs in a Beckman SW41 rotor in 6-30% (W/V) sucrose density-gradient columns in the same buffer. The contents of each tube was analysed by ISCO as described in Chapter 2.

Fig. 4-3

Comparison of stability of glutaraldehyde-fixed virus in 10 mM phosphate buffer, PH 7.0, (traces a) of AMV isolates H4 (panel A), N20 (panel B), S30 (panel C), S40 (panel D) and W1 (panel E) and their corresponding native virus preparations after incubation for 16 hr at 25°C in 10 mM phosphate buffer, pH 7.0, (traces b) and carbonate buffer, pH 9.6 (traces c). In each experiment, 50 µl (100µg) of freshly prepared native or fixed virus in 10 mM phosphate buffer, pH 7.0, was mixed with 9 volumes of the appropriate buffer and incubated for 16 hr at 25°C. Centrifugation conditions were as in Fig. 4-2.

Fig. 4-2

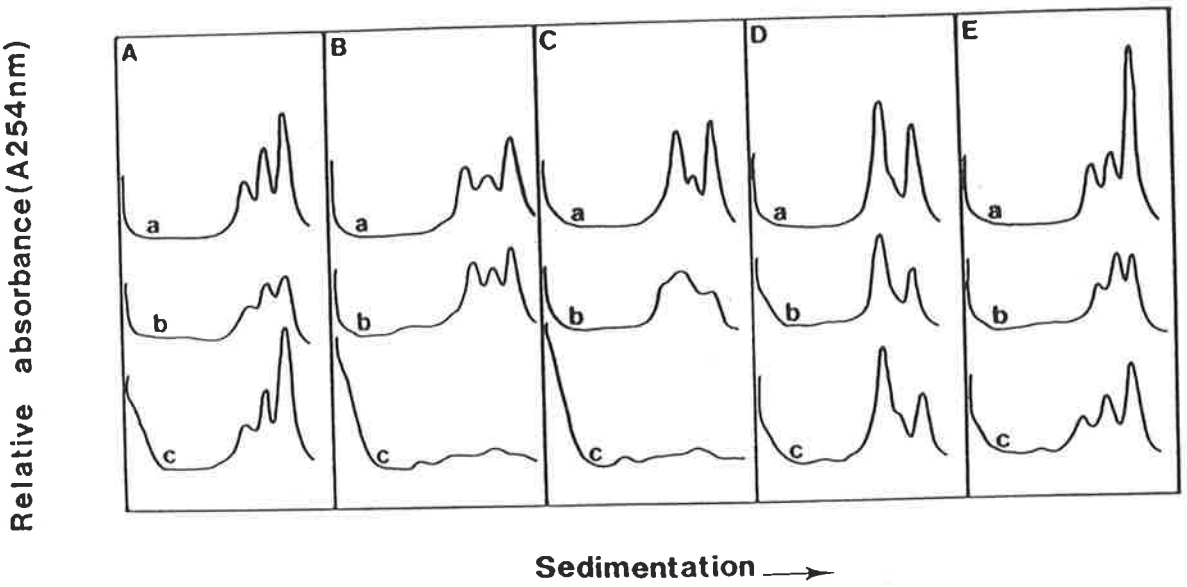
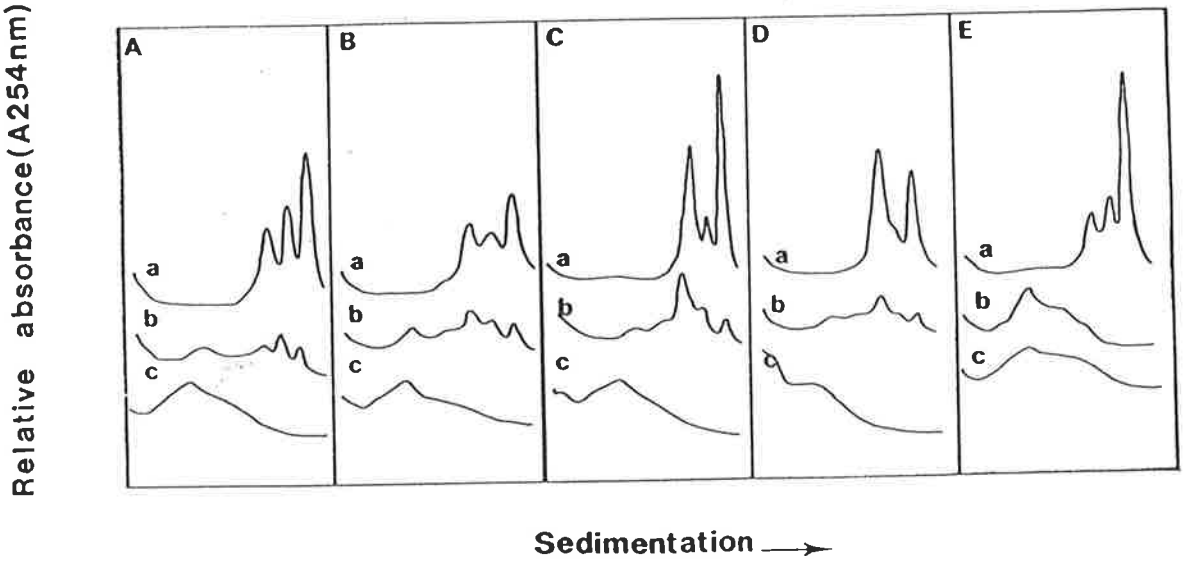

Relative absorbance(A254nm)
A
B
C
D
E
a
b
c
Sedimentation

Fig. 4-3


Relative absorbance(A254nm)
A
B
C
D
E
a
b
c
Sedimentation

result of differences in propagation host or extraction procedure used, as claimed by Lister and Bancroft, (1969). However, the inoculum of AMV-N20 used in the experiments had a different history of passage in N clevelandii. The isolate used for analytical centrifugation had been passaged only four times in N.clevelandii, while the isolate used for sucrose density-gradient analysis had a history of sixteen passages in this host over a period of 2 years. The change in component ratio between these two isolates of AMV-N20 could be a reflection of a host passage effect (Yarwood, 1979) possibly induced by a mutation in Tb-RNA. This RNA has a regulatory role in determining particle ratio of AMV (Dingjan-Versteegh et al., 1972; Hartmann et al., 1976).

B. Relative stability of AMV isolates

A virus preparation of each isolate was subjected to different treatments and the nucleoprotein composition of each preparation was analysed by sucrose density-gradient centrifugation. The preparation of each isolate in 10 mM phosphate buffer, pH 7.0, kept at 4°C was used as a control.

When the preparation of each isolate incubated in PEN buffer at 4°C for four months was analysed, no nucleoprotein corresponding to any of the AMV components was detected in preparations of the S30 and N20 isolates (Fig.4-2, panels B and C traces c, respectively). Preparations of the other three isolates, however, showed only slight signs of degradation (Fig 4-2 panels A, D and E traces c). These data suggest that under the storage conditions, S30 and N20 isolates are less stable than the other three isolates of AMV. Incubation at 25°C in 10 mM phosphate buffer , pH 6.5, resulted in reduction in the amounts of the B-components of all isolates (Fig. 4-2 traces b). Besides, under these conditions only the preparation of the S30 isolate showed any sign of degradation (Fig 4-2 panel C trace b). Incubation of preparations of all isolates in 10 mM phosphate buffer, pH 7.0, at 25°C resulted in degradation of almost all the nucleoprotein components of AMV-W1 (Fig 4-3 panel E, trace b). In preparations of all other isolates, major peaks were observed but the heights of all were reduced drastically, especially peaks corresponding to the B-components, and slower sedimenting material was detected in preparations of all the isolates (Fig. 4-3, Traces b). Incubation of preparations of the isolates in carbonate

buffer, pH 9.6, resulted in degradation of all the components and appearance of slower sedimenting material (Fig. 4-3, traces c). However, glutaraldehyde-stabilised particles of all the isolates, similarly treated, remained intact (Fig. 4-3 , traces a).

The coat protein subunits of intact S30 particles also had lower stability compared to those of the W1 isolate when incubated at 25°C in the presence of 1 mM $CaCl_2$ (Fig. 4-4, lanes 2-6 and 7-11, respectively). The induced $CaCl_2$ degradation of AMV coat protein was studied in detail and the results are presented in Appendix **2**.

C. Comparison of electrophoretic mobility of AMV coat protein

Under reduced conditions, the coat proteins of the five AMV isolates migrated at slightly different rates (Fig. 4-5, panel A) suggesting that they may differ slightly in size. On the other hand, under non-reduced conditions the isolates showed different electrophoretic behaviour (Fig. 4-5, panel B). Isolates N20 and W1 had small amounts of a material corresponding to a polypeptide with Mr. of 30,000 (Fig 4-5, panel B, lanes 2 and 5) when compared to the other isolates (Fig.4-5, panel B, lane 1, 3 and 4). However, isolates N20 and W1 had a greater amount of protein migrating as bands similar in mobility to ovalbumin (Mr. 43,000). In preparations of all the isolates except N20, material of mobilities corresponding to higher Mr. than 43,000 were also observed. Isolates S40 and W1 also contained material corresponding to Mr. between 94,000 and 67,000 (Fig.4-5, panel B, lanes 4 and 5) which were not detected in preparations of isolates H4 and S30. The differences in electrophoretic behaviour of these isolates under non-reduced conditions could be a reflection of the different numbers of disulphide bonds either intramolecularly or extramolecularly.

D. Comparison of RNAs from AMV isolates

When unfractionated RNAs from purified preparations of all isolates were electrophoresed in 1.5 % agarose gels under non-denaturing conditions RNAs 1 and 2 showed similar mobilities (Fig. 4-6, panels A, B and C). However, the proportion of each RNA varied between the isolates. In preparations of all isolates, RNAs 1 and 2 were in greater amounts than RNA 3 which varied in amounts from isolate to isolate (Fig.4-6). Whereas isolate S30 and S40 contained high concentrations of RNA3, the other three

Fig. 4-4

Comparison of the stability of coat protein subunits in intact S30 and W1 AMV particles in the presence of 1mM $CaCl_2$. Protein from virus preparations of S30 AMV (Tracks 2-6) and W1-AMV (Tracks 7-11) were incubated in the presence of 1 mM $CaCl_2$ at 25°C for 12, 24, 36, 48 and 60 hr respectively. Equal volumes of each sample were mixed with Laemmli's sample buffer and heated at 95°C for 5 min. Protein markers as described in Chapter 2 were run in track 1 and 0.5 μg of protein was electrophoresed in each of the traucks 1-11.

Fig. 4-5

Comparison of electrophoretic mobilities of AMV coat proteins from isolates H4 (1), N20 (2), S30 (3), S40 (4) and W1 (5) under reduced (panel A) and non-reduced (panel B) conditions. Samples (reduced) were prepared by mixing equal volumes of highly purified virus preparations with equal volumes Laemmli's sample buffer and were heated at 95°C for 5 min. For analysis under non-reducing conditions, 2-mercaptoethanol was omitted from the sample buffer and the samples were not heated. The molecular weight markers were the same as those described in Chapter 2; 12% polyacrylamide gels with 5% stacking layers were used. The electrophoresis was at 100V until the protein moved through the stacking gel and then at 180V for 60 min. The gels were stained with silver.

Fig.4-4


94
67
43
30
20
14
1 2 3 4 5 6 7 8 9 10 11

Fig.4-5

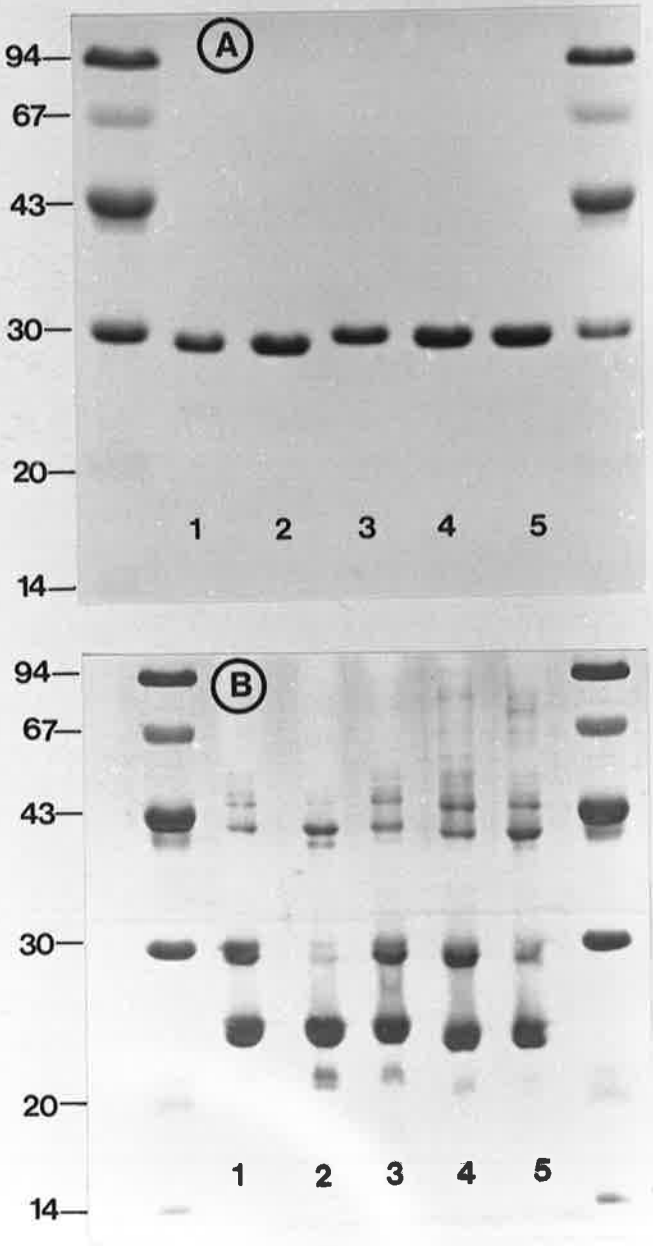
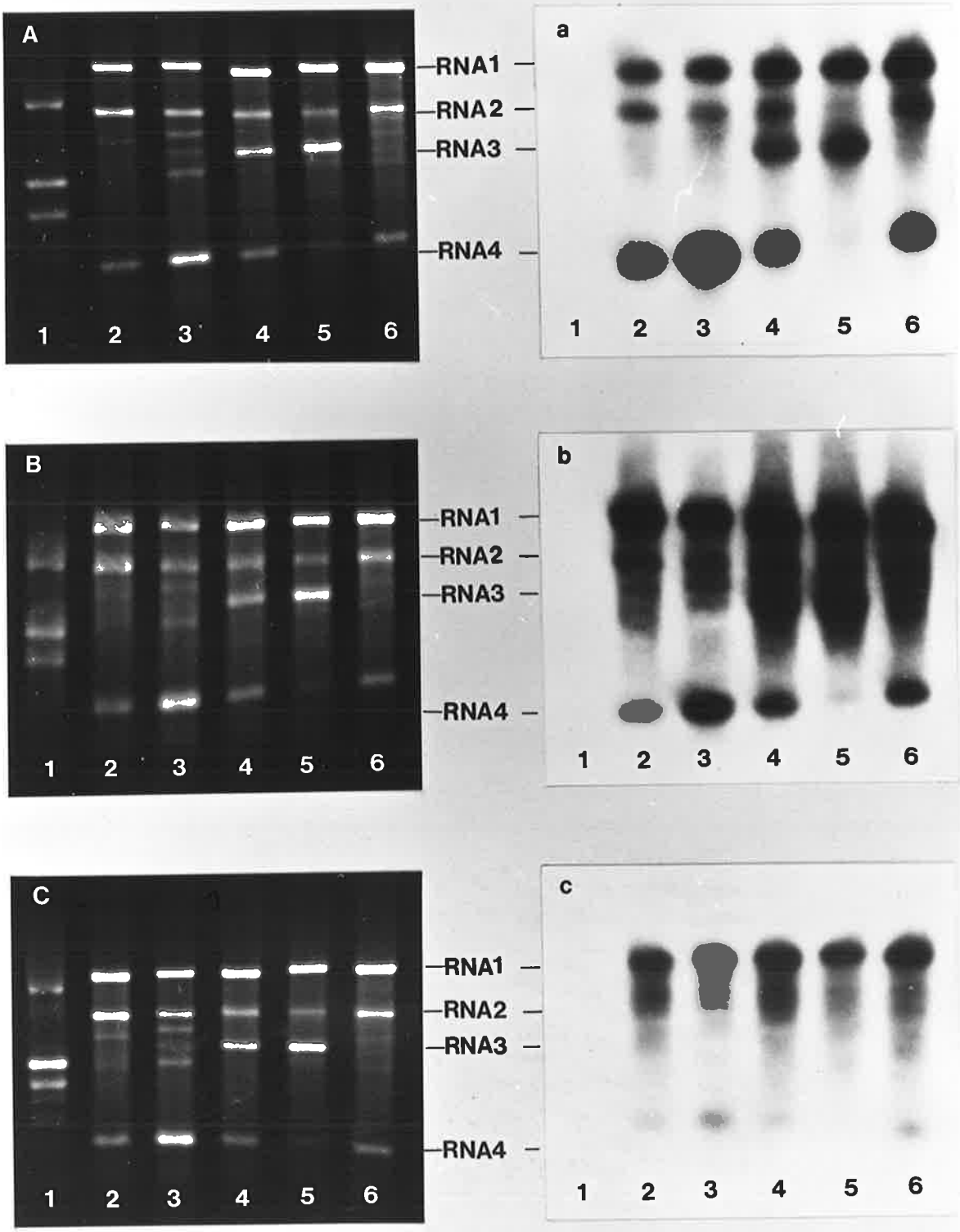

A
94
67
43
30
20
14
1 2 3 4 5
B
94
67
43
30
20
14
1 2 3 4 5

Fig. 4-6

Northern-blot hybridization of AMV-RNAs from isolates H4 (tracks 2), N20 (track 3), S30 (track 4), S40 (track 5) and W1 (track 6) with cDNAs prepared against total RNAs of N20 (Panel a), S30 (Panel b) and W1 (Panel c). Panels A, B and C are gels which were photographed in UV light before transfer to nitrocellulose. Panels a, b and c were autoradiographed for 36, 24 and 72 h, respectively, at -70C with intensifying screens. Total RNA extracted from healthy N.clevelandii was electrophoresed in track 1. Total RNA of each isolate was extracted from partially purified virus preparations as described in Chapter 2; 1 μg of RNA was electrophoresed in each track.


A
RNA1
RNA2
RNA3
RNA4
1 2 3 4 5 6
a
1 2 3 4 5 6
B
RNA1
RNA2
RNA3
RNA4
1 2 3 4 5 6
b
1 2 3 4 5 6
C
RNA1
RNA2
RNA3
RNA4
1 2 3 4 5 6
c
1 2 3 4 5 6

isolates contained only traces of this RNA. In RNA preparations of the H4, N20 and W1 isolates additional segments of RNA with intermediate mobility between RNAs 2 and 3 were also detected (Fig 4.6, lanes 2, 3 and 6, panels A, B and C). Isolate N20 and W1 also had RNA segments migrating faster than RNA 3. None of these RNA segments were observed in the total RNA extracted from N. clevelandii, a plant host from which all the isolates were propagated (Fig. 4-6, lane 1, panels A, B and C).

E. RNA-cDNA hybridization tests

RNAs of all the isolates hybridized strongly with cDNA prepared against their homologous cDNAs as well as heterologous cDNAs (Fig.4-6, panels a, b and c). This indicates that all the isolates have sequence similarities. In studying the sequence homology between biologically distinct AMV isolates by using competition hybridization experiments, similar results were obtained by Bol et al., (1975). As cDNAs prepared from the RNAs of the other AMV isolates did not cross-hybridize with total RNA extracted from N. clevelandii (Fig. 4-6, lane 1 in panels a, b and c), confirms that the RNA segments present in stained electrophoretograms (Fig. 4-6, panels A-C) are virus specific. Cross hybridization of cDNA prepared against S30 isolate, which lacks these extra segments, with all the RNAs of H4, N20 and W1 isolates, provided further evidence that these RNAs have AMV RNA sequences (Fig. 4-6, panels B and b lanes 2, 3 and 6).

As the RNA 4 of S40 AMV did not hybridize clearly in the above experiments (Fig. 4-6, lane 5 in panels a, b and c) a further experiment was conducted by using cDNA prepared against RNA4 of this isolate which had been purified by preparative gel electrophoresis. This cDNA cross hybridized strongly with top a-RNA fractions of all the other isolates in dot-blot hybridization tests. This indicates that there is sequence homology between the coat protein genes of all the five AMV isolates (Fig. 4-7).

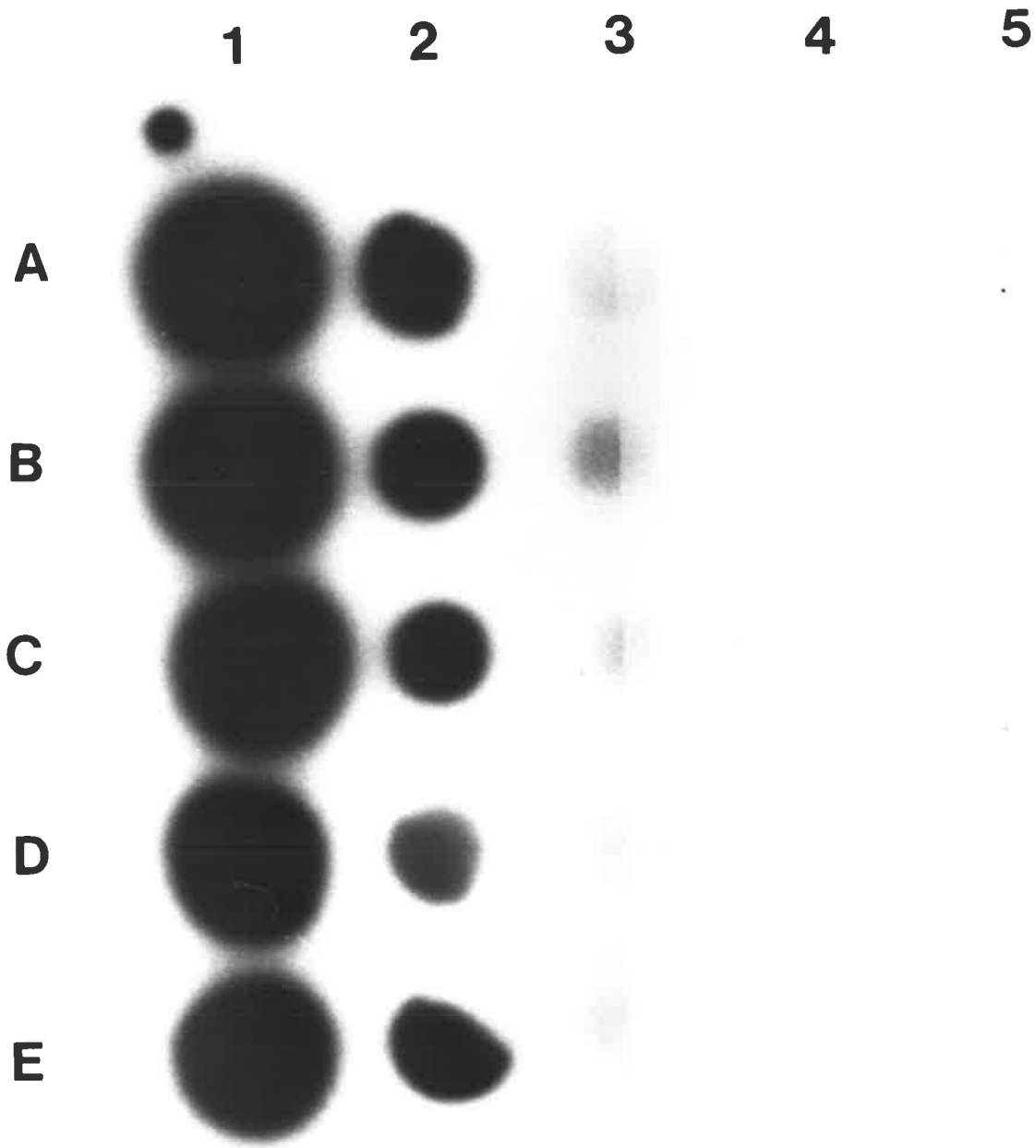
III Conclusion

The results presented in this Chapter enables the following conclusions to be made :

1 - The five biologically distinct AMV isolates were also distinguishable by their nucleoprotein component ratios.

Fig. 4-7

Dot-blot hybridization of Top a component-RNA fractions of AMV-isolates S40 (A), S30 (B), W1 (C), N20 (D) and H4 (E) with cDNA prepared against gel-purified RNA4 of AMV-S40. Concentrations of 20ng, 2ng, 200pg, 20pg and 2pg of RNA in TE buffer were spotted in columns 1-5, respectively. Hybridization was done at 68^{0}C overnight and autoradiography was for 20 hr at -70^{0}C using an intensifying screen.


1
2
3
4
5
A
B
C
D
E

2 - The particles of the AMV isolates had different stabilities during storage or incubation at various temperatures under different pH conditions.

3 - Their coat proteins were not distinguishable during gel-electrophoresis under reduced conditions, but they exhibited different electrophoretic patterns under non-reduced conditions, suggesting that they differ in the number of disulphide bonds.

4 - All five AMV isolates contained RNAs 1-4 similar in electrophoretic mobility. However, the concentrations of RNAs 3 and 4 differed widely. Also some of the isolates contained additional RNA segments with viral RNA related sequences.

5 - Northern-blot and dot-blot hybridization showed that despite biological and physicochemical differences, all the five AMV isolates have sequence homology.

Chapter 5

Immunogenicity and antigenicity of native, glutaraldehyde-fixed virus and isolated coat protein of AMV

I Introduction

AMV is unstable under physiological conditions (Tremaine and Chidlow, 1974) which makes it a weak immunogen (Rybicki and Von Wechmar, 1985). The poor immunogenicity of viral particles is assumed to be due to the breakdown of the virus in the animal and it is known that the proteins in their monomeric forms are poorly immunogenic (Marbrook and Matthews, 1966; Loor, 1967; Reichlin et al., 1970; Hirata et al., 1972). For plant viruses, it has also been shown that the removal of RNA from viral particles reduces their immunogenicity. For example, intact tobacco mosaic virus (TMV) or turnip yellow mosaic virus (TYMV) particles are more immunogenic than their protein rods or empty protein shells devoid of RNA, respectively, (Marbrook and Matthews, 1966).

The immunogenicity of some small proteins can be considerably enhanced by polymerisation (Reichlin, 1980) and this has been successfully shown for mammalian cytochrome c (Reichlin et al., 1970). It has also been shown that the immunogenicity of some plant viruses can be enhanced by cross-linking the protein of the viral particles with either formaldehyde or glutaraldehyde (Hollings and Stone, 1962; Von Wechmar and Van Regenmortel, 1968; Francki and Habili, 1972; Van Regenmortel and Lelarge, 1973; Rybicki and Von Wechmar, 1981; Van Regenmortel, 1982). Although the mechanism of the reactions of formaldehyde and glutaraldehyde are similar (Habeeb and Hiramoto, 1968; Reichlin, 1980), the reaction with formaldehyde is more reversible (Fraenkel-Conrat, 1969).

It has been found that any chemical modification involving cross-linking of proteins will have an effect on the conformation of the protein (Reichlin, 1980). For example, conformational changes in bovine serum albumin (BSA) due to fixation with formaldehyde caused about 8% reduction in its ability to react with antiserum prepared against native BSA (Habeeb, 1969). A similar effect was claimed to occur with formaldehyde-treated brome

mosaic virus (BMV) using indirect ELISA (Rybicki and Von Wechmar, 1981). This was interpreted as evidence that the antigenic structure of the viral particles was changed (Rybicki and Von Wechmar, 1981; Devergne et al., 1981). It was also shown that DAS-ELISA can differentiate between fixed virus and its native form (Rybicki and Coyne, 1983).

It appears that aldehyde-fixation has the ability of converting a poor immunogen into a potent one, but it limits the exposure of some epitopes to the immunized animal and consequently affects the quality of the antiserum. It has been shown that antisera prepared against formaldehyde-fixed TMV free of contaminating protein oligomers, did not react with depolymerized viral protein (Van Regenmortel and Lelarge, 1973).

In this chapter, the immunogenicity and antigenicity of native and glutaraldehyde-fixed AMV and its isolated coat protein have been compared. Also, the results of experiments aimed at revealing changes in the antigenic structure of AMV due to fixation are presented.

II Experimental

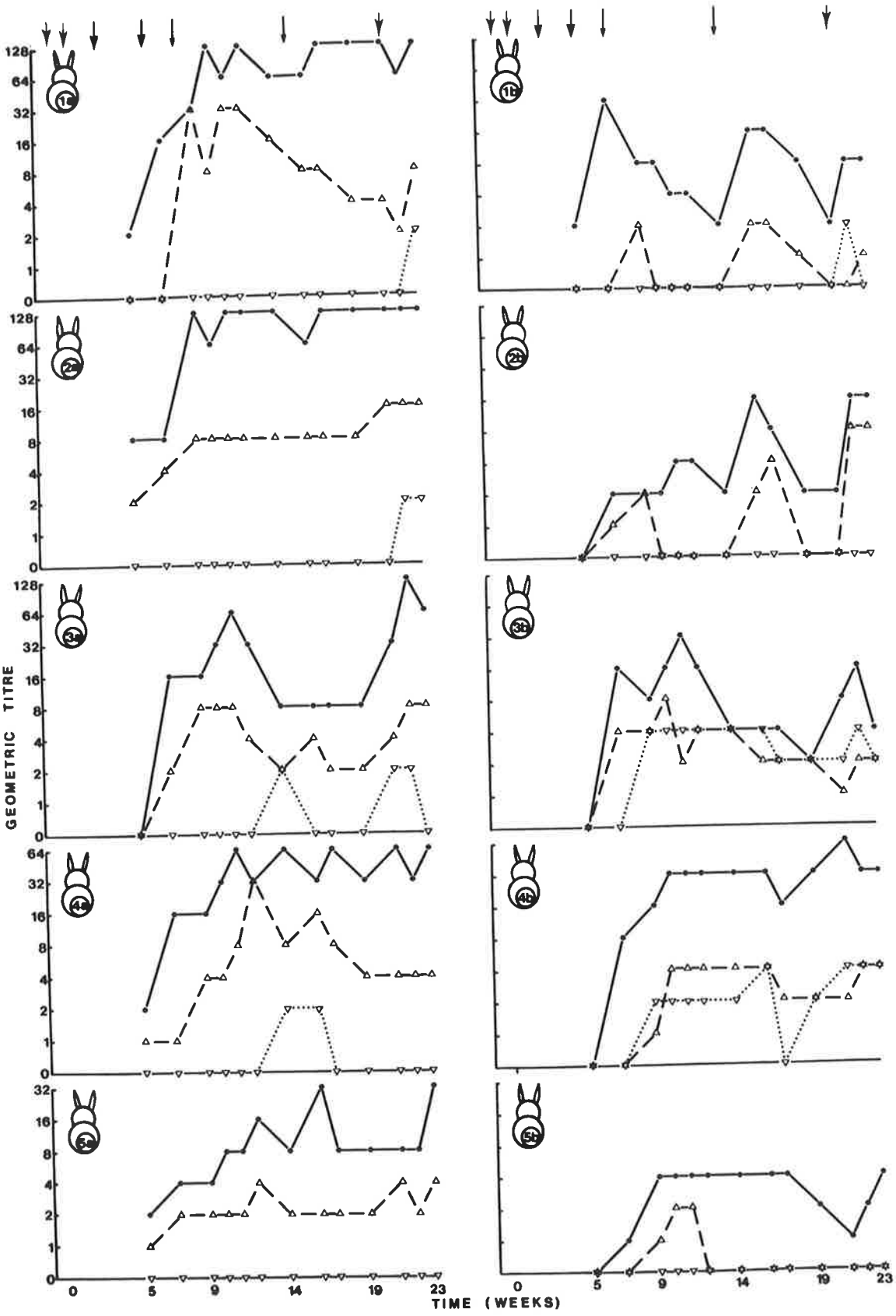
A. Comparison of immunogenicity of native and glutaraldehyde-fixed AMV

1. Using rabbits as experimental animals

One rabbit was immunized with a highly purified preparation of either glutaraldehyde-fixed or native form of each AMV isolate. Each animal was immunized 7 times over a period of 21 weeks using three different routes of injection as described in Chapter 2 and the rabbits were bled at intervals as indicated in Fig. 5-1. The antisera were titrated against 1mg/ml of native, glutaraldehyde-fixed virus, or isolated coat protein preparation in gel-immunodiffusion tests. Antisera against fixed antigen of S30, S40 and H4 reached maximum titres of 1: 128, and against W1 and N20 of 1: 64 and 1: 32, respectively, when tested against their homologous fixed antigens (Fig.5-1, rabbits 1a-5a, respectively). Antisera against the native virus preparations of the same virus isolates reached maximum titres of 1: 2, 1: 4, 1: 8, 1: 4 and 1: 2 when tested against their homologous native antigens (Fig.5-1, rabbits 1b-5b, respectively). When antisera against

Fig. 5-1

Antibody titres in sera from rabbits immunized with glutaraldehyde-fixed virus preparations of AMV S30 (1a), S40 (2a), H4 (3a), W1 (4a), N20 (5a) and native virus preparations of S30 (1b), S40 (2b), H4 (3b), W1 (4b) and N20 (5b) AMV isolates. The antisera were titrated by gel-immunodiffusion against 1mg/ml of homologous native (△– – – – –△), or glutaraldehyde-fixed (•———•) virus and isolated coat protein preparations (▽·····▽). Titration against both native and glutaraldehyde-fixed virus preparations was done in 0.75% agar in 10 mM phosphate buffer, pH 7.6, and against protein preparations in 0.75% agarose in 10mM Na acetate buffer, pH 7.6 containing 100mM $CaCl_2$. Each well was charged with 12 µl of the appropriate reagent and the plates were incubated at 25°C for 5 days before recording the results. Arrows indicate the times and routes of each injection; the first two were administered intravenously, each with 250 µg of virus, the third and fourth subcutaneously, each with 500 µg of virus, the fifth and sixth intramuscularly with 500 µg and 1 mg, respectively, and the last injection was administered intravenously with 500 µg of virus. (The geometric titre is the reciprocal of the maximum antiserum dilution producing a positive reaction).


GEOMETRIC TITRE
TIME (WEEKS)
1c
1t
2c
2t
3c
3t
4c
4t
5c
5t

the glutaraldehyde-fixed antigens were titrated with native virus preparations of their homologous isolates, they showed lower titres. However, when antisera to native virus were titrated with glutaraldehyde-fixed virus of the same isolates, all antisera showed higher titres (Fig.5-1). The results summarised in Fig.5-1 show that antigen fixation enhanced the immunogenicity of all the virus isolates irrespective of whether fixed or native AMV preparations were used as test antigens. As only one rabbit was injected with each antigen, it cannot be concluded that the apparently poor immunogenicity of some of the antigens such as AMV-N20 (Fig.5-1, rabbits 5a and 5b), was significant. It could have been a reflection of the differences in the responses of the rabbits due to their genetic backgrounds, which has been well demonstrated (Van Regenmortel and Von Wechmar, 1970).

Generally, the titres of antisera were higher when the gel-immunodiffusion tests were done in agarose than in agar gels (Table. 5-1). The differences were much greater when native virus preparations were used as test antigens (Table. 5-1). However, the fixed virus was always a better test antigen irrespective of the immunogen used (Fig. 5-1).

All except rabbits 3a and 4b (Fig. 5-1) produced maximum antibody responses 6-14 weeks after the initial injections and further injections failed to increase the antibody titres. Rabbits 3a and 4b produced a maximum response 23 and 21 weeks after immunization, respectively (Fig. 5-1).

When antisera prepared against either native or glutaraldehyde-fixed AMV were titrated with preparations of AMV coat protein, the titres were very low and antisera from some rabbits failed to recognise the coat protein subunits from their homologous viruses (Fig. 5-1, rabbits 2b, 5a and 5b). In some rabbits, antibodies capable of recognising protein subunits of their homologous viruses appeared only after prolonged immunization (Fig. 5-1, rabbits 1a, 1b, and 2a). In yet others, such antibodies appeared intermittently (Fig. 5-1, rabbits 3a, 4a, 4b). Only one of the rabbits (Fig. 5-1, rabbit 3b) produced antibodies which recognised the subunits relatively early during immunization (6 weeks after initial injection) and maintained the titre till the end of the experiment. Although only one rabbit was used for immunization of each antigen the titre of none of the antisera exceeded 1:128 even after glutaraldehyde-fixation of the viral antigen.

Table 5-1 : Comparison of antiserum titres tested against their homologous antigens by gel-immunodiffusion tests using agar and agarose .

		Antisera titres determined in[a] :			
		Agar[b]		Agarose[b]	
Antiserum to :	Weeks after immunization	Fixed Virus	Native Virus	Fixed Virus	Native virus
S30 Fixed virus	10	64	2	128	16
	24	128	4	128	64
S30 Native virus	8	16	-[c]	32	8
	24	8	-	8	4
N20 Fixed virus	17	32	2	64	4
	24	32	4	32	16
N20 Native virus	17	4	-	4	4
	24	4	-	8	4

[a]Reciprocals of maximum dilution of antisera producing visible immunoprecipitin lines when tested against a preparation of their homologous antigens .

[b]0.75 % agar or agarose in 10 mM phosphate buffer, pH 7.6 .

[c]Indicates that no precipitin line was detected in gel-immunodiffusion tests with undiluted antisera .

2. Using chickens as experimental animals

A chicken was immunized with a highly purified preparation of either glutaraldehyde-fixed or native virus of AMV isolates S30 and S40 as described in Chapter 2. Although each chicken received a total of 3.5mg of each immunogen in seven injections over a period of 15 weeks, the maximum titre of the antisera did not exceed 1: 32 when glutaraldehyde-fixed virus of AMV-S40 was used as immunogen. The maximum titre of antiserum from the chicken injected with glutaraldehyde-fixed virus of AMV-S30 was only 1: 4 (Table 5-2). These observations indicate that AMV is also a poor immunogen in chickens.

Table 5-2 : Comparison of reactivity of native and glutaraldehyde-fixed virus preparations of two AMV isolates by gel-immunodiffusion tests using antisera raised in chickens[a].

Antiserum to AMV isolate :	Weeks after immunization	Antiserum titre determined against : Fixed virus	Native virus
S30 Fixed virus	11	4[b]	-[c]
	13	2	2
S30 Native virus	11	4	-
	13	4	4
S40 Fixed virus	11	16	8
	13	8	8
S40 Native virus	11	16	16
	13	32	16

[a] Chickens were immunized over periods of 15 and 17 weeks with antigen from S40 and S30, respectively, as described in Chapter 2. The results in the above table only shows the titration of sera from two bleedings.

[b] Maximum reciprocal of titre in gel-immunodiffusion test using 200µg / ml of homologous antigen.

[c] Indicates that no precipitin line was detected in gel-immunodiffusion tests with undiluted serum.

B. Immunogenicity of isolated viral coat protein using rabbits as experimental animals

The soluble proteins from two AMV isolates (S30 and S40) were prepared as described in Chapter 2 and Appendix **1**. The characteristics of protein preparations used are presented in Appendix **1** (Fig. 1, panel D and Fig. 3, panels B and E). Two rabbits were injected with each of the two AMV protein subunit preparations as described in Chapter 2. The animals were bled at intervals and the sera were titrated against 1mg/ml isolated coat protein, native and glutaraldehyde-fixed virus preparations of their homologous isolates in gel-immunodiffusion tests (Fig. 5-2). All antisera reacted much more strongly with glutaraldehyde-fixed than with native virus preparations irrespective of which isolate was used as test antigen. The AMV coat protein preparations from the homologous virus isolates reacted very weakly, to a maximum titre of only 1:2 with antisera against AMV S30 raised in rabbit 2a, and antisera raised in rabbit 2b failed to react after dilutions (Fig. 5-2). Antisera prepared against S40 (Fig. 5-2, rabbits 1a and 1b) however, reached titres of 1 : 8.

C. Detection of antibodies reacting with protein subunits in antisera from rabbits immunized with native or fixed AMV

All early or late bleeding antisera tested from rabbits immunized with either fixed or native AMV had titres ranging from 1: 4 to 1: 128 when assayed by immunodiffusion tests against preparations of fixed viruses (Tables 5-3 and 5-4). When the same antisera were titrated against native virus, the titres were almost always lower and sometimes failed to react altogether (Tables 5-3 and 5-4). Moreover, the titres were lower still when tested against preparations of viral protein (Tables 5-3 and 5-4). Nevertheless, all the antisera tested in tables 5-3 and 5-4 reacted positively when tested with native or fixed virus, or with viral protein preparation in indirect ELISA (Tables 5-3 and 5-4).

It is interesting to note that when antisera were tested with fixed virus preparations, the titres were usually much higher than with either native virus or viral protein preparations in gel immunodiffusion tests, but the reverse was true in indirect ELISA (Tables 5-3 and 5-4). For example, a serum with a titre of 1: 32 in gel diffusion tests had a

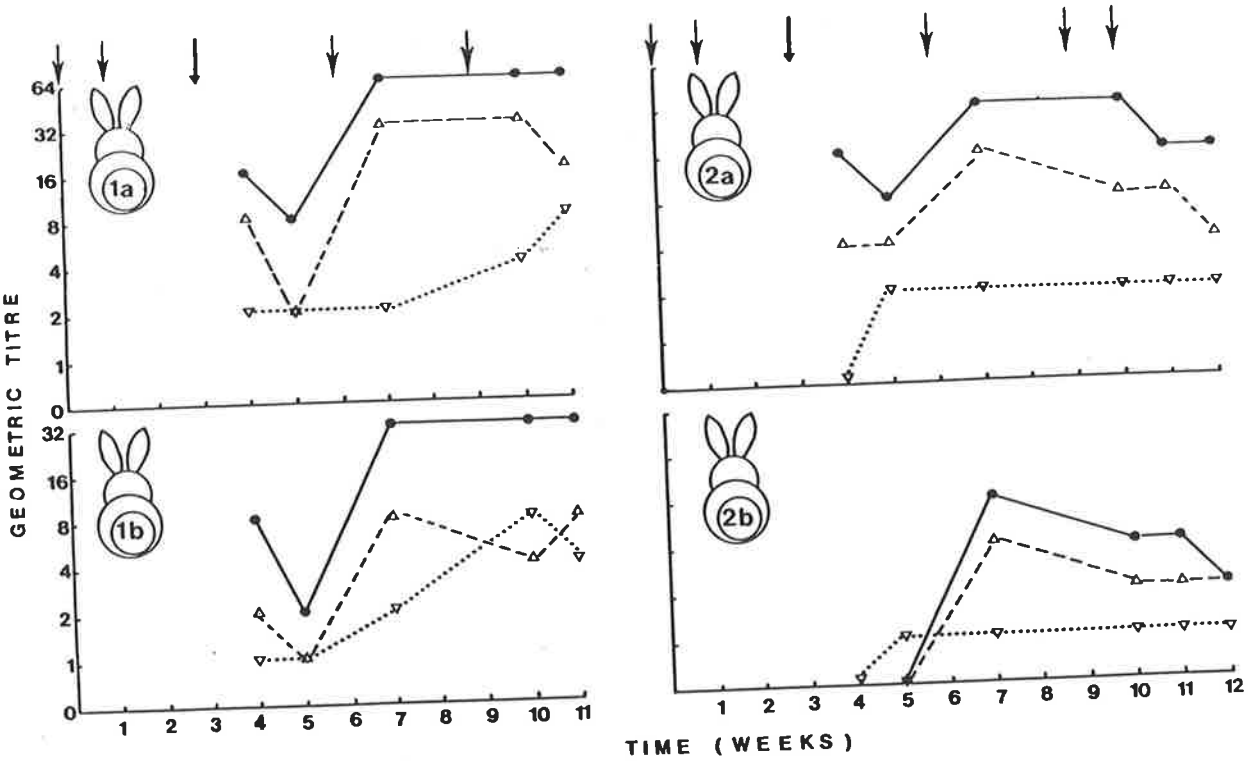


Fig. 5-2

Antibody titres in sera from rabbits immunized with protein preparations from the S40 (1a and 1b) and S30 (2a and 2b) isolates of AMV. The antisera were titrated by gel-immunodiffusion tests against 1mg/ml of homologous native (△_ _ _△) or glutaraldehyde-fixed (•————•) virus or isolated coat protein preparations (▽····· ▽). Titration against virus preparations was done in 0.75% agarose in 10 mM phosphate buffer, pH 7.6, and against protein preparations in 0.75% agarose in 10 mM Na-acetate, pH 7.6, containing 100 mM $CaCl_2$. Each well was charged with 12 µl of the appropriate reagent and the plates were incubated at 25°C for 5 days before recording the results. Arrows indicate the times and route of each injection. Each rabbit was immunized by an initial intravenous injection of 250 µg viral protein in 10 mM Na-acetate, pH 7.6, containing 100 mM $CaCl_2$ and a further four injections of 250, 500, 500 µg, and 2 mg of protein. Rabbits 2a and 2b recieved an additional intravenous injection of 2mg protein, 11 weeks after commencement of immunization. All injections were done interavenously except the third which was administered intramuscularly with an equal volume of Freund's complete adjuvant.

Table 5-3 : Comparison of antigenicity of native, glutaraldehyde-fixed and isolated coat protein preparations of AMV by indirect ELISA and gel-immunodiffusion tests using antisera from early bleedings from immunized rabbits[a].

Antisera against AMV isolates :	Type of immunogen	Weeks after immunization	Test antigen: Fixed antigen ELISA[b]	Fixed antigen Gel-diffusion	Native antigen ELISA	Native antigen Gel-diffusion	Isolated protein ELISA	Isolated protein Gel-diffusion
H4	Fixed virus	11	10^{3}[c]	64[d]	10^{6}	8	10^{7}	-[e]
	Native	11	10^{2}	32	10^{7}	2	10^{7}	4
N20	Fixed virus	16	10^{3}	32	10^{5}	2	10^{6}	-
	Native	16	10^{2}	4	10^{6}	-	10^{7}	-
S30	Fixed virus	9	10^{3}	128	10^{6}	32	10^{6}	-
	Native	7	10^{2}	32	10^{6}	-	10^{7}	-
S40	Fixed virus	9	10^{2}	128	10^{6}	8	10^{6}	-
	Native	16	10^{3}	16	10^{6}	2	10^{6}	-
W1	Fixed virus	11	10^{2}	64	10^{6}	8	10^{7}	-
	Native	10	10^{2}	32	10^{6}	4	10^{7}	2

[a] Early bleeding antiserum is here defined as the first bleeding in which the maximum titre reached or exceeded 1 : 16 (except for N20 native antisera) when tested with glutaraldehyde-fixed virus (see Fig. 5-1 for responses of rabbits during 24 weeks following initial immunization).

[b] Indirect ELISA formats 3, 4 and 5 were done as described in Chapter 2 (Fig.2-1) with the three antigens, respectively, at a concentration of 1µg/ml. For coating of native and glutaraldehyde-fixed virus preparations, 10mM phosphate buffer, pH 7.0 was used. For coating the coat protein preparations, 10 mM Na-acetate, pH 6.0, containing 100 mM $CaCl_2$ was used.

[c] Figures indicate the reciprocal maximum dilution of each antiserum at which the optical density at 405 nm against homologous antigens was below 0.1 after 1 hr substrate hydrolysis at 25°C.

[d] Maximum reciprocal of titre in gel-immunodiffusion tests using 1mg/ml of homologous antigen.

[e] Indicates that no precipitin line was detected in gel-immunodiffusion tests with undiluted antiserum.

Table 5-4 : Comparison of antigenicity of native, glutaraldehyde-fixed and isolated coat proteins of AMV by indirect ELISA and gel-immunodiffusion tests using antisera from late bleedings from immunised rabbits[a].

Antisera against AMV isolates :	Type of immunogen	Weeks after immunization	Test antigen: Fixed virus ELISA[b]	Fixed virus Gel-diffusion	Native virus ELISA	Native virus Gel-diffusion	Isolated protein ELISA	Isolated protein Gel-diffusion
H4	Fixed virus	22	10^{2}[c]	128[d]	10^{7}	8	10^{7}	2
	Native	22	10^{2}	16	10^{6}	2	10^{6}	4
N20	Fixed virus	23	10^{3}	32	10^{6}	4	10^{6}	-[e]
	Native	23	10^{3}	4	10^{6}	-	10^{6}	-
S30	Fixed virus	23	10^{4}	128	10^{6}	8	10^{6}	2
	Native	23	10^{2}	8	10^{6}	1	10^{7}	-
S40	Fixed virus	23	10^{2}	128	10^{6}	16	10^{7}	2
	Native	23	10^{2}	16	10^{6}	8	10^{6}	-
W1	Fixed virus	23	10^{2}	64	10^{6}	4	10^{7}	-
	Native	23	10^{3}	32	10^{6}	4	10^{7}	4

[a] Late bleeding antiserum is here defined as the antiserum which collected 22-23 weeks after initial immunization (see Fig. 5-1, for responses of rabbits during 24 weeks following initial immunization.

[b] Indirect ELISA formats 3, 4 and 5 were done as described in Chapter 2 (Fig.2-1) with the three antigens, respectively, at a concentration of 1µg/ml. For coating of native and glutaraldehyde-fixed virus preparations, 10 mM phosphate buffer, pH 7.0, was used. For coating the coat protein preparations, 10 mM Na-acetate, pH 6.0, containing 100 mM $CaCl_2$ was used.

[c] Figures indicate the reciprocal maximum dilution of each antiserum at which the optical density at 405 nm against homologous antigens was below 0.1 after 1 hr substrate hydrolysis at 25°C.

[d] Maximum reciprocal of titre in gel-immunodiffusion tests using 1mg/ml of homologous antigen.

[e] Indicates that no precipitin line was detected in gel-immunodiffusion tests with undiluted antiserum.

titre of 10^6 in ELISA when native virus was used as test antigen, whereas the same serum had a titres of 1:128 but only 10^3 in gel-diffusion and ELISA, respectively, when tested with fixed virus (Table 5-3, antisera to fixed S30 AMV).

Despite the lack of detectable antibodies reacting with protein preparations in gel-immunodiffusion tests in antisera prepared in rabbit 2b in Fig.5-1, such antiserum, as well as antisera to glutaraldehyde-fixed virus (Fig. 5-1, rabbit 2a) or to isolated coat protein (Fig. 5-2, rabbit 2a) successfully recognised the homologous coat protein subunits in western blots (Fig. 5-3, panels b, c and a, respectively). It is known that antibodies can bind non-specifically to viral proteins with basic domains in western immunoblotting (Dietzgen and Francki, 1987). However, detection of isolated AMV protein by antiserum to glutaraldehyde-fixed virus was specific because the same antisera could not detect the isolated protein of cucumoviruses under similar conditions (Fig. 5-4).

Although antibodies specific to host plant antigens were not detected by gel-immunodiffusion or ELISA in any of the antisera, (Chapter 6) small amounts of such antibodies were detected by western blotting of extracts from uninfected N.clevelandii plants in which all viruses were propagated and from which they were purified (Chapter 2). The antisera to S40 glutaraldehyde-fixed AMV (Fig. 5-3, panel c), to native AMV (Fig.5-3, panel b) and to its isolated coat protein (Fig.5-3, panel a) did recognise traces of host plant antigens. However, different antisera detected antigens with different electrophoretic mobilities (Compare lanes 2 in Fig.5-3, panels a,b and c).

D. Antigenic reactivity of various conformations of AMV as determined by indirect ELISA

In immunodiffusion tests, the glutaraldehyde-fixed virus always reacted at higher dilutions of antisera irrespective of which type of immunogen was used for its production (Fig. 5-1 and 5-2). As the gel-immunodiffusion test is not a sensitive assay method (Van Regenmortel, 1982), the indirect ELISA was used for comparing the reactivity of native and glutaraldehyde-fixed virus and isolated coat protein preparations of each AMV isolate. One set of antisera collected at early, and one at late stages of immunization was used in these experiments. Results of the ELISA experiments which have been compared to those

Fig. 5-3

Detection of AMV coat protein by western blotting with polyclonal antisera. A preparation of coat protein from AMV-S40 (track 1) and a crude preparation of host plant proteins from uninfected Nicotiana clevelandii leaf tissue (track 2) were electrophoresed in 12% polyacrylamide gels and the proteins were transfered to nitrocellulose. Proteins remaining in the gel were stained with silver (panel d) and those transfered to nitrocellulose were probed with antibodies to isolated coat protein (panel a), native virus (panel b) and glutaraldehyde-fixed virus preparations (panel c). The plant protein preparation was obtained by grinding 1 g of N. clevelandii leaf tissue in 1 ml of 10 mM phosphate buffer, pH 7.0, and after centrifugation at 12,000 g for 10 min the supernatant was retained as the protein preparation and mixed with an equal volume of Laemmli buffer. (Samples of 0.5 µg of each viral protein or 0.5 µl of the plant protein preparation were electrophoresed in each track). Track 3 was loaded with prestained protein markers (Sigma).

Fig. 5-4

Specific detection of AMV coat protein by antisera to glutaraldehyde-fixed virus. Preparation of protein from three strains of cucumber mosaic virus (track 1-3), tomato aspermy virus (track 4), the S40 isolate of AMV (track 5) and a crude preparation of host plant protein from uninfected N.clevelandii (track 6) were electrophoresed in a polyacrylamide gel and the proteins were transferred to nitrocellulose. Proteins remaining in the gel were stained with silver (panel A) and those transferred to nitrocellulose were probed with antibodies to glutaraldehyde-fixed AMV isolate (panel B). The plant protein preparation was obtained as described in Fig. 5-3. (Samples of 0.5 µg of each viral protein or 0.5 µl of the plant protein preparation were electrophoresed in each track).

Fig.5_3

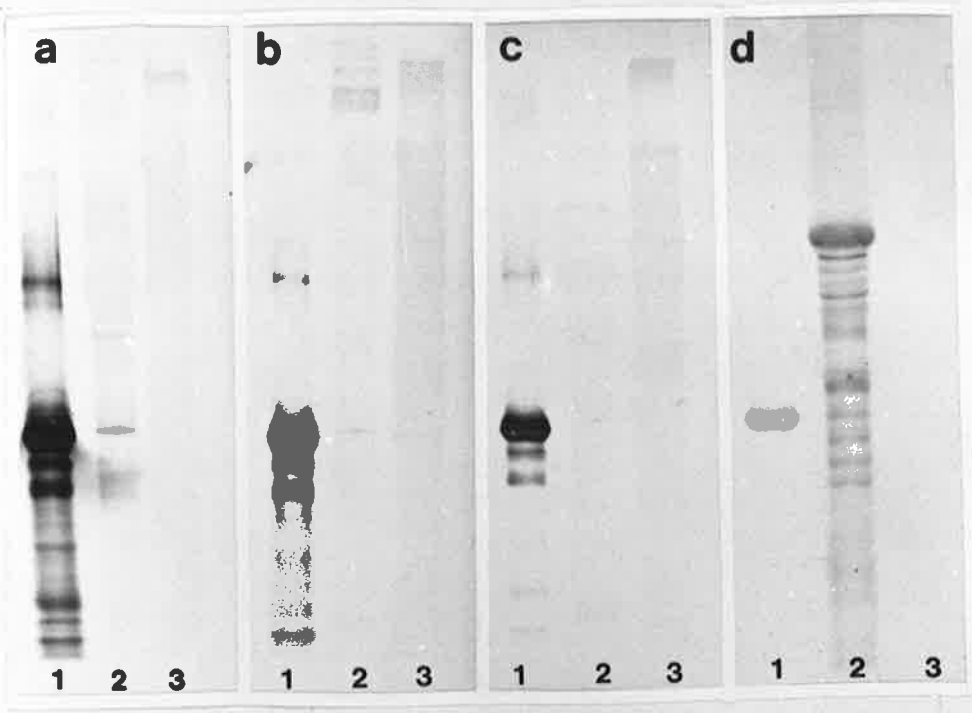
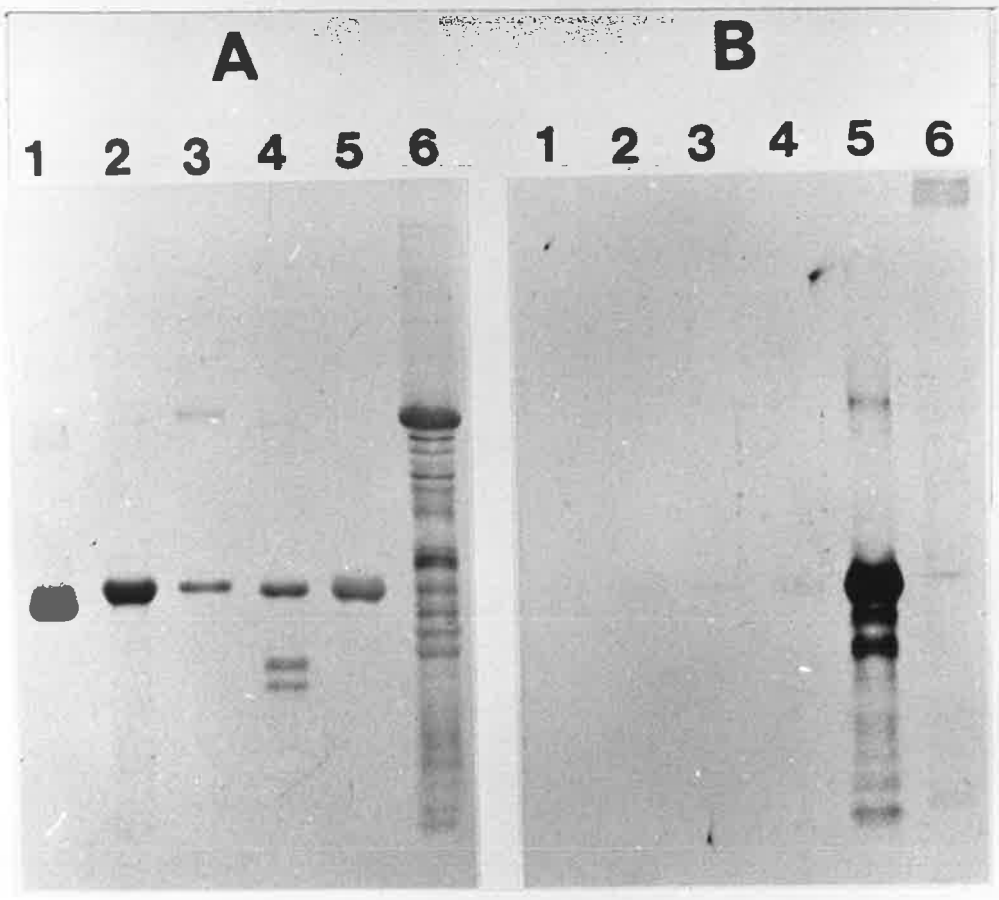


Fig.5_4



obtained from gel-immunodiffusion tests with the same antisera are presented in Tables 5-3 and 5-4. From these data it appears contrary to the results of immunodiffusion tests, that the indirect ELISA titres obtained using fixed virus as test antigen were very much lower than those with either native virus or coat protein preparations. However, antisera to isolated coat protein (Table 5-5) showed higher reactivities in similar ELISA tests than those with antisera to either native or glutaraldehyde-fixed virus preparations (Tables, 5-4 and 5-3). Antisera against isolated coat protein of the S30 isolate raised in rabbits 2a and 2b (Fig. 5-2), showed the same titre in ELISA when tested with native or glutaraldehyde-fixed virus or isolated coat protein (Fig.5-5, C and D). Antisera to isolated coat protein of S40 had similar titres in ELISA when native or glutaraldehyde-fixed virus were used as test antigens, but in similar test they had higher titres with the isolated coat protein preparations (Table 5-5 and Fig. 5-5, A and B). Despite, the similar ELISA titres of the four antisera raised against the coat proteins when tested against native or fixed virus preparations or against isolated coat proteins, the antibody binding was more efficient with native virus or isolated coat protein than the glutaraldehyde-fixed virus (Fig. 5-5).

The observation that the glutaraldehyde-fixed virus reacted weakly in ELISA is in direct contrast to the results obtained from gel-immunodiffusion tests (Fig. 5-1, 5-2 and 5-5 and Tables 5-3, 5-4 and 5-5). It was also surprising to note the higher reactivity of isolated coat protein in indirect ELISA than in gel-diffusion tests (Tables 5-3, 5-4 and 5-5).

E. Specificity of binding of native and glutaraldehyde-fixed antigen to antibodies

Glutaraldehyde-fixed antigens had different reactivities in gel-immunodiffusion tests to those in indirect ELISA. In immunodiffusion tests, fixed virus reacted better than either native virus or isolated coat protein preparations but much less efficiently in indirect ELISA (Fig. 5-1, 5-2 Tables 5-3 and 5-4). The possibility of non-specific binding of fixed virus preparation to antibodies was investigated in experiments by using anti-AMV IgG precoated microtitre wells and ^{35}S-labelled native and glutaraldehyde-fixed virus antigens as follows.

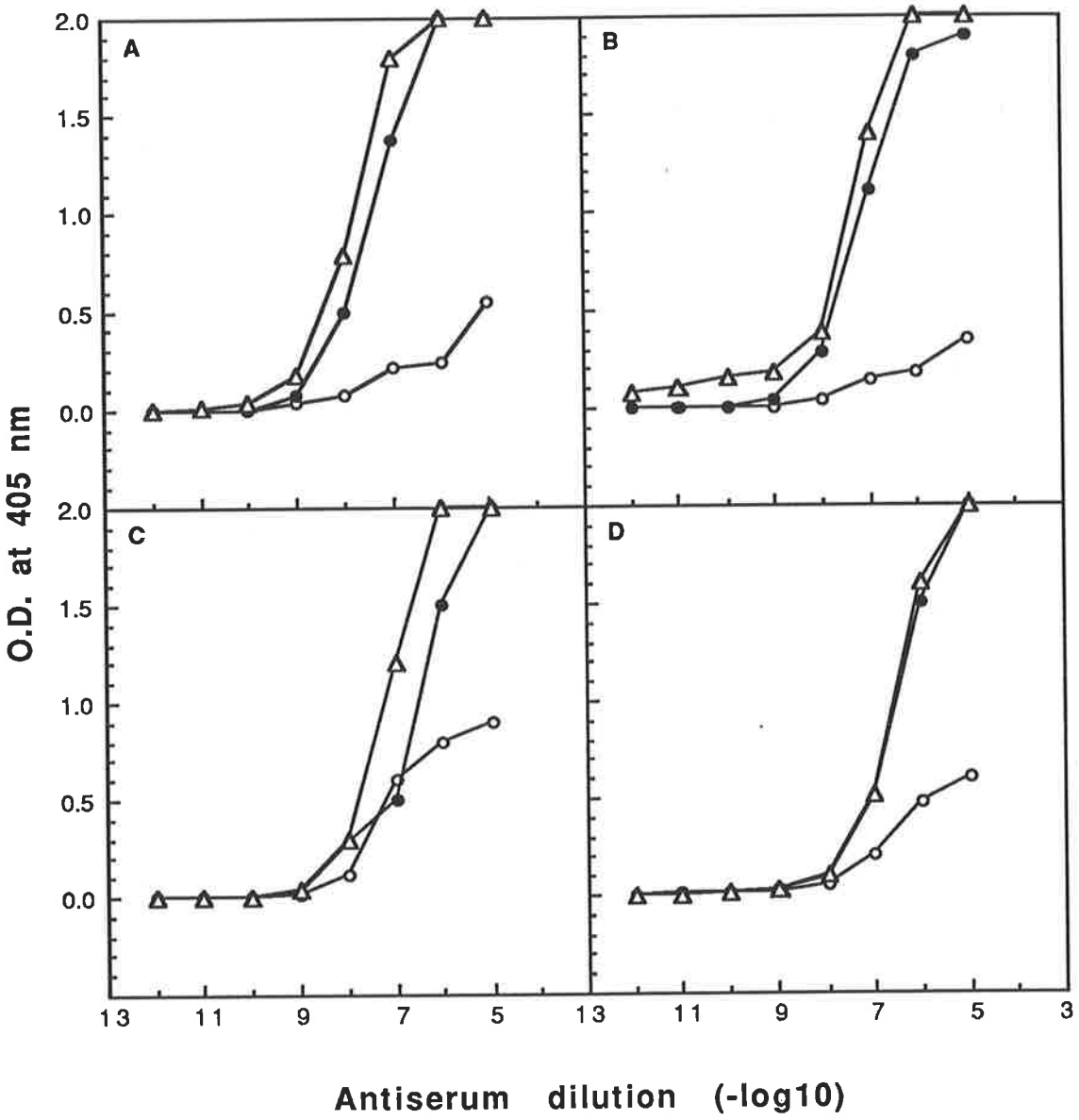


Fig. 5-5

Antibody titres in sera from rabbits immunized with isolated coat protein preparations of S40, rabbits 1a (A) and 1b (B) or S30, rabbits 2a (C) and 2b (D). The antisera were titrated against native (—●—) and glutaraldehyde-fixed virus (—o—) or isolated coat protein preparations (—△—) at concentration of 1 µg / ml using ELISA formats 3, 4 and 5, respectively, as described in Chapter 2 (Fig.2-1). For coating of native or glutaraldehyde-fixed virus preparations, 10 mM phosphate buffer, pH 7.0, and isolated coat protein preparations, 10 mM Na-acetate, pH 6.0, containing 100 mM CaCl2 were used. The substrate was hydrolysed for 1 h at room temperature before recording the results .

Table 5-5 : Comparison of antigenicity of native, glutaraldehyde-fixed and isolated coat protein preparations of AMV by indirect ELISA and gel-immunodiffusion tests with antisera to isolated coat protein[a].

Antisera to AMV isolates:	Rabbit No[b]	Time after immunization	Test antigens: Fixed virus ELISA[c]	Fixed virus gel-diffusion	Native virus ELISA	Native virus gel-diffusion	Isolated protein ELISA	Isolated protein gel-diffusion
S40	1a	11	10^{6}[d]	64[e]	10^{7}	32	10^{8}	8
S40	1b	10	10^{6}	64	10^{7}	8	10^{9}	4
S30	2a	11	10^{7}	16	10^{7}	8	10^{7}	2
S30	2b	11	10^{7}	4	10^{7}	1	10^{7}	1

[a] See Fig. 5-2 for responses of rabbits during the immunization.

[b] 1a, 1b, 2a and 2b are the numbers of the rabbits immunized with isolated coat protein preparations (Fig. 5-1).

[c] Indirect ELISA formats 3, 4 and 5 were done as described in Chapter 2 (Fig. 2-1) with the three antigens, respectively, at a concentration of 1µg/ml. For coating of native and glutaraldehyde-fixed virus preparations, 10mM phosphate buffer, pH 7.0, was used. For coating the coat proteins preparations, 10 mM Na-acetate, pH 6.0, containing 100mM $CaCl_2$ was used.

[d] Figures indicate the reciprocal maximum dilution of each antiserum at which the optical density at 405 nm against homologous antigens was below 0.1 after 1 hr substrate hydrolysis at 25°C.

[e] Maximum reciprocal of titre in gel-immunodiffusion tests using 1mg/ml of homologous antigen.

Anti-AMV-IgGs were purified from two successive bleedings of the same rabbit immunized with a preparation of glutaraldehyde-fixed AMV-W1 (Fig. 5-1, rabbit 4a , 6th and 7th bleedings, respectively). In gel-immunodiffusion tests, the first antiserum had similar titres when tested with either native or glutaraldehyde-fixed virus but, antiserum from the other bleeding reacted with a 2-fold higher dilution with fixed antigen than with native virus (see Fig. 5-1, rabbit 4a). As shown in Fig. 5-6, the glutaraldehyde-fixed virus was trapped by antibodies more efficiently than the native virus irrespective of the type of anti-AMV IgG. The difference in binding of the two forms of antigen to antibodies reflected the results (Fig. 5-6) obtained in gel-immunodiffusion tests (Fig. 5-1, rabbit 4a), that both antigenic forms of AMV reacted similarly with the antiserum from the 6th bleeding, but quite differently with antiserum from the 7th bleeding. The antibody binding was virus-specific because negligible AMV was detected in microtitre wells coated with anti-TMV IgG.

These results suggest that the low ELISA titres observed when glutaraldehyde-fixed virus was used as test antigens could not be due to poor antigen-antibody recognition. Hence further experiments were conducted to find the reason for these observations by measuring the adsorption of glutaraldehyde ^{35}S-labelled antigens to microtitre wells. It was shown that the ability of AMV to adsorb to the microtitre wells was greatly diminished by glutaraldehyde-fixation of the virus and that this accounted for its apparent poor reactivity. The experiments demonstrating this are presented in Appendix 2.

F. The difference in stability of native and glutaraldehyde fixed virus

The results presented in Appendix 2 show that the inability of glutaraldehyde-fixed AMV to adsorb to microtitre plates can account for the apparent poor reactivity in indirect ELISA. As Figs. 5-1 and 5-2 show, the native virus was always a weaker test antigen in gel-immunodiffusion tests despite its better reactivity in indirect ELISA (Tables 5-3, 5-4 and 5-5). It was thought that this could be due to the instability of the native antigen which undergoes degradation during gel-immunodiffusion tests. Temperature or interaction of the antigen with the agar or agarose gel could account for such degradation. The effects of temperature and pH conditions on stability of native and glutaraldehyde-fixed virus

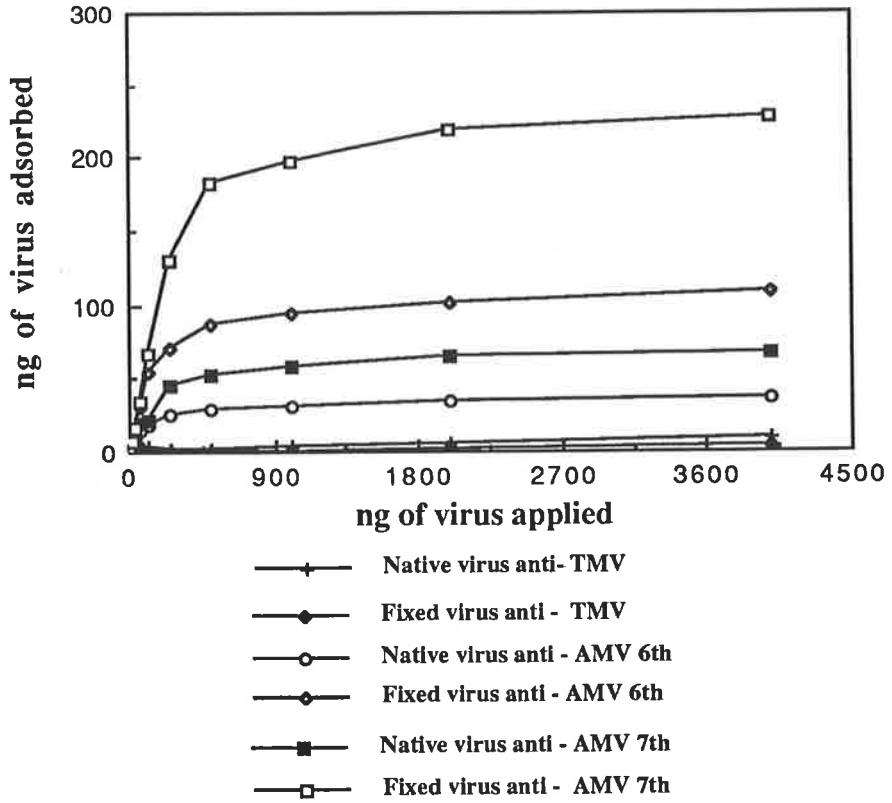


Fig. 5-6:

Specific binding of native and glutaraldehyde-fixed W1-AMV to two antisera obtained from two successive bleedings (6th and 7th bleedings) of a rabbit immunized with a fixed virus preparation of W1-AMV (Fig.5-1, rabbit 4a). Each microtitre well was filled with 300 µl of IgG (2.5µg/µl) in carbonate coating buffer, pH 9.6, and incubated at 25°C for 3 hrs. After washing, increasing amounts of ^{35}S-labelled native or glutaraldehyde-fixed W1-AMV in 10 mM phosphate buffer, pH 7.0, were added to the wells and incubated for 16 hr at 4°C. Wells were washed and the radioactivity determined as described in Chapter 2. The IgG purified from anti-TMV antisera were used as controls.

preparations of all five AMV isolates were already demonstrated in Chapter 4 (Fig. 4-3). It was demonstrated that native AMV was easily degraded when incubated at 25°C in carbonate buffer, pH 9.6, while under similar conditions the glutaraldehyde-fixed virus preparations of all the isolates tested remained intact (Fig. 4-3 compare traces c and a). Signs of degradation also appeared in native virus preparations of all isolates, when incubated at 25°C in 10 mM phosphate buffer, pH 7.0 (Fig. 4-3, traces b and Fig. 7 in Appendix **2**).

The effect of the interaction of both glutaraldehyde-fixed AMV and the native virus of all the isolates in agarose gel was studied by electrophoresis. Native and glutaraldehyde-fixed virus of each isolate was subjected to electrophoresis in 10 mM phosphate buffer, pH 7.0, at 4°C. After electrophoresis, the gel was first stained with ethidium bromide for RNA and then for protein with Coomassie blue. Fig. 5-7 shows that the fixed virus moved faster than the native virus. Two discret components were observed in preparations of the glutaraldehyde-fixed virus of all the isolates when stained either for RNA or protein. However, the native virus preparations of all the isolates migrated as more heterogeneous bands with slower mobility. Preparations of all the native viruses when stained for RNA, showed the presence of smears indicating the degradation of virus particles.

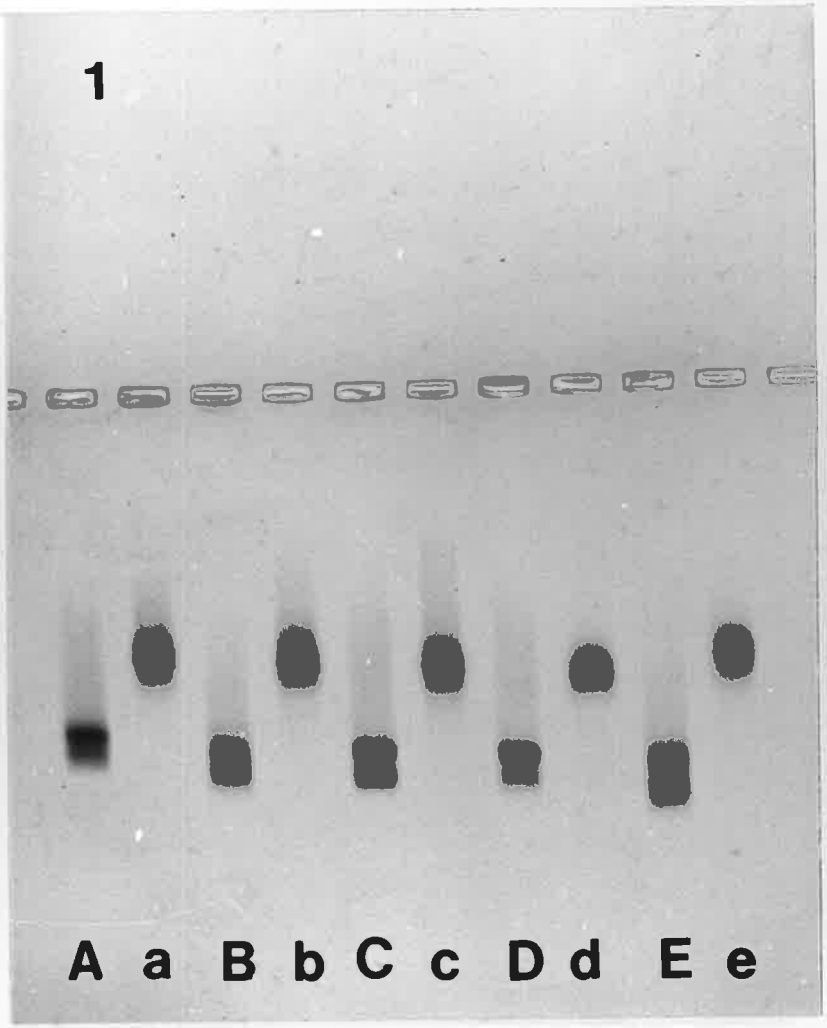
The poor reactivity of native AMV in gel-immunodiffusion compared to glutaraldehyde-fixed AMV may be due to degradation of native AMV, but this cannot be used to explain the better reactivity of fixed antigen with antibodies coating microtitre wells (Fig. 5-6 and Appendix **2** Fig. 4). These experiments were conducted at 4°C, and 10 mM phosphate buffer, pH 7.0, was used for dilution of viral antigen. As shown in Fig 6, traces a, in Appendix **2**, native AMV as well as glutaraldehyde-fixed virus are stable at this temperature.

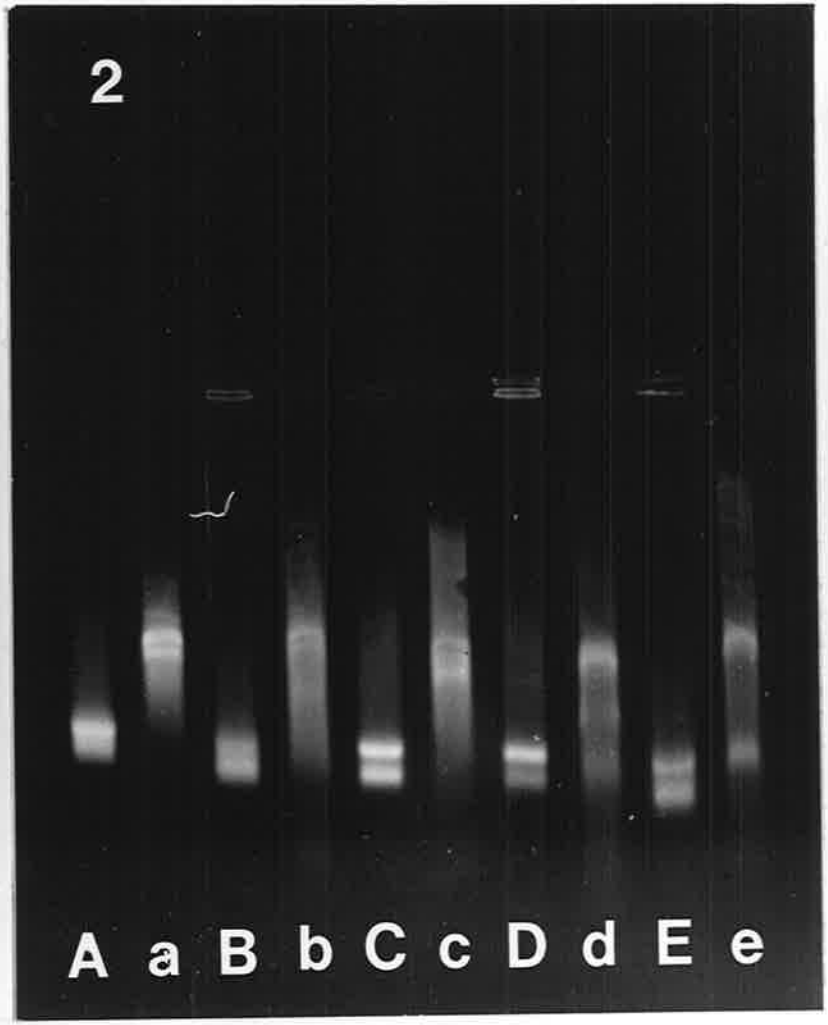
G. The antigenic similarity of native with glutaraldehyde-fixed AMV

It was mentioned in the introduction to this chapter that fixation of an antigen may change its conformation. Although conformational changes have not been observed with fixation of BSA (Habeeb, 1969), the possibility of differentiating native AMV from glutaraldehyde-fixed virus of the same isolate was investigated in gel-immunodiffusion

Fig. 5-7

Agarose gel electrophoresis of native (traces a-e) and glutaraldehyde-fixed virus preparations (traces A-E) of H4, N20, S30, S40 and W1-AMV isolates, respectively. Agarose gel (1.5%) was prepared in 10mM phosphate buffer, pH 7.0. After equilibrating the temperature of the apparatus and electrophoresis buffer to 4°C, 15 µl (15µg) of each preparation was loaded per well and electrophoresis was allowed to proceed at 4°C in 10 mM phosphate buffer, pH 7.0. After 3 hr electrophoresis at 100V, the gel was first stained with ethidium bromide and photographed under ultraviolet light (panel 2). The gel was then stained with coomassie blue, destained until the protein bands appeared as described in Chapter 2, and then photographed (panel 1). Bromophenol blue in 50% glycerol was used as tracking dye.


1
A a B b C c D d E e


2
A a B b C c D d E e

tests using a mixture of their corresponding antisera raised in rabbits. When mixtures of antisera against native or glutaraldehyde-fixed AMV of each isolate was reacted under optimal conditions against native and glutaraldehyde-fixed antigens in separate wells, no spurs were observed (Fig. 5-8). This indicates that fixation did not induce any detectable new epitopes. However, both antisera prepared against the isolated coat protein of AMV-S40 raised in rabbits 1a and 1b (Fig. 5-2), were able to differentiate the glutaraldehyde-fixed AMV from the native form of the same virus isolate (Fig. 5-9, A and B). On the contrary, only one of the antisera against the isolated protein of AMV-S30 (Fig. 5-2, rabbit 2a) could differentiate glutaraldehyde - fixed virus from its native form (Fig. 5-9, C and D). The abilities of these antisera to differentiate these antigens varied when the distances between the reactants in the gel were increased (Fig. 5-10), and only antisera to AMV-S40 raised in rabbit 1b could differentiate glutaraldehyde-fixed virus from its native forms (Fig.5-10, C and D). The increase in the distance between wells may have resulted in greater dilution of reactants (Grogan et al., 1963) and probably the level of differentiative antibody varied in these antisera. Despite the differentiative ability of some of the antisera to isolated protein, the antisera were completely exhausted when they were cross-absorbed with either native or glutaraldehyde-fixed AMV of the same isolate using the intra-gel cross absorption technique (Fig. 5-10, A-F).

All the antisera prepared against native and glutaraldehyde-fixed virus of all the isolates failed to differentiate the glutaraldehyde-fixed AMV from their native forms by formation of spurs(Fig. 5-8, panels 1-5). However, the glutaraldehyde-fixed AMV differed from native AMV by its stronger reactivity in gel-immunodiffusion tests as it always reacted at higher titres of antisera (Fig. 5-1 and 5-2). These results are in contrast with the observations by Habeeb (1969) who reported that formalinization of BSA reduces its binding ability with antibodies to native BSA. However, experiments with indirect ELISA indicate that the fixed AMV was far less reactive (Table 5-3, 5-4 and 5-5). A similar observation was reported with formalinized BMV and such results were interpreted as evidence for change in antigenicity due to the fixation (Rybicki and Von Wechmar, 1981). However, my experiments show that the poor reactivity of glutaraldehyde-fixed AMV in this test may not be due to conformational changes of the antigen which affect

Fig. 5-8

Gel-immunodiffusion tests in agarose between mixtures of antisera to native and glutaraldehyde-fixed virus preparations of AMV isolates W1 (panel 1), N20 (panel 2), H4 (panel 3), S30 (panel 4) and S40 (panel 5) and their homologous fixed (wells A) and native virus preparations (wells a). The antisera wells (FU) were filled with equal volumes (7.5µl) of each antiserum. Antigen wells were filled with 15 µl of 1 mg/ml of the antigens in 10 mM phosphate buffer, pH 7.0. The plates were incubated for 5 days at 25°C before recording the results.

Fig. 5-9

Comparison of antigenic properties of glutaraldehyde-fixed (C, D) and native (c, d) virus preparations of AMV-S40 (panels A and B) and AMV-S30 (panels C and D) using antisera to isolated coat protein. Antisera prepared in rabbits against isolated coat protein of AMV-S40 (1a,1b) and AMV-S30 (2a,2b) were used. Agarose (0.75%) in 10 mM phosphate buffer, pH 7.4, was used. The distances between the antigen wells were 2mm and antigen and antiserum wells 4 mm. Antigens were used at a concentration of 1mg/ml and each well was charged with 15 µl of each reactant. The plates were incubated at 25°C for 5 days before recording the results.

Fig.5_8

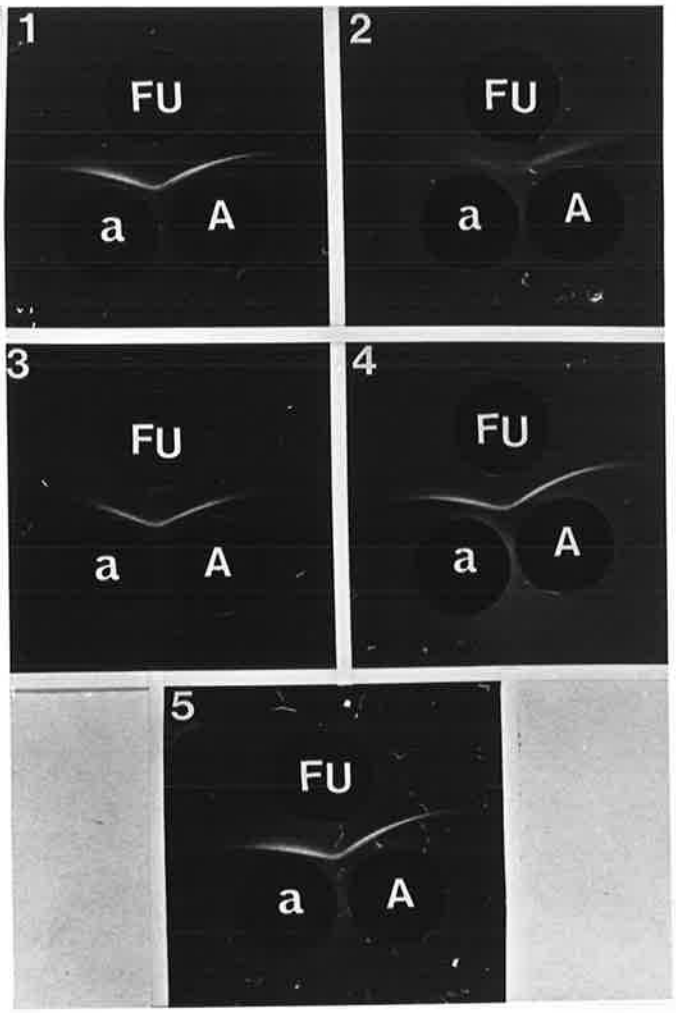
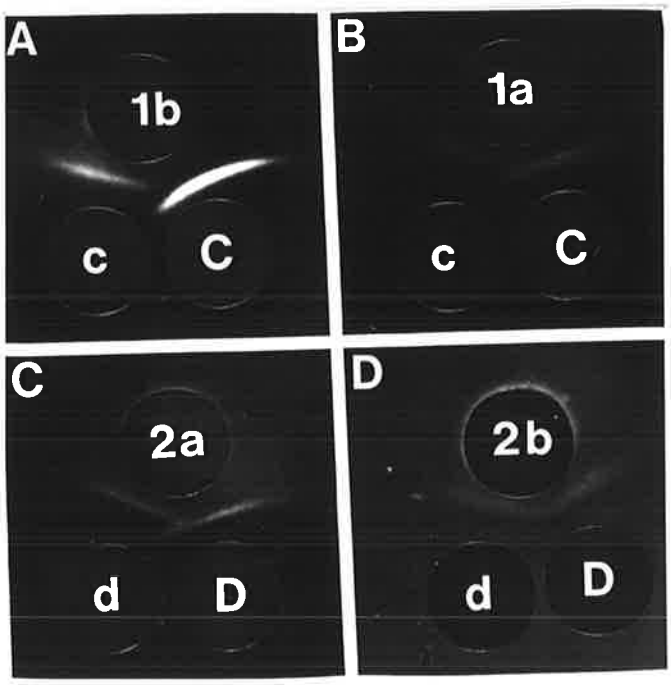


Fig.5_9



antigen-antibody recognition, but rather provide evidence that fixation alters the binding ability of AMV to the microtitre wells (see Appendix 2).

H. Possibility of formation of new epitopes by glutaraldehyde-fixation

It is known that the α-amino group of lysine is the principal side chain of proteins reacting with glutaraldehyde (Habeeb and Hiramoto, 1968; Korn et al., 1972). As AMV coat protein is rich in lysine (Krall, 1975 ; Krall et al., 1976 ; Collot et al., 1976 ; Castle et al., 1979), it was thought that the reaction of glutaraldehyde with these residues may create new epitopes not present on native AMV. However, it was shown that a mixture of antisera to native and glutaraldehyde-fixed AMV placed in the same well could not distinguish these two forms of AMV in gel-immunodiffusion tests (Fig. 5-8). Also, the glutaraldehyde-fixed poly-L-lysine was not recognised by any of the antisera raised against glutaraldehyde-fixed AMV in either rabbits or chickens (data not shown). Furthermore, the intragel cross-absorption of all antisera to glutaraldehyde-fixed AMV with glutaraldehyde-fixed poly-L-lysine did not change the reactivity or the titre of any of the antisera (data not shown). These observations provide evidence that none of the antisera contained antibodies with an ability to recognise fixed poly-L-lysine.

I. Antigenic comparison of isolated coat protein with native and glutaraldehyde-fixed virus

The AMV protein preparations used as immunogens were prepared by the $CaCl_2$ method (Appendix 1), and hence were required to be suspended in 100 mM $CaCl_2$ to remain soluble. Under these conditions, the native virus degraded and the glutaraldehyde-fixed virus precipitated. Consequently it was not possible to study the serological relationship between isolated protein and intact AMV particles in immunodiffusion tests. The procedure of Kelly and Kaesberg, (1962) was used for preparation of virus soluble protein as described in Chapter 2. The protein prepared by this method remains soluble in the presence of 5 mM SDS. This concentration of SDS is believed to have an adverse effect on antigen-antibody binding in ELISA (Halfman et al, 1986) but has been used by Moed and Veldstra, (1968) to reveal different AMV antigenic sites in gel-immunodiffusion tests.

When antisera to isolated coat proteins was used to compare coat protein preparation of AMV-S40 with its homologous native virus in gel-immunodiffusion tests, both antigens were shown to share common antigenic determinants (Fig. 5-11, panel A). Such epitopes which are present on both polymerised and non-polymerised proteins are known as metatopes (Van Regenmortel, 1982). When the comparison was made with antiserum from rabbit 1a (Fig. 5-2), there was a spur between the two antigenic forms of AMV indicating that the native virus possesses unique antigenic determinants which were not present on the isolated coat protein (Fig. 5-11, panel A). Such determinants which are present only on polymerised protein but not on isolated protein are known as neotopes (Van Regenmortel, 1982). A similar test with antisera from another rabbit (Fig.5-2, rabbit 1b) also produced a spur (Fig. 5-11, panel A). However, the spur indicated the reaction of antibodies with the isolated coat protein but not with the native virus preparations. Such antigenic determinants which are only present on the isolated protein, but are masked after polymerisation are known as cryptotopes (Jerne, 1960).

The antiserum from rabbit 1a (Fig. 5-2) showed the same reactivity when the glutaraldehyde - fixed AMV was replaced by native virus (Fig. 5-11, panel B) revealing two types of epitopes, metatopes and neotopes. But antiserum from rabbit 1b (Fig. 5-2) could no longer reveal the presence of cryptotopes (Fig. 5-11, panel B).

III Conclusion

The results presented in this Chapter enable the following conclusions to be drawn :

1 -The native AMV is poorly immunogenic and glutaraldehyde-fixation enhanced its immunogenicity .

2 - Antibodies recognising epitopes on native and fixed AMV particles, as well as on isolated coat protein subunits, were elicited in rabbits irrespective of which of the above antigens were used as immunogen.

3 - In immunodiffusion tests, titres of antisera prepared against the three immunogens were highest when fixed AMV and lowest when viral coat protein subunit preparations were used as test antigen. The titres were intermediate when tested against native virus preparations. It appears that the strong reactivity of glutaraldehyde-fixed virus

Fig. 5-10

Comparison of antigenic properties of glutaraldehyde-fixed (F) and native (U) AMV-S40 (panels A,B,C and D) and AMV-S30 (panels E and F) in gel-immunodiffusion tests. The antisera raised against isolated coat protein of AMV-S40 (rabbits 1a and 1b) and AMV-S30 (rabbits 2a) were used. Each antiserum was used unadsorbed (1a,1b and 2a) or after intragel cross-absorption with either glutaraldehyde-fixed (1aF,1bF and 2aF) or native (1aU,1bU and 2aU) preparations of the same virus isolate. For intra-gel cross-absorption, 15 µl of 2mg/ml of the appropriate antigen was added to the well and incubated at 25°C for 16 hr before recharging the wells with antiserum. The antigen wells were charged with 15µl of 0.5 mg/ml of the appropriate antigen. Agarose (0.75%) was prepared in 10 mM phosphate buffer, pH 7.6. The antigen wells were 10 mm apart from each other and 6 mm from the antiserum wells. The plates were incubated at 25°C for 5 days before recording the results.

Fig. 5-11

Comparison of antigenic properties of isolated coat protein preparation (P) with native virus (U) (panel A) and glutaraldehyde-fixed virus (F) (panel B) of AMV-S40 in gel-immunodiffusion tests. Undiluted antisera to isolated coat protein of AMV-S40 raised in rabbits 1a and 1b were used for all comparisons. Agarose gel (1%) was prepared in 10 mM phosphate buffer, pH 7.6 and coat protein was isolated by the method of Kelly and Kaesberg, (1962) as described in Chapter 2. Tests antigens were used with concentrations of 1mg/ml and each well was filled with 15µl of each reactant. Plates were incubated at 25°C for 5 days before recording the results.

Fig.5_10

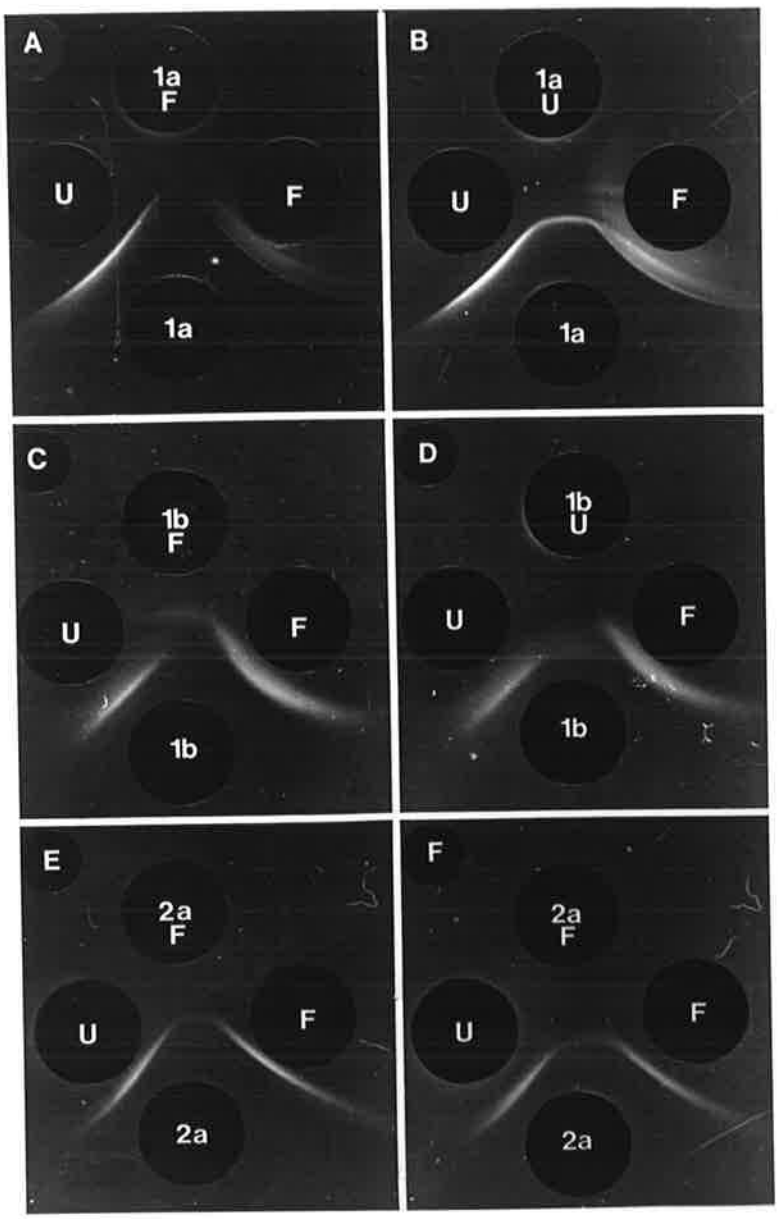
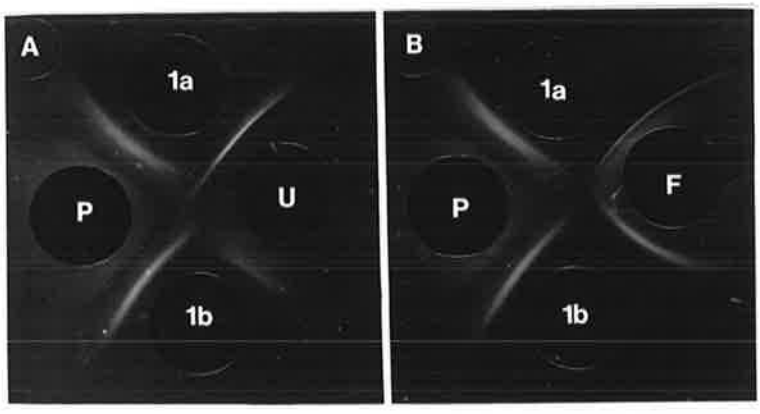


Fig.5_11



is due to its enhanced stability whereas native virus is prone to at least particle degradation during the tests.

4 - Contrary to the results in immunodiffusion tests, in indirect ELISA, highest titres were observed using coat protein and lowest with glutaraldehyde-fixed virus preparations as test antigens. This was shown to be due to the impaired binding to ELISA microtitre plates of virus following fixation (see also Appendix 2). It was shown that fixation actually enhanced the binding of AMV to specific antibodies.

5 - Antisera elicited to native and glutaraldehyde-fixed AMV as well as isolated coat protein preparations recognised AMV coat protein in western blots.

6 - As observed by Rybicki and Von Wechmar (1981), antisera to bromoviruses were difficult to titrate against coat protein preparations in immunodiffusion tests and they recommended that they were better done by indirect ELISA. The experiments described in this chapter confirms this conclusion.

7 - Immunodiffusion tests with antisera to the isolated coat protein preparations revealed the presence of metatopes, cryptotopes and neotopes on various antigenic forms of AMV. This was in agreement with the observation of Moed and Veldstra, (1968).

8 - Tremaine and Chidlow, (1974) have pointed out that if particles of a virus and its protein subunits share related epitopes, it can not be assumed that the antibodies reacting with the subunits were completely protein-induced or that the antibodies reacting with the intact virus particles were completely particle induced. Results presented in this chapter support this concept.

9 - Antisera to native or glutaraldehyde-fixed virus failed to differentiate these two antigenic forms of AMV, but antisera to isolated coat protein reacted with epitopes present on fixed antigen that could not be detected on native virus particles.

Chapter 6

Attempts to differentiate biologically distinct isolates of AMV with polyclonal antisera

I Introduction

It has been generally accepted that isolates of AMV which differ widely in pathogenicity or geographical origin are serologically very similar (Bancroft et al., 1960; Tomaru et al., 1968; Paliwal, 1982; Walter and Kuszala, 1985; Hiruki and Miczynski, 1987), and there are no reports of AMV isolates that are serologically easily distinguishable (Jaspars and Bol,1980). However, serological evidence for strain differences between AMV isolates in the form of precipitin "spurs" in gel-immunodiffusion tests has been reported (Van Vloten-Doting et al., 1968; Roosien and Van Vloten-Doting, 1983). In contrast to other workers who prepared their antisera against native virus particles (Bancroft et al., 1960; Tomaru et al., 1968; Paliwal, 1982; Walter and Kuszala, 1985; Hiruki and Miczynski,1987), this differentiative antiserum was prepared by immunization of rabbits with virus which had been heated for 1 hr at 30°C before injection (Van Vloten-Doting et al, 1968).

It has been shown in Chapter 5 of this thesis, and also by Moed and Veldstra, (1968) as well as Halk (1986), that the antigenic structure of AMV, like that of TMV and BMV (Von Wechmar and Van Regenmortel, 1968; Van Regenmortel and Lelarge, 1973), can expose at least three different types of conformational antigenic determinants, **neotopes, metatopes** and **cryptotopes**. The properties of the antisera which were prepared against glutaraldehyde-fixed, native and isolated coat protein of different AMV isolates were described in the previous Chapter. It was shown that the antisera exhibited different reactivities against various antigenic conformations of AMV in gel-immunodiffusion and indirect ELISA tests (Chapter 5).

In this Chapter are presented the results of comparative serological studies on five biologically distinct AMV isolates using their different antigenic conformations.

II Experimental

A. Use of polyclonal antisera to glutaraldehyde fixed-virus

1. Gel-diffusion

It was shown that fixation of virus particles with glutaraldehyde enhanced the immunogenicity of all AMV isolates (Chapter 5, Fig. 5-1). When antiserum from each immunized rabbit collected at a late stage of immunization (Table 5-4) was used for serological comparisons of the AMV isolates, no specificity was observed and none of the antisera were capable of differentiating heterologous from homologous virus isolates as judged by spur formation. This was irrespective of whether fixed or native virus preparations were used as test antigens (Figs. 6-1 and 6-2, panel A). However, titration of antisera against homologous and heterologous virus isolates showed some minor differences, nevertheless the serological differentiation index (SDI) did not exceed 1 (Tables 6-1 and 6-2).

It is known that early bleeding antisera are usually more specific than those obtained after prolonged immunization (Tremaine and Wright, 1967; Kassanis and Phillips, 1970; Crumpton, 1974; Shepard et al., 1974; Van Regenmortel, 1982). To determine if antisera to fixed AMV collected at early stages of immunization possessed any differentiative ability, one antiserum from an early bleeding of each immunized rabbit (Table 5-3) was also selected for comparing the five AMV isolates. It was shown that the specificity of the antisera against fixed antigen was not influenced by time after immunization as both antisera collected at early or late bleedings exhibited the same properties and were not able to differentiate any of the heterologous virus isolates (Fig. 6-1, Tables 6-1 and 6-2). This was judged by the lack of any spur formation between homologous and heterologous antigens (Fig. 6-1) and the failure of SDIs to exceed 2 (Tables 6-1 and 6-2).

As the antisera to fixed viruses had low titres when tested against viral protein preparations in gel-immunodiffusion tests (Fig. 5-1), they were not useful for comparative serology of the AMV isolates using their corresponding coat protein preparations as test antigens.

Fig. 6-1

Immunodiffusion tests in agarose gels between antisera to glutaraldehyde-fixed (F) and native (U) virus preparations of AMV-S30 and preparation of 0.5 mg/ml of fixed virus (capital letters) and native virus (small letters) of S30 (A,a), S40 (B,b), N20 (C,c), H4 (D,d) and W1(E,e) AMV isolates. Well H was filled with a concentrated protein preparation from healthy N. clevelandii plants. Early bleeding antisera against fixed virus were diluted 1: 8 and 1: 4 when used for testing of fixed and native virus preparations, respectively. Late bleeding antisera against fixed virus were diluted 1: 32 and 1: 8 for testing of fixed and native virus preparations, respectively. Early bleeding antisera aginst native virus were always diluted 1: 2 irrespective of the type of test antigen. The wells were charged with 15 µl of each reactant and the plates were incubated at 25°C for 5 days before recording the results.

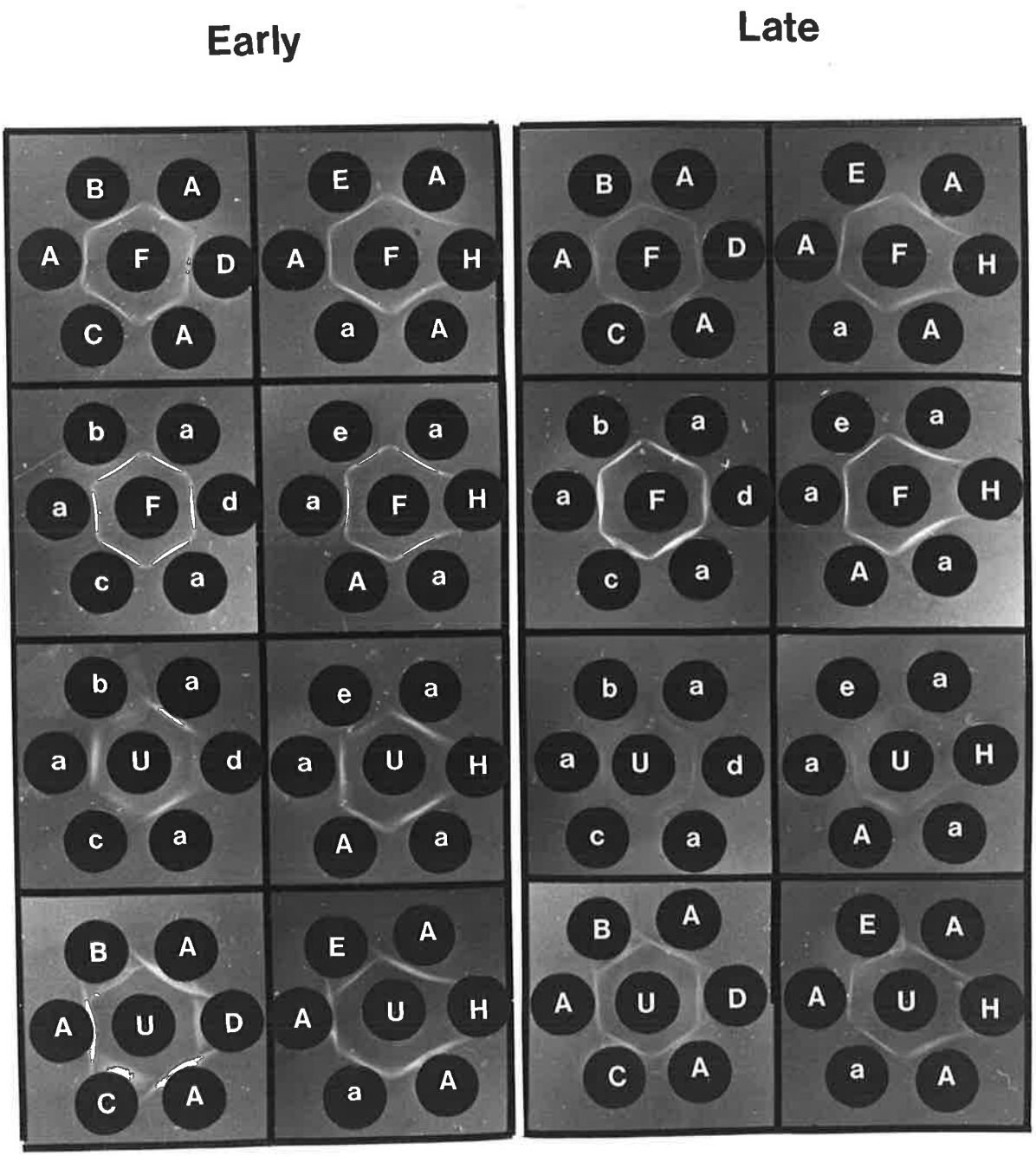

Early
Late
B A A F D C A
E A A F H a A
b a a F d c a
e a a F H A a
b a a U d c a
e a a U H A a
B A A U D C A
E A A U H a A

Fig. 6-2

Immunodiffusion tests in agar gels between antisera to glutaraldehyde-fixed (panel A) and native virus (panel B) preparations of S30 AMV and preparations of 1 mg/ml of homologous fixed virus (a) and preparations of glutaraldehyde-fixed virus preparations of the H3 (b), N20 (c), N3 (d), LA (e), NI (f), H2 (g), S40 (h), H4 (i), H5 (j), HI (k), and W1 (l) AMV isolates (see Chapter 3, Table 3-4 for details of virus isolates). Well m contained an antigen preparation from uninfected N. clevelandii. Antiserum against glutaraldehyde-fixed virus was diluted 1 : 8 and antiserum to native AMV was used undiluted. Wells were filled with 15 µl of each reactant and incubated for 5 days at 25°C before recording the results.

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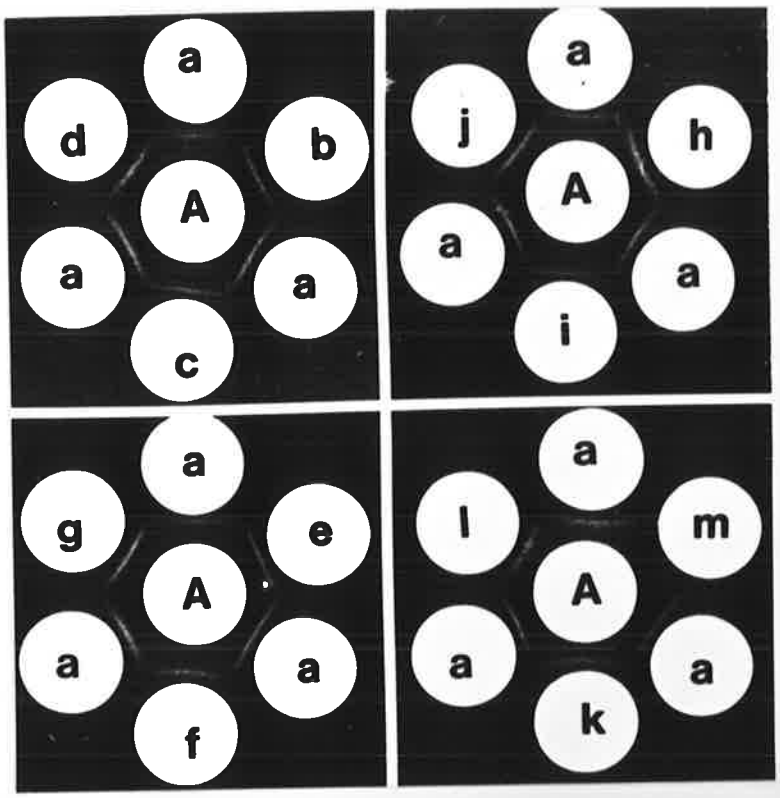
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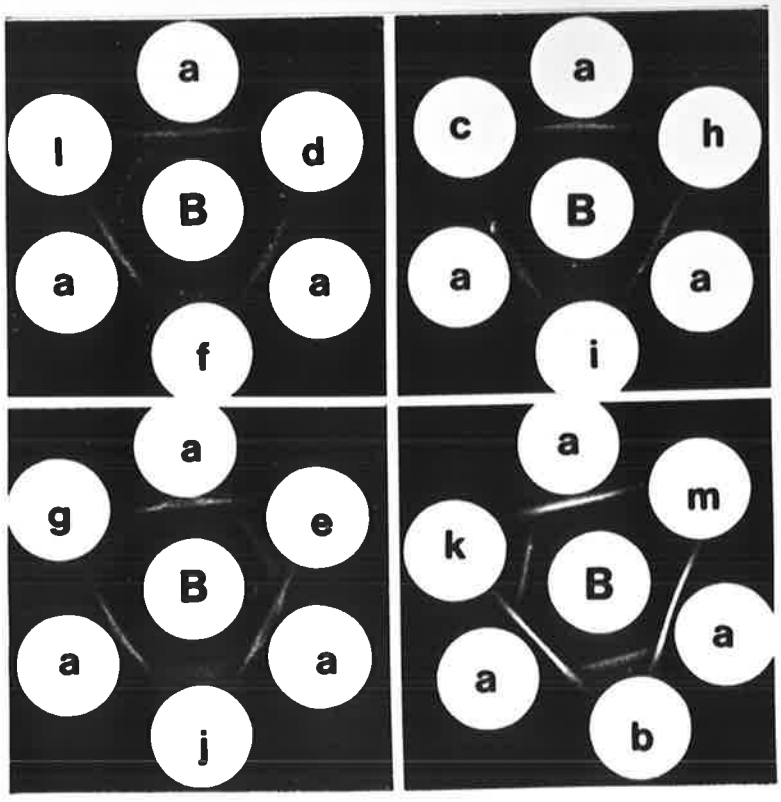
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Table 6-1: Homologous and heterologous titres determined by gel-immunodiffusion of AMV isolates using antisera to glutaraldehyde-fixed virus particles and fixed virus preparation as test antigens[a].

Antisera to fixed virus of :		Antigen (glutaraldehyde-fixed virus particles)				
		H4	N20	S30	S40	W1
H4	Early[b]	64[c]	64	64	64	64
	Late	64	64	64	64	64
N20	Early	32	32	32	32	32
	Late	64	64	32	64	32
S30	Early	64	64	64	64	64
	Late	128	128	128	128	128
S40	Early	128	128	128	128	128
	Late	256	256	256	256	256
W1	Early	64	64	64	32	64
	Late	64	64	64	64	64

[a]Homologous and heterologous virus preparations were used at concentrations of 200 µg/ml. Antisera and antigens were diluted in 10 mM phosphate buffer, pH 7.0. Wells were charged with 15 µl of each reactant and plates were incubated for 5 days at 25°C before recording the results.

[b]Early and late bleeding antisera are as defined in Tables 5-3 and 5-4.

[c]Reciprocals of maximum antiserum dilution producing a visible immunoprecipitin line. Homologous titres are underlined.

2. Indirect ELISA

For serological comparison of AMV isolates using indirect ELISA antisera from early stages of immunization only were used. As native virus particles of AMV readily degrade in carbonate buffer, pH 9.6, (Appendix 2), 10 mM phosphate buffer, pH 7.0, was used as coating buffer. It was shown that the native virus preparations of the different AMV

Table 6-2: Homologous and heterologous titres determined by gel-immunodiffusion of AMV isolates using antisera to glutaraldehyde-fixed virus particles and native virus preparations as test antigens[a].

Antisera to fixed virus of :		Antigen (native virus preparation)				
		H4	N20	S30	S40	W1
H4	Early[b]	16[c]	8	4	4	4
	Late	16	8	8	16	8
N20	Early	4	4	4	4	4
	Late	8	8	8	8	8
S30	Early	8	8	8	8	8
	Late	16	16	16	16	16
S40	Early	16	16	16	16	16
	Late	32	32	32	32	32
W1	Early	8	8	8	8	8
	Late	8	8	8	8	8

[a]Homologous and heterologous virus preparations were used at concentrations of 200 µl/ml. Antisera and antigens were diluted in 10 mM phosphate buffer, pH 7.0. Wells were charged with 15µl of each reactant and plates were incubated for 5 days at 25°C before recording the results.

[b]Early and late bleeding antisera are as defined in Tables 5-3 and 5-4.

[c]Reciprocals of maximum antiserum dilution producing a visible immunoprecipitin line. Homologous titres are underlined.

isolates exhibited different binding properties to ELISA microtitre plates which gave false indications of serological differences between the AMV isolates (Appendix 2). Furthermore, the glutaraldehyde-fixed virus was not able to adsorb efficiently to the microtitre plates in 10 mM phosphate buffer, pH 7.0, (Tables 5-3, 5-4 and Appendix 2). As both native and glutaraldehyde-fixed AMV could be efficiently adsorbed to the ELISA plates in carbonate buffer, pH 9.6, (see Appendix 2), this buffer was used for adsorption of viral antigen. However, for adsorption of isolated coat protein preparations to the ELISA plates, 10 mM Na-acetate, pH 6.0, containing 100mM $CaCl_2$ was found to be

satisfactory (Appendix 2) and was therefore used as coating buffer. Preparations of native and glutaraldehyde-fixed virus of tobacco ringspot virus (TRSV) were used as negative controls in these experiments.

In indirect ELISA as in the gel-immunodiffusion tests, none of the antisera differentiated glutaraldehyde-fixed of homologous from heterologous virus isolates suggesting that all isolates are antigenically similar (Fig. 6-3, 1A-5A). When native virus preparations were used as test antigens, antisera to H4, N20 and S40 AMV failed to differentiate any of the virus isolates (Fig. 6-3, 1B, 2B and 4B). However, antisera to S30 AMV did differentiate this isolate from the four heterologous isolates (Fig. 6.3, 3B) and antiserum to W1-AMV differentiated W1-AMV from H4, N20 and S30 AMV but not from S40 AMV (Fig. 6-3, 5B).

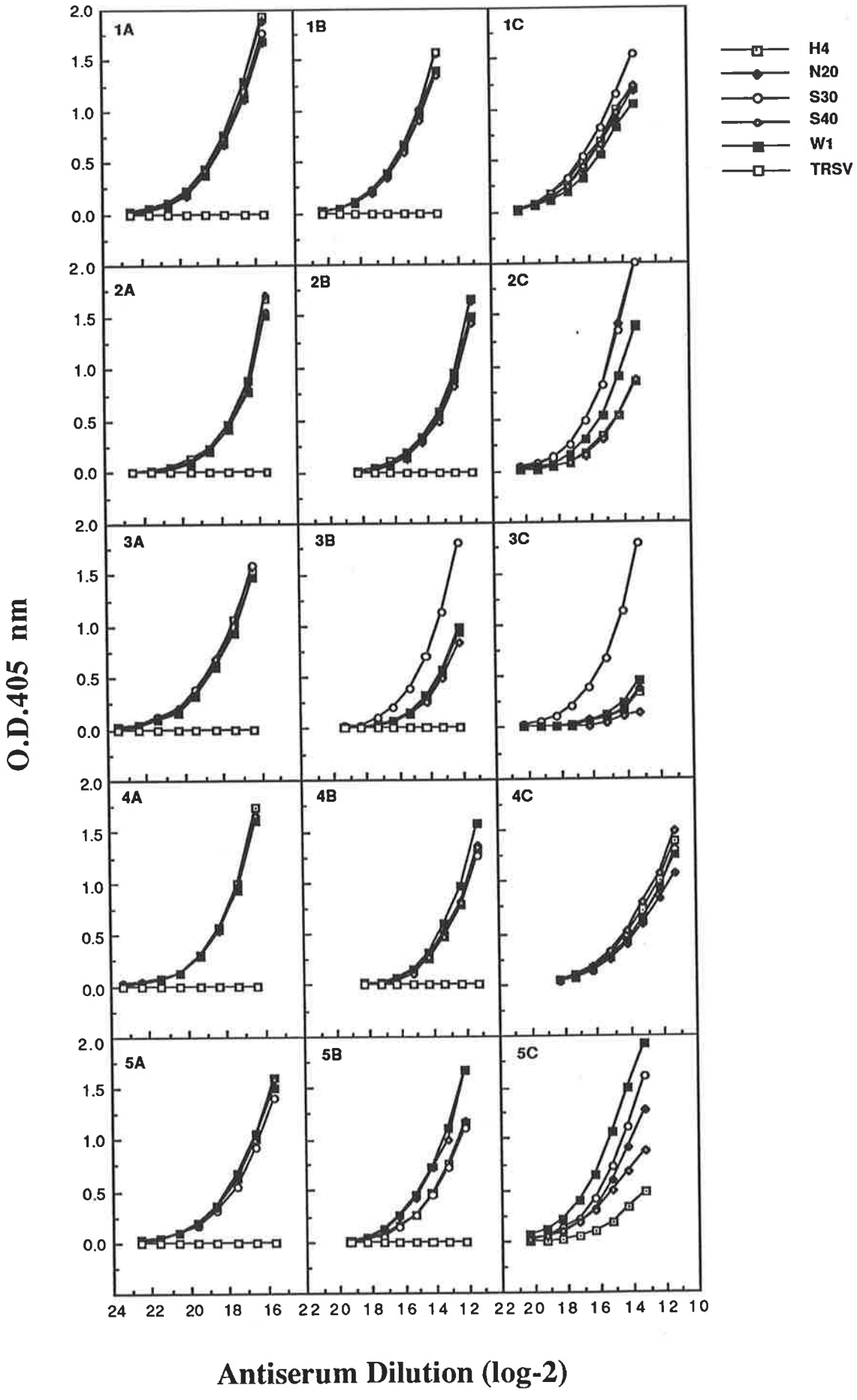
Using isolated coat protein preparations as test antigens, antisera to AMV-S30 and W1 differentiated all heterologous isolates (Fig. 6-3, 3C and 5C). Antisera to AMV-N20 could differentiate all heterologous protein preparations except the S30 isolate (Fig. 6-3, 2C). Antisera to H4 and S40 were not as differentiative as other antisera (Fig. 6-3, 1C and 5C).

3. DAS-ELISA

This test is known to be strain specific with the ability of differentiating closely related viruses from each other (Van Regenmortel , 1982). However, when it was used for serological comparison of AMV isolates using glutaraldehyde-fixed virus as test antigen, it failed to establish any significant differences between homologous and heterologous viruses, despite using early bleeding antisera (Fig. 6-4). The antisera to glutaraldehyde-fixed virus of all the five isolates not only failed to differentiate the glutaraldehyde-fixed virus preparations of these isolates, but they also failed to establish significant serological differences between the homologous and heterologous preparations of glutaraldehyde-fixed virus of six other biologically distinct AMV isolates (Fig. 6-4, a-e) which were characterised in Chapter 3.

Fig. 6-3

Serological comparison of AMV isolates using glutaraldehyde-fixed (A) and native (B) virus preparations and isolated coat protein (C) of AMV isolates by indirect ELISA (formats 5, 3 and 4, respectively, see Fig. 2-1 for description of ELISA). Antisera were prepared against glutaraldehyde-fixed virus preparations of the H4 (1), N20 (2), S30 (3), S40 (4) and W1 (5) AMV isolates. For coating of native and glutaraldeyde-fixed virus preparations, carbonate buffer, pH 9.6, and for coating of isolated coat protein 10 mM Na-acetate, pH 6.0, containing 100 mM $CaCl_2$ were used. [A preparation of tobacco ringspot virus (TRSV) was used as control antigen in A and B]. Native and glutaraldehyde-fixed virus preparations were used at concentrations of 0.5 mg/ml. The coat proteins were used at concentrations of 50 ng/ml with antisera to H4, S40 and N20, and 25 ng/ml with antisera to the S30 and W1 AMV isolates. In all tests, a preparation of 200 µl of each antigen was applied per microtitre well and the tests were done as described in Chapter 2.


1A
1B
1C
2A
2B
2C
3A
3B
3C
4A
4B
4C
5A
5B
5C
H4
N20
S30
S40
W1
TRSV
O.D.405 nm
Antiserum Dilution (log-2)

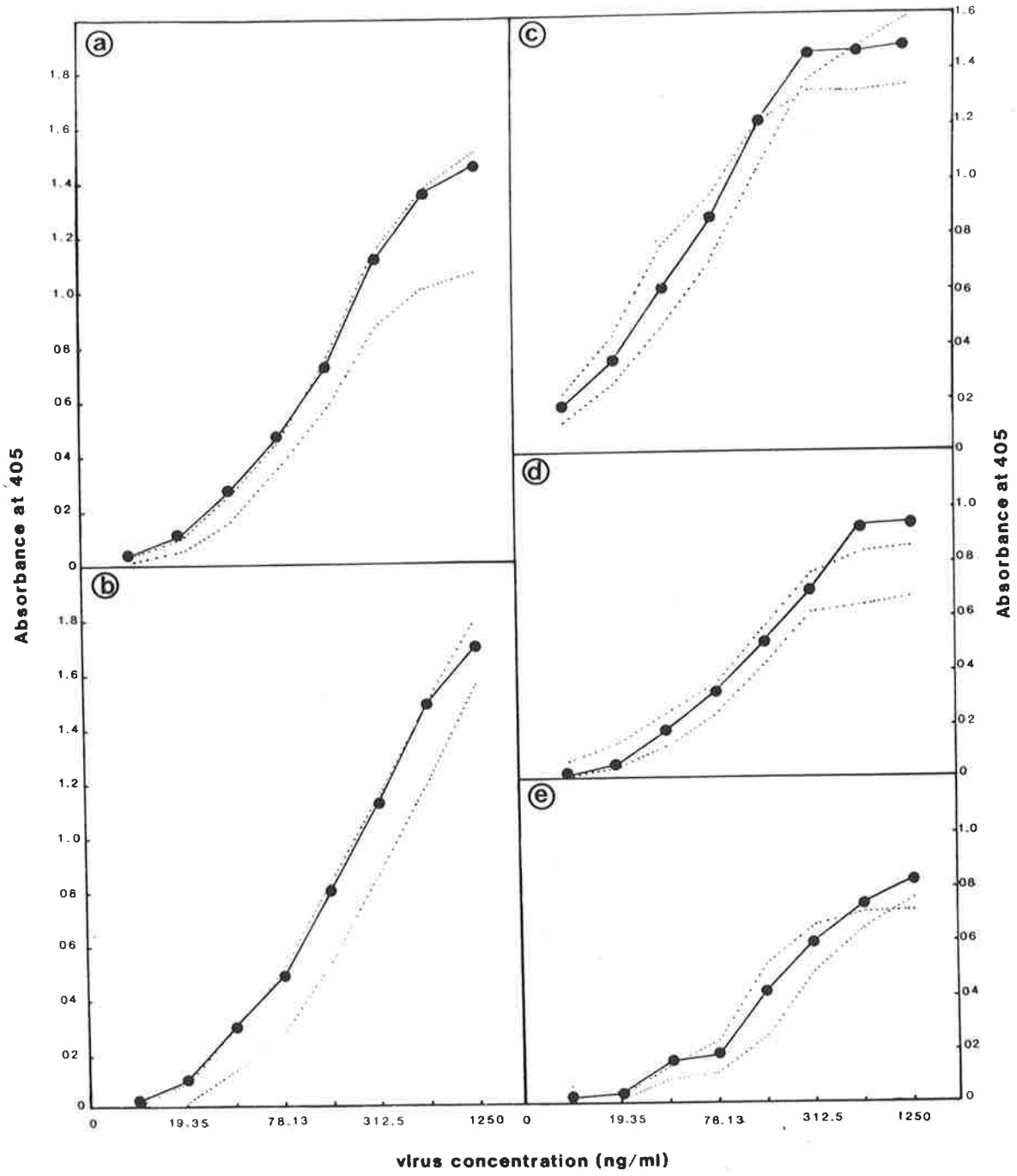


Fig. 6-4

Reactions in DAS-ELISA between antisera to AMV isolates S30 (a), S40 (b), H4 (c), N20 (d) and W1 (e) and purified preparations of their homologous glutaraldehyde-fixed virus preparations (●——●). The broken lines indicate the limits (·····)of the reactions between the antisera and preparations of ten heterologous virus isolates. (See Chapter 3, Table 3-4 for details of virus isolates).

B. Polyclonal antisera to native virus preparations

1. Gel-immunodiffusion tests

Antisera to native virus particles showed lower titres when compared to antisera prepared to glutaraldehyde-fixed virus particles of the same strains, irrespective of the type of the test antigen used (Fig. 5-1). When such antisera were used for serological comparisons of the AMV isolates, antiserum to AMV-S30 was the only one able to differentiate native virus preparations of homologous from heterologous isolates, however, the specificity of this antiserum was changed after prolonged immunization (Fig. 6-1). Early bleeding antiserum of S30 AMV revealed SDI differences of 1-2 (Table 6-3) and differentiated it from all the other isolates by formation of pronounced spurs (Fig. 6-1). Although antiserum from a late bleeding showed no differences in SDI (Table 6-3), all viral antigens except AMV-W1 were differentiated by this antiserum as indicated by spur formation when homologous and heterologous virus preparations were placed in adjacent wells (Fig. 6-1). Antisera to the native virus preparations of the other isolates were not able to differentiate any of the virus isolates (Fig. 6-5, Table 6-3). When each antiserum was titrated against native virus preparations of the other AMV isolates, the SDIs were between 1 and 2 (Table 6-3).

Antisera to native virus preparations were better at differentiating heterologous AMV isolates when preparations of fixed viruses were used as test antigens (Table 6.4). As it was shown in Chapter 5, the fixed virus was always a better test antigen in gel-immunodiffusion tests and always reacted to higher titres with antisera irrespective of whether raised against native or fixed virus preparations or to preparations of the coat proteins (Figs. 5-1 and 5-2). Also, it was shown that none of the antisera raised against fixed or native virus preparations were able to differentiate between the two antigenic forms of each AMV isolate in gel-immunodiffusion tests. This suggests that antigenically, these two forms of AMV are similar (Figs. 5-8, 6-1 and 6-5). Using fixed virus preparations and antisera to native virus, the SDIs did not exceed 4 and the differences in SDIs were correlated with the formation of spurs between homologous and heterologous antigens (Table 6-4, Fig. 6-1, 6-5). Such specificity was usually associated with antisera from both

Table 6-3: Homologous and heterologous titres determined by gel-immunodiffusion of AMV isolates using antisera to native virus particles and native virus preparations as test antigens[a].

Antisera to native virus of :		Antigen (native virus particles)				
		H4	N20	S30	S40	W1
H4	Early[b]	4[c]	4	4	4	4
	Late	16	16	16	16	16
N20	Early	16	16	16	8	8
	Late	16	16	16	8	8
S30	Early	4+[d]	8+	16	4+	8+
	Late	4+	4+	4	4+	4
S40	Early	4	2	2	4	4
	Late	8	8	4	8	4
W1	Early	16	16	16	8	32
	Late	4	4	4	4	8

[a] Homologous and heterologous virus preparations were used at concentrations of 200 µg/ml. Antisera and antigens were diluted in 10 mM phosphate buffer, pH 7.0. Wells were charged with 15µl of each reactant and plates were incubated for 5 days at 25°C before recording the results.

[b] Early and late bleeding antisera are as defined in Tables 5-3 and 5-4.

[c] Reciprocals of maximum antiserum dilution producing a visible immunoprecipitin line. Homologous titres are underlined.

[d] +Indicates the presence of pronounced spurs between homologous and heterologous reactions.

early and late bleedings (Table 6-4). The formation of such differentiative spurs was not affected when antisera were cross-absorbed in gels with a concentrated protein preparation of healthy N. clevelandii, the host plant in which virus was propagated for purification (Fig. 6-6).

Fig. 6-5

Immunodiffusion tests in agarose gels between antisera to a native virus preparation of AMV-H4 (U) and preparations of glutaraldehyde-fixed (capital letters) and native virus preparations (small letters) of AMV-H4 (A,a), N20 (B,b), S40 (C,c), S30 (D,d) and W1 (E,e). Well H was filled with a protein preparation from uninfected N.clevelandii. Test antigens were used at a concentration of 1 mg /ml and antisera were diluted 1 : 2. Each well was filled with 15 µl of each reactant. The plates were incubated for 5 days at 25°C before recording the result.

Fig. 6-6

Intragel-cross absorption of antisera prepared against native virus preparations of AMV-W1 (A) and H4 (B) with protein preparations from uninfected N. clevelandii. The antiserum wells were filled with 15µl of freshly extracted (1:1 w/v in 10 mM phosphate buffer, pH 7.0) leaf tissue. After 16 h of incubation at 25°C the same wells were filled with undiluted antisera. Preparations of 1 mg/ml of glutaraldehyde-fixed virus of AMV-W1 (a), AMV-H4 (b) and S30-AMV (c) were used as test antigens. The wells were charged with 15µl of each reactant and incubated at 25°C for 5 days before recording the results.

Fig.6_5

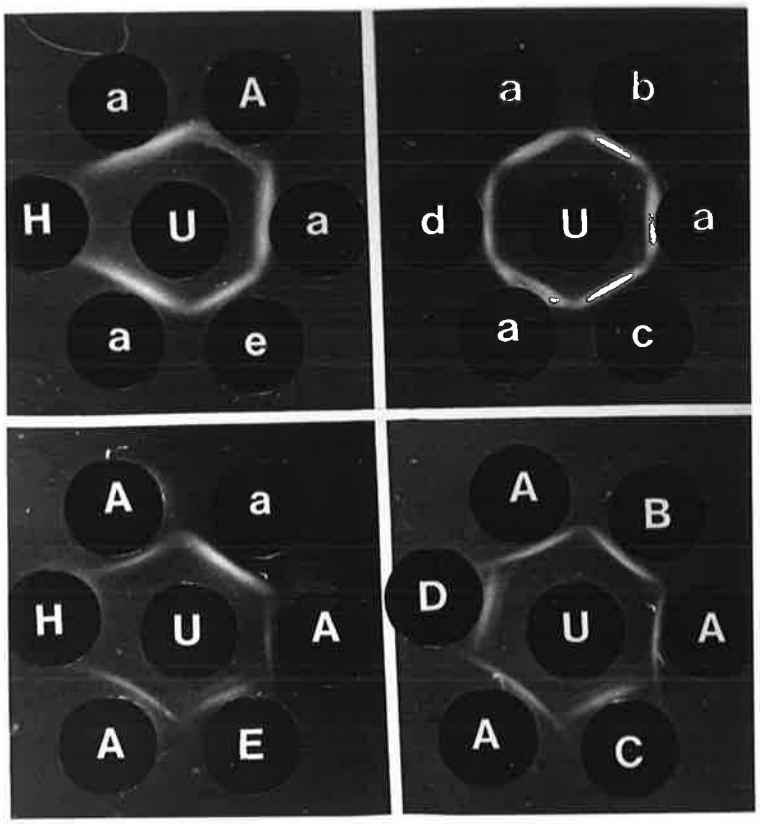


Fig.6_6

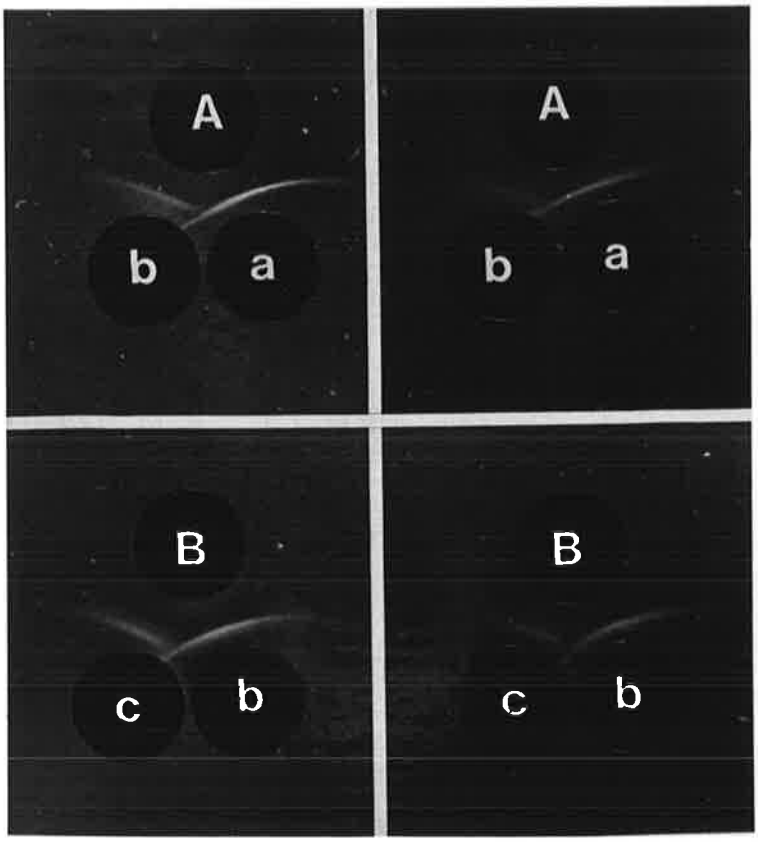


Table 6-4: Homologous and heterologous titres determined by gel-immunodiffusion of AMV isolates using antisera to native virus particles and fixed virus preparations as test antigens[a].

Antisera to native virus of		Antigen (glutaraldehyde-fixed virus preparation)				
		H4	N20	S30	S40	W1
H4	Early[b]	32[c]	32	4[+d]	8[+]	4[+]
	Late	32	32	4[+]	8[+]	16[+]
N20	Early	16	16	8	4[+]	4[+]
	Late	16	16	16	4[+]	4[+]
S30	Early	4[+]	8[+]	32	8[+]	8[+]
	Late	8[+]	8[+]	32	8[+]	4[+]
S40	Early	16	16	16	16	8
	Late	32	32	32	32	32
W1	Early	4[+]	64	32	64	64
	Late	8[+]	64	64	64	64

[a]Homologous and heterologous virus preparations were used at concentrations of 200µg/ml. Antisera and antigens were diluted in 10 mM phosphate buffer, pH 7.0. Wells were charged with 15µl of each reactant and plates were incubated for 5 days at 25°C before recording the results.

[b]Early and late bleeding antisera are as defined in Tables 5-3 and 5-4.

[c]Reciprocals of maximum antiserum dilution producing a visible immunoprecipitin line. Homologous titres are underlined.

[d]+Indicates the presence of pronounced spurs between homologous and heterologous reactions.

Using glutaraldehyde-fixed virus and antisera to native virus, the antisera against different isolates showed different levels of specificity. Antisera to AMV-S30 was very specific and differentiated the S30 isolate not only from the other four AMV isolates (Fig. 6-1, Table 6-4), but also from seven other biologically distinct AMV-isolates (Fig. 6-2, panel B) whose properties are described in Chapter 3. Under similar conditions, but using antisera to glutaraldehyde-fixed virus of the same isolates, no serological differences between the isolates were detected when their fixed virus preparations were used in the

immunodiffusion tests (Fig. 6-2, panel A). Antisera to AMV-H4 differentiated it from all the heterologous isolates except AMV-N20 (Fig. 6-5, Table 6-4). However, antiserum to AMV-N20 could differentiate this virus from two isolates, W1 and S40, antiserum to AMV-W1 could only differentiate it from one isolate, H4 (Table 6-4) but antiserum to AMV-S40 was not able to differentiate any of the other isolates (Table 6-4).

When each antiserum was cross-absorbed with each of the heterologous antigens, all AMV-specific antibodies were removed from some of the antisera. However, others still retained antibodies to some of the virus isolates. Such isolate-specific antibodies were useful for establishing minor serological differences between isolates. Data summarised in Table 6-5 show that all the five AMV isolates except W1 and S40 are not serologically identical. However, their differences could be detected by only some of the antisera. These observations also confirm the presence of the serological differences between the isolates previously detected by spur formation (Table 6-4, Figs. 6-1 and Fig. 6-5).

Like the antisera to glutaraldehyde fixed virus, all the antisera raised against native virus preparations of all the five isolates reacted very poorly with isolated coat protein in gel-immunodiffusion tests (Fig. 5-1). Hence, they were not useful for serological comparisons of the isolates using their coat protein preparations as test antigen.

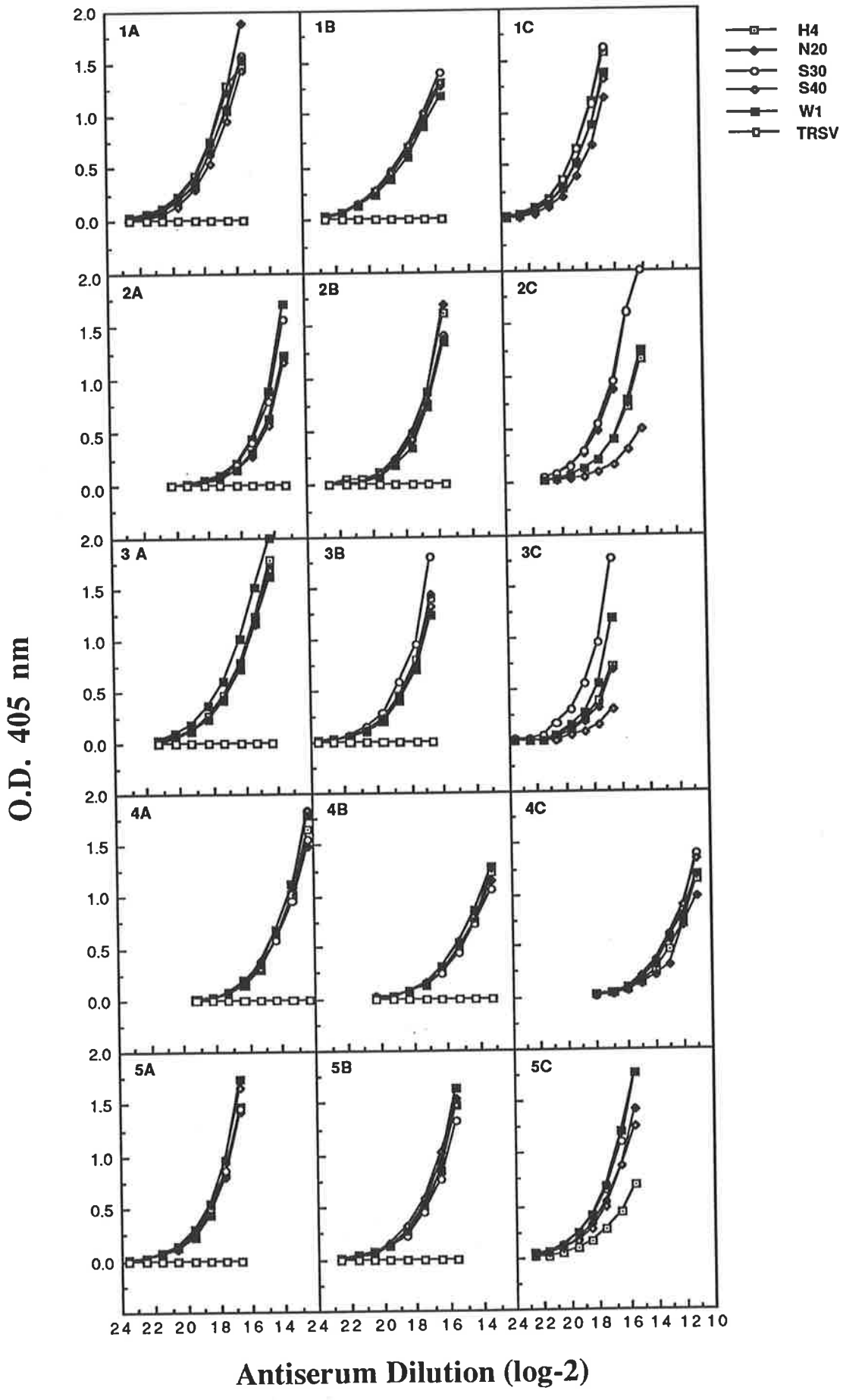
2. Indirect ELISA

When glutaraldehyde-fixed or native virus preparations were used as test antigens, none of the antisera were able to reveal significant serological differences between the homologous and heterologous reactions (Fig. 6-7, 1A-5A and 1B-5B).

Like antisera to glutaraldehyde-fixed virus (Fig. 6-3), when preparations of isolated coat proteins were used as test antigens, some of the antisera could differentiate between some of the AMV isolates. Antiserum to S30 AMV differentiated it from all the other isolates (Fig. 6-7, 3C). Antiserum to N20 AMV differentiated this isolate from H4, S40 and W1 but not from S30 AMV. Similarly, antiserum to W1-AMV differentiated this isolate from H4, N20 and S40 but not from S30-AMV (Fig. 6-7, 2C and 5C). Antisera to S40 and H4 were not able to differentiate significantly these isolates from any of the others (Fig. 6-7, 1c and 4C).

Fig. 6-7

Serological comparison of AMV isolates using glutaraldehyde-fixed (A) and native (B) virus preparations, and coat protein preparations (C) by indirect ELISA (formats 5, 3, and 4, respectively, (see Fig. 2-1, for description of ELISA). Antisera were prepared against native virus preparation of the H4 (1), N20(2), S30(3), S40(4) and W1(5) AMV isolates. The conditions of the experiments were the same as those described in Fig. 6-3 except that concentration of coat proteins were reduced to 25 and 20 ng/ml when antisera to H4 and S30 were used, respectively.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1B
1C
2A
2B
2C
3 A
3B
3C
4A
4B
4C
5A
5B
5C
H4
N20
S30
S40
W1
TRSV
2.0
1.5
1.0
0.5
0.0
24 22 20 18 16 14 24 22 20 18 16 14 24 22 20 18 16 14 12 10
O.D. 405 nm
Antiserum Dilution (log-2)

Table 6-5 : intragel-cross absorption of antisera[a] prepared against native virus particles of AMV isolates with glutaraldehyde-fixed virus.

Antiserum To AMV isolate	Cross-adsorbed with :	Antigen (glutaraldehyde-fixed virus)				
		H4	N20	S30	S40	W1
H4	H4 (control)	-[b]	-	-	-	-
H4	N20	-	-	-	-	-
H4	S30	+[c]	+	-	-	-
H4	S40	+	+	-	-	-
H4	W1	+	+	-	-	-
N20	N20(control)	-	-	-	-	-
N20	H4	-	-	-	-	-
N20	S30	-	-	-	-	-
N20	S40	+	+	+	-	-
N20	W1	+	+	+	-	-
S30	S30(control)	-	-	-	-	-
S30	H4	-	-	+	+*[d]	+*
S30	N20	-	-	+	+*	+*
S30	S40	+*	+*	+	-	-
S30	W1	+*	+*	+	-	-
W1	W1(control)	-	-	-	-	-
W1	H4	-	+	+	+	+
W1	N20	-	-	-	-	-
W1	S30	-	-	-	-	-
W1	S40	-	-	-	-	-

[a] For intragel cross-absorption, 1 mg/ml of glutaraldehyde-fixed antigen was used: 15 µl was added to the central wells and plates were incubated at 25°C for 16 hr. Then the same well was filled with 15 µl of antiserum diluted in 50% glycerol and the surrounding wells were filled with preparations of 200 µg/ml of homologous and heterologous glutaraldehyde-fixed virus . The plates were incubated at 25°C for 7 days before recording the results.

[b] Indicates no precipitin line was formed.

[c] Indicates formation of precipitin line.

[d] Indicates the formation of precipitin spur between homologous and heterologous antigens.

C. Polyclonal antisera to isolated coat protein

1. Gel-immunodiffusion tests

Polyclonal antisera raised against isolated coat protein of two of the AMV isolates (Fig. 5-2) were used for comparing the serological properties of all the AMV isolates using their various antigenic forms. When native virus preparations were used as test antigen, both the antisera against isolated coat protein of AMV-S40 were able to differentiate AMV S40 from isolates H4, N20 and S30 by formation of pronounced spurs (Fig. 6-8). However, the maximum SDIs were only 1 (Table 6-6). Both antisera raised against isolated coat protein of AMV-S40 had the same differentiative properties (Table 6-6). However, when the antisera were cross-absorbed in the gels with native virus preparation of individual heterologous isolates, all the antibodies were absorbed from the serum of rabbit 1b (data not shown). However, the antiserum from rabbit 1a showed that native virus preparations of S40 and W1 have common specific antigenic determinants which are not present on native virus particles of H4, N20 and S30 (Table 6-7). This confirms the differences between these isolates observed by spur formation (Fig. 6-8, Table 6-6). After intragel cross-absorption of this antiserum with native virus preparations of either N20, S30 and H4, antibodies reacting only with preparations of W1 and S40 were retained and the precipitin line showed complete fusion (Table 6-7, Fig. 6-9).

When glutaraldehyde-fixed virus was substituted for the native antigen, only antisera raised in rabbit 1a could differentiate S40 AMV from isolates H4 and N20 (Table 6-6). Antiserum raised in the other rabbit, (1b), failed to differentiate any of the isolates from S40 (Table 6-6).

Antiserum from rabbit 1a was not able to reveal the serological differences between the isolates S30 and S40 as no spurs were formed in immunodiffusion tests (Fig. 6-8). However, when the antiserum was cross-absorbed with glutaraldehyde-fixed virus preparations of each of the heterologous isolates, N20 and W1 were differentiated from H4, S30, and S40. Under the same condition antiserum from rabbit 1b had similar but not identical differentiating properties (Table 6-8). The results presented in Table 6-8 also show the presence of unique antigenic determinants on glutaraldehyde-fixed virus particles

Table 6-6: Homologous and heterologous titres determined by gel-immuodiffusion of AMV isolates using antisera prepared against viral coat protein and native and glutaraldehyde-fixed virus and preparations of their coat proteins as test antigens.

Rabbit No[a]	Isolate used for Immunization	Antigen (glutaraldehyde fixed virus)[b]					Antigen (native virus preparation)[b]					Antigen (isolated coat protein)[c]				
		H4	N20	S30	S40	W1	H4	N20	S30	S40	W1	H4	N20	S30	S40	W
1a	S40	64+[d]	64+	64	128[e]	128	32[f]+	32+	32+	64	64	4+	4	8	8	8
1b	S40	64	32	64	64	32	8+	8+	8+	8	8	8	4	8	8	4
2a	S30	16+	16+	32	16+	16+	8+	8+	16	8+	4+	2	2	4	4	2
2b	S30	4+	4+	8	4+	4+	4	4+	4	1+	1+	1	1	1	1	1

[a]Details of immunization of rabbits are presented in Fig. 5-2.

[b]Gel-diffusion tests were done in 0.75% agarose in 10 mM phosphate buffer, pH 7.6. Wells were charged with 12-15 µl of each reactant and were incubated at 25°C for 5 days before recording the results.

[c]Gel-diffusion tests were done in 0.75% agarose in 10 mM Na-acetate, pH 7.6, containing 100 mM $CaCl_2$. All test antigens were used at concentration of 0.25 mg/ml.

[d]Reciprocals of maximum antiserum dilution producing a visible immunoprecipitin line.

[e]Homologous titres are underlined.

[f]+Indicates the presence of pronounced spurs between adjacent wells containing homologous and heterologous antigens.

Table 6-7: Intragel-cross absorption of antisera prepared against isolated coat protein of AMV isolates with native virus preparations.

Antisera to AMV isolate	Cross absorbed with :	Test antigen (native virus)				
		H4	N20	S30	S40	W1
S40[a](1a)	S40	-[b]	-	-	-	-
	H4	-	-	-	+[c]	+
	N20	-	-	-	+	+
	S30	-	-	-	+	+
	W1	-	-	-	-	-
S30[d](2a)	S30	-	-	-	-	-
	H4	-	-	+	-	-
	N20	-	-	+	-	-
	S40	-	-	+	-	-
	W1	-	-	+	-	-

[a]Antiserum from rabbit 1b was exhausted of antibodies by cross-absorption with any of the heterologous antigens.

[b]Indicates the absence of any precipitin line.

[c]Indicates the appearance of precipitin line with complete fusion when the preparation of heterologous isolate was loaded in the adjacent well to that of the homologous isolate.

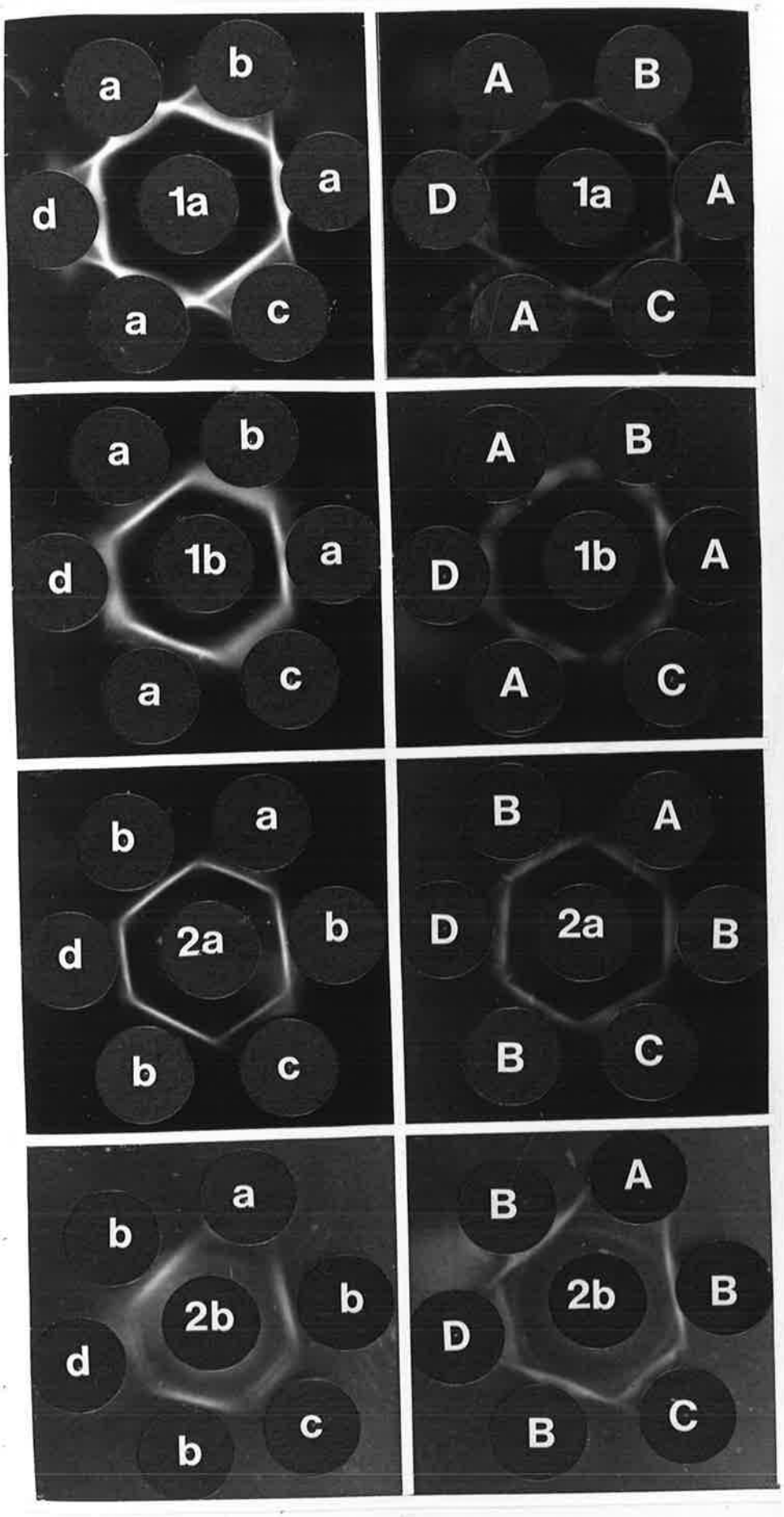
[d]Antisera from rabbits 2a and 2b reacted similarly.

of S40. Although no spur was formed between the glutaraldehyde-fixed virus preparations of AMV S30 and S40 when tested against antiserum 1a (Fig. 6-8), cross-absorption tests showed that these two isolates are not identical (Table 6-8).

Antisera against isolated coat protein of AMV-S30 raised in both the rabbits (Fig. 6-8) differentiated isolate S30 from all the others when fixed virus preparations were used as test antigens (Table 6-6), as did the antiserum to native virus preparations (Table 6-4). Although antiserum raised in rabbit 2a differentiated native virus preparations of all the AMV isolates, antiserum from rabbit 2b failed to differentiate the S30 isolate from H4 (Table 6-6). Nevertheless, cross-absorption of the antiserum from rabbit 2a with glutaraldehyde-fixed virus preparation of each heterologous isolate showed that this strain possesses unique antigenic determinants not present on any of the other isolates (Table 6-

Fig. 6-8

Immunodiffusion tests in agarose gels using antisera against isolated coat protein preparations of AMV-S40 (1a,1b) and AMV-S30 (2a,2b). Preparations of native (small letters) and glutaraldehyde-fixed virus (capital letters) of AMV-isolates S40 (a,A), S30 (b,B), H4 (c,C), and N20 (d,D) were loaded in the peripheral wells. Test antigens were used at concentrations of 0.25 mg/ml. Antisera from rabbits 1a and 1b were diluted 1: 8, antiserum from rabbit 2a was diluted 1:4 and antisera from rabbit 2b was used undiluted when the glutaraldehyde-fixed virus preparations were used as test antigens. In all the other comparisons using native virus preparations, all the antisera were used undiluted. The wells were charged with 15µl of each reactant and plates were incubated at 25°C for 5 days before recording the results.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2a
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2b
b
d
c
b
B
A
2b
B
D
B
C

Table 6-8: Serological comparisons of AMV isolates using intragel cross-absorbed antisera raised against isolated coat protein with glutaraldehyde-fixed virus preparations.

Antisera[a] to AMV isolates:	Cross absorbed with:	antigen (glutaraldehyde-fixed virus)				
		H4	N20	S30	S40	W1
S40(1a)	S40	-[b]	-	-	-	-
	H4	-	-	+[c]	+	-
	N20	+*[d]	-	+*	+	-
	S30	+*	-	-	+	-
	W1	+*	-	+*	+	-
S40(1b)	S40	-	-	-	-	-
	H4	-	-	-	+	-
	N20	+	-	+*	+	-
	S30	+*	-	-	+	-
	W1	+	-	-	+	-
S30(2a)	S30	-	-	-	-	-
	H4	-	-	+	-	-
	N20	-	-	+	+*	-
	S40	-	-	+	-	-
	W1	-	-	+	-	-
S30(2b)	S30	-	-	-	-	-
	H4	-	+*	+	-	-
	N20	-	-	-	-	-
	S40	-	+*	+	-	-
	W1	-	+*	+	-	-

[a] 1a,1b,2a,2b are the numbers of the rabbits immunized with isolated coat protein preparation (Fig. 5-2).

[b] Indicates that no precipitin line was formed after cross-absorption of antisera.

[c] Indicates that the presence of a precipitin line was formed after cross-absorption which fused completely with the homologous antigen when loaded in an adjacent well.

[d] * Indicates that a pronounced spur was formed between the homologous and heterologous antigen when they were placed in adjacent wells.

8). However, after cross-absorption of antiserum from rabbit 2b with a preparation of glutaraldehyde-fixed N20, all the antibodies were removed (Table 6-8). These data confirmed previous observations that glutaraldehyde-fixed S30 particles posses unique antigenic determinants. Antisera prepared in rabbit 2a also revealed the presence of such

unique antigenic determinants on native virus preparations of this isolate (Table 6-7), but antisera raised in rabbit 2b, showed that only fixed virus of N20 could remove all reacting antibodies recognising glutaraldehyde-fixed virus particles of the S30 isolate (Table 6-8).

Titration by immunodiffusion tests using coat protein preparations as test antigen showed very little difference between any of the five AMV isolates (Table 6-6). However, one of the antisera against AMV-S40 (1a) differentiated the protein preparations of AMV-S40 from H4 by the formation of a spur (Fig. 6-10). However, this was not reproducible when the protein was prepared by longer dialysis against 0.1M $CaCl_2$ which probably causes proteolysis (Appendix **2,** Fig. 3). This indicates that the proteolysis may affect antigenicity as has been demonstrated with cauliflower mosaic virus (CaMV) and some potyviruses (Du Plessis et al.,1980, Hiebert et al.,1984). When preparations of coat proteins of the S30 or S40 isolates were loaded adjacent to preparations of any of the other isolates no spurs were formed (Fig. 6-11). The lack of any significant serological differences between coat protein preparations of these isolates in gel-diffusion tests indicate that they are antigenically similar. This was also confirmed by cross-absorption tests of all four antisera (data not shown).

2. Indirect ELISA

When glutaraldehyde-fixed virus was used as test antigen, only antiserum against S30 AMV raised in rabbit 2b could differentiate all the heterologous isolates (Fig. 6-12, 4A) and all the other antisera failed to establish any significant differences between the homologous and heterologous isolates (Fig. 6-12, 1A-3A). The same results were obtained when native virus preparations were used as test antigens (Fig. 6-12, 1B-4B). However, when isolated coat protein preparations were used as test antigens, antisera against S40 AMV raised in rabbit 1a could detect minor serological differences between isolated coat protein preparations of S40 and those of S30, H4 and N20, while protein preparation of AMV-W1 reacted stronger than the homologous preparation (Fig. 6-12, 1C). Antiserum raised in rabbit 2b differentiated protein preparations of all isolates from that of the S30-AMV (Fig. 6-12, 4C), but antiserum raised in rabbit 2a could differentiate

Fig. 6-9

Intragel-cross absorption of an antiserum raised against isolated coat protein of AMV-S40 (1a) with a native virus preparation of AMV-N20. The central wells (1a) were filled with 15µl of native virus preparation of AMV-N20 (1mg/ml) and incubated for 16 hr at 25°C. The same wells were then filled with undiluted antiserum from rabbit 1a. The native virus preparations of AMV-S40 (A), S30 (B), N20 (D), H4 (C) and W1 (E) at concentrations of 1 mg/ml were loaded in the surrounding wells. Each well was charged with 15µl of each reactant and incubated at 25°C for 5 days before recording the results.

Fig. 6-10

Immunodiffusion tests using antiserum to an isolated coat protein preparation of AMV-S40 (1a) and the coat protein preparations of homologous and heterologous isolates. The coat proteins were prepared by the $CaCl_2$ method using two dialysis times of 12 hr (panels 1) or 48 hr (panels 2) against 100 mM $CaCl_2$ as described in Appendix **1**. Preparations of 0.25 mg/ml of coat proteins from S40 (A), S30 (B), H4 (C), and W1 (E) were used as test antigens. Wells were filled with 15 µl of each reactant and incubated at 25°C for 5 days before recording the results.

Fig.6_9

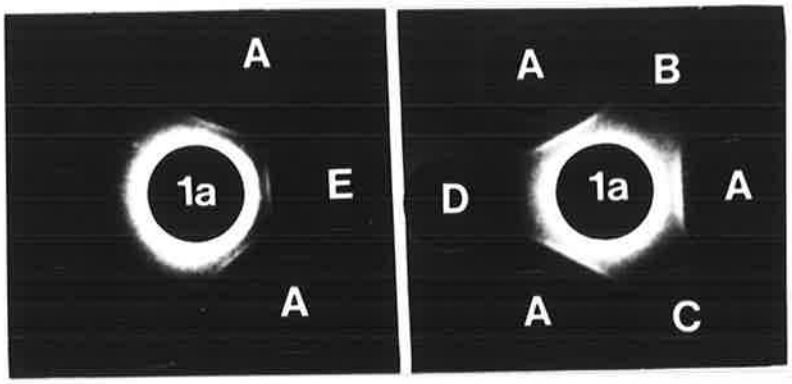


Fig.6_10

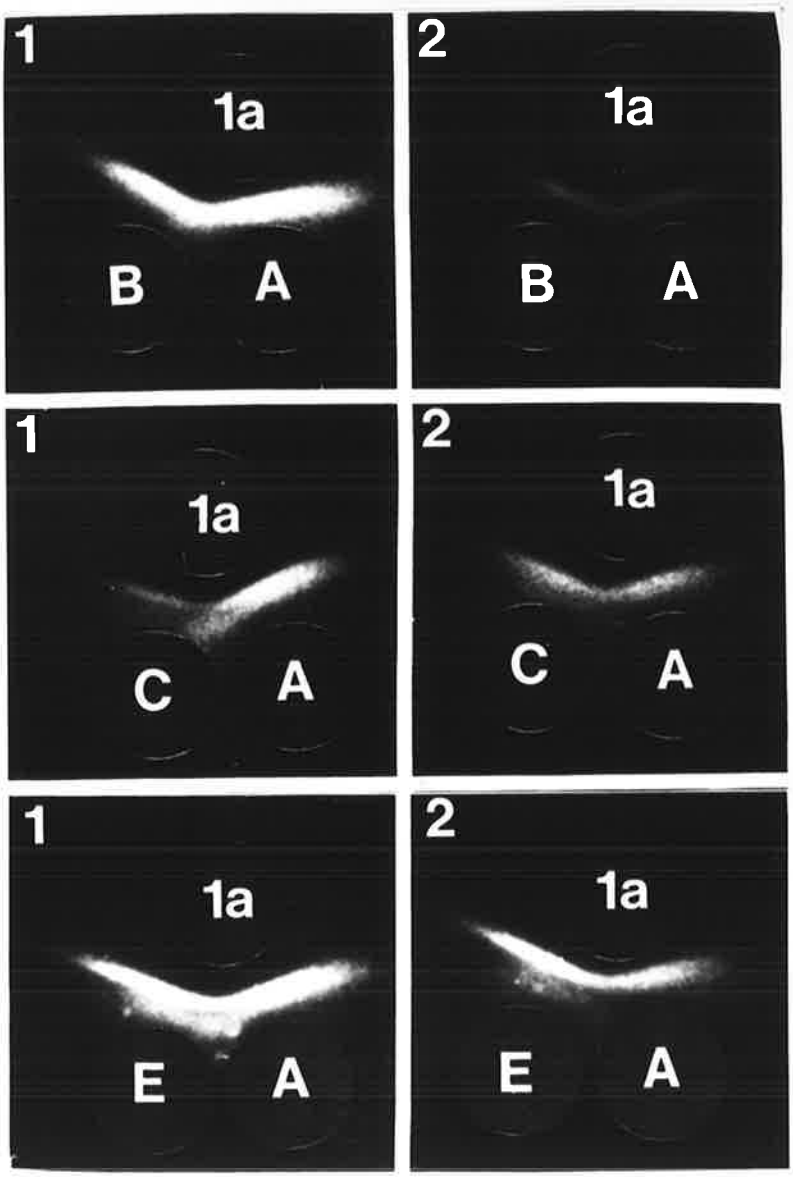
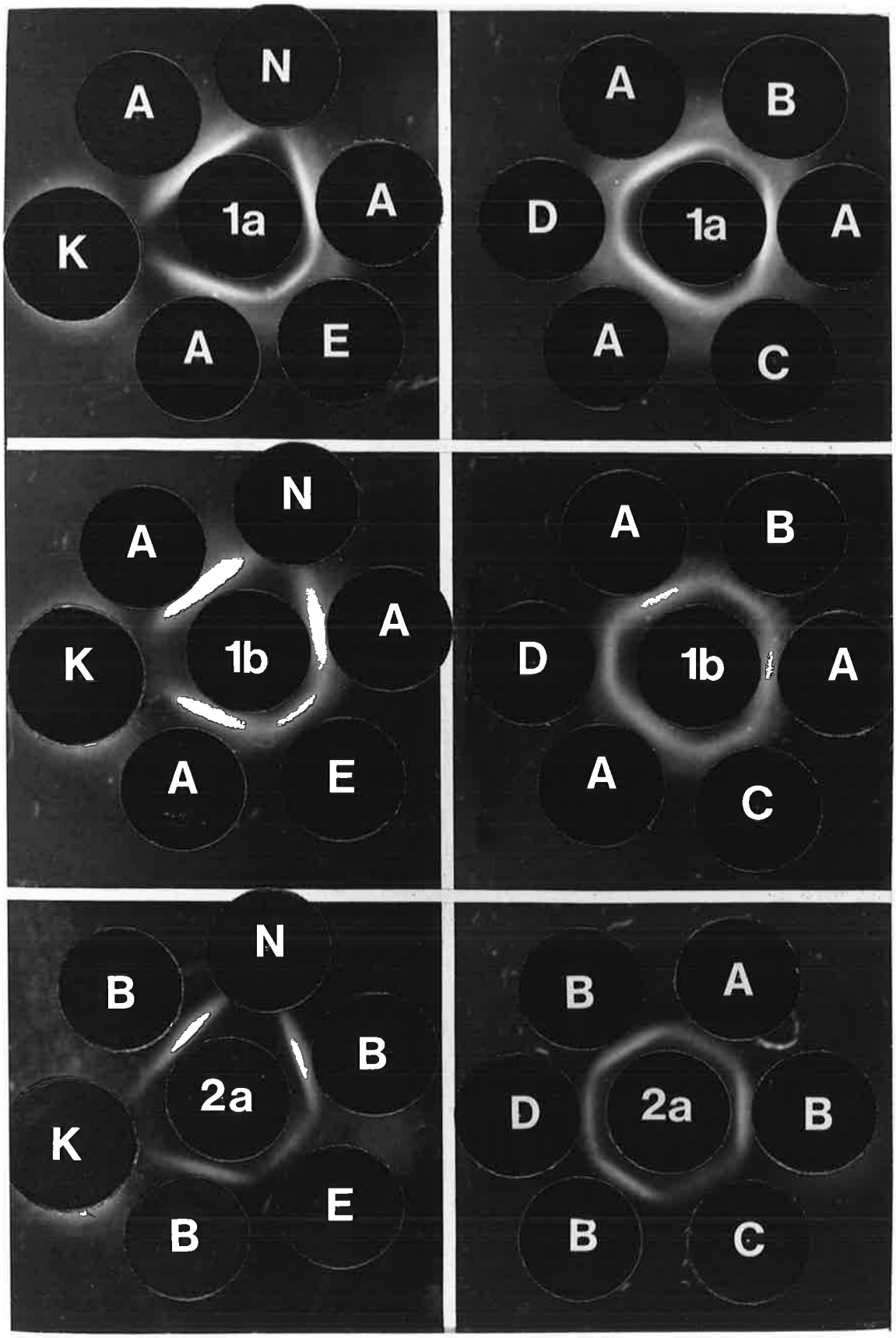


Fig. 6-11

Immunodiffusion tests in agarose gels using antisera to isolated coat proteins of S40 (1a, 1b) and S30 (2a) AMVs. Undiluted antisera were loaded in the central wells and 0.25 mg/ml preparations of coat protein of AMV isolates S40 (A), S30 (B), N20 (D), H4 (C) and W1 (E) were loaded in the sourrounding wells. Well K was loaded with a protein preparation from uninfected N.clevelandii and well N with buffer (10 mM Na-acetate, pH 6.0, containing 100 mM $CaCl_2$). The gel was prepared in 10 mM Na-acetate buffer, pH 7.6, containing 100 mM $CaCl_2$. Each well was loaded with 12 µl of each reactant and incubated at 25°C days before recording the results.


N
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1a
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K
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E
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E
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1b
A
A
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N
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2a
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2a
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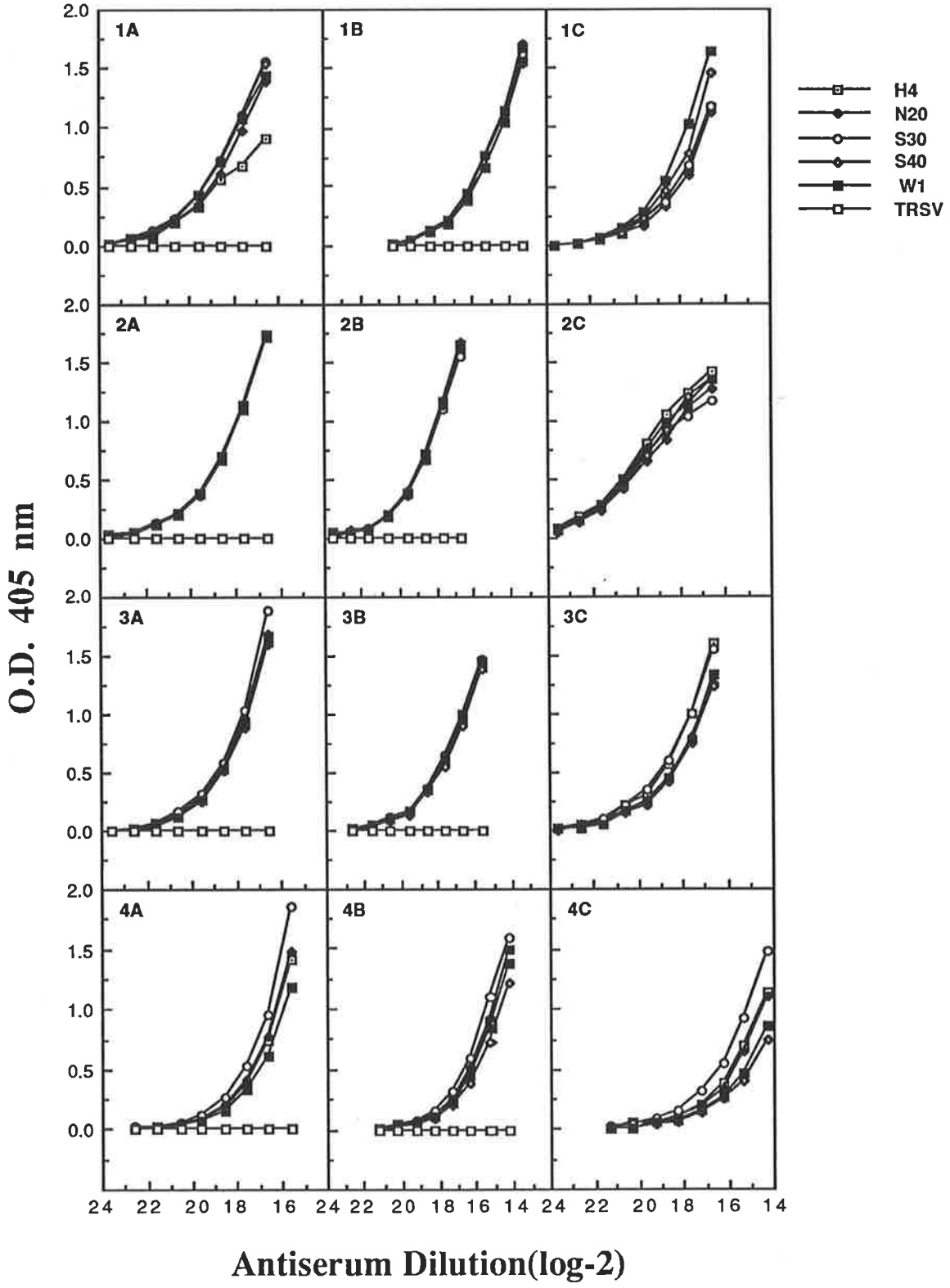
C

Fig. 6-12

Serological comparisons of glutaraldehyde-fixed (A), and native (B) virus preparations and coat protein preparations (C) of AMV isolates in indirect ELISA (formats of 5, 3 and 4, respectively, see Fig. 2-1). Antisera against isolated coat protein preparation of AMV-S40 raised in rabbits 1a (1), 1b (2) and antisera against isolated coat protein of AMV-S30 raised in rabbits 2a (3) and 2b (4) were used. Conditions for coating viral antigen were as described in Fig. 6-7.


1A
1B
1C
2A
2B
2C
3A
3B
3C
4A
4B
4C
H4
N20
S30
S40
W1
TRSV
O.D. 405 nm
Antiserum Dilution(log-2)

protein preparations of S30-AMV from, those of S40 and W1 but not of H4 (Fig 6-12, 3C).

3. DAS-ELISA

This test was used for the serological comparison of the five AMV isolates using coat protein preparations as test antigens. Both antisera raised against S40 coat protein differentiated N20 and H4 from S40, while isolated coat protein of S30 and W1 reacted stronger than the homologous S40 (Fig. 6-13,1a and 1b). However, antisera against coat protein of S30 differentiated all heterologous isolates from that of S30 (Fig. 6-13, 2a and 2b).

4. Western immunoblotting

The results presented in Figs. 6-3, 6-7, 6-12 and 6-13 show that the isolated coat protein of some of the AMV isolates are more readily serologically distinguishable than their corresponding native or glutaraldehyde-fixed virus preparations. These observations suggest that either the differentiative epitopes are on the non-polymerised form of AMV coat protein subunits which are masked after polymerisation **(cryptotopes)** or they are located internally, and are exposed only when protein partially denatures after binding to the microtitre wells. These possibilities were investigated by western immunoblotting. The method has been used recently for studying serological relationships between plant viruses by Burgermeister and Koenig, (1984), Rybicki and Von Wechmar, 1982), Hunter et al., (1989), Shukla et al., (1989a) and Clarke et al., (1989). AMV proteins dissociated in Laemmli buffer from virus particles or prepared by the $CaCl_2$ method (Appendix 2) were electrophoresed on 12% SDS-polyacrylamide gels. After transfer to nitrocellulose (Chapter 2), the proteins were reacted with antisera against S40 raised in rabbit 1a (Fig. 6-14, A) or S30 in rabbit 2a (Fig. 6-14, B). The results in Fig. 6-14 show that both antisera reacted with all protein fragments of the homologous and all the heterologous isolates. These observations show that this technique is not able to reveal the minor serological differences between the coat proteins of these isolates.

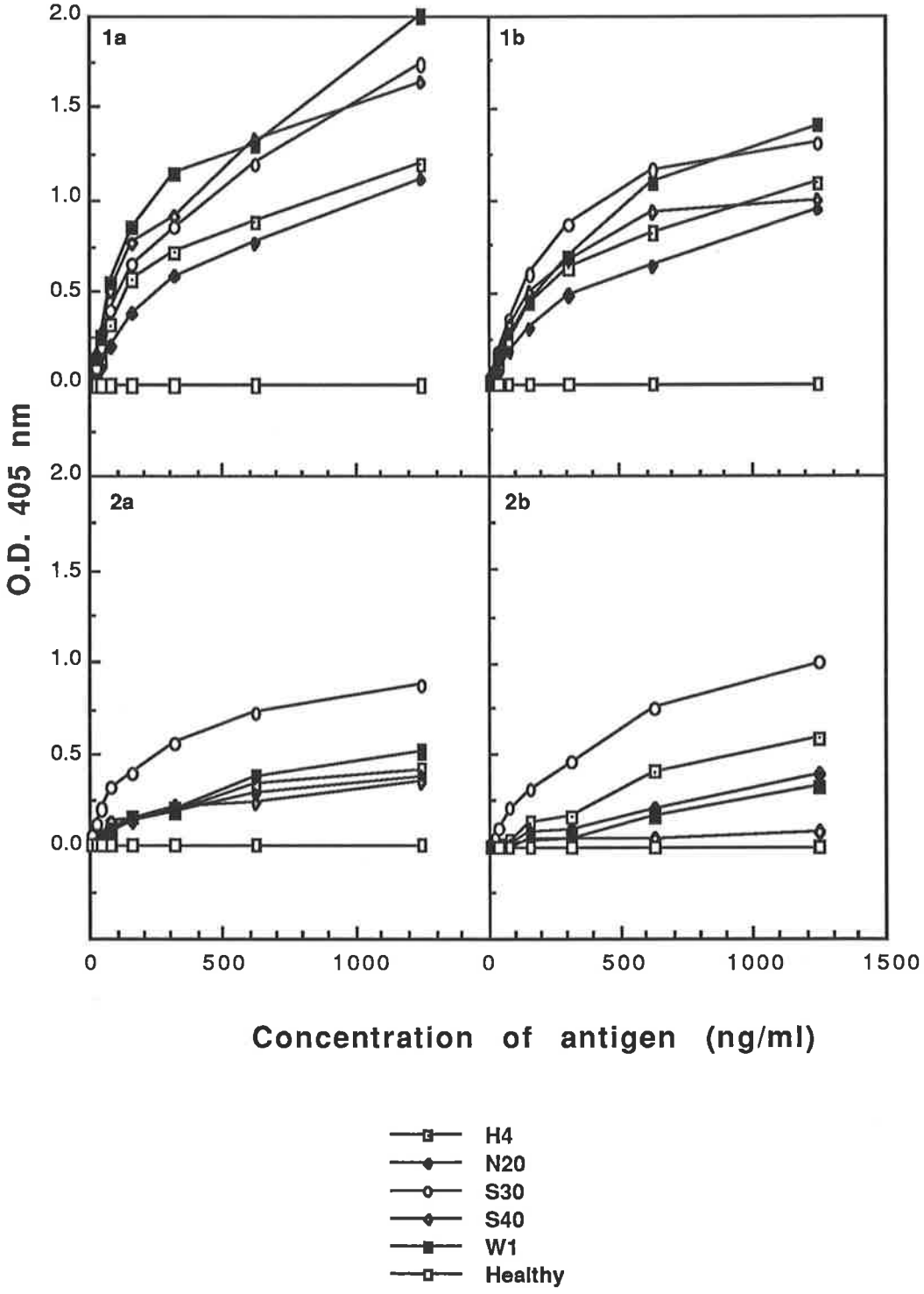


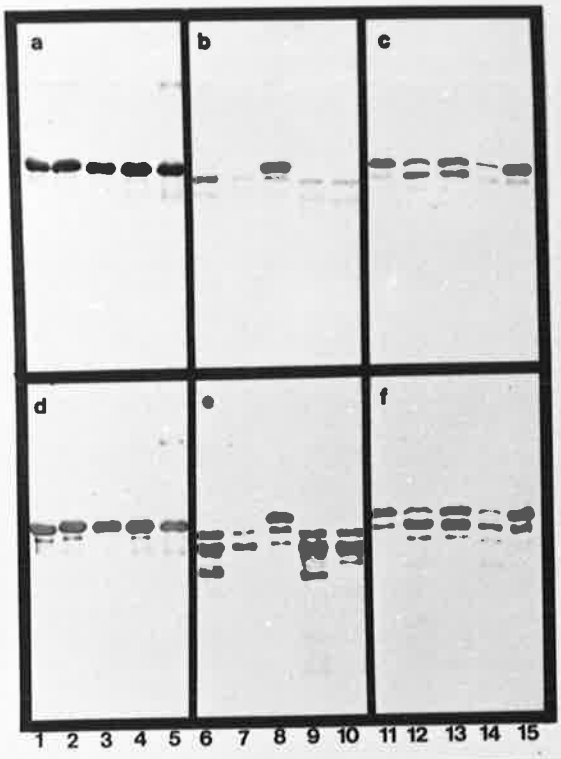
Fig. 6-13

Reactions in DAS - ELISA between antisera to isolated coat protein of AMV-S40 (1a and 1b) and AMV-S30 (2a and 2b) with isolated coat protein preparations of all five AMV isolates. An extract from uninfected N. clevelandii was used as a control test antigen.

Fig. 6-14

Analysis of coat proteins from AMV isolates H4, N20, S30, S40 and W1 (from left to right in each panel, respectively) by polyacrylamide gel electrophoresis (panels a-c) and western immunoblotting (panels d-f). Proteins were dissociated directly from the respective virus preparations (panels a and d), from proteins prepared by $CaCl_2$ degradation (panel b, e), and proteins prepared as in b and e except that the initial dialysis of virus against 1M $CaCl_2$ was reduced from 48 hr to 12 h and the dialysis of the protein from 48 to 36 (panels c and f, see Appendix 1 for protein preparation). Gels shown in panels a-c were stained with silver after electrophoretic transfer of the proteins to nitrocellulose and the transfers (panels d-f) were probed with antisera to coat proteins of the S40 (panel A) and S30 (panel B) AMV isolates. Antisera from rabbits 1a and 2a in Fig. 5-2 bled 11 weeks after commencement of immunization were used at dilutions of 1 : 10,000 and 1 : 5000 in panels A and B, respectively.

A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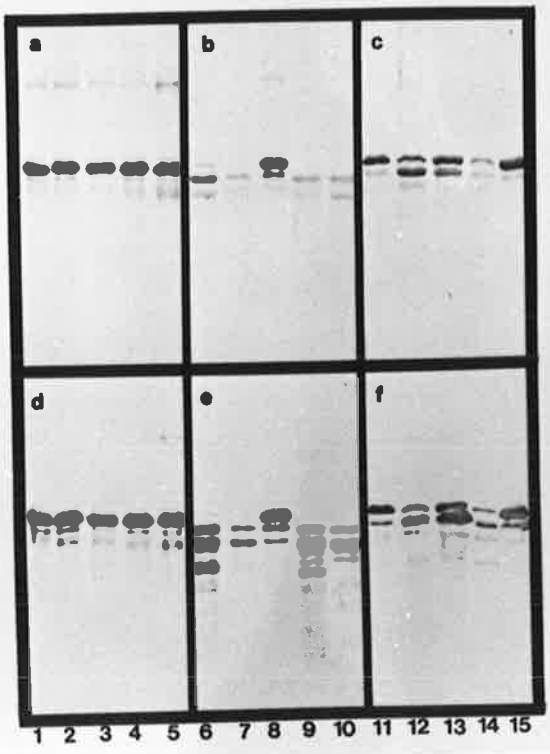
B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D. Preparation of Strain specific and group specific polyclonal antisera

Intragel-cross absorption of antisera to native or isolated coat proteins showed that native or glutaraldehyde-fixed virus of some AMV isolates have antigenic determinants which are either unique or shared among some but not all of the isolates (Table 6-5). AMV-S30 was the only isolate which had unique antigenic determinants on its glutaraldehyde-fixed virus particles. The possibility of obtaining monospecific polyclonal antisera against this isolate was studied by cross-absorption of antisera in tubes. Antiserum raised against native virus preparation of this isolate was added to a mixture of glutaraldehyde-fixed virus preparations of all the four heterologous isolates. After precipitation, the remaining antibodies reacted only with the glutaraldehyde-fixed virus of this isolate (Fig. 6-15, A). Cross-absorption of antiserum to a native virus preparation of AMV-W1 with glutaraldehyde-fixed virus preparation of isolate H4, resulted in an antiserum which reacted with the homologous antigen as well as with glutaraldehyde-fixed virus preparation of N20, S30 and S40 but not the H4 isolate (Fig. 6-15, B). It is shown in Table 6-5 that AMV-H4 possesses antigenic determinants similar to N20, but different from W1, S30 and S40. After cross-absorption of antiserum to a native virus preparation of the H4 isolate with a mixture of glutaraldehyde-fixed virus of isolates S30, S40 and W1, an antiserum was obtained which reacted only with glutaraldehyde-fixed virus preparations of N20 and H4 (Fig. 6-15, C).

III Conclusion

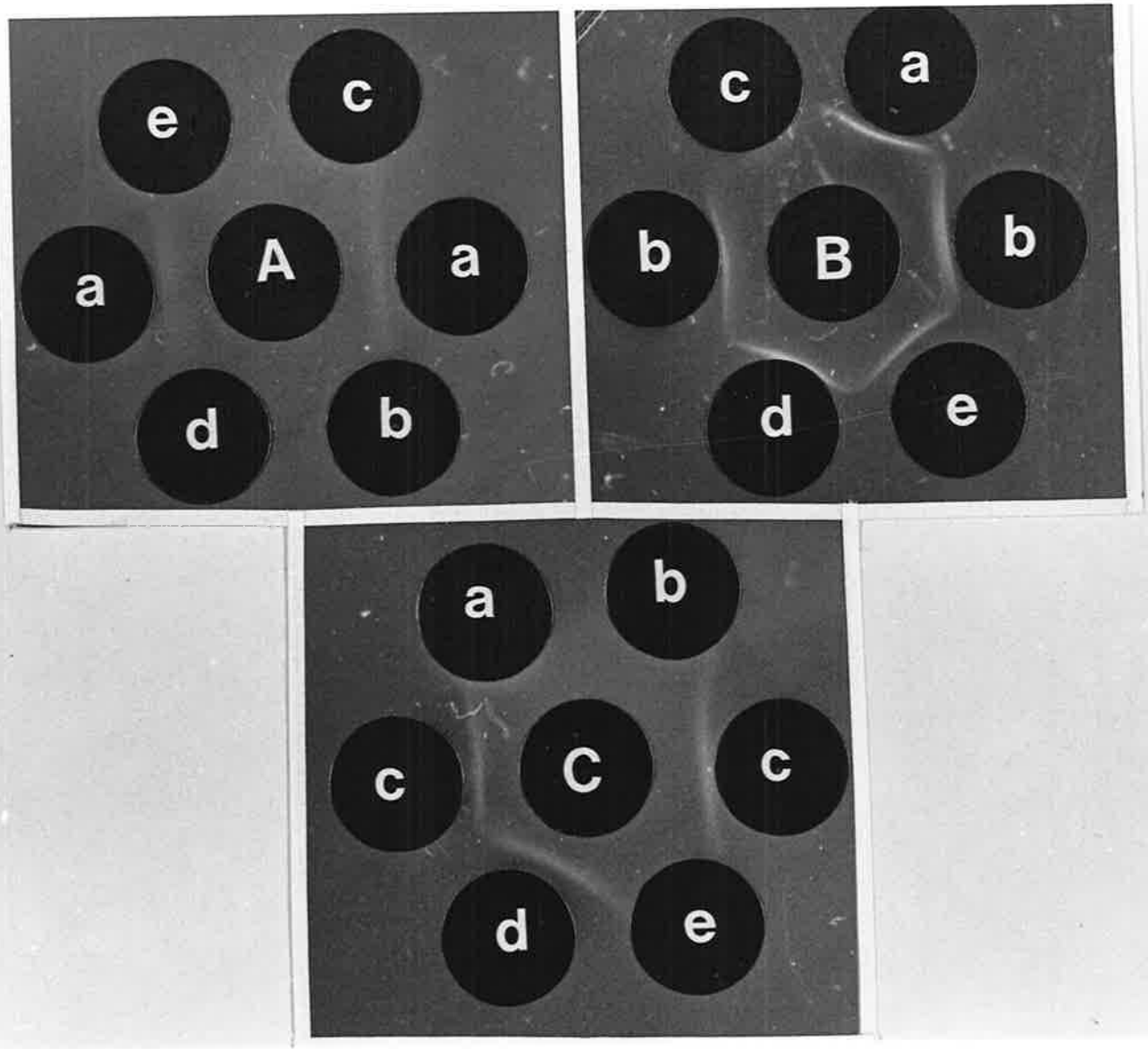
The results presented here allow the following conclusions to be drawn:

1 - The five biologically distinct AMV isolates studied were shown to have minor differences in their antigenic structures. These differences were more readily revealed with antisera to native virus or isolated coat protein than with antisera to glutaraldehyde-fixed virus. However, the differences were more clearly seen when fixed virus preparations were used as test antigens.

2 - The minor differences in antigenic structure of the AMV isolates were easier to detect in immunodiffusion tests than by various forms of ELISA or western immunoblotting. However, some differences between some of the virus isolates were

Fig. 6-15

Preparation of monospecific or group specific polyclonal antisera by cross-absorption. Preparations of glutaraldehyde-fixed virus of S30(a), H4(c), N20(d), S40(e) and W1(b) were reacted with cross-absorbed antisera raised against native AMV of S30(A), W1(B) and H4(C) isolates. Antiserum to native AMV-S30 had been absorbed with a mixture of 0.6mg/ml (150μg of each heterologous) of glutaraldehyde-fixed virus of H4, S40, N20 and W1. Antiserum to native W1 was adsorbed with a glutaraldehyde-fixed virus preparation of H4 (150μg/ml of antiserum). Antiserum to native H4 was absorbed with preparations of glutaraldehyde-fixed virus of S30, W1 and S40 isolates (150 μg of each antigen /ml of the antiserum). Mixtures of antisera and antigens were shaken for 1 hr at 37°C and then subjected to centrifugation at 12,000g for 10 min. The supernatants were retained. Wells were filled with 15μl of each reactant and the plates were incubated for 5 days at 25°C before recording the results.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detected by ELISA where isolated coat protein subunit preparations were used as test antigens, irrespective of the type of antiserum used.

3 - The presence of specific epitopes on particles of some of the AMV isolates provided a basis for obtaining monospecific or group specific antisera.

Chapter 7

Production and characterization of a panel of monoclonal antibodies (McAbs) for differentiation of AMV isolates

I Introduction

It was shown in Chapter 6 that polyclonal antibodies raised against glutaraldehyde-fixed virus of five biologically distinct AMV isolates failed to differentiate any antigenic differences between them in gel-immunodiffusion tests. However, antisera to native virus preparations or their isolated coat proteins were able to reveal some antigenic differences between the isolates, particularly when glutaraldehyde-fixed virus was used as test antigen (Chapter 6). If an antiserum can differentiate two antigens from each other, one approach of obtaining monospecific polyclonal antibodies is by cross-absorption with heterologous antigens to remove the cross-reacting antibodies (Rao, 1982). This was actually done with the AMV antisera as described in Chapter 6. AMV is not a potent immunogen (Chapter 5), and the antisera against native virus or isolated coat protein preparations, which were the only differentiative one, had low titres (Chapter 5). Hence, the monospecific antisera obtained had a low titre.

The other approach for obtaining monospecific antibodies is by hybridoma technology. It was shown that the AMV isolates do have specific epitopes (Chapter 6) and hence the preparation of isolate specific monoclonal antibodies seemed to be a feasible approach.

This chapter describes the production, characterization and utilization of a panel of McAbs raised against a mixture of native virus preparations of the five AMV isolates.

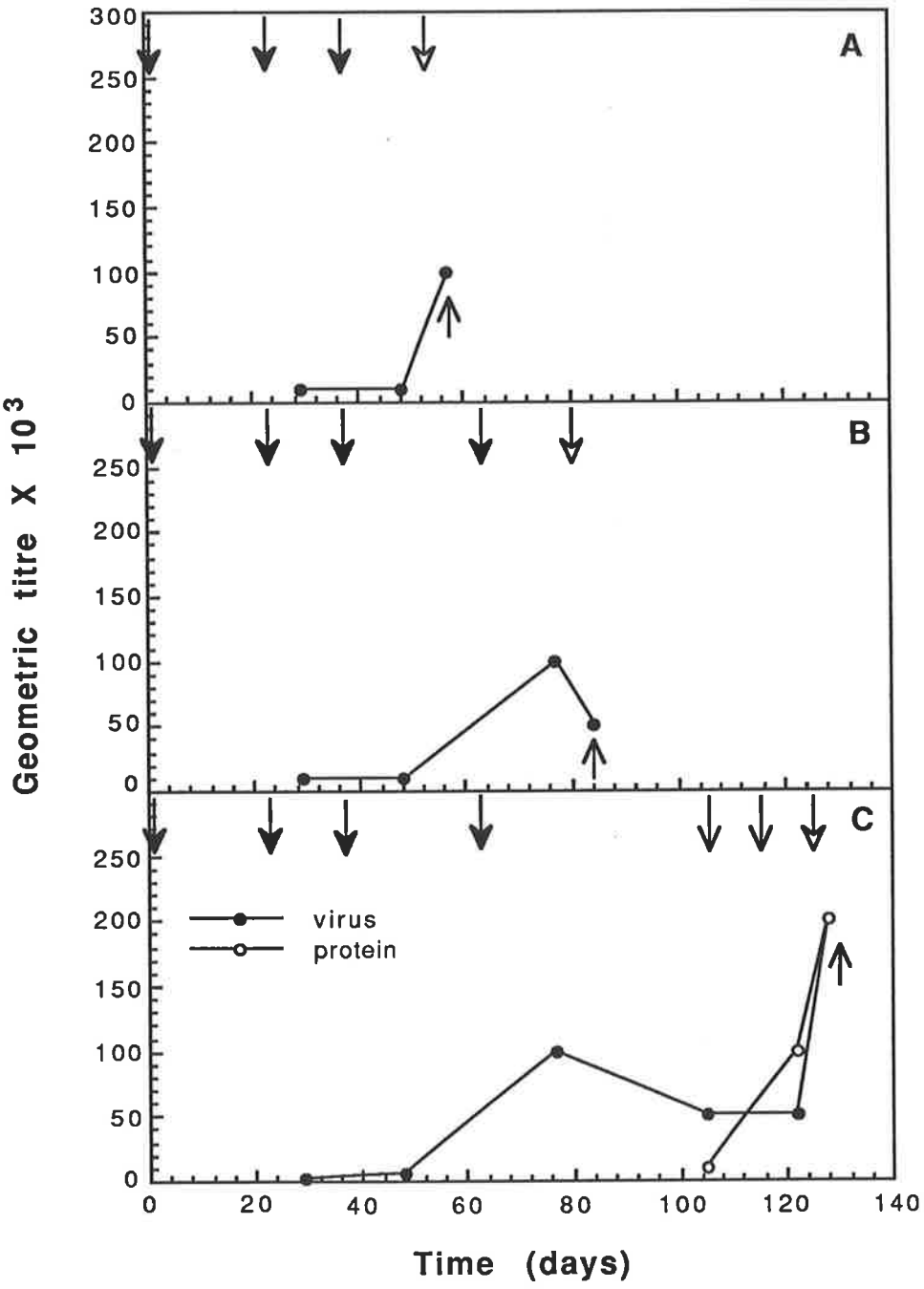
II Experimental

A. Immunization of mice

Three BALB /c mice were immunized differently as was described in Chapter 2 (Table 2-5) and is shown in Fig. 7-1 . Mice A and B were injected only with mixtures of native virus preparations of the five AMV strains. Mouse C, in addition to injections with

Fig. 7-1

Antibody titres of sera immunized with mixtures of native virus preparations (mice A and B) and similar injections followed by a series containing mixtures of isolated coat protein preparations (mouse C). The mixtures used as immunogens contained equal amounts of virus or isolated coat protein preparations of AMV isolates H4, N20, S30, S40 and W1. The downward arrows indicate the intraperitonial injections of the native virus preparations (⇓) or isolated coat proteins (⇓). For each injection, 200 µg of a purified mixed virus preparation in 10 mM phosphate buffer, pH 7.0, or 200µg of an isolated coat protein preparation in 10 mM Na-acetate, pH 6.0 containing 100 mM $CaCl_2$ was used. Each immunogen aliquotes was emmulsified with an equal volume of complete adjuvant before injection. The downward arrows (⇓) show the time of intravenous booster injections. The upward arrows (⇑) indicate the time of sacrificing of each mouse. For titration of the sera, 1µg/ml of a mixture of either native or isolated coat protein preparations were used. The sera from mice A and B were titrated by ELISA format 8 and the sera from mouse C were titrated by ELISA formats 6 and 8 (see Fig. 2-1 for details of ELISA formats). (The geometric titre is the reciprocal of the maximum antiserum dilution producing a positive reaction).


Geometric titre X 10^3
300
250
200
150
100
50
0
A
B
C
virus
protein
0
20
40
60
80
100
120
140
Time (days)

native virus preparations as mice A and B, also received injections of a mixture of isolated protein preparations of the five AMV isolates as described in Chapter 2 (Table 2-5). Each animal was bled at intervals as indicated in Fig. 7-1 and antisera were titrated as described in Chapter 2. Data in Fig.7-1 show that mice A and B showed a slightly better response than mouse C at early stages of immunization, but after the 4th injection the titres of sera from all three mice were similar. Antisera titres of sera from mice A and B at the time of sacrifice did not exceed $1x10^5$ when tested against a mixture of native virus preparations of all the isolates (Fig. 7-1, A and B). The titre of mouse C at the time of sacrifice was 2×10^5 irrespective of the type of test antigen used (Fig. 7-1, C).

The three mice also responded differently to the final intravenous booster. Three days after this injection only antiserum from mouse B showed a lower titre (5×10^4) compared to that before the injection, which was (1×10^5) (Fig. 7-1, B). This is of normal occurrence (Sander and Dietzgen, 1984). It has been suggested that the assembly of the activated B-lymphocytes in the spleen accounts for this difference (Sander and Dietzgen, 1984 and references therein). However, the titre of the antisera from the other two mice increased after this intravenous injection (Fig. 7-1, A and C) which show that the decrease in antibody titre following an intravenous injection is not a general phenomenon and varies between individual animal. The sera from mice A and C had titres of 1×10^5 and 2×10^5 respectively, against native virus preparation after the final intravenous injection.

B. Generation of hybridoma clones

After sacrificing each mouse, the spleen cells (1×10^8 from mouse A, 1.14×10^8 from mouse B and 2×10^8 from mouse C) and the appropriate number of myeloma cells (2×10^8, $7.7x10^7$ and 8×10^7 for mice A, B and C, respectively) were mixed and fused as described in Chapter 2 and were plated out in tissue culture plates and kept in a humid atmosphere of 5% CO_2 at 37°C in an incubator. After 3-4 weeks post-fusion, the supernatants from the wells with growing hybridoma cells were screened as described in Chapter 2. The results show that 321 of a total 480 wells used in the three fusions contained growing hybridoma cells and all of these secreted AMV specific antibodies (Table 7-1). The detection of a significantly high proportion of AMV secreting wells was

Table 7-1: Frequency of hybridoma cultures secreting anti-AMV antibodies following fusion.

No. of fusion	Total wells	Number of wells growing hybridoma cultures	Number of wells containing antibody secreting hybridomas[a]
1	192	48	48
2	144	144	144
3	144	129	129
Total	480 (100%)	321 (66.8%)	321 (66.8%)

[a]The supernatant from fusions 1 and 2 were screened by ELISA formats 6, 7 and 8 and supernatants from fusion 3 were screened by ELISA format 6 (see Fig. 2-1).

made possible because of the screening strategy used, which provided diverse antigenic conformations of AMV in the tests (Fig. 2-1, panels 6,7 and 8). The selected hybridoma cultures were further cloned 2-3 times by limited dilution cloning followed by screening as before (Chapter 2). Finally the desired clones were expanded and McAbs produced either *in vitro* or *in vivo* as described in Chapter 2.

C. Differentiation of AMV isolates by McAbs in indirect ELISA

From the three cell fusions, a panel of 15 McAbs was selected to analyse the possibility of differentiating various antigenic forms of the five AMV isolates. All McAbs were shown to be AMV-specific because they did not react with glutaraldehyde-fixed virus preparations of tomato aspermy virus (TAV) or cucumber mosaic virus (CMV) in indirect ELISA format 7 (see Fig. 2-1). They also failed to react when tested with dissociated coat protein preparation of TAV, native virus preparations of galinsoga mosaic virus (GMV) and protein preparations from uninfected N. clevelandii leaves in indirect ELISA format 6 (see Table 7-2). McAb 1 also detected AMV protein but not, tobamo, sobemo, bromo or cucumoviruses by western immunoblotting (Fig. 7-2). McAb5 also did not react with an

Table 7-2: Specificity of monoclonal antibodies determined by indirect ELISA[a].

		Test antigens[b]				
McAb	Isotype	GMV	Leaf extract	TAV(protein)	MCMV[c](Fixed)	TAV(Fixed)
1	IgG1	-[d]	-	-	-	-
2	IgM	-	-	-	-	-
3	IgM	-	-	-	-	-
4	IgG1	-	-	-	-	-
5	IgG1	-	-	-	-	-
6	IgG1	n.d[e].	-	n.d.	n.d.	-
7	IgM	-	-	-	-	-
8	IgM	-	-	-	-	-
9	IgG1	n.d.	n.d.	n.d.	n.d.	n.d.
10	IgM	-	-	-	-	-
11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12	n.d.	n.d.	-	n.d.	n.d.	n.d.
13	IgG1	-	-	-	-	-
14	n.d.	n.d.	-	n.d.	-	-
15	IgG1	n.d.	-	n.d.	n.d	-

[a]ELISA formats were as described in Fig. 2-1 (Chapter 2).

[b]TAV protein (1.5µg/ml), galinsoga mosaic virus (1µg/ml) and protein extract from uninfected N. clevelandii were applied directly to the ELISA plates (ELISA format 3, Fig. 2-1) in 10 mM Na-acetate, pH 6.0 containing 100 mM $CaCl_2$ or 10 mM phosphate buffer, pH 7.0, respectively. Glutaraldehyde-fixed virus preparations of TAV and MCMV (10µg/ml) were applied to the ELISA wells (ELISA format 7, Fig. 2-1) previously coated with purified IgG (1.5µg/ml) from antisera raised to the same form of antigen.

[c]The M strain of CMV (Mossop and Francki, 1977) was used in these experiments.

[d]Indicates that the A405 nm was in the range of background.

[e]Means not tested.

extract from uninfected N.clevelandii in agarose gel immunodiffusion tests or with native virus preparations of CMV in gel-immunodiffusion tests (Fig. 7-3). As it has been shown in Tables 7-2 and 7-3, the McAbs obtained belong to the IgG1 or IgM subclasses. More than 60% of the McAbs obtained did react with most of the five AMV immunogens, i.e. they were directed against group specific dominant epitopes (Table 7-3). However, some McAbs were able to detect minor unique antigenic differences and could thus differentiate between isolates (Table 7-3). AMV isolate S30 can be identified specifically by McAb 3 using native virus as test antigen; N20 by McAbs 4 and 6 with fixed virus; W1 by McAb 9 with native virus; H4 by McAb 11 with protein subunits or McAb 12 with native virus; and S40 by McAb 10 with either native or fixed virus. The different differentiation capacities of the different McAbs depended on which ELISA format was used, which points to a functional heterogeneity of the McAbs (Mierendorf and Dimond, 1983). Besides the McAbs which differentiated between AMV isolates, others were selected that reacted with all 5 isolates (5, 13, 14, 15) but not in all three ELISA formats (Table 7-3).

D. Differentiation of epitope type

Antibodies recognising different types of epitopes have been observed for several plant viruses, including tobamoviruses, nepoviruses and ilarviruses (Van Regenmortel et al., 1985; Dietzgen, 1986a, b; Halk, 1986). In the case of polyclonal antisera to AMV, three types (**neotopes**, **metatopes** and **cryptotopes**) have been described by Moed and Veldstra, (1968), and confirmed to exist in this thesis (Chapter 5). The panel of McAbs listed in Table 7-3 can be divided into 7 types, based on their reactivity in the three indirect ELISA formats used:

1 -McAb 11 reacts with a cryptotope, i.e. a hidden epitope that is expressed only after depolymerization or denaturation of the antigen.

2 -McAb 4, 9, 10, and 15 are specific for neotopes, i.e. epitopes created by the quarternary structure of AMV, present on the surface of fixed and native virus particles, but absent on disassembled protein subunits.

3 -McAb 14 also reacts with a neotope, which is however only present on fixed AMV and therefore owes its existence to the effect of glutaraldehyde-fixation.

Fig. 7-2

Determination of specificity of McAb1 for detection of AMV by western immunoblotting. Preparation of AMV-W1 (Lane 7), tomato aspermy virus (V-strain) (Lane 6), cucumber mosaic virus (M-strain) (Lane 5), cucumber mosaic virus (G-strain) (Lane 4), broad bean mottle virus (Lane 3), tobacco mosaic virus (Lane2) and southern bean mosaic virus (Lane 1) were subjected to polyacrylamide-gel electrophoresis and then transferred to nitrocellulose. The ammonium sulphate precipitated McAb 1 at concentration of 0.5 µg/ml was used for probing the blot. Lanes 1-6 were loaded with 0.75 µg of the corresponding virus and lane 7 with 1µg of the virus preparations. The polyacrylamide-gel was run initially at 100V until the protein passed through the stacking gel and then at 180 V for 60 min and electrotransfer was done at 100V for 70 min.

Fig. 7-3

Immunodiffusion tests in agarose gel using McAb 5 and McAb 8. Central wells were filled with undiluted ascites fluid of the appropriate McAb. Preparations of native (small letters) and glutaraldehyde-fixed (capital letters) AMV-S40 (a,A), N20 (b,B), H4(c,C) S30 (d,D) and W1 (e,E) were loaded in the surrounding wells. Wells f and F were filled with native and glutaraldehyde-fixed virus preparations of L-CMV, respectively. Well H was filled with a protein extract from an uninfected N. clevelandii. The wells were filled with 12 µl of 1 mg/ml of each viral antigen and plates were incubated for 5 days at 25°C before recording the results.

Fig.7_2

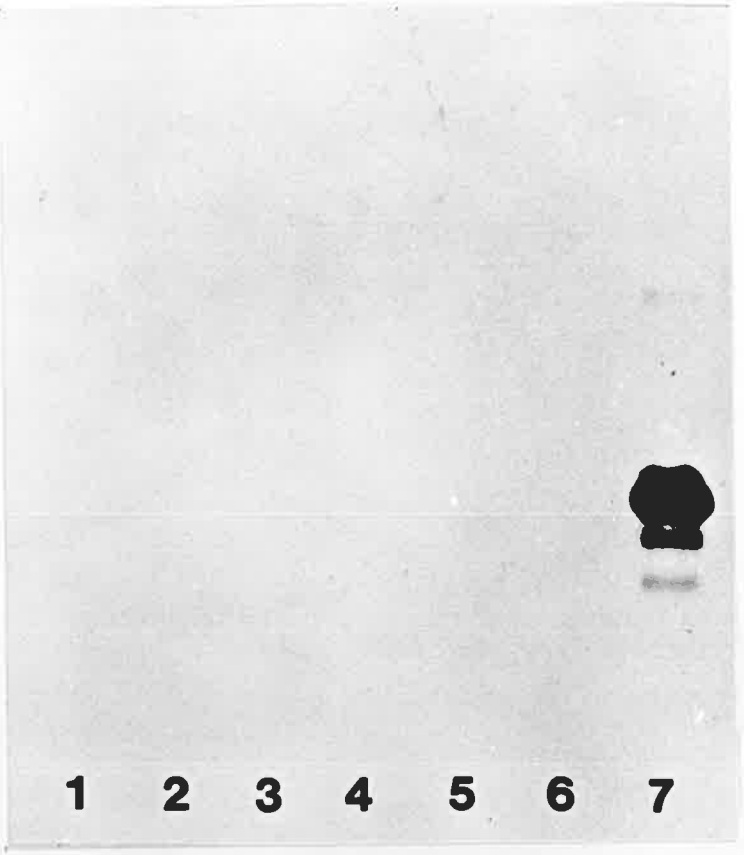


Fig.7_3

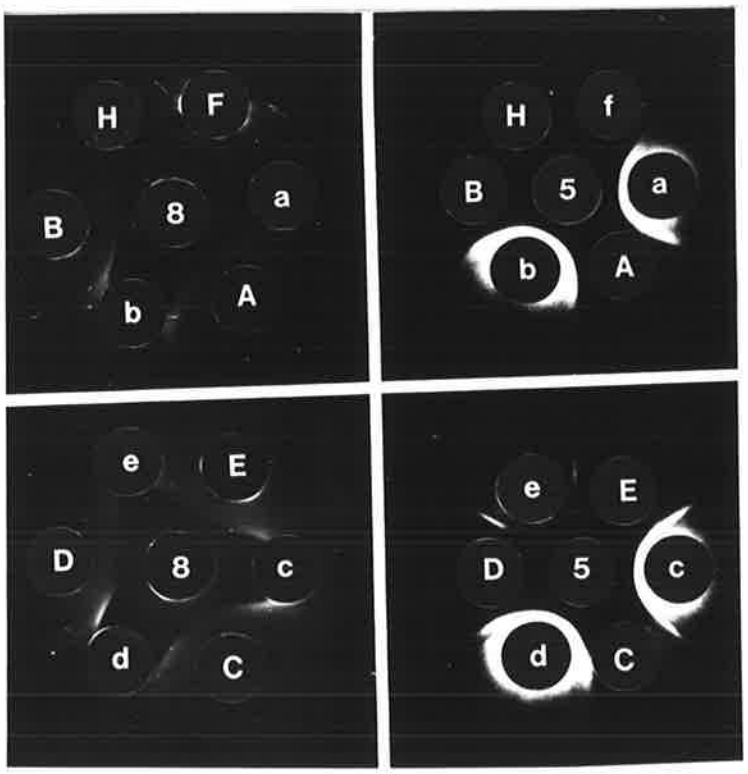


Table 7-3 : Differentiation of AMV isolates by monoclonal antibodies .

		Protein subunits					Native virus					Fixed virus					Antibody specificity	
McAbs	Isotype	H4	N20	S30	S40	W1	H4	N20	S30	S40	W1	H4	N20	S30	S40	W1	ELISA format[a]	AMV isolates
1	IgG1	+[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	1,2,3	All isolates
2	IgM	+	+	+	+	+	-[c]	-	-	-	-	-	-	+	+	-	3	S30, S40
3	IgM	+	+	+	+	+	-	-	+	-	-	-	-	+	+	-	2	S30
4	IgG1	-	-	-	-	-	-	+	-	+	-	-	+	-	-	-	3	N20
5	IgG1	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	1,2	All isolates
6	IgG1	-	+	-	-	+	+	+	-	+	-	-	+	-	-	-	3	N20
7	IgM	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	2	W1[d]
8	IgM	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	1	N20 , W1
9	IgG1	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	2,3	W1
10	IgM	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	2,3	S40
11	n.d[e].	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	H4
12	n.d.	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	2	H4
13	IgG1	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	2	All isolates
14	n.d.	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	3	All isolates
15	IgG1	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	2,3	All isolates

[a] ELISA formats 6, 7 and 8 (see Fig.2-1, Chapter 2) were used with protein subunits, native and glutaraldehyde-fixed virus preparations, respectively.

[b] Indicates A405 nm more than 3 times the background, and [c] indicates that A405 nm is in the range of the background.

[d] Differentiation based on non-reactivity of single isolates.

[e] n.d. = not determined.

4 -McAb 12 and 13 are specific for yet another type of neotopes, which apparently is destroyed by fixation with glutaraldehyde; these McAbs react with the native virus only.

5 -McAbs 1, 3, 6 react with epitopes exposed on dissociated protein subunits, native and glutaraldehyde-fixed virions, the so-called metatopes. These particular metatopes appear to be independent of the conformation of the antigen and are stabilised by fixation with glutaraldehyde.

6 -McAb 2 detects a metatope which is stabilised either by direct binding to the microplate surface or glutaraldehyde-fixation.

7 -McAb 5 also appears to react with a metatope, which is, however, destroyed or masked by fixation.

E. Precipitating McAbs

Of the 15 McAbs listed in Table 7-3, only two were found to react by precipitating in agarose immunodiffusion tests. McAb 5 reacted with an epitope common to all 5 isolates and specifically precipitated native AMV but not native CMV or a protein extract from uninfected N.clevelandii (Fig. 7-3). It did not react when the same isolates had been fixed with glutaraldehyde. This specificity is in agreement with the reactivity exhibited in indirect ELISA (Table 7-3). This indicates either that glutaraldehyde-fixation had altered the reacting epitopes or that the antibody reacts with an epitopes which appears after degradation of AMV in the gel (Chapter 5). The latter possibility seems unlikely because this McAb did not react with AMV coat protein preparations of any of the five AMV isolates in immunodiffusion tests (Table 7-3).

McAb 8 precipitated AMV only after the isolates had been fixed with glutaraldehyde (Fig. 7-3). Antibody concentration in hybridoma supernatants of McAb 8 cultures must have been very low because polyethylene glycol had to be incorporated (Goding, 1986; Van Regenmortel et al 1985) at the agarose gels before precipitin lines could be detected. However, addition of polyethylene glycol was not necessary when the antibodies were purified by affinity chromatography or when undiluted ascites fluids were used. Like McAb5, the McAb8 was not able to precipitate isolated coat protein from any of the AMV isolates.

McAb8 reacted strongly in immuno-diffusion tests with glutaraldehyde-fixed virus preparations of a number of CMV isolates, but not with protein extracts from uninfected N. clevelandii (Fig. 7-3, 7-4, 7-5 and 7-6). However, this McAb was not able to detect glutaraldehyde-fixed TAV, broad bean mottle virus (BBMV), bovine serum albumin, TRSV, TMV, red clover necrotic mosaic virus (RCNMV), southern bean mosaic virus (SBMV) or velvet tobacco mottle virus (VTMoV) in similar tests (Fig.7-5) but not glutaraldehyde-fixed poly -L-lysine (data not shown). McAb8 also showed heterospecific reactivity, as it recognised native particles of many CMV isolates, but not native particles of any of the AMV isolates or BBMV, VTMoV, TRSV, SBMV, TMV or SMBV in similar tests (Fig.7-5 and 7-6). McAb 8 was not able to precipitate native virus preparations of TAV or RCNMV in similar tests (data not shown).

When native and glutaraldehyde-fixed virus preparations of CMV isolates were loaded in adjacent wells, McAb 8 differentiated fixed from native particles by the formation of pronounced spurs (Fig. 7-4 and 7-6). Cross-absorption of this antibody in the gel with glutaraldehyde-fixed virus removed all the reactive antibodies (Fig. 7-4). However, after cross-absorption with native virus preparation antibodies were retained which only reacted with glutaraldehyde-fixed virus (Fig. 7-4). All reactions of McAb 8 were reproducible when either ascites or column purified preparations were used.

F. Detection of AMV with McAbs in western immunoblotting

The reactivity of McAbs 1 and 5, both of which reacted with dissociated coat protein subunits in ELISA (Table 7-3), were further analysed by western immunoblotting. Both McAbs were specific for AMV because no cross-reactivity was detected with coat proteins of members of the tobamo, bromo, or cucumovirus groups (Fig. 7-2) and (Table 7-2).

A marked difference in migration of the coat proteins of all five AMV isolates were detected when they were separated by SDS-polyacrylamide gel electrophoresis under reduced versus non-reduced conditions, indicating the presence of disulphide bonds (Chapter 4, Fig. 4-5). McAb 5 reacted with AMV coat protein in its reduced and non-

Fig. 7-4

Reactions of McAb 8 in agarose gel with native (U) and glutaraldehyde-fixed virus (F) preparations of two isolates of cucumber mosaic virus (CMV). Ascites fluid was dilipidized and diluted 1:4 in 10 mM phosphate buffer, pH 7.0, and loaded directly in antibody wells (8) in panels 1 and 4. Antibody wells in panels 2 and 5 were first loaded with 15µl of preparations of glutaraldehyde-fixed AMV and those in panels 3 and 6 with native virus. After 16 hr incubation at 25°C, wells in panels 2, 3, 5 and 6 were then recharged with 15 µl of 1 : 4 dilution of ascites fluid. Native and glutaraldehyde-fixed virus preparations of CMV isolate TF was loaded in panels 1, 2 and 3. Antigen wells in panels 4, 5 and 6 were filled with native or glutaraldehyde-fixed virus preparations of Fny isolate of CMV. All antigens were used at concentrations of 1 mg/ml. The plates were incubated at 25°C for 5 days before recording the results.

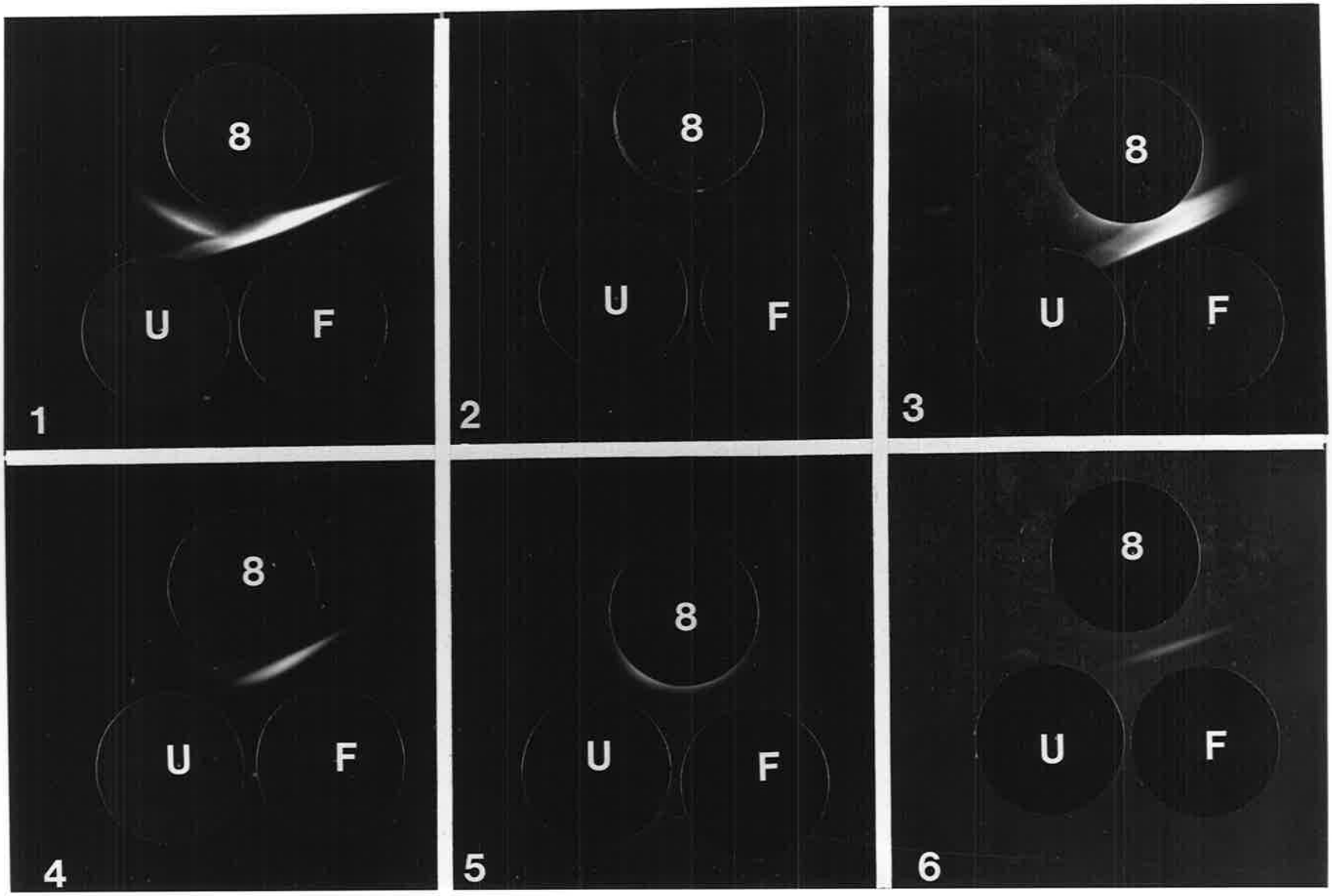

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Fig. 7-5

Reactions of native preparations of different virus isolates from different taxanomic groups with McAb 8 in gel-immunodiffusion tests. Antibody wells (8) were filled with column purified preparations of McAb 8 at concentrations of 1 mg/ml. AMV isolates W1, S40, H5, HI, N3, LA, N20, S30 and H4 were loaded in wells a, e, g, h, i, j, u, p, and s, respectively, (see Chapter 2 for characterization of AMV isolates). Preparations of cucumber mosaic virus isolates Fny, M, U, 2a, Tn, YWA, T, L, TF, Lp and Lc were loaded in wells b, d, f, k, l, v, x, m, o, t and r, respectively. Preparations of tobacco ringspot virus, tobacco mosaic virus, broad bean mottle virus and southern bean mosaic virus were loaded in wells c, w, y and n, respectively. All antigens were used at concentrations of 1 mg / ml and 15 µl of each reactant was loaded in each well. Plates were incubated at 25°C for 5 days before recording the results.

Fig. 7-6

Reactions of a variety of antigens with McAb 8 in gel-immunodiffusion tests. Native (small letters) or glutaraldehyde-fixed (capital letters) virus preparations of cucumber mosaic virus isolates EWA, (a,A), TF(b,B), Fny(D), M(G), T(H), U(I), Wc(K), Q(L), Tn(N), Lc(O), Lv (P), B14(R), and Nan(T); velvet tobacco mottle virus (c,C); tomato aspermy virus (E); tobacco ringspot virus (F); southern bean mosaic virus (J); broad bean mottle virus (Q); tobacco mosaic virus (S) and red clover necrotic mosaic virus (M) were loaded in surrounding wells. The central wells were filled with preparation of delipidized ascites fluid (8). Well U was filled with a preparation of glutaraldehyde-fixed bovine serum albumin. All antigens were used at a concentration of 1 mg/ml and each well was filled with 15µl of each reactant. The plates were incubated at 25°C for 5 days before recording the results.

Fig.7_5

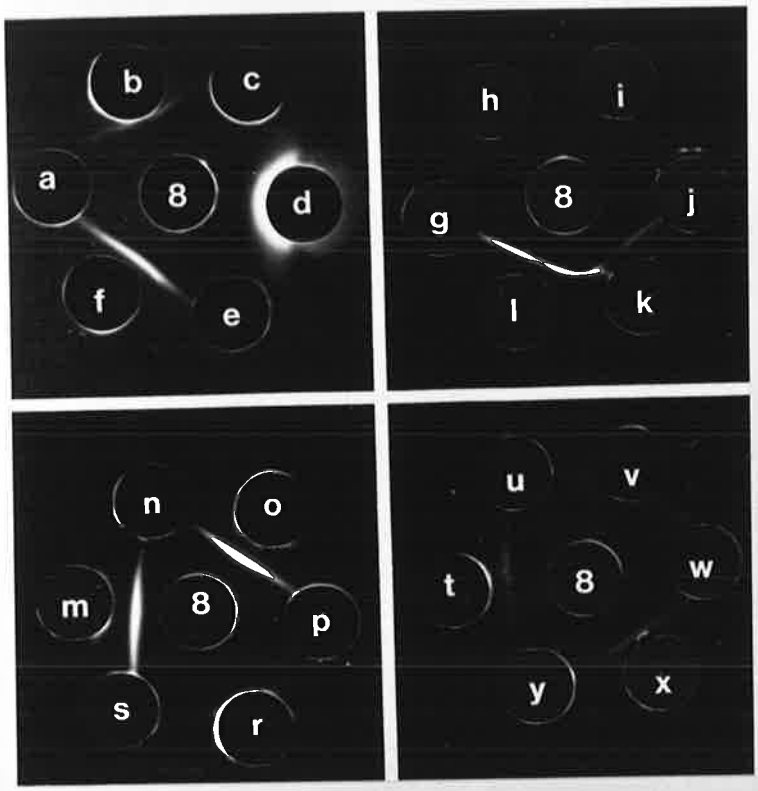
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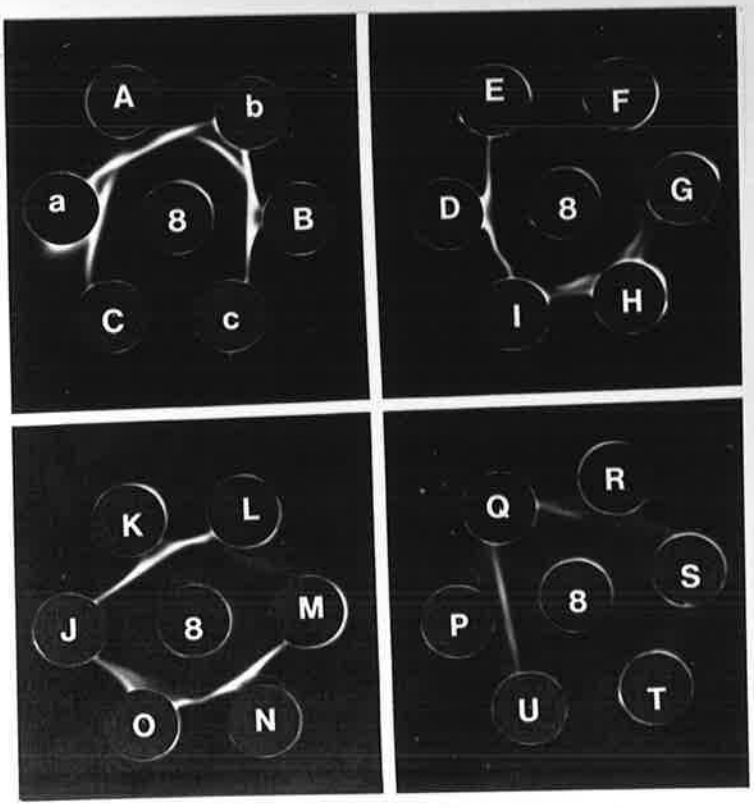
Fig.7_6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reduced states (Fig. 7-7) which indicates that the conformation of the epitope recognised was independent of the stabilising disulphide bonds.

$CaCl_2$-dissociated AMV coat proteins were found to be susceptible to proteolytic degradation, with protein from isolate AMV-S30 being the most stable one (see Appendix 1, Fig. 3). Even though both McAbs 1 and 5 detected epitopes on the coat proteins of all the five AMV isolates (Table 7-3), they appeared to be specific for different epitopes (Fig. 7-8). McAb 5 (Fig. 7-8, panel 2) reacted only with intact coat protein subunits, whereas McAb 1 (Fig. 7.8, panel 1) reacted with intact, as well as coat protein degradation products of all AMV isolates. Both McAbs also reacted with a higher molecular weight protein which appeared to be a dimer of the coat protein. Mouse polyclonal antisera exhibited a reactivity similar to McAb 1 (Fig. 7.8, panel 1) and pre-immune mouse serum showed no detectable reactions (data not shown).

G. Limit of detection of AMV by western immunoblotting using McAbs

The detection limit of AMV coat protein in immunoblots using 100 ng/ml of McAb 5 as probe was reached when 244pg of virus was applied per lane. The same sensitivity was obtained when the separated proteins were stained with silver nitrate directly in the polyacrylamide gel (Fig. 7-9, B and A, respectively).

H. Comparison of sensitivity of McAb and polyclonal antibodies in biotinylated ELISA

The McAb 5 which reacts with native virus and isolated coat protein preparations of all five AMV isolates and McAb 1 which reacts with all the antigenic forms of AMV in various ELISA formats (Table 7-3) were chosen. McAb 5 was produced *in vivo* and McAb1 was produced *in vitro*, but both were purified by affinity chromatography. The polyclonal antisera raised in a rabbit against glutaraldehyde-fixed virus preparation of S30 AMV isolate (Chapter 5, Fig. 5-1) was further purified by ion-exchange chromatography (Chapter 2). McAb 8 and polyclonal IgG were biotinylated and their sensitivity for detecting the native form of the S40 AMV isolate were compared in ELISA formats 10, 11 and 12 (see Fig.2-1). As Fig.7-10 shows, neither of the two McAbs were able to trap any viral particles. This is a common feature of many McAbs which are damaged by

Fig. 7-7

Detection of coat protein of AMV under reduced and non-reduced conditions by McAb 5. Highly purified virus preparations of AMV-S30 and W1 under reduced (lanes 1 and 3) and non-reduced (lanes 2 and 4) conditions were subjected to 12% polyacrylamide-gel electrophoresis. Lane 5 was loaded with 1 μg of Pharmacia protein markers. Electrophoresis was done at 7 mA/gel for 16 hr. One gel was stained with silver (B) and a sister gel was transferred to nitrocellulose at 100V for 4 hr and probed with McAb 5 (A). Each lane was loaded with 5μg of AMV and 1μg/ml of a highly purified preparation of McAb 5 was used for probing the blot.

Fig. 7-8

Comparison of reactivity of McAbs 1 and 5 in western immunoblots. Preparations of virus or coat protein of AMV isolates S30(A, a respectively), N20(B, b), S40(C, c), W1(D, d) and H4(E,e) were subjected to 12% polyacrylamide-gel electrophoresis. The electrophoresis was done initially at 100V until the protein passed through the stacking gel and then at 180V for 60 min. The gel was transferred to nitrocellulose at 100V for 70 min. Affinity purified McAbs 1 (panel 1) and 5 (panel 2) at concentrations of 100μg/ml were used for probing the blots. Each track was loaded with 0.5 μg of either dissociated virus or isolated coat protein. The coat protein was isolated as described in Appendix **1**.

Fig.7.7

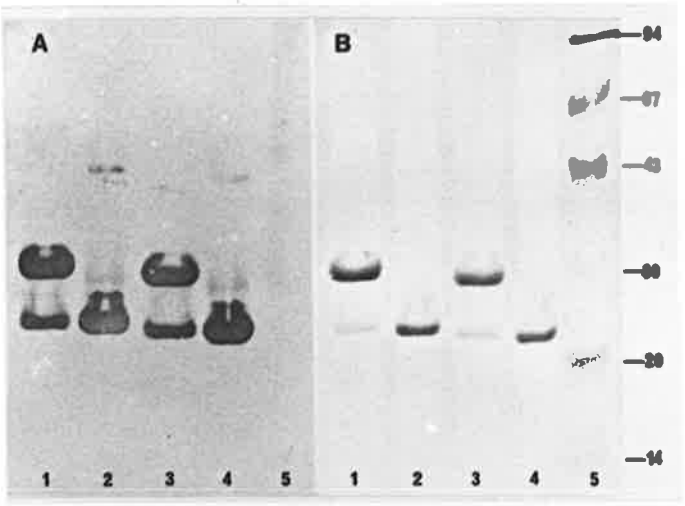


Fig.7.8

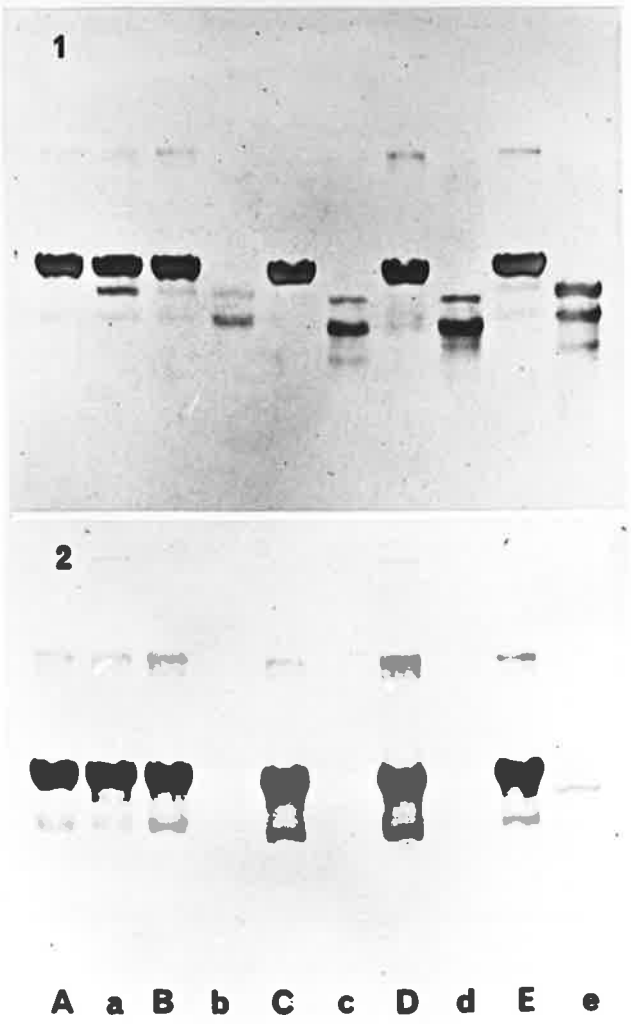
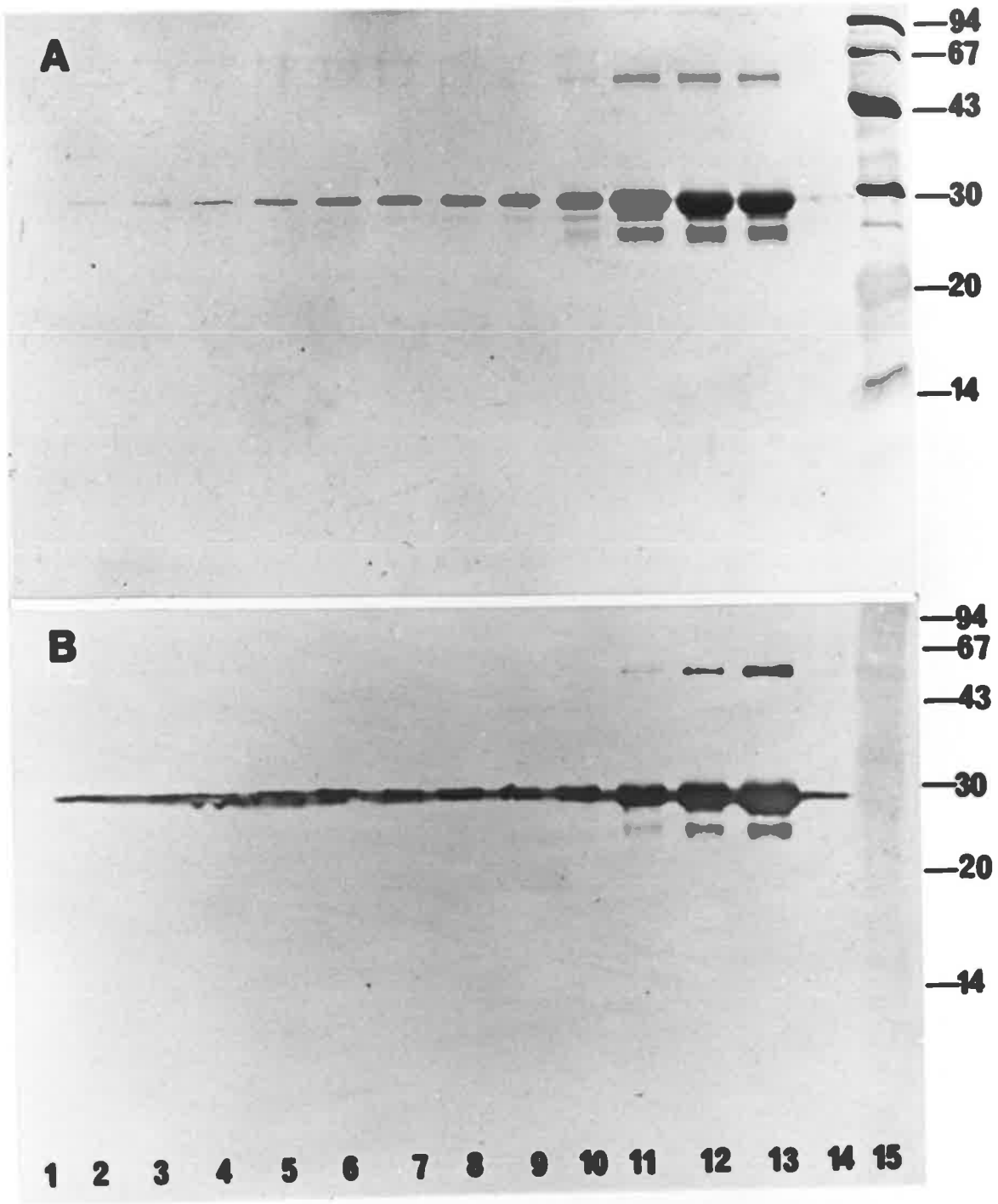


Fig. 7-9

Comparison of sensetivity of detecting AMV coat protein on polyacrylamide-gels stained with silver to that on western immunoblots using McAb 5. Preparations of highly purified N20 AMV isolate were mixed with equal volumes of Laemmli (1970) sample buffer and after heating at 90°C for 5 min were subjected to polyacrylamide-gel electrophoresis using a 12% gel. Lanes 13-1 were loaded with samples containing AMV in a two-fold dilution series, respectively. (In lane 13, 1µg of AMV was loaded and hence 244pg was loaded in lane 1). Lane 15 was filled with 0.5 µg of Pharmacia protein markers and lane 14 was left empty. Electrophoresis was done initially at 100V until the protein passed through the stacking gels and then at 180V for 60 min. One gel was stained with silver (panel A) and a sister gel (panel B) was transferred to nitrocellulose at 100V for 70 min and probed with 100 ng/ml of highly purified McAb 5.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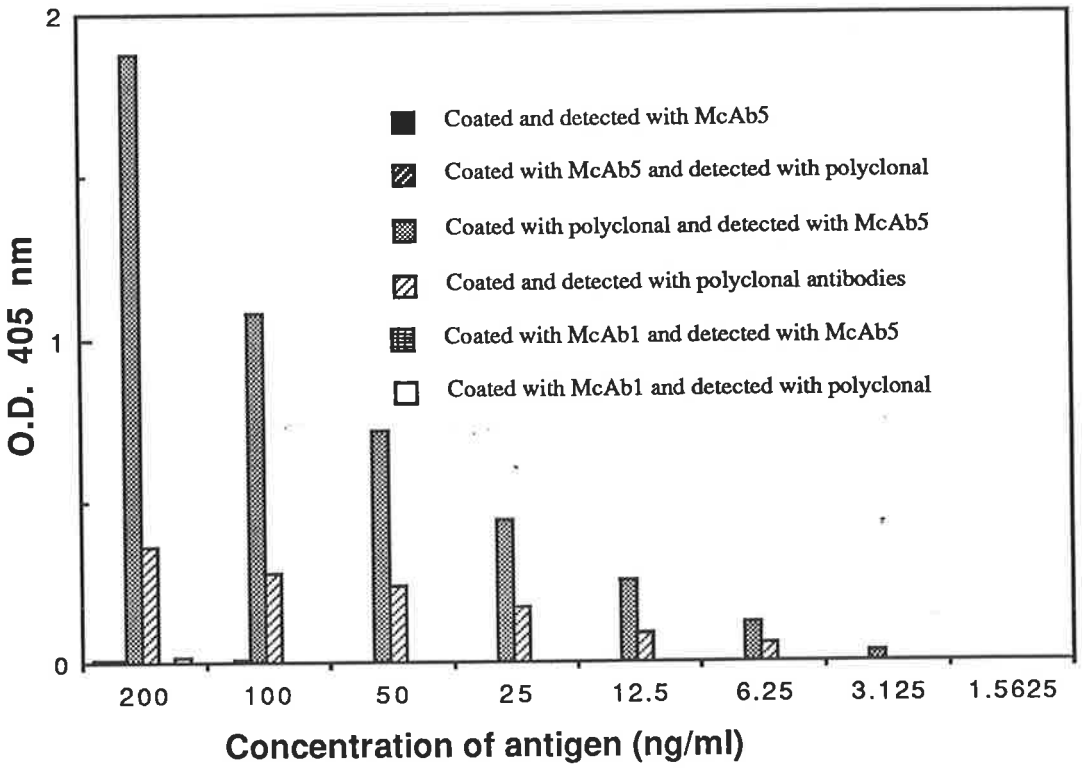

Fig. 7-10

Comparison of detecting AMV by biotinylated McAb and polyclonal IgG using ELISA formats 10, 11 and 12 (see Fig. 2-1 for details of formats). Wells were coated with either affinity purified McAbs 1 or 5 or polyclonal IgG raised against glutaraldehyde-fixed virus. Native virus preparations of AMV-S40 were used as test antigen.

adsorption to the microtitre wells (Martin, 1987). Biotinylated polyclonal antibodies were able to detect as low a concentration as 12.5 ng/ml of AMV. However, when the same antibody was used for trapping antigen, and McAb 5 was used as probe, the limit increased to 3.125 ng /ml.

III Conclusion

The results presented here allow the following conclusions to be drawn:

1 - A panel of stable hybridoma clones secreting specific antibodies to AMV were established. Some of the McAbs were able to differentiate between biologically diverse, but serologically closely related AMV isolates as they reacted with unique isolate-specific epitopes on their coat protein subunits, native virus or glutaraldehyde-stabilized virus particles in indirect ELISA.

2 - The presence of cryptotopes, neotopes and metatopes were recognized by some of the McAbs revealing a complex picture of the antigenic structure of this virus as previously shown with polyclonal antisera.

3 - Masking or altering of AMV antigenic determinants by glutaraldehyde-fixation was demonstrated as one McAb reacted only with native but not glutaraldehyde-fixed virus particles of all five AMV isolates in both ELISA and gel-immunodiffusion tests.

4 - The ability of glutaraldehyde-fixation for retaining epitopes prone to conformational changes was also demonstrated as one McAb reacted only with fixed but not native particles of any of the five AMV isolates.

5 - The usefulness of McAb for detection of AMV was demonstrated as they showed higher sensitivity in ELISA when compared to polyclonal antibodies.

6 - One McAb which reacted with fixed but not native AMV also reacted with fixed or native CMV and fixed particles of several other viruses in different taxonomic groups. This heterospecific reactivity demonstrates the danger of relying on McAb for diagnostic purposes.

Chapter 8

General Discussion

I The importance of AMV in South Australia

The widespread of AMV in the lucerne stands surveyed indicates that the virus is well established in South Australia (Fig. 3-1). Because of the widespread host range of the virus, it seems that it could become an important pathogen of a number of other crops besides lucerne. Already AMV has been isolated from a number of other crops including the forage legumes Medicago scutellata (L.) Miller (snail medic), M. truncatula Gaerth. (barrel medic), Trifolium repens L. (white clover) and Trifolium. subterraneum L.(subteranean clover) as well as the grain legume Lens culinaris Medik. (lentil). AMV has also been isolated from non-leguminous crops such as Lactuca sativa L. (lettuce), Solanum tuberosum L. (potato) (R. Francki and D. Dall, personal communication) and Ocimum basilicum (common basil).

The effect of AMV on lucerne stands around South Australia is not known; however, it has been shown in field trials that it can reduce the herbage production of barrel medic by more than 50%, but the seriousness of the damage depended on the AMV isolate (Dall et al., 1989). Nevertheless, in other countries it has been shown that AMV can cause significant losses in lucerne crops (Gibbs, 1962; Frosheiser, 1969; Crill et al., 1970; Hemmati and McLean, 1977; Tu and Holmes, 1980; Ohki et al., 1986; Bailiss and Ollennu, 1986; Miczynski and Hiruki, 1987; Hiruki and Miczynski, 1987). It seems that because of its wide host range and potential to cause significant damage, measures should be taken for its control in this state. The possible means of control will be discussed later in this Chapter.

II General properties of AMV isolates studied in this thesis

AMV isolates collected from lucerne showed considerable variation in their wide host ranges and symptom expression. Results presented in Table 3-5 show that each of the twelve biologically characterised isolates of AMV could be differentiated by their reactions on one of a number of host plant species. Their different symptoms on bean and tobacco

indicate the possible occurrence of mutations on their respective RNAs 2 and 3 as these genome segments have been shown to carry determinants for such reactions (Dingjan-Versteegh et al., 1972; Hartmann et al., 1976). Isolates which differed in their host ranges and symptomatology were also distinct in their particle length ratios (Fig. 4-1). These differences could be a reflection of nucleotide base sequence differences on their RNA3 as this RNA has been shown to regulate this property (Dingjan-Versteegh et al., 1972; Hartmann et al., 1976). However, any such differences must be relatively small because all the AMV RNAs tested showed strong sequence homology as determined by northern-blot hybridization (Fig. 4-6 a, b and c). Based on dot-blot hybridization tests, the nucleotide sequences of their corresponding coat protein genes also appears to be strongly conserved (Fig. 4-7). This is in agreement with data on two other AMV isolates (425 and S) whose coat protein genes have been completely sequenced (Barker et al., 1983; Ravelonandro et al., 1984). Despite such coat protein gene sequence homology, the capsids of these viruses showed different stabilities and the isolated coat proteins different solubilities (Fig. 1, Appendix **1**). As AMV particles retain their structure by RNA-protein interactions (Jaspars, 1985), the physical differences observed between these isolates could be a reflection of differences in such interactions.

There is no information available as to the role of AMV coat protein in symptom induction. However, it is known that in other viruses such as TMV, the coat protein gene affects symptom expression (Saito et al., 1987; Knorr and Dawson, 1988; Dawson et al., 1988). There is also evidence that the host range of poliovirus is determined by a short amino acid sequence in its coat protein neutralization antigenic site 1 (Murray et al., 1988). With the availability of recently developed techniques such as construction of full length infectious cDNA (Ahlquist et al., 1984; Dawson et al., 1986; Allison et al., 1988; Vos et al., 1988; Dore and Pink, 1988; Hamilton and Bulcombe, 1989), site-directed mutagenesis (Gardiner et al., 1988) and *in vitro* recombination (Saito et al., 1987; Knorr and Dawson, 1988) it is possible to investigate the role of the AMV coat protein gene in symptom induction and host range recognition.

The sizes of RNAs 1-4 were indistinguishable between the AMV isolates studied (Fig. 4-6, A, B and C), however, their relative amounts varied considerably. The most

variable encapsidated segments were RNAs 3 (Fig. 4-6, A, B and C). Although all the AMV isolates encapsidated RNAs 1-4 some, such as isolates N20, H4 and W1, also encapsidated additional segments (Fig. 4-6 A, B and C). It had been previously shown that some AMV isolates encapsidated more than 13 different sized RNA segments (Bol and Lak-Kaeshoek, 1974). An extra RNA segment, with electrophoretic mobility slightly faster than RNA 3 (RNA 3') has been observed in RNA preparations of AMV strains S and B (Walter and Kuszala, 1985). The additional segment was shown to encode for a protein called P'3 *in vitro*, but its function *in vivo* was not elucidated. The presence of AMV sequences on the additional RNA segments observed in preparation of isolates studied in this thesis (Fig. 4-6, a, b and c), shows that they are all AMV related but their role and origin was not investigated. They may have originated by partial transcription of other parts of the genomic segments. Similar but shorter RNA segments have been found in RNA preparations of other viruses (Hilman et al., 1985, 1987). It is not known whether the additional RNA segments observed in the AMV preparations were present originally in the field isolates or whether they originated in the green house after repeated passage. It has been shown that due to repeated passage of cymbidium ringspot virus, a small RNA with virus related-sequences appeared in the plant host (Burgyan et al., 1989). If the extra RNA segments detected in my AMV isolates originated after repeated passage, then one possibility would be that the viral population remained heterogeneous even after five local lesion passages. Such an example has been reported for TMV (Garcia-Arenal et al., 1984).

The difference in host range and symptomatology of the AMV field isolates from their corresponding local lesion isolates (Table 3-6) suggests that field isolates of AMV may contain mixtures of virus variants. With regard to the current theory of "quasispecies" introduced by Domingo et al., (1985) and the experimental evidence published for TMV (Goelet et al., 1982; Garcia-Arenal et al., 1984; Rodriguez-Cerezo and Garcia-Arenal, 1989), it seems that this is not a unique feature of AMV isolates. The presence of such heterogeneity may play an important role in the survival of viruses under field conditions in that selection of variants may be capable of breaking host plant resistance. Such an example has been reported for TMV where, after the release of a resistant plant variety in

the field, in a period of one year, variants were selected which were able to infect the new resistant cultivar (Pelham et al., 1970).

III Effect of glutaraldehyde-fixation on immunogenicity and antigenicity of AMV

In studying the comparative immunogenicity of glutaraldehyde-fixed versus native virus particles in this thesis, glutaraldehyde was used as the fixative reagent. Although formaldehyde has also been used for the same purpose by other workers (see Chapter 1), it is known that its reaction is more reversible than glutaraldehyde (Fraenkel-Conrat, 1969). The results presented in Fig. 5-1 show that fixation enhanced the immunogenicity of AMV, but not to the same extent as that of Q- CMV (Francki and Habili, 1972). However, fixation of some isolates of CMV such as CMV-Y failed to enhance its immunogenicity (Kaper and Waterworth, 1981). The maximum titres of antisera obtained against glutaraldehyde-fixed AMV in this study did not exceed 1 : 128 when titrated against glutaraldehyde-fixed virus (1mg/ml) in gel-immunodiffusion tests, in spite of the long period of immunization (Fig. 5-1). Although antisera with a titre of 1:128 (in gel-immunodiffusion) against native AMV particles was not obtained, such antisera have been produced by other workers (Forster et al., 1985; Paliwal, 1982; Pesic et al., 1988; Avgelis and Katis, 1989). The fact that the intact particles of AMV share antigenic determinants with those of isolated coat protein subunits makes it impossible to determine if the glutaraldehyde-fixed or native AMV particles degrade in the body of the animals. However, in indirect ELISA isolated coat protein was detected with antisera to native AMV as efficiently or better than with antisera to glutaraldehyde-fixed virus (Tables 5-3, 5-4) irrespective of the titre of the antiserum (Fig. 5-1). The titre of antisera to AMV raised against glutaraldehyde-fixed virus was much higher than antisera to native AMV. This suggests that the native virus particles do break up in the body of the animals and are consequently weaker immunogens.

Glutaraldehyde-fixed AMV was always a better test antigen in gel-immunodiffusion tests irrespective of the type of antiserum used (Fig. 5-1, 5-2). The better reactivity of fixed virus may be explained by its enhanced stability. Evidence to support this is

presented in Fig. 4-3 and Fig. 7 in Appendix 2. If this explanation is correct, then probably the agarose gel has less effect on degradation of native AMV than agar (Table 5-1). The experiments in Fig. 5-7 show that in agarose, the electrophoretic mobility of native virus particles is different from those which were glutaraldehyde-fixed. If degradation of native AMV in gel is responsible for its poor antigen-antibody interaction, a similar phenomenon cannot be invoked to explain the results where it was shown that fixed virus particles were trapped more efficiently than native ones to antibodies attached to ELISA microtitre wells (Fig. 5-6, Fig. 4 in Appendix 2). However, it is possible that degradation of native AMV can also occur during antigen-antibody interactions. Although conformational changes in the antigen due to antibody binding has been observed (Crumpton, 1966), there is no published evidence for degradation of viral particles due to antibody-antigen interactions.

IV Does fixation change the antigenicity of AMV?

The antigenic reactivity of different types of polyclonal antisera raised against different conformations of AMV determined by indirect ELISA, revealed that glutaraldehyde-fixed virus appeared to be the least reactive antigen. These results were irrespective of the type of immunogen used for production of the antisera (Tables 5-3, 5-4, 5-5 and Fig. 5-5). These results appeared to be in direct contrast to the observation obtained in gel-immunodiffusion tests (Figs. 5-1 and 5-2). However, results of experiments presented in Appendix 2 demonstrate that the apparent poor antigenicity of glutaraldehyde-fixed virus was a reflection of poor adsorption of fixed virus to microtitre plates and not to any change in the antigenicity of the viral particles as has been claimed for BMV by Rybicki and Von Wechmar, (1981).

Antigenic reactivity of a protein molecule has been defined as its capacity to bind specifically to the functional attachment sites of certain immunoglobulin molecules (Van Regenmortel, 1986b). The titration of different polyclonal antisera against native and glutaraldehyde-fixed virus preparation as well as isolated coat proteins in gel-immunodiffusion tests, showed that glutaraldehyde-fixed virus always reacted better than native virus, irrespective of the type of immunogen used for injecting the rabbits (Figs. 5-1

and 5-2). Fixed virus was also more efficiently bound than native virus to antibodies attached to microtitre plates (Fig. 4 in Appendix 2). Furthermore, polyclonal antisera produced against native or glutaraldehyde-fixed AMV preparations were not able to differentiate between glutaraldehyde-fixed and native AMV particles of their respective AMV isolates in gel-immunodiffusion tests (Fig.5-8). These observations suggest that the glutaraldehyde-fixation had failed to significantly change the antigenic structure of the AMV particles.

Antisera to isolated coat proteins differentiated glutaraldehyde-fixed from native AMV particles by formation of pronounced spurs in immunodiffusion tests (Fig.5-9). Surprisingly, the additional epitopes were detected on glutaraldehyde-fixed rather than native AMV particles. The apparent discrepancy between the data in Fig.5-8 and 5-9 may be explicable by differences in quality of antisera used in these two experiments. Qualitative differences in antisera were actually revealed when reacted in indirect ELISA against different antigenic conformation of AMV particles (compare Tables 5-3, 5-4 with 5-5 and Fig. 5-5). Anti-protein sera, probably contained antibodies directed against the entire antigenic surface of the isolated coat protein subunits. It is known that the isolated coat protein of TMV and potato virus X have at least 5 and 4 antigenic determinants, respectively, (Van Regenmortel, 1982), however, the number of antigenic determinants on AMV coat protein is not known. When glutaraldehyde-fixed and native AMV were compared with these antisera to isolated coat protein preparations, both the antisera demonstrated the possession of a number of common antigenic determinants (Fig. 5-9). However the presence of epitopes on glutaraldehyde-fixed virus particles which do not appear to be present on native particles (Fig. 5-9), indicates changes in configuration of these epitopes on the native particles presumably as a result of viral degradation. The data in Figs 4-2, 4-3, 5-7 and Fig.6 in Appendix 2 indicate that the native virus particle is not as stable as the fixed virus and easily degrades even in 10 mM phosphate buffer, pH 7.0, if it is incubated at 25°C. The reason why antisera to native and glutaraldehyde-fixed virus failed to differentiate between these two antigenic forms of AMV could be due to the lack of antibodies to such epitopes or the presence of steric hindrance as a result of binding of an antibody to nearby epitopes (neotopes created by juxtaposition of coat protein subunits).

McAb 8 which only recognised glutaraldehyde-fixed AMV but not native virus particles in gel-immunodiffusion tests, may have been directed against a conformational epitope retained as a result of fixation. This McAb also precipitated both native and glutaraldehyde-fixed virus particles of a number of CMV isolates (Fig. 7-5). Like AMV, CMV is not a very stable virus and is also prone to degradation during gel-immunodiffusion tests (Scott, 1968). The presence of the epitope on glutaraldehyde-fixed CMV but not native CMV, was revealed by both the presence of precipitin spurs in immunodiffusion tests as well as by intragel cross-absorption tests. When cross-absorption was done with glutaraldehyde-fixed CMV, all reacting antibodies were removed, but when native CMV was used, antibody which reacted only with glutaraldehyde-fixed CMV was retained (Fig. 7-4). It is possible that this antibody has multispecificity or it is a mixture of two McAbs. As has been suggested by Al Moudallal et al., (1982), the concept of multispecificity of combining sites of an antibody has only slowly become established. Although, this concept contradicts the very notion of immunological specificity some experimental data support such a concept. Recently, a McAb with multispecificity was described for potyviruses (Shukla et al., 1989b). Antibodies with multispecific reactivity also has been described for other non plant viral antigens (Richards and Konigsberg, 1973; Richards et al., 1975; Cameron and Erlanger, 1977; Lane and Koprowski, 1982). The possibility of mixed antibodies in McAb 8 seems remote, because they have similar isotypes (IgM) determined by both gel-immunodiffusion and indirect ELISA. Furthermore, the corresponding hybridoma secreting this antibody was obtained after four cycles of limited dilution cloning. Nevertheless, if this McAb is a mixture it still indicates that fixation has preserved one conformational antigenic determinant on CMV particles which is common to AMV.

McAb 5 on the other hand, precipitated native AMV but not glutaraldehyde-fixed particles in gel-immunodiffusion tests (Fig. 7-3). This McAb also recognised both native virus particles and isolated coat protein subunits in indirect ELISA (Table 7-3). Its reactivity with isolated coat protein was confirmed by western immunoblotting as it reacted with intact but not $CaCl_2$ degraded coat protein (Fig. 7-8). The lack of reactivity of this McAb with glutaraldehyde-fixed virus could be simply interpreted as the result of a

conformational change of an epitope. It is also possible that fixation had masked that particular epitope. However, it is possible that McAb 5 can recognise an epitope on the native virus particles which had been induced by binding of the antigen to rabbit polyclonal antibodies used to trap the particles to the microtitre wells (Table 7-3). The capacity of antibody to induce conformational changes in protein antigens has been demonstrated by Crumpton, (1966). Such a change would not be expected to occur on glutaraldehyde-fixed particles because of their enhanced stability. Possibly, one way of resolving the behaviour of McAb5 would be to attempt to detect such an epitope by immunogold labeling as has been done with certain epitopes on TMV (Dore et al., 1988). On the basis of results presented in this thesis, I conclude that if fixation induces any antigenic changes to AMV particles, such changes do not significantly reduce the antigenic reactivity of AMV.

The possibility of creating new epitope(s) by glutaraldehyde-fixation was also investigated in this thesis. It was assumed that, because of the high number of lysine residues in AMV coat protein (Krall et al., 1976; Collot et al., 1976; Castel et al., 1979) glutaraldehyde may induce new antigenic determinants by cross-linking of these residues (Habeeb and Hiramoto, 1968; Korn et al., 1972). If this were so, then glutaraldehyde-fixed poly-L-lysine should have been detected by antisera raised to glutaraldehyde-fixed AMV. As was mentioned in Chapter 5, none of the antisera raised against glutaraldehyde-fixed particles of AMV were able to detect poly-L-lysine.

V Serological properties of AMV isolates studied in this thesis

The overall conclusion from the serological studies in this thesis of the five biologically distinct AMV isolates, indicate that they are serologically related but not identical. However, the degrees of relationship detected between the isolates varied depending on type of serological test, antigenic conformation of AMV and type of immunogen used for production of test antisera. The close serological relationships between these isolates is in agreement with those of other reported isolates of AMV (Bancroft et al., 1960; Tomoru et al., 1968; Paliwal, 1982; Walter and Kuszala, 1985; Hiruki and Miczynski, 1987). However, in contrast with the finding of these workers the results presented in this thesis show that the antigenic structures of the biologically distinct

AMV isolates are not identical and are serologically distinguishable if sufficiently sophisticated methods for their discrimination are used. In gel-immunodiffusion tests, when the antisera to glutaraldehyde-fixed virus preparations were used and native or fixed AMV served as test antigens, no detectable serological differences were established based on the absence of precipitin spur formation (Fig. 6-1, 6-2 A) or differences in SDI values (Tables 6-1 and 6-2). However, when the same preparations were reacted against antisera raised to native virus or isolated coat protein preparations, the isolates could be differentiated (Figs. 6-1, 6-2 B and 6-8).

It seems that, in the light of these observations the majority of antibodies in antisera raised against glutaraldehyde-fixed virus were directed against neotopes, possibly due to their immunodominance (Lubeck and Gerhard, 1981). This is supported by observations that antisera with high titres against glutaraldehyde-fixed and native AMV were relatively inefficient in recognising isolated coat protein subunits in gel-immunodiffusion tests (Fig. 5-1). Nevertheless, antibodies to other epitopes, such as metatopes, were also present in antisera to fixed AMV, but probably they were in lower relative concentrations. These antibodies reacted with isolated coat protein subunits in indirect ELISA and by western immunoblotting (Tables 5-3, 5-4 and Fig. 5-3). It seems possible that antibodies directed against metatopes were unable to bind efficiently to glutaraldehyde-fixed AMV particles due to competition with neotopes or to steric hindrance.

The majority of antibodies in antisera raised to native virus particles appears to have been directed against metatopes. This conclusion is supported by observations that antisera to native virus, despite having lower titres in gel-immunodiffusion tests, reacted equally or sometimes better with native virus or isolated coat protein preparations in indirect ELISA (Tables 5-3 and Table 5-4).

Native AMV is known as one of the most unstable viruses (Bol and Veldstra, 1969; Jaspars and Bos, 1980) and therefore probably degrades under physiological conditions in the bodies of rabbits (Tremaine and Chidlow, 1974). Consequently, in antisera prepared against native virus preparations, in addition to antibodies directed against metatopes and neotopes, some antibodies directed against cryptotopes are also produced. These antisera generally failed to differentiate the different AMV isolates when their native virus particles

were compared (Table 6-3), probably because the particles lost their integrity during the test and the antigenic determinants exposed are shared between all isolates. The reason for the failure of most antisera raised against native AMV particles to differentiate these antigens of the different virus isolates could be due to only a minor proportion of their antibodies being directed against metatopes. This is supported by the observation that only antisera to native S30 were differentiative under these conditions (Fig. 6-1) and the particles of S30 AMV were shown to be the least stable of any of the isolates studied (Fig. 4-2 and Fig. 5, Appendix 1). Because of its instability, the virus probably had been readily degraded in the body of the animal to expose its antigenic determinants on the coat protein subunits.

Antisera raised to native particles of AMV-S40 failed to differentiate any of the five AMV isolates in gel-immunodiffusion tests, irrespective of the type of virus preparation used as test antigen (Tables 6-3 and 6-4). It is possible that either S40 AMV lacks any specific epitopes or that the corresponding region on its native particles is not immunogenic. However, the best explanation is probably that the native particles of this isolate are very stable in the animals body. As a result of the stability, the quality of antisera raised against this antigen was similar to that of antisera produced against glutaraldehyde-fixed virus preparations. This conclusion is supported by data showing that antisera to isolated coat protein preparations of S40 AMV were very differentiative (Fig. 6-8).

Using antisera raised against native virus particles, but employing glutaraldehyde-fixed virus preparations as test antigens, the AMV isolates were most readily differentiated. The differences between some isolates was such that it was possible to obtain isolate-specific or group-specific polyclonal antisera (Fig. 6-15). This indicates that some epitopes on the surfaces of AMV particles are isolate-specific. The reactions of some of the McAbs also support this conclusion (Table 7-3). The antisera to isolated coat protein preparations were the most differentiative in gel-immunodiffusion tests when either native or glutaraldehyde-fixed virus preparations were used as test antigens (Fig. 6-8). This observation probably explains the reason why, for other workers, two isolates of AMV had been differentiated in gel-immunodiffusion tests by an antiserum produced against a virus

preparation which had been heated at 30°C (Van Vloten-Doting et al., 1968; Roosein and Van Vloten-Doting, 1983) and hence had presumably been degraded.

The majority of antibodies in antisera raised against the isolated AMV coat proteins had presumably been directed against both metatopes and cryptotopes. These antibodies failed to differentiate AMV isolates in gel-immunodiffusion tests when their corresponding coat proteins were used as test antigens (Fig. 6-11). However, when native or glutaraldehyde-fixed virus preparations were used as test antigens, the serological differences between these isolates were readily revealed (Fig. 6-8). It has been observed that TMV mutants were only serologically differentiable when their intact particles rather than their respective isolated coat protein preparations were compared (Dore et al., 1987b). Similar observations have also been made with potyviruses and bromoviruses (Shepherd et al., 1974; Rybicki and Von Wechmar, 1981). On the basis of the observations with TMV, it was suggested that in intact virus particles, amino acid exchanges at protein-protein or protein-RNA interfaces can affect virus assembly, therefore modifying the surface structure. It has also been suggested that conformational changes arising from the quaternary structure will not play a role in the antigenic structure of the monomeric protein subunits (Dore et al., 1987b). The same suggestions can also be applied to AMV.

In the potyvirus, bromovirus and cucumovirus groups, it is known that the viruses appeared to be serologically more closely related when their coat protein preparations rather than their intact particles were compared (Sheperd et al., 1974; Rybicki and Von Wechmar, 1981; Lawson, 1967; Mink 1969). Shepherd et al., (1974) suggested that the isolated coat proteins of potyviruses were more closely related because cryptotopes are more stable during evolution than the epitopes present on the surfaces of the virus particles. It was assumed that such conformation was essential for the specific folding and aggregation of the protein chains and the majority of the mutations altering such structure could be lethal for the virus. However, recent studies with potyviruses have shown that the N-termini of the coat protein subunits contains the virus-specific epitopes and are located on the surfaces of the particles (Shukla et al., 1988). It seems that the difficulty in differentiating potyviruses using coat protein preparations is due to the highly conserved core antigens being involved in the reactions.

In contrast to the above mentioned observations, AMV isolates are more readily differentiated by using coat protein preparations and antisera to either fixed or native virus or coat protein preparations in indirect ELISA (Figs. 6-3, 6-7 and 6-12). In studying the serological relationships between AMV isolates using their coat proteins, they were prepared by the $CaCl_2$ method (Appendix 1). Such coat protein preparations undergo proteolysis, the extent of which was shown to vary between isolates (Fig. 3 in Appendix 1). Therefore, the observed differences in serological properties of coat proteins from the different isolates could also be a reflection of differences in their degree of degradation by $CaCl_2$ and consequent cleavage in their antigenic determinants. The effect of such proteolysis on the antigenic properties was observed in gel-immunodiffusion tests where the viral coat proteins at two different levels of degradations were compared (Fig. 6-10). In a western immunoblotting experiment, McAb 5 failed to detect degraded coat protein preparation of all the isolates, but recognised intact coat proteins of all the AMV isolates obtained directly from intact particles, as well as $CaCl_2$ coat protein preparations of AMV-S30 which had remained intact (Fig. 7-8). This demonstrated that antigenic determinants can be lost during $CaCl_2$ degradation of the protein. The effect of proteolysis has also been shown to affect the antigenic properties of other plant viruses such as CaMV (Du Plessis et al., 1980) and some potyviruses (Hiebert et al., 1984).

The type of serological test also played an important role in detecting serological differences between AMV isolates. The indirect ELISA was not a suitable method because of differences in binding properties of the native virus particles of different isolates to microtitre plates in 10 mM phosphate buffer, pH 7.0 as coating buffer (see Appendix 2). However, when carbonate buffer, pH 9.6, was used for coating, antigens of all the isolates bound uniformly, irrespective of whether they were fixed or native. Under these conditions, native virus particles lost their integrity, but glutaraldehyde-fixed virus remained intact (see Appendix 2, also Fig. 4-3). In these experiments only native virus particles of S30-AMV were differentiated from those of the other isolates, but only when antisera to glutaraldehyde-fixed virus was used (panel 3B in Fig. 6-3). The native and glutaraldehyde-fixed viruses of other isolates were indistinguishable in this test irrespective of the type of antisera used (Fig. 6-3, 6-7, 6-12). However, in similar tests, when

comparison was made at the level of isolated coat protein preparation, serological differences between some of the isolates were revealed. This was irrespective of the type of antisera used (Fig. 6-3, 6-7 and 6-12).

The discrepancy in the results obtained in indirect ELISA compared to gel-immunodiffusion tests must be due to the basic differences of these two systems. In gel-immunodiffusion tests, the antibody-antigen interaction is different from the indirect ELISA in which one of the reactants is immobilized (Stevens et al., 1986).

Some McAbs produced in this study had the ability to serologically differentiate between certain AMV isolates (Table 7-3). However, they demonstrated that the isolate-specific epitopes are located on different conformations of AMV. For example McAb 11 differentiated AMV-H4 from all the other isolates, but only when their isolated coat proteins were compared. As this McAb did not react with any other epitope located on native or glutaraldehyde-fixed virus preparations of any of the isolates, it seems that it recognised a cryptotope. One possibility is that this cryptotope is specific to AMV-H4. However, because of the differential $CaCl_2$ degradation of AMV coat proteins such a conclusion must be tentative.

McAb 10 differentiated AMV-S40 from all the other isolates only when native or glutaraldehyde-fixed virus preparations were used as test antigens (Table 7-3). As native AMV-S40 particles are relatively stable, and hence the recognised epitope was probably retained on native virus during the test. The stability of native AMV-S40 virus particles may also explain why polyclonal antisera raised against such particles, like antisera to its glutaraldehyde-fixed virus, lacked specificity.

McAbs 13 reacted only with native virus particles whereas McAb 14 reacted only with glutaraldehyde-fixed particles of all isolates. As these McAbs did not react with any of the isolated coat protein preparations, they must recognise neotopes (Table 7-3). This indicates that these two conformations of AMV possess different antigenic determinants.

Despite employing different versions of indirect ELISA, which exposed various antigenic conformations of AMV, only three isolate-specific McAbs were obtained (Table 7-3). This may be due to the immunization strategy used. As the mixture of native virus preparations of all five AMV isolates served as immunogen, it is possible that the B-

lymphocytes of immunized mice only responded to immunodominant epitopes which were probably similar in all the isolates. Although mixtures of the isolated coat proteins of all five isolates were also injected at later stages of immunization, the hybridomas were screened only with isolated coat proteins as test antigen. In hind-sight of the results obtained with polyclonal antisera, there may have been better chances of obtaining isolate-specific McAbs if each mouse was injected with coat protein preparation of only one virus isolate.

Among the assays which were used for the comparative serology of AMV isolates, western immunoblotting was the least satisfactory test for differentiating the isolates (Fig. 6-14). This has also been observed with viruses of other groups where cross-reactivity has been observed, even with viruses from different taxonomic groups (Burgermeister and Koenig, 1984). The antigenic similarity between AMV isolates revealed in this test provide additional evidence that the isolate-specific antigenic determinants are conformational-dependent.

VI Speculation on conformational types of isolate-specific antigenic determinants

AMV, like many other plant viruses, is known to have a number of different types of antigenic determinants (Chapters 5 and 7 of this thesis; Moed and Veldstra, 1968; Halk, 1986). Cryptotopes are believed to be present only on the surfaces of the protein subunits which are hidden after particle assembly (Van Regenmortel, 1982). Isolated subunits also have other determinants which are, however, exposed on the surfaces of intact viral particles. These antigenic determinants have been referred to as metatopes if their specificity remains similar on isolated coat protein subunits and intact particles. However, due to inter-subunits bonds or RNA-protein interactions on intact particles, some of these antigenic determinants undergo conformational changes and consequently are changed and are then referred to as neotopes. Other kinds of neotopes are also present on intact particles which owe their existence to juxtaposition of the viral coat protein subunits (Van Regenmortel, 1982). The presence of all four antigenic determinants associated with AMV was confirmed by studies with both polyclonal antisera and McAbs (Fig.5-11, Table 7-3).

On the basis of the experimental evidence from gel-immunodiffusion tests with polyclonal antisera, it seems that the neotopes originated by juxtaposition of subunits are similar between all the five AMV isolates studied (Figs 6-1, and 6-2, panel A). These neotopes are presumably also immunodominant, particularly when glutaraldehyde-fixed virus was used as immunogen. The antigenic determinants present on surfaces of isolated coat protein subunits are also similar between the AMV isolates (Fig. 6-11). On the other hands the type of neotopes originating by conformational changes in isolated coat protein after polymerisation appear to be isolate-specific (Fig. 6-1. 6-2 panel B).

VII The status of the five AMV isolates studied in this thesis

Throughout this thesis I have referred to each AMV isolate as an "isolate" rather than "strain". As was mentioned in the introduction to this thesis, terms such as "strains", "variants" and "serotypes" are all ill defined and different virologists have different perceptions of them (Price, 1964; Gibbs and Harrison, 1976; Van Regenmortel and Von Wechmar, 1970; Bos, 1983; Walkey, 1985). Even in the guide-lines for the identification and characterization of plant viruses published by a study group of the I.C.T.V., such terms have not been adequately defined (Hamilton et al., 1981). As AMV isolates were collected directly from the field during this study it seemed justified to refer to them as isolates rather than strains or variants of AMV. Now that differences in the biological properties of all the isolates and physical as well as serological properties of the five most biologically distinct isolates have been established, the status of at least these five isolates should be changed to strains. However, it has been shown that most of the AMV isolates examined can be differentiated by their host ranges and symptom expressions (Table 3-5). The situation with the description of new strains of AMV is probably best summed up by Bawden (1964) who remarked that it is valueless to describe new strains of such variable viruses as AMV. If the definition of Van Regenmortel and Von Wechmar, (1970) be adopted to the AMV isolates studied in this thesis, the five AMV isolates can also be considered as five serotypes because all are serologically distinguishable. However, because of their serological close relationships, it is suggested that the use of this term be

avoided for variants within the AMV group because of the practical difficulties of detecting the differences.

The attempt to classify the biologically characterised AMV isolates into clusters (Fig. 3-5) which could be considered as strains on the basis of their reactions on plants as attempted by Crill et al., (1971), convinced me that this is impossible unless completely arbitrary criteria are used. I conclude that the host range and variability of AMV is so great that any such classification would fail to serve any useful purpose.

VIII Are AMV and CMV serologically related?

AMV and CMV share a lot of common features (Chapter 1), but no serological relationships between these viruses have been detected with polyclonal antisera. However, McAb 8 recognised glutaraldehyde-fixed virus particles of both AMV and CMV (Fig. 7-5, 7-6). It was shown that the epitope recognised by this antibody was not induced by lysine aldehyde bridges as the antibody failed to recognise the glutaraldehyde-fixed poly-L-lysine in similar tests (data not shown). Moreover, the antibody recognised native particles of CMV but not AMV. This possibility had to be eliminated because the coat proteins of both these viruses are rich in lysine residues (Habili and Francki, 1974b; Krall et al., 1976; Collot et al., 1976; Castel et al., 1979) and it is known that aldehyde cross-links these residues on protein molecules (Habeeb and Hiramoto, 1968). It seems that the epitope shared by AMV and CMV does not involve cross-links between lysine residues but that it has been stabilised by glutaraldehyde-fixation and it is presumably an epitope which has been evolutionarily conserved. Similar or a different epitope was also recognised on native CMV, but not AMV. Heterospecificity has been observed with McAbs raised against TMV or potato virus X (PVX) where higher affinity was observed with antigens of a strain of the same virus which was not used for immunization (Al Moudallal et al., 1982; Torrence et al., 1986). The heterospecific phenomenon is not only a feature of McAbs as it has also been observed with polyclonal antisera (Loor, 1971; Rao et al., 1987). However, the heterospecific reactivity observed with these polyclonal antibodies have been detected with viruses within the same taxonomic group. In polyclonal antisera, due to the presence of a large and varied population of antibodies, the potential cross-reactivity of each individual

antibody can easily be masked (Lane and Koprowski, 1982). Consequently, heterospecificity is more commonly observed with McAbs.

Van Regenmortel, (1982) has suggested that heterospecific antibodies appear as a result of the unfolding of otherwise hidden epitopes of an antigen in the immunized animal and will only react with a heterologous antigen when the particular epitope is rendered more accessible than in the homologous antigen. The recognition of the epitope on native CMV but not on native AMV, may be because of AMV being less stable than CMV in gel-immunodiffusion tests. Such instability may be the cause of alteration of particular epitopes to which this antibody has been directed. Recently, it was shown that a McAb cross-reacted with the 3A proteins of TAV and CMV (Mackenzie and Tremaine, 1988). On the basis of this observation, Mackenzie and Tremaine, (1988) suggested that the 3A protein is conserved between these two viruses even though their capsid proteins show only a limited serological relationship. If such a conclusion can be drawn, then it can also be extended to a hypothesis that the observed cross-reactivity between AMV and CMV indicates evolutionary relationship. However, one must consider that such an assumption can only be made if the binding of these two viruses to the antibody is due to the conformational similarity of the epitope concerned, and not the multispecificity of the functional sites of the antibody (Lane and Koprowski, 1982). If the cross-reactivity is due to conformational stability of the epitope, it is possible to link their presence with a particular biological function which would place evolutionary constraints on any change. It has also been suggested by Rybicki and Von Wechmar (1981) that the conformation-specific antibodies are evolutionarily much more conserved between bromoviruses than sequence-specific antibodies. It would seem worthwhile to investigate the serological cross-reactivity between other members of the Tricornaviridae using their various antigenic conformations and corresponding antisera. The results presented in this thesis clearly demonstrate how different serological results can be obtained experimentally with different antigenic conformations of a virus and their corresponding antisera.

Based on reactions of McAbs raised against CMV (Porta et al., 1989), no McAb was found which was able to recognize all the CMV isolates from a collection of virus strains belonging to the major serotypes. It was suggested that a mixture of at least two McAbs is

necessary for the detection of all isolates from the different serotype groups. However, the reaction of McAb 8 with CMV isolates from two distinct serotypes indicates the possibility of using this antibody as a universal agent for identification of CMV. However, the heterospecific reactivity of McAb 8 also demonstrates the danger of using McAbs for epidemiological investigations, because of its recognition of CMV and hence possibly other viruses. Furthermore, this finding emphasizes the importance of intensive screening of McAbs before releasing them for diagnostic purposes. The finding of a hybridoma secreting antibody with the ability of recognising all CMV isolates demonstrates the importance of using different test antigens in the screening strategy. As has been suggested by Van Regenmortel, (1986a), McAbs can be obtained which react weakly (or not at all in a particular type of assay) with the virus strain used for immunization, but which have a high degree of affinity for other strains.

IX Taking advantage of the genetic stability of the AMV coat protein

The very wide biological variation among AMV isolates raises an important question regarding control of the virus by selection and breeding for resistance. Screening for resistance will be extremely difficult because of plants being immune to some isolates of the virus and susceptible to others. For example, five of the twelve AMV isolates tested failed to infect Rutgers tomato whereas the others infected and some even killed the plants of this cultivar (Table 3-4). On the other hand, Gross Lisse tomato was immune to all but one of the virus isolates, which was so virulent that it killed the plants. Moreover, the isolate which infected Gross Lisse tomato failed to infect Rutgers plants. Nevertheless, based on the results of experiments in this thesis, two approaches can be pursued with confidence for the control of AMV.

A. Seed indexing scheme based on serological techniques

AMV is a seed-borne virus (Jaspars and Bos, 1980), by which the long distance spread of the virus can be achieved. The introduction of AMV in both Australia and New Zealand has been partly blamed to the importation of AMV-infected seeds from the U.S.A. (Garran and Gibbs, 1982; Forster et al., 1985). Seed lines have been reported to be up to

10% (Hemmati and McLean, 1977; Garran and Gibbs, 1982) and occasionally as high as 17-26% infected (Frosheiser, 1974; Tosic and Pesic, 1975). Even much lower levels of seed infection are sufficient to establish AMV in a crop which can then spread quickly by aphid vectors and can remain as a long-lived source of infection in perennial plants such as lucerne. The high incidence and longevity of AMV in alfalfa seeds suggest that infected seeds play an important role in the epidemiology of AMV (Frosheiser, 1974; Hemmati and McLean, 1977). Consequently, as a preventive measure it is important to screen seed for AMV. To do this, a reliable screening system is necessary. Results presented in Chapter 6 and 7 show that AMV isolates with wide biological properties are serologically very closely related when assayed by different serological techniques. This is in agreement with work on other AMV isolates reported by Bancroft et al, (1960) based on serological precipitin ring tests and by Paliwal, (1982) using immunodiffusion. Thus serology can be used with confidence to detect any AMV strains with unusual biological properties even by DAS-ELISA (Fig. 6-4) which is known to often have narrow specificity (Koenig, 1978; Lister and Rochow, 1979; Uyemoto, 1980). Nucleic acid hybridization experiments also showed that isolates with wide biological differences have strong sequence homology (Fig. 4-6 a, b and c). This assay can probably also be used for the detection of AMV with confidence, although it probably offers no advantages over serological methods. Although hybridization assays are believed by some to be much more sensitive than DAS-ELISA, a comparative study of ELISA and dot-blot hybridization for detecting AMV in alfalfa pollen, revealed that both techniques had similar sensitivities (Pesic and Hiruki, 1988). If a more sensitive assay than DAS-ELISA is needed, other versions of ELISA can be used as has been demonstrated by Al Moudallal et al., (1984). McAbs could also be employed to provide higher sensitivities as was demonstrated in Fig. 7-10. Although, most McAbs are labile and tend to lose their activity when directly attached to the solid phase (Martin, 1987), in conjunction with polyclonal antibodies they can provide a sensitive serological system (Fig. 7-10).

B. Development of coat protein mediated resistant plants

For developing resistance to AMV, the coat protein mediated genetically engineered cross-protection is a promising approach. Tomato and tobacco transgenic plants expressing the coat protein gene of AMV have already been developed (Tumer et al., 1987; Loesch-Fries et al., 1987; Van Dun et al., 1987). It seems that the AMV coat protein gene is evolutionarily highly conserved. The observation of strong sequence homology between coat protein genes of different AMV isolates studied in this thesis, as well as the serological studies indicate the presence of such genetic stability. DNA or protein sequencing of coat protein genes or coat proteins of other isolates of AMV also support this conclusion (Krall et al.,1976; Collot et al., 1976; Castel et al., 1979; Barker et al., 1983; Ravelonandro et al., 1984). With the genetic stability of this gene among different isolates, it seems that transgenic plants expressing AMV coat protein should protect against a wide variety of AMV isolates. Such an opportunity does not appear to exist between strains of some other plant viruses such as tobacco rattle virus where it has been shown that the transgenic plants expressing the coat protein gene of one strain did not protect against infection with some heterologous isolates. This was almost certainly due to the lack of sequence homology between their coat protein genes determined by hybridization experiments (Van Dun and Bol, 1988). However, if this approach is going to be pursued with AMV, it is important to select a coat protein of an AMV isolate which is not transmissible by aphids. This is of paramount importance because of the danger of encapsidation of other pathogenic RNAs by the expressed coat protein already present in the transgenic plant. A number of examples of transcapsidation have been reported for plant viruses (Dodds and Hamilton, 1976). For example, it has been demonstrated that carrot mottle virus, which is naturally non transmissible by carrot-willow aphid (Cavariella aegopodii), is transmitted from a plant doubly infected with carrot red leaf virus (Waterhouse and Murant, 1983; Murant et al., 1985). The transmission is achieved because the RNA of carrot mottle virus is packaged in the coat protein of carrot red leaf virus. Also, the ability of a virus to encapsidate a viroid has been demonstrated (Francki et al., 1986).

X Why the coat protein gene of AMV is evolutionarily conserved

As was mentioned in the introduction to this thesis, the coat protein of AMV plays at least three functions in the life cycle of the virus, encapsidation of newly formed RNA, programming of the viral RNA polymerase for plus-strand synthesis (Nasuth and Bol, 1983; Houwing and Jaspars, 1987) and activation of the genome (Bol et al., 1971; Smit et al., 1981). With other viruses, there is evidence that the coat protein of the virus has a role in its vector transmissibility (Mossop and Francki, 1977; Stanley et al., 1989; B. Chen personal communication) and a similar role of AMV coat protein also seems certain. In some viruses, such as the geminiviruses which are transmitted by the whitefly Bemisia tabaci, the coat protein gene between the isolates has been more strongly conserved than any other part of the genome (Hamilton et al., 1984). It is assumed that the structural features of the surface of their particles are required for transmission by this vector species (Roberts et al., 1984). The presence of at least two common epitopes between all these viruses were revealed by two McAbs (Thomas et al., 1986). Consequently, during evolution, mutants with changes in this gene presumably failed to survive. As far as the importance of coat protein of AMV for its aphid transmission is concerned, this virus is not completely dependent for its survival on vector transmission, as it can be transmitted mechanically as well as through seeds and pollen (Jaspars and Bos, 1980). The significance of the evolutionary conservation of the coat protein gene to the survival of the AMV is not absolutely clear. Whatever the reason is, at least it is unlikely to be concerned with activation of the AMV genome because the coat protein of some Ilarviruses which lack any sequence homology with AMV (Cornelissen et al., 1984), can replace AMV coat protein for its genome activation (Gonsalves and Garnsey, 1975a).

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Appendix 1

Preparation of soluble, biologically active alfalfa mosaic virus coat protein and its $CaCl_2$-induced degradation.

(This has been published as a paper in the Journal of Virological Methods).

Preparation of soluble, biologically active alfalfa mosaic virus coat protein and its $CaCl_2$-induced degradation

I Summary

A method for the preparation of soluble protein from five biologically distinct alfalfa mosaic virus (AMV) isolates is described. Highly purified AMV was dissociated with 1 M $CaCl_2$ in 10 mM sodium acetate, pH 6.0, and the precipitated RNA was removed by centrifugation. The protein was dialysed against 10 mM sodium acetate, pH 6.0 containing 0.1 M $CaCl_2$. If the salt concentration was reduced further, proteins from some AMV isolates precipitated. Proteins prepared by this method were shown to be immunoreactive and to activate the infectivity of the AMV genome. However, during prolonged exposure of the protein to buffers containing 0.1 M $CaCl_2$, it undergoes slow proteolysis thereby losing its ability to activate the AMV genome but not its immunoreactivity.

II Introduction

Alfalfa mosaic virus (AMV) has a tripartite genome consisting of three single-stranded RNAs (RNAs 1, 2 and 3) of positive sense. The partial transcript of RNA3 (RNA4) encoding the coat protein gene is also encapsidated with protein subunits of Mr. 24.3 x 10^3. Each of the four RNAs is encapsidated separately to form quasispherical particles about 18 nm in diameter, plus a series of bacilliform particles of the same width but differing in length depending on the RNA encapsidated. AMV is unusual in that for infectivity, inocula must contain either RNA4 or coat protein in addition to the three genomic RNAs (Jaspars, 1985).

A number of methods have been described for preparing coat protein from purified AMV with various degrees of success (Kelly and Kaesberg, 1962; Hull, 1969; Kruseman et al., 1971; Gonsalves and Garnsey, 1975). We wished to prepare proteins from five biologically distinct but serologically similar isolates of AMV. However, we found that whereas satisfactory preparations from some of the virus isolates could be obtained by some of the previously published methods, no one method was suitable for the preparation of protein from all the five isolates. Thus we set out to develop a method which could be

used for all the AMV isolates. We required that the method should provide soluble protein preparations free of viral RNA which were immunoreactive and capable of activating the AMV genome (Bol et al., 1971; Smit et al., 1981). This paper describes a method using $CaCl_2$ to dissociate AMV into RNA and protein which remains undegraded if prolonged exposure to low molarity $CaCl_2$ is avoided.

III Materials and Methods

A. Virus isolates and their purification

Five AMV isolates (H4, N20, S30, S40 and W1) shown to be biologically distinct (Hajimorad and Francki, 1988), were propagated in Nicotiana clevelandii and purified by differential and sucrose density-gradient centrifugation as described by Hajimorad and Francki, (1988).

B. Isolation of virus genomic RNAs

AMV genomic RNA (RNAs 1, 2 and 3) devoid of the subgenomic RNA4 was prepared as follows. Virus preparations were mixed at 0°C, with an equal volume of 60 mM $MgSO_4$ and 10 mM NaH_2PO_4 adjusted to pH 7.0 with NaOH. The solution became turbid and after 2 h was layered over 10% sucrose dissolved in 10 mM phosphate buffer, pH 7.0, containing 30 mM $MgSO_4$ and centrifuged for 30 min at 35000 x g. Under these conditions the nucleoprotein components containing RNAs 1, 2 and 3 were pelleted whereas most of that containing RNA4 remained in the supernatant (Hull and Johnson, 1968; Van Vloten-Doting and Jaspars, 1972). The pellets were resuspended in 10 mM phosphate buffer, pH 7.0, and dialysed against the same buffer overnight at 4°C. RNA was isolated by phenol extraction (Peden and Symons, 1973) but still contained small amounts of RNA4 when analysed by agarose gel electrophoresis (Francki et al.,1986). Thus the RNA was subjected to one cycle of preparative gel electrophoresis (Rao and Francki, 1982) from which only RNAs 1, 2 and 3 were recovered. RNA4 was not detected in such preparations when subjected to further agarose gel electrophoresis. The preparations were not infectious also indicating that they were devoid of RNA4.

C. Spectrophotometry

Concentrations of purified AMV and its RNA were determined in a Beckman DU 8B spectrophotometer using $E^{0.1\%}_{260nm}$ of 5 and 25, respectively. Protein concentration was determined using $E^{0.1\%}_{280nm}$ of 0.7 (Jaspars and Bos, 1980).

D. Infectivity assays

Infectivity of AMV RNA and added protein preparations were assayed on half-leaves of French bean plants (Phaseolus vulgaris cv. Hawkesbury Wonder). Mixtures of RNA and protein in 10 mM Tris buffer, pH 8.3, containing 1 mM EDTA were incubated for 30 min at room temperature before inoculation.

E. Polyacrylamide gel electrophoresis of proteins

AMV preparations, or protein preparations from which salts were removed by microdialysis (Overall, 1987) against 50 mM Tris-HCl, pH 6.8, containing 0.05% SDS, were heated for 5 min at 90-100°C in 62.5 mM Tris-HCl, pH 6.8, containing 5% 2-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue. The dissociated proteins were then separated on 12% slab gels using the SDS-discontinuous buffer system (Laemmli, 1970). The separated proteins were detected by staining with silver nitrate (Wray et al., 1981).

F. Serological techniques

Antisera were produced in rabbits subjected to periodic immunization with AMV proteins, and the animals were bled through the ear vein at regular intervals (see Results and legend to Fig. 2).

Antiserum titres were determined by immunodiffusion tests. Native or glutaraldehyde-fixed virus preparations (Hajimorad and Francki, 1988) were suspended in 10 mM phosphate buffer, pH 7.0, and reacted in petri dishes containing 0.75% agarose in phosphate buffer, pH 7.6, containing 0.02% sodium azide. To retain them in solution, virus protein preparations, were suspended in 10 mM sodium acetate, pH 6.0, containing 0.1 M $CaCl_2$ and were reacted in 1% agarose in 10 mM sodium acetate buffer, pH 7.6, containing 0.1 M $CaCl_2$. Some of the antisera were also titrated by an indirect enzyme-

linked immunosorbent assay (ELISA). Antigens in 10 mM sodium acetate buffer, pH 6.0, containing 0.1 M $CaCl_2$ were applied to polystyrene microtitre plates (Nunc, Denmark) and incubated at 25°C for 3 h. The plates were then washed, blocked with 1% bovine serum albumin in 0.1 M NaCl, incubated with dilutions of the antisera to be titrated, and probed with affinity purified goat anti-rabbit serum labelled with alkaline phosphatase as described by Dietzgen and Francki, (1988).

Immunoreactive polypeptides separated by polyacrylamide gel electrophoresis were detected by Western blotting as described by Dietzgen and Francki, (1988) using nitroblue tetrazolium/phenazine methosulphate/5-bromo-4-chloro-indolyl phosphate as substrate (Ey and Ashman, 1986).

IV Results

A. Attempts to prepare RNA-free soluble protein from different isolates by previously used methods

Spectrophotometric analysis showed that the yield and quality of proteins prepared from the five AMV isolates by LiCl degradation (Francki et al., 1966; Francki and McLean, 1968) varied considerably (Fig. 1A). All the preparations had adsorption maxima below 280 nm and low 280/260 nm ratios indicating the presence of contaminating RNA. Light scattering as indicated by the absence of clear absorbance minima between 240 and 250 nm and high absorption between 300 and 320 nm, especially in preparations of the N20, S30 and S40 virus isolates, showed that the material was aggregated (Fig. 1A). Reducing the temperature to -70°C or raising it to +4°C after addition of LiCl did not significantly change the properties of the proteins recovered. Similar unsatisfactory results were obtained when attempts were made to prepare soluble protein from another seven AMV isolates with distinct biological properties (Hajimorad and Francki, 1988).

Using the $MgCl_2$ method (Kruseman et al., 1971) but without 2-mercaptoethanol, reasonably good protein preparations (280/260 nm ratios of more than 1.35) in satisfactory yields, were obtained from the H4 and W1 AMV isolates (Fig. 1B). However, very little material of any kind was recovered from virus preparations of the S40 isolate, and highly aggregated material was obtained from virus of the N20 and S30 isolates (Fig. 1B).

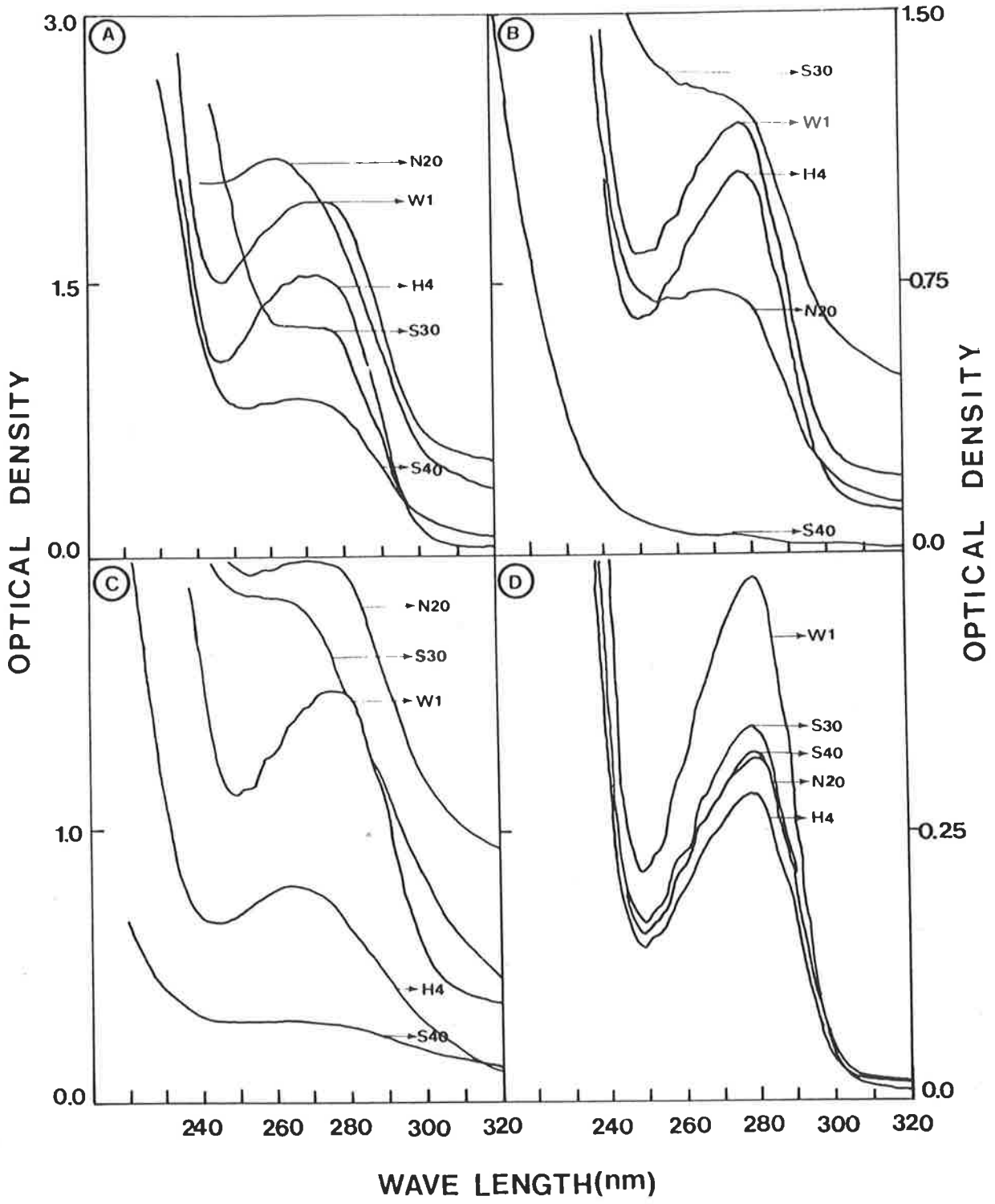


Fig. 1

Ultraviolet absorbance spectra of proteins isolated from five AMV isolates (H4, N20, S30, S40 and W1) by : A, degradation with LiCl (Francki et al., 1966); B, $MgCl_2$ degradation (Kruseman et al., 1971); C, $CaCl_2$ degradation (Gonsalves and Garnsey, 1975); and D, as in C but the final dialysis was against 0.1 M $CaCl_2$ and not water as described in the text.

Soluble protein relatively free of RNA was also recovered from preparations of the W1 AMV isolate by the $CaCl_2$ method described by Gonsalves and Garnsey (1975) (Fig. 1C). However, virus of the S40 isolate was almost completely lost again, and the other isolates failed to yield satisfactory preparations (Fig. 1C).

B. Modification of the $CaCl_2$ method to prepare soluble proteins from five AMV isolates

A modification of the $CaCl_2$ method of Gonsalves and Garnsey (1975) was shown to yield soluble proteins from preparations of all five AMV isolates (Fig. 1D). Purified virus was dialysed against 1M $CaCl_2$ for 48 h at 4°C. The precipitated RNA was removed by centrifugation at 12000 x g for 10 min and the supernatant was subjected to centrifugation at 400,000 x g for 30 min to remove any intact or only partially degraded virus particles. The uppermost 3/4 of the supernatant was aspirated from the tubes and dialysed for 48 h at 4°C against 0.1 M $CaCl_2$ and 10 mM sodium acetate, pH 6.0 with three changes of the buffer. The preparation was again centrifuged at 400,000 x g for 30 min to remove insoluble material. Ultraviolet spectra of protein preparations from all five AMV isolates had 280/260 nm ratios above 1.45 and low absorbance above 300 nm indicating that the preparations contained soluble protein essentially free of RNA. However, reducing the $CaCl_2$ concentration below 0.1 M resulted in precipitation of the protein. Dialysis of this protein against 0.1 M NaCl or LiCl resulted in its precipitation.

Rabbits immunized with protein prepared as described above from AMV isolates S30 and S40, produced antibodies which reacted with their homologous antigens as well as preparations of native or glutaraldehyde-fixed virus particles in gel-diffusion tests (Fig. 2). It is interesting that titres of the antisera were almost always higher when tested with virus particles than with homologous coat protein preparations, and highest with fixed virus particles (Fig. 2). Although the highest homologous titres of the antisera when tested against protein preparations were only between 1/1 and 1/8 in immunodiffusion tests (Fig. 2), they performed satisfactorily in ELISA. For example, antisera collected from the rabbits immunized with AMV S40 and bled 11 weeks (rabbit 1a, Fig. 2) and 10 weeks (rabbit 1b, Fig. 2) after the first injection, had titres of 1/8 in immunodiffusion tests but in

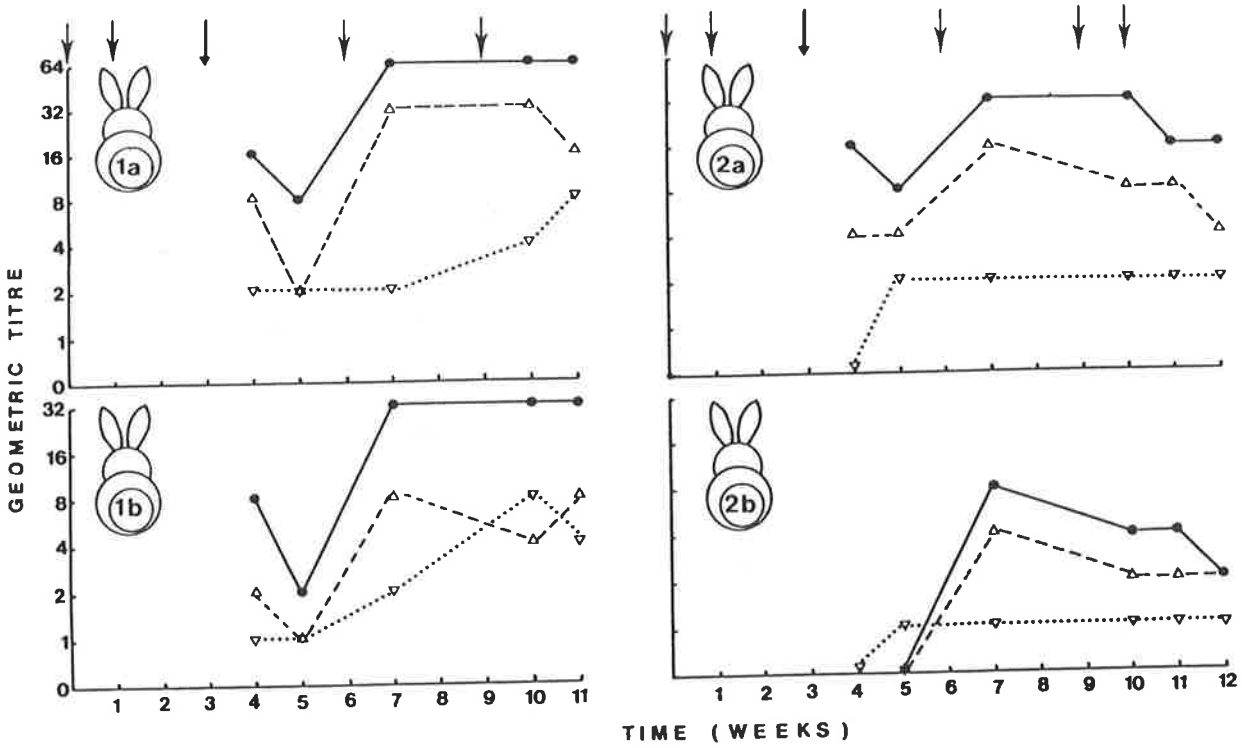


Fig. 2

Antibody titres in sera from rabbits immunized with protein preparations from the S40 (1a and 1b) and S30 (2a and 2b) isolates of AMV. Each rabbit was immunized by an initial intravenous injection of 250 µg viral protein and a further injections of 250, 500, 500 µg and 2 mg of protein 1, 3, 6, and 9 weeks thereafter. All injections were intravenous except the third which was administered intramuscularly with the protein emulsified in Freund's complete adjuvant. Rabbits 2a and 2b recieved an additional intravenous injection of 2 mg protein, 11 weeks after commencement of immunization. Each antiserum was titrated by gel-immunodiffusion tests against either 1 mg/ml of native (△ - - △) or glutaraldehyde-fixed (●——●) homologous virus preparations, or against 1 mg/ml viral protein (▽····▽) as described in Materials and Methods.

excess of 1 x 10^{-7} and 1 x 10^{-10}, respectively, in ELISA. Similarly, antisera from rabbits immunized with AMV S30 (rabbits 2a and 2b) and collected 11 weeks after the first immunization, had titres of 1/2 (rabbit 2a, Fig. 2) and 1/1 (rabbit 2b, Fig. 2) in immunodiffusion tests, yet their titres were in excess of 10^{-6} in ELISA. Antisera from all four rabbits also reacted with protein, virus and fixed virus preparations of all five AMV isolates but not with leaf extracts of virus-free N.clevelandii, the plant from which the viruses had been purified.

Unexpectedly, protein from only the S30 AMV isolate was able to activate infectivity of genomic AMV RNA (data not shown). Proteins from all five isolates were analysed by PAGE and compared to those obtained by direct dissociation of the corresponding virus preparations. Fig. 3 shows that proteins dissociated directly from each virus preparation consisted largely of a single polypeptide of about Mr 30,000 and only traces of smaller polypeptides were detected (Fig. 3, Panel A). A polypeptide of Mr 30,000 was detected only in protein isolated from AMV S30 which, however, also contained some smaller polypeptides (Fig. 3, Panel B). The proteins from all the other virus isolates contained only polypeptides smaller than Mr 30,000 (Fig. 3, Panel B) indicating that they had been degraded. However, although degraded, the polypeptides separated from all five AMV isolates were immunoreactive as shown in Western blots probed with antibodies to AMV S30 protein (Fig. 3, Panels D and E). When polyacrylamide gels were stained directly with silver (data not shown), the intensity of the bands representing the degradation products were similar to those in the Western blots (Fig. 3, Panels D-F). Similar results were obtained with antibodies to the S40 AMV protein (results not shown) indicating that antibodies elicited by a highly degraded AMV protein could recognise the undegraded proteins of all five AMV isolates. It seemed significant that the AMV S30 protein preparation was the only one which could activate the AMV genome and contained undegraded coat protein.

C. Effect of exposure of AMV protein to $CaCl_2$ on its integrity

By decreasing the time of the initial dialysis of AMV preparations against 1M $CaCl_2$ from 48 to 12 h and the subsequent dialysis of the protein against 10 mM sodium acetate, pH 6.0, containing 0.1 M $CaCl_2$ from 48 to 36 h, the proteins of all the virus isolates had spectra very similar to those in Fig. 1D (data not shown) but were far less degraded (compare Panels B and C in Fig. 3). Such protein preparations were effective in activating the infectivity of AMV genomic RNAs. For example, in one experiment, a preparation of 10 µg/ml of W1 AMV genomic RNA failed to produce any local lesions when inoculated to French bean. However, inocula containing 10 µg/ml of the RNA and 10 µg/ml of each of the H4, N20, S30, S40 and W1 protein preparations produced 15, 8, 7, 16 and 19 lesions per half-leaf, respectively. The protein preparations alone, failed to produce any lesions.

In an endeavour to further reduce the degradation of AMV protein isolated by dissociating virus with $CaCl_2$, virus preparations were mixed with an equal volume of 2 M $CaCl_2$ and it was shown that after 3 h at 0^0C all the RNA had precipitated and could be removed from the protein by low speed centrifugation. The supernatant was then centrifuged at 400,000 x g for 30 min to remove any other particular materials and was dialysed for 24 h against 10 mM sodium acetate buffer, pH 6.0, containing 0.1 M $CaCl_2$. Such preparations yielded recoveries of over 40% of the viral protein with no evidence of RNA contamination (data not shown) or protein degradation (Fig. 4). Exposure of AMV preparations to 1 M $CaCl_2$ for periods up to 48 h yielded similar amounts of protein of similar quality. However, prolongation of the final dialysis step to reduce the concentration of $CaCl_2$ to 0.1 M, increased the amount of degradation of the viral proteins (data not shown). This indicates that the proteolysis takes place only at relatively low $CaCl_2$ concentrations.

$CaCl_2$ also induced the proteolysis of AMV protein in intact virus particles. It was shown that $CaCl_2$ could be added to virus preparations up to a concentrations of 1 mM without any signs of the virus dissociating into protein and RNA. Addition of this concentration of the salt to virus preparations of the S30 AMV isolate whose protein was shown to be relatively resistant to proteolysis (Fig. 3), resulted in significant degradation of

Fig. 3

Analysis of coat proteins from AMV isolates H4, N20, S30, S40 and W1 (from left to right in each panel, respectively) by polyacrylamide gel electrophoresis (panels A-C) and Western blotting (panels D-F). Proteins were dissociated directly from the respective virus preparations (panels A and D), from proteins prepared by $CaCl_2$ degradation (panels B and E; see spectra in Fig. 1D and text for details), and proteins prepared as in B and E except that the initial dialysis of virus against 1M $CaCl_2$ was reduced from 48 to 12 h and the dialysis of the protein from 48 to 36 h (panels C and F). Gels shown in A-C were stained with silver after electrophoretic transfer of the proteins to nitrocellulose and the transfers (panels D-F) were probed with antibodies to coat protein of the S30 AMV isolate (serum from rabbit 2a in Fig. 2 bled 11 weeks after commencement of immunization at a dilution of 1: 5000). (Samples of 0.5 μg of protein were electrophoresed in each track).

Fig. 4

Degradation of AMV (isolate N20) coat protein during preparation by the $CaCl_2$ method. Protein markers (phosphorylase b, Mr. 94,000; bovine serum albumine, 67,000; ovalbumine, 43,000; carbonic anhydrase, 30,000 and soybean trypsin inhibitor, 20100) were separated in track 1 and protein dissociated directly from a virus preparation (control) in track 2. Coat protein was prepared from the same virus preparation by mixing with an equal volume of 2 M $CaCl_2$ for either 3 h (tracks 3, 5, 7 and 8) or 48 hr (tracks 4 and 6) without dialysis against 0.1 M $CaCl_2$ (tracks 3 and 4), with dialysis for 24 h at 4^0C (tracks 5 and 6), and additional dialysis for 48 h at 4^0C (track 7) and at 25^0C (track 8). (Samples of 0.5 μg of protein or virus were electrophoresed in each of the tracks 2-8).

Fig.3

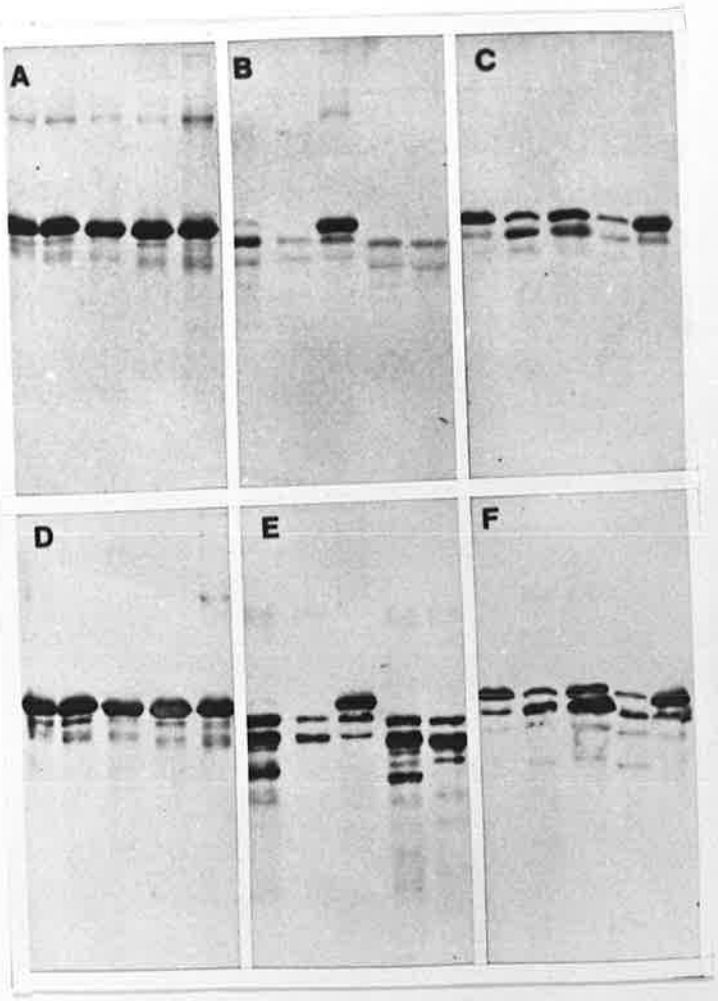
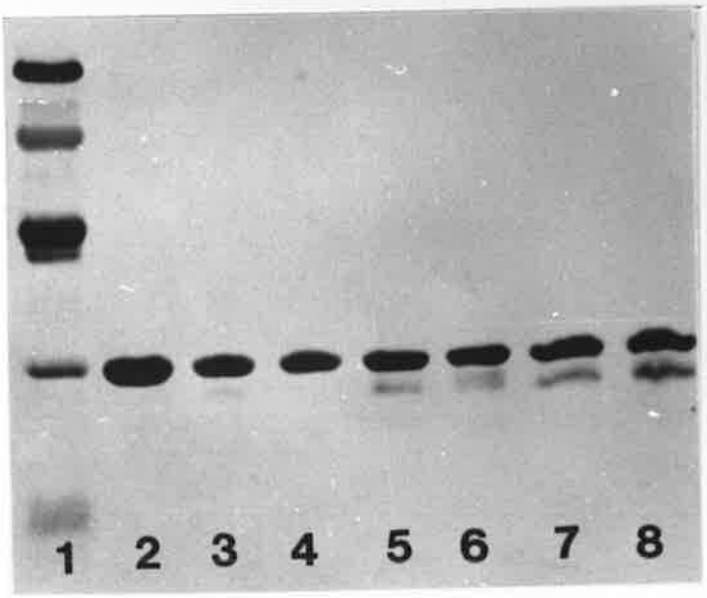


Fig. 4



its coat protein with time (Fig. 5). The degradation was evident at 25°C but not at 4°C and was inhibited by EGTA, an agent which chelates Ca^{2+} preferentially.

D. The chosen routine method for preparing intact AMV protein

Purified AMV preparations were mixed with equal volumes of 2 M $CaCl_2$ and left overnight at 4°C. The precipitated RNA was removed by centrifugation for 10 min at 12,000 x g and any other insoluble material for 30 min at 400,000 x g. The supernatant was dialysed for 24 h against 10 mM sodium acetate buffer, pH 6.0, containing 0.1 M $CaCl_2$ and the preparation was again centrifuged at 400,000 x g to remove any insoluble materials.

Protein from all five AMV isolates used were recovered in yields of about 50%, they had 280/260 nm ratios between 1.38-1.53, and were shown to be essentially undegraded (Fig. 6).

E. The purity of AMV protein preparations

The degradation of AMV coat protein in preparations of either the virus or isolated protein took place during incubation in the presence of low concentrations of $CaCl_2$ (Figs. 4 and 5). This degradation had the characteristic of an enzymatic reaction because it proceeded faster at 25°C than at 4°C. The most likely source of proteolytic enzymes in these preparations would be from the host plants which had not been eliminated during virus purification. However, only AMV protein was detected by silver-staining in electrophoretograms of our virus or protein preparation. Nevertheless, although no antibodies specific to host plant antigens were detected by ELISA in antisera to AMV protein preparations used as immunogens, small amounts of such antibodies were detected in Western blots of extracts from inflected plants using antisera from rabbits hyperimmunized with native or glutaraldehyde-fixed AMV, or isolated viral proteins (data not shown). However, different antisera detected antigens with different electrophoretic mobilities (data not shown). Thus it must be concluded that our virus and viral protein preparations did contain trace amounts of host plant proteins, some of which may have proteolytic enzymes which require Ca^{2+} for activity.

Fig. 5

Slow degradation of coat protein of intact S30 AMV particles in the presence of low $CaCl_2$ concentrations. Protein from virus preparations was incubated in the presence of 1 mM $CaCl_2$ at 4°C (tracks 2-5), and 25°C (tracks 6-9) and in the presence of 1 mM EGTA at 25°C (track 10-13) for 24, 36, 48 and 60h, respectively. (Protein markers as described in Fig. 4 were run in track 1 and 0.5 µg of protein was electrophoresed in each of the tracks 2-13).

Fig. 6

Polyacrylamide gel electrophoretic analysis of virus preparations and their proteins isolated from AMV by the finally adopted $CaCl_2$ dissociation method (see text for details). Proteins dissociated directly from AMV preparations of isolates S30, S40, W1, N20 and H4 were electrophoresed in tracks 2, 4, 6, 8 and 10 respectively, and those from the corresponding coat protein preparations in tracks 3, 5, 7, 9 and 11. (Protein markers as detailed in Fig. 4 were separated in track 1 and 0.5 µg of protein was electrophoresed in each of the tracks 2-11).

Fig.5

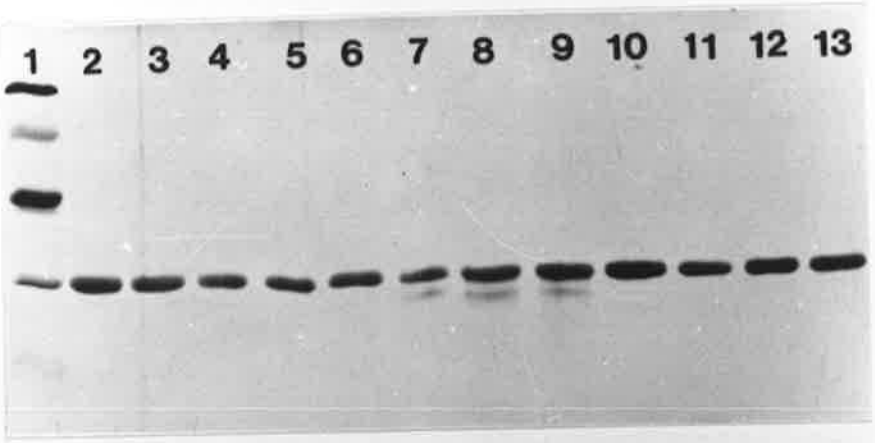
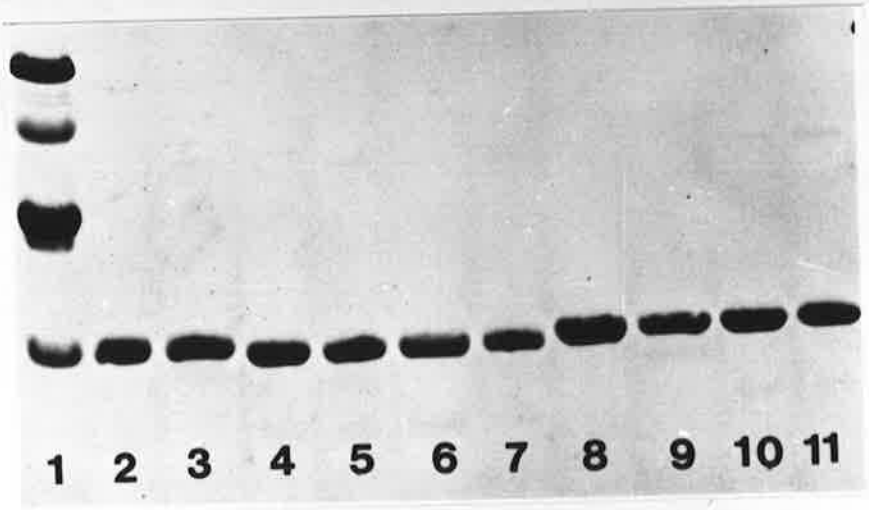


Fig.6



V Discussion

The method for AMV coat protein preparation described here is a modification of that used by Gonsalves and Garnsey (1975) for isolating protein from three different ilarviruses, two bromoviruses and one isolate of AMV. Although Gonsalves and Garnsey (1975) did not check the proteins for their integrity, they did show that the ilarvirus and AMV proteins were capable of activating their homologous and heterologous genomes. In our hands, this method was suitable for the preparation of protein from only one out of the five AMV isolates used. Although the integrity of AMV coat protein is essential for activating the AMV genome, as previously shown (Bol et al., 1974; Zuidema et al., 1983), it is not necessary for eliciting polyclonal antibodies capable of recognising intact and partially degraded AMV protein as well as native and glutaraldehyde-fixed virus particles. Our modification of the procedure ensures that AMV protein is essentially free of RNA and remains soluble by maintaining it in a buffer containing $CaCl_2$ at a concentration of at least 0.1 M.

The size of the AMV coat protein subunit as determined by polyacrylamide gel electrophoresis has been reported to be in the range of Mr about 27,000 to 30,000 (see Hull, 1971, and references therein). These values and that of 30,000 obtained here, however, appear to be overestimated because the nucleotide sequence of AMV RNA3 from three strains of the virus indicate that the coat protein consists of between 218 and 221 amino acids and hence Mr about 24,000 (Brederode et al., 1980; Barker et al.,1983; Ravelonandro et al.,1984). Heterogeneity of AMV coat protein had been observed previously and whereas some components appeared to be degradation products, Hull (1971) concluded that at least two components occurred *in vivo* and suggested that the larger component was necessary for the formation of hexamers and the smaller one for the pentamers of AMV particles. Joshi et al., (1984) have also detected, *in vivo*, small amounts of a protein smaller than the coat protein with antiserum to AMV.

Results in this paper show that undegraded AMV coat protein can be isolated from AMV preparations but that it does undergo slow degradation *in vivo* by what appears to be a $CaCl_2$-dependent enzymatic reaction. Attempts to avoid this degradation by dialysing $CaCl_2$ prepared protein against either LiCl or NaCl were unsuccessful because the protein

began to precipitate when the salt concentrations were reduced to below 0.5 and 1.0 M, respectively (unpublished data). We cannot exclude the possibility that the degradation is catalysed by a contaminating host plant protease because traces of plant proteins were detected in the AMV preparations. However, this does not seem very likely because the contaminating proteins detected were not consistently the same ones from preparation to preparation. It may be worth considering the possibility that AMV coat protein can self-cleave to yield smaller polypeptides such as those detected in infected plants by Joshi et al., (1984) and may be functional *in vivo*.

The method described here yields biologically active protein from a range of distinct AMV isolates. Its only disadvantage is that the protein must be suspended in buffer containing 0.1 M $CaCl_2$ to remain soluble. Unfortunately this concentration of the salt allows the protein to degrade slowly. We have found it convenient to prepare the protein just before use. However, intact protein can be prepared from purified virus which has been stored in 10 mM phosphate buffer, pH 7.0, containing 50% glycerol at -20^{0}C for many months without signs of degradation.

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Appendix 2

Binding properties of alfalfa mosaic virus to polystyrene and its significance to indirect ELISA

(This has been submitted for publication as a paper in the Journal of Virological Methods).

Binding properties of alfalfa mosaic virus to polystyrene and its significance to indirect ELISA

I Summary

The adsorption properties of native and glutaraldehyde-fixed alfalfa mosaic virus (AMV) antigens to the polystyrene of ELISA plates was studied using [^{35}S]-labeled virus preparations. It was shown that adsorption was a temperature-dependent, relatively slow process which varied between different AMV isolates. The amount of antigen adsorbed was dependent on the type and pH of the suspending buffer. Although native virus adsorbed very efficiently at high pH when the particles had dissociated, significant amounts also adsorbed at pH 7.0, or lower. Glutaraldehyde-fixed virus whose particles retained their integrity even at pH as high as 9.6, however, adsorbed much more efficiently than native virus above pH 9.0 but hardly at all around pH 7.0. The wide variation in adsorption of AMV antigen to microtitre plates under even slightly different conditions, calls for extreme caution in interpreting serological results from indirect ELISA when antigen is used to coat the microtitre plates.

II Introduction

While investigating the relationships among isolates of alfalfa mosaic virus (AMV), we observed that serological differences between a number of isolates were difficult to detect by the double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) or Ouchterlony immunodiffusion tests (Hajimorad and Francki, 1988) . However, in indirect ELISA, where virus antigen was adsorbed directly to the microtitre wells in neutral pH buffer to avoid dissociation of virus particles, much larger serological differences were apparent. It was also observed that the sensitivity of the indirect ELISA was much lower when glutaraldehyde-fixed AMV was used to coat the plates instead of native virus. This is in direct contrast to our experience with Ouchterlony immunodiffusion tests (Hajimorad and Francki, 1989). These unexpected results led us to investigate the adsorption to microtitre plates of five native and glutaraldehyde-fixed particles of AMV

isolates under a variety of conditions including time, temperature and pH, which is the subject of this report.

III Materials and Methods

A. Virus isolates and their purification

AMV isolates H4, N20, S30, S40 and W1 which are readily distinguished by their biological properties (Hajimorad and Francki, 1988) were propagated in *Nicotiana clevelandii* and purified by differential centrifugation, and when necessary, also by sucrose density-gradient centrifugation as described by Hajimorad and Francki (1988).

B. Serology

Antisera raised to native and glutaraldehyde-fixed AMV preparations and $CaCl_2$-dissociated coat proteins were the same as those used previously (Hajimorad and Francki, 1988, 1989; and unpublished data). IgG from rabbit antiserum was prepared as described by Clark and Adams (1977).

In indirect ELISA, 200 µl aliquots of purified AMV preparations were applied directly to the microtitre wells and incubated for 3 hr at 25°C. The wells were then blocked with 350 µl of blocking solution [0.1 M NaCl containing 1% (w/v) BSA] and incubated for 1 hr at 25°C. Serial two-fold dilutions (200 µl) of antisera in sample buffer (PBS-Tween, pH 6.0, containing 2% (w/v) PVP and 0.2g NaN_3 per litre) was applied to each well and incubated overnight at 4°C. Then 200 µl of preparation of 1:1000 affinity purified goat anti-rabbit IgG labeled with alkaline phosphatase (Sigma) in conjugate buffer [PBS-tween, pH 7.4, containing 2% (w/v) PVP, 0.2% (w/v) BSA and 0.2g NaN_3 per litre] was applied per well and incubated for 3 hr at 25°C. Finally, 200µl of 1 mg/ml of p-nitrophenyl phosphate (Sigma) in substrate solution (97 ml diethanolamine and 0.2g NaN3 per litre, pH 9.8) was applied to each well and after the required time, the optical density was measured with a Bio-Rad Model 1550 EIA reader. After each step, the ELISA plates were rinsed three times, each time for 3 min with 350 µl of PBS-Tween (0.8g NaCl, 0.2g KH_2PO_4, 1.15g Na_2HPO_4, 0.2g KCl, 0.5 ml Tween-20 and water to 1 litre, pH 7.4).

The parameters of the indirect ELISA were optimised as described by Jaegle and Van Regenmortel, (1985).

C. Preparation of [^{35}S]-labelled AMV

Labelled virus was prepared essentially as described by Francki (1968). *N. clevelandii* plants inoculated with AMV 4-5 days previously, were removed from the pots and their roots were washed free of soil. Six to eight small plants were placed with their roots in 100 ml beakers and the roots were moistened with 0.5-1 ml of water containing 3-5 mCi carrier-free Na2 [^{35}S]O4. Plants were prevented from wilting by adding minimal amounts of water to the roots for about 12 h and were then immersed in about 20 ml of water and maintained at 25°C under artificial light for 4-5 days. Virus was extracted and purified from the entire plants as already described (Hajimorad and Francki, 1988).

Analysis of fractions collected from sucrose-gradient tubes after the final step of the purification procedure, showed that about 97% of the [^{35}S] in the fractions contained virus as determined by monitoring absorption at 254nm and assaying the fractions by DAS-ELISA as described by Clark and Adams (1977). The purified virus was stored in PEN buffer (10 mM phosphate, 1 mM EDTA and 1 mM sodium azide, pH 7.0) at 4°C or in 10 mM phosphate buffer, pH 7.0, and 50% (v/v) glycerol at -20°C.

D. Assay for binding of viral protein to ELISA plates

Microtitre ELISA plates from Nunc (Denmark) were cut into single rows of wells with a band saw. Samples of 200 µl of [^{35}S]-labeled AMV in the appropriate buffer, were loaded into each well. Each sample was dispensed in duplicate using wells in rows from two different ELISA plates. The rows of wells were then incubated for the required time at 25°C. Each well was rinsed individually three times, each time for 3 min with 350 µl of PBS-Tween and then air dried. Each well was excised with a hot scalpel, placed in a tube and filled with 200µl of Soluene-350 (Packard Instrument Co. Inc., Illinois). After 2-3 hr at room temperature, 3 ml of scintillation fluid [4% (w/v) 2,5 diphenyloxazole (PPO) in toluene] was added to each tube and the radioactivity determined in a Packard Tricarb 3320 Scintillation Spectrometer .

Virus concentrations were determined spectrophotometrically ($E^{0.1\%}_{260\ nm}$ =5) (Jaspars and Bos, 1980). Specific radioactivity was determined by dispensing 200 µl samples of a preparation into ELISA plate wells, the virus was dried under an infra-red lamp and the radioactivity determined as described above. Background radioactivity was determined by counting plate wells in which 200 µl of the appropriate buffer was dispensed and then treated as in the experiment in hand.

IV Results and discussion

A. Attempts to trace serological relationships among AMV isolates by indirect ELISA

When ELISA plates were coated with purified preparations of the five different AMV isolates in 10 mM phosphate buffer, pH 7.0, and then probed with antibodies to native virus of the S30 isolate, the homologous reaction was significantly higher than any of the heterologous reactions (Fig.1A). This suggested that the S30 isolate was serologically readily distinguishable from the other isolates. However, when antibodies to native virus of the N20 isolate were used as the probe, the results were unexpected in that the reaction with the S30 isolate antigen was still stronger than with either the homologous or all the other heterologous antigens (Fig.1B). Moreover, the S30 antigen always reacted the strongest irrespective of the antiserum to which AMV isolate was used (data not shown). When the ELISA plates were coated with purified AMV preparations in carbonate buffer, pH 9.6, (1.59gm Na_2CO_3, 2.93gm $NaHCO_3$ and 0.2gm sodium azide per litre of distilled water), however, all the virus isolates reacted strongly when probed with antiserum to either the S30 (Fig. 1C), the N20 (Fig.1D) or to any of the other virus isolates (data not shown). These results suggested that all the AMV isolates were serologically similar.

Glutaraldehyde-fixed AMV is a more efficient test antigen in Ouchterlony immunodiffusion tests irrespective of whether antisera to native or fixed AMV or to the coat protein subunits are used (Hajimorad and Francki, 1988, 1989, and unpublished data). However, when we used fixed AMV preparations to coat the plates for indirect ELISA, the reactions were very much weaker than with native virus. This was irrespective of whether

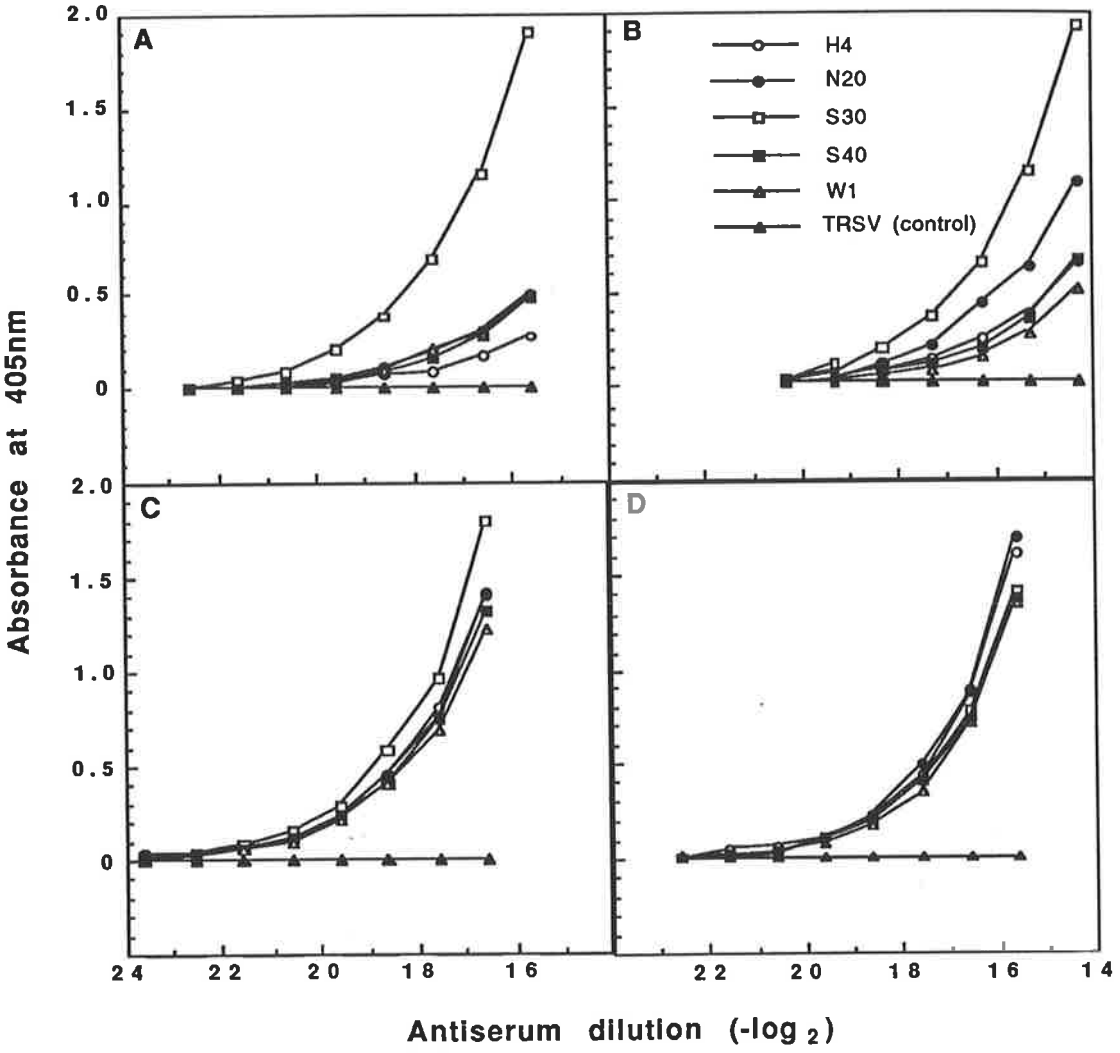


Fig. 1

Serological reactions of five different AMV isolates as determined by indirect ELISA. The microtitre wells were coated with 0.5µg/ml purified AMV in 10 mM phosphate buffer, pH 7.0 (panels A and B) or in carbonate buffer, pH 9.6 (panels C and D), and were probed with antisera to native S30 (panels A and C) or N20 (panels B and D) AMV isolates. [A preparation of tobacco ringspot virus (TRSV) was used as a coating antigen control].

antiserum to fixed (Fig.2A) or native virus (Fig.2B) or to the coat protein subunits (Fig.2C) was used.

B. Differences in the adsorption properties of protein from AMV to ELISA plates

The anomalous results reported above prompted us to investigate the possibility that they could be explained by differences in the adsorption properties of the various antigens used to coat ELISA plates. This was done by directly measuring the adsorption of [^{35}S]-labeled antigens to ELISA plates.

Fig. 3A shows that adsorption of native S30-AMV in 10 mM phosphate buffer, pH 7.0, to ELISA plates is much more efficient than any of the other isolates. On the other hand, virus adsorption of all the five isolates in carbonate buffer, pH 9.6, was very similar (Fig. 3B). Thus it appears that the results of the indirect ELISA may be a reflection of the adsorption properties of the virus isolate (compare Figs. 1A and B to 3A and Figs. 1C and D to 3B).

Fig. 4A shows that glutaraldehyde-fixation reduced the ability of AMV to adsorb to ELISA plates. Moreover, it was shown that it was easier to wash off the adsorbed fixed than the native virus (data not shown). Similar results were obtained with all the other four AMV isolates tested (data not shown). However, when the plates were first coated with antibody, fixed AMV adsorbed more efficiently than native virus (Fig. 4B). Similar results were obtained with antisera to native or fixed AMV or to coat protein with all virus isolates tested (data not shown). These results indicate that fixation of AMV reduces the ability of its protein to bind to ELISA plates but increases its affinity for antibodies.

C. Parameters affecting adsorption of protein from AMV to ELISA plates

Adsorption of AMV protein to ELISA plates was shown to be slow (Fig. 5A and B). The amount of virus adsorbed to the plates was still increasing after 20 h (Fig. 5A). Virus protein adsorption was proportional to the virus concentration applied (Fig. 5B and C) up to at least 20 µg/ml, (4 µg in 200 µl per well), the highest concentration tested (Fig. 5B). The rate of virus protein adsorption was affected by temperature so that for example at

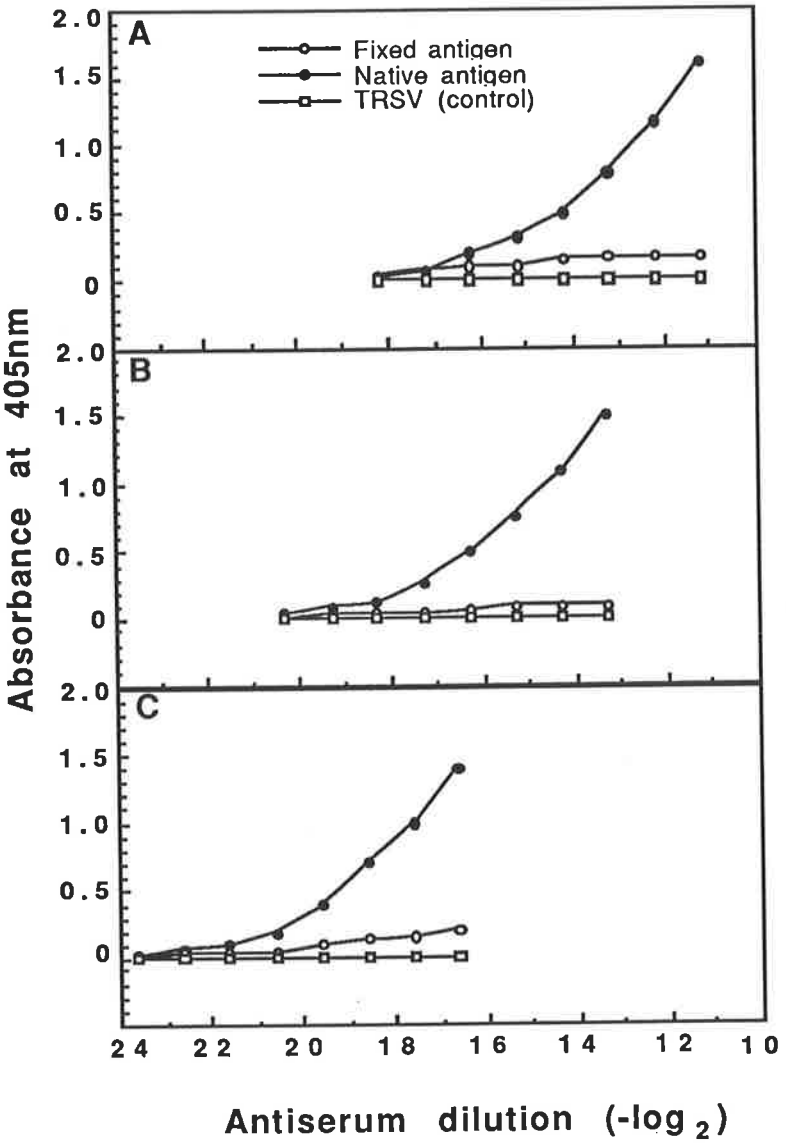


Fig. 2

Serological reactions of native and glutaraldehyde-fixed AMV preparations as determined by indirect ELISA. The microtitre wells were coated with 0.5 µg/ml of purified fixed or native W1 AMV (panels A and B) and S40 AMV (panel C) in 10mM phosphate buffer, pH 7.0, and probed with antisera prepared to fixed (panel A) and native (panel B) W1 AMV, and with antiserum to a coat protein preparation of the S40 isolate of AMV (panel C). [A preparation of tobacco ringspot virus (TRSV) was used as a coating antigen control].

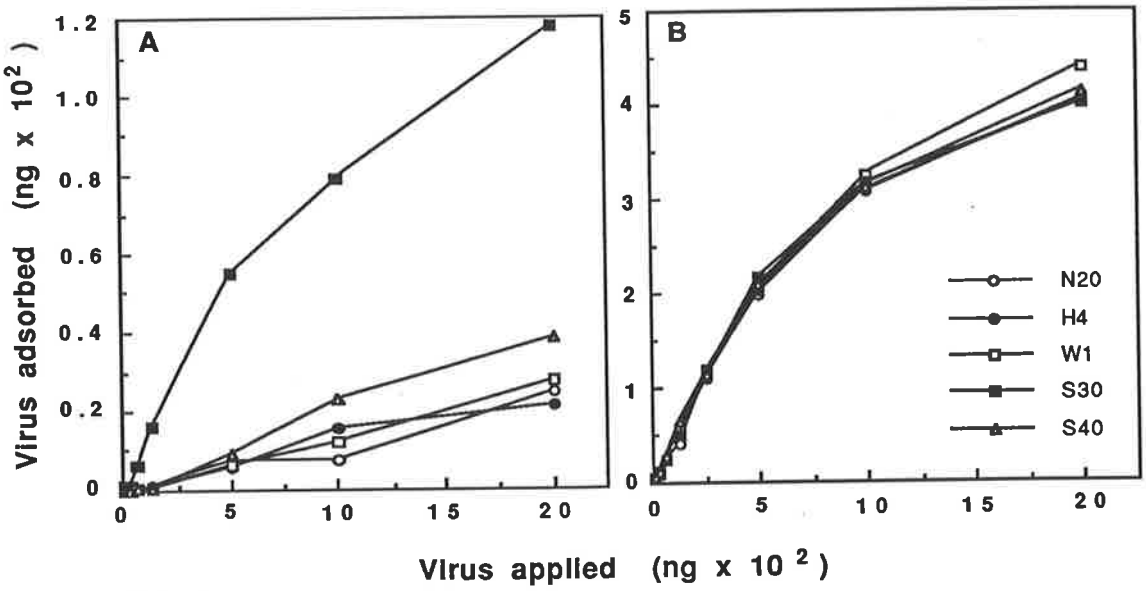


Fig. 3

Adsorption of [^{35}S]-labeled preparations of the five AMV isolates to ELISA plates. The preparations were suspended in 10mM phosphate buffer, pH 7.0 (panel A), or carbonate coating buffer, pH 9.6 (panel B).

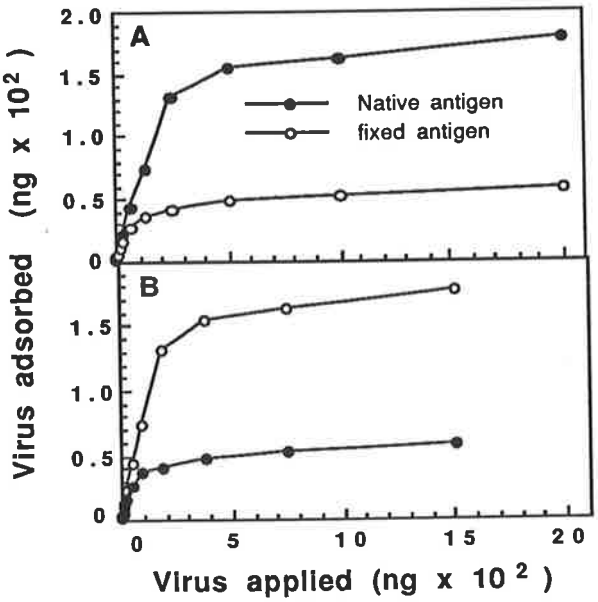


Fig. 4

Adsorption of [^{35}S]-labeled preparations of native and glutaraldehyde-fixed W1 AMV to ELISA plates. The labeled virus was applied directly to the microtitre wells (panel A) or to wells which had been pre-coated with antibodies to fixed W1 AMV (panel B).

25°C, the amount adsorbed in 3 h was greater than that adsorbed during 16 h at 4°C (Fig. 5C).

Both the pH and type of buffer in which the AMV was suspended, affected the adsorption of AMV to ELISA plates (Fig. 5D). In phosphate buffer, adsorption of native virus was more efficient than the fixed virus, and the adsorption increased with pH between 6.5 and 8.0 (Fig. 5D). However, the adsorption of both fixed and native virus at pH 8.0,was very much higher in carbonate than in phosphate buffer (Fig. 5D). In the carbonate buffer, adsorption of both the fixed and native virus was similar at, pH 8.0, and 8.5, but at higher pH, the fixed AMV was adsorbed much more readily (Fig. 5D). In the pH range between 5.5 and 6.0, adsorption of both native and fixed virus was very poor, and no significant differences were observed when the viruses were suspended in either 10 mM phosphate buffer or 10 mM acetate buffer (data not shown).

D. The effect of temperature and pH on AMV nucleoprotein stability

It seemed that the adsorption of AMV to ELISA plates may be a function of virus particle integrity during incubation in that intact particles may have lower affinity for the plastic than virus degraded into coat protein subunits. The stability of AMV particles was therefore investigated under differing conditions of temperature and pH.

The proportion of the nucleoprotein components in preparations of the same virus isolate was always similar (data not shown) but was different and characteristic for the five AMV isolates studied (Fig. 6, panels A-E, traces a). The nucleoprotein components of all isolates were shown to be reasonably stable at 4°C in either 10 mM phosphate buffer at pH between 6.0 and 7.0 (data not shown), although even in PEN buffer which has been recommended for storing AMV (Van Vloten Doting and Jaspars, 1972), preparations of some isolates showed signs of degradation after 4 months at 4°C, and the particles of N20 and S30 isolates were completely degraded (Fig. 6, panels A-E, traces c). When the temperature of freshly prepared virus (Fig. 6, panels A-E, traces a) was raised to 25°C for 16 h, some degradation, especially in the preparation of the S30 isolate, was evident (Fig. 6, panels A-E, traces b). Moreover, degradation was significantly greater at pH 8.0 than at

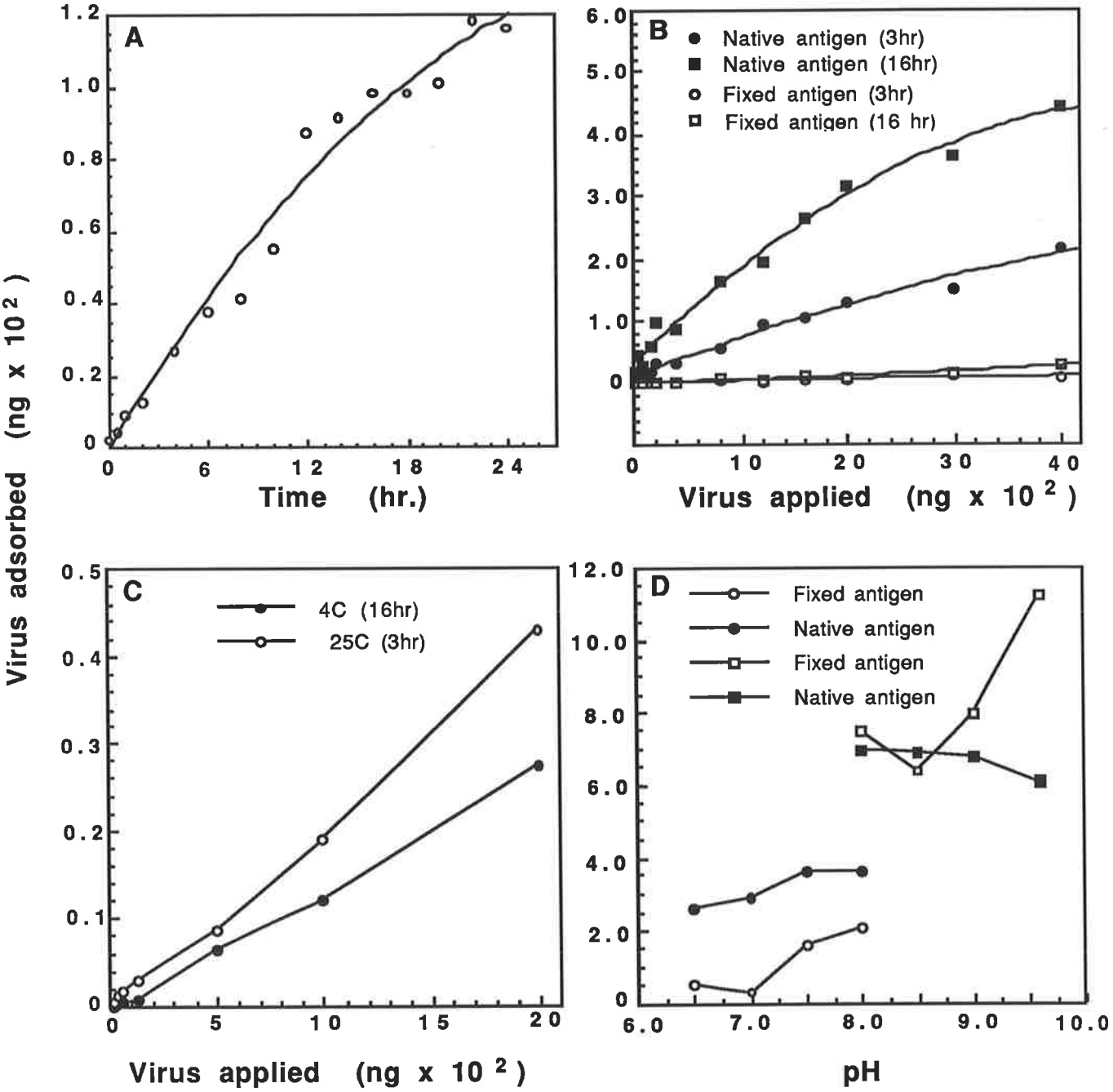


Fig. 5

Effects of various conditions on adsorption of [^{35}S]-labeled W1 AMV to ELISA plates. Panel A - Effect of incubation time at 25°C on adsorption of native virus (1µg/well) suspended in 10mM phosphate buffer, pH 7.0. Panel B - Effect of time and the amount of native and glutaraldehyde-fixed virus preparations in 10mM phosphate buffer, pH 7.0, on adsorption. Panel C - Effect of time and temperature on adsorption of native virus suspended in 10mM phosphate buffer, pH 7.0. Panel D - Effect of buffer type and pH on adsorption of native and glutaraldehyde-fixed virus (2µg/well) in 10mM phosphate buffer (pH 6.5-8.0) and carbonate coating buffer (pH 8.0-9.6).

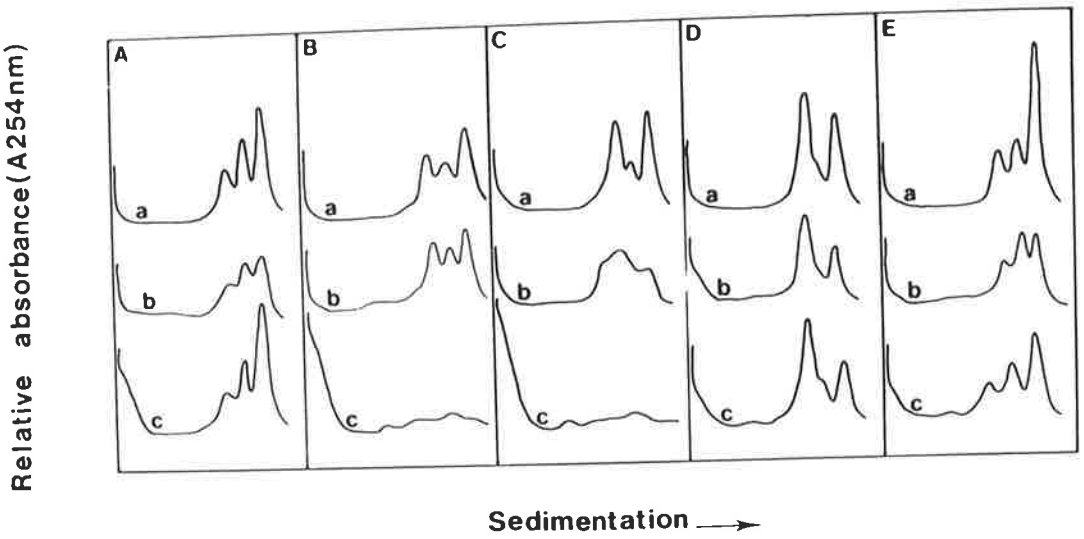
Fig. 6

Sedimentation profiles of five AMV isolates. Purified preparations (100 µg virus) of isolates H4 (panel A), N20 (panel B), S30 (panel C), S40 (panel D) and W1 (panel E) were centrifuged in 6-30% (w/v) sucrose density gradients for 2 hr at 35,000 rpm in a Beckman Spinco SW 41 Rotor. Traces a, are of freshly purified virus in 10 mM phosphate buffer, pH 7.0; traces b, are the same preparation incubated at 25°C for 16hr in 10 mM phosphate buffer, pH 6.5; and traces c, are similar preparations stored at 4°C for 4 months in PEN buffer (The sucrose density-gradients were prepared in the same buffers as those used for suspending the virus).

Fig. 7

Sedimentation of native (panel A) and fixed W1 AMV preparations (panel B) after storage for 16 h in 10 mM phosphate buffer, pH 7.0 at 4°C (traces a), pH 7.0 at 25°C (traces b) and pH 8.0 at 25°C (traces c). (The sucrose density-gradient centrifugation conditions were the same as in Fig. 6).

Fig.6


A
B
C
D
E
a
b
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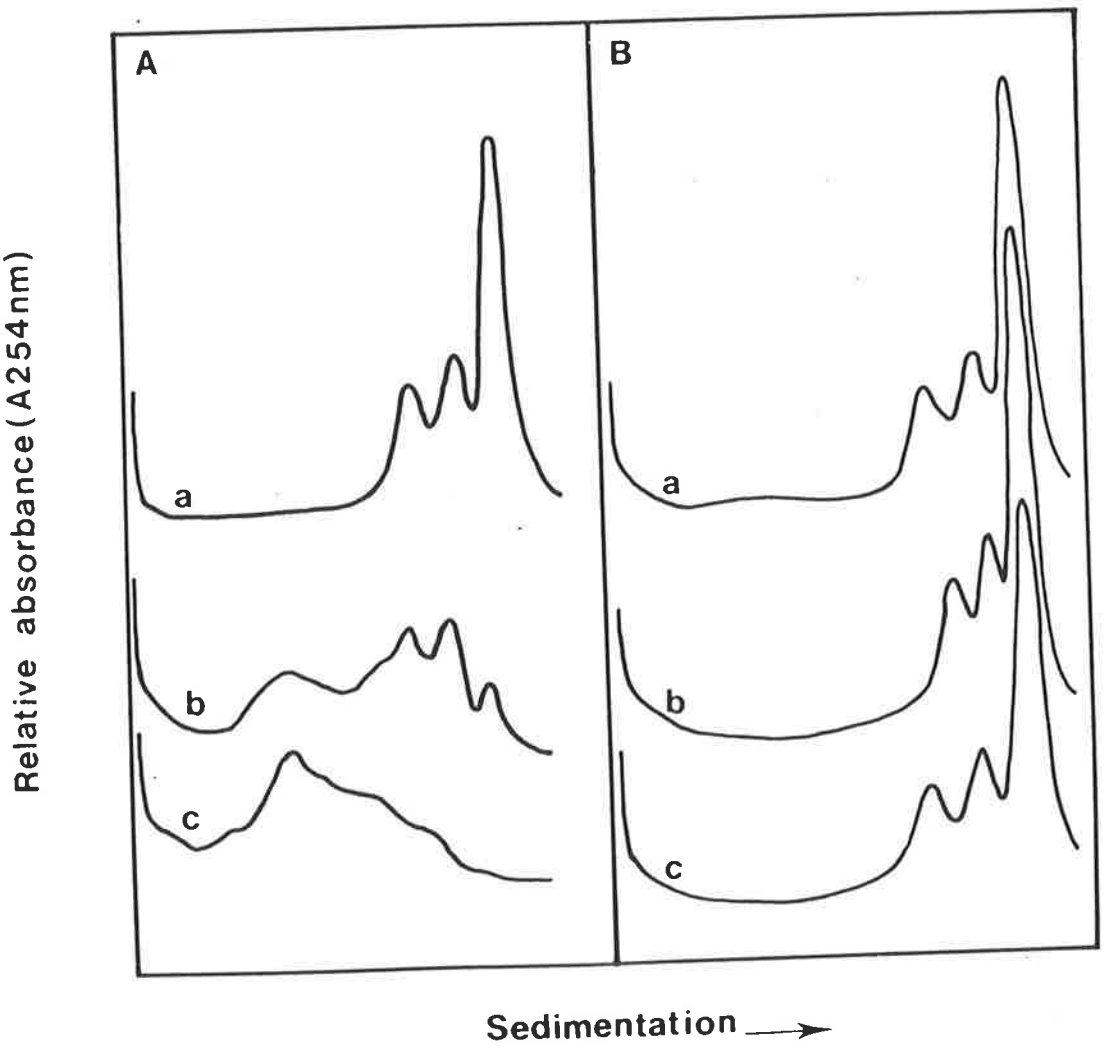
Relative absorbance(A254nm)
Sedimentation

Fig.7


A
B
a
b
c
Relative absorbance(A254nm)
Sedimentation

7.0 or 6.5 (compare panel E, trace b in Fig. 6 with panel A, traces b and c in Fig. 7). Under similar conditions, no particle degradation was detected in preparations of fixed virus (compare panels A and B in Fig. 7).

Native virus of the S30 isolate had the strongest affinity for ELISA plates when suspended in 10 mM phosphate buffer, pH 7.0, (Fig. 3A) and the particles of this isolate also appear to be the least stable (Fig. 6, panel C). Furthermore, in carbonate buffer, pH 9.6, in which nucleoproteins of all the isolates degraded completely (data not shown), all the isolates had a very similar affinity for ELISA plates (Fig. 3B), and three to ten-fold greater than in the phosphate buffer, pH 7.0, (compare panels A and B in Fig. 3). These data suggest that adsorption of protein from native AMV to ELISA plates is dependent on the particles becoming dissociated. However, this cannot account for the observed increase in adsorption of fixed AMV with increase in pH (Fig. 5D) because no particle degradation was observed in such preparations, even when suspended in carbonate buffer, pH 9.6 (data not shown).

E. The effect of $CaCl_2$ on adsorption of protein from AMV to ELISA plates

Native AMV is known to dissociate into RNA and soluble protein in 10 mM acetate buffer, pH 6.0, containing 1 M $CaCl_2$ (Hajimorad and Francki, 1989). When suspended in this medium, adsorption of all five AMV isolates to ELISA plates was very efficient, but even so, AMV S30 adsorbed more readily than any of the other isolates (Fig. 8A). When 500 ng of AMV S30 was applied to each microtitre well, nearly 100% of virus was adsorbed whereas adsorption of the other isolates was between 50 and 80% (Fig. 8A). When similar amounts of any of the other isolates were applied in the same buffer but without $CaCl_2$, less than 2% of the virus was adsorbed (data not shown).

The greatly increased adsorption of native AMV to ELISA plates in the presence of $CaCl_2$ could be explained by the salt-induced dissociation of the virus particles. However, we have also observed that the addition of $CaCl_2$ to fixed AMV results in much greater increases in virus adsorption (Fig. 8B). Moreover, the adsorption capacity of ELISA plate wells in the presence of $CaCl_2$, was much greater when fixed virus was used. For

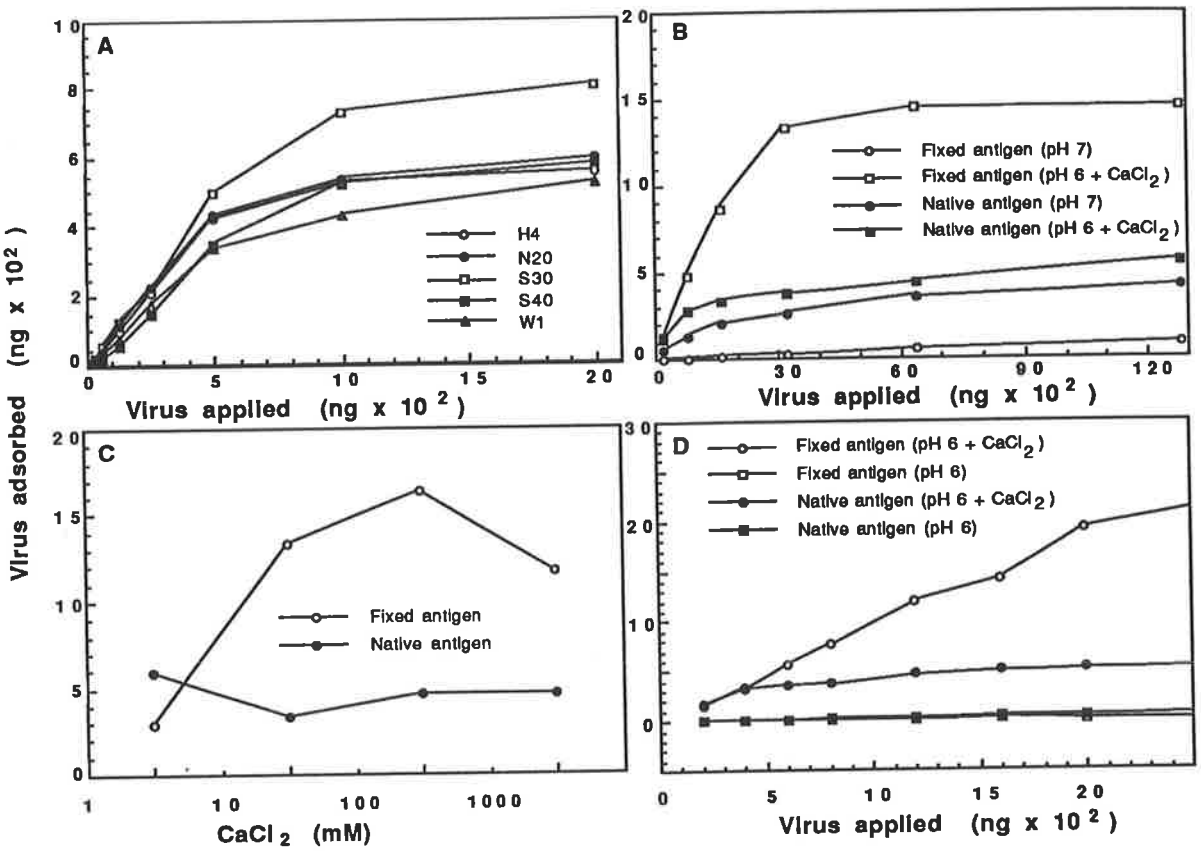


Fig. 8

Effect of glutaraldehyde-fixation and $CaCl_2$ on the adsorption of [^{35}S]-labelled AMV preparations. Panel A - adsorption of native virus preparations of five AMV isolates suspended in 10 mM acetate buffer, pH 6.0, containing 1 M $CaCl_2$. Panel B - adsorption of native and fixed W1 AMV preparations suspended in either 10 mM acetate buffer, pH 6.0, containing 1 M $CaCl_2$ or 10 mM phosphate buffer, pH 7.0. Panel C - adsorption of native and fixed W1 AMV (2µg/ml) in 10 mM acetate buffer, pH 6.0, containing various concentrations of $CaCl_2$. Panel D - adsorption of native and fixed W1 AMV in 10 mM acetate buffer, pH 6.0, with or without 100 mM $CaCl_2$.

example, in the presence of 1 M $CaCl_2$, ELISA wells appeared to be saturated with about 1.5 μg W1-AMV/well when fixed virus was used (Fig. 8B), but only about 0.6 μg of virus/well with native virus (Fig. 8A). The optimum concentration of $CaCl_2$ for maximum adsorption of fixed virus was about 100 mM whereas different concentrations of the salt had similar effects on adsorption of native virus (Fig. 8C). However, $CaCl_2$ always increased the adsorption of both native and fixed virus protein (Fig. 8D).

Fixed virus particles migrated towards the cathode during electrophoresis in agarose gels (data not shown) indicating that they retained a net negative charge. Although they formed fine precipitates in 10 mM acetate buffer with high concentrations of $CaCl_2$, the virus particles did not dissociate. This was ascertained by failure to detect RNA spectrophotometrically in supernatants after the precipitated material was removed by slow-speed centrifugation (data not shown). Thus it seemed possible that the increased adsorption of fixed AMV to the plastic of ELISA plates could have been due to Ca^{2+} acting as bridges between the plastic surface and virus particles. However, this seems unlikely because when the ELISA plate wells were pre-treated by incubation for 16 h in 10 mM acetate buffer, pH 6.0, containing 1 M $CaCl_2$ and then rinsed three times with distilled water, the adsorptive properties of the wells were not changed significantly (data not shown).

V Conclusions

Results presented in this paper reveal some of the problems of interpreting serological data obtained from indirect ELISA where the virus antigen is adsorbed directly to microtitre wells. It was established that adsorption of native AMV particles depends on the type and pH of the suspending buffer as well as the temperature of incubation. Under most conditions the adsorption was a relatively slow process and that some isolates of AMV adsorbed more readily to the plastic than others. Efficiency of adsorption appeared to be correlated with the increased instability of the virus particles. This conclusion is also supported by the observation that in carbonate buffer, pH 9.6, when all the isolates dissociated completely, their antigens adsorbed efficiently and to the same extent. The

preferential binding of dissociated tobacco mosaic virus antigen to a solid phase has also been reported by Dore et al., (1988).

Experiments with AMV particles fixed with glutaraldehyde, which stabilizes them so that they remain intact even when suspended in carbonate buffer, pH 9.6, revealed that their adsorption properties to microtitre wells were drastically changed. Fixation reduced adsorption greatly when the virus was suspended in buffers at neutrality or below. However, this cannot be interpreted as a consequence of increased particle stability because when AMV stabilized by fixation was suspended in carbonate buffer at pH above 9.0, it adsorbed much more efficiently than native virus particles which had dissociated completely.

Although at present the physical phenomena involved in the adsorption properties of AMV antigen to microtitre wells remain obscure, it seems amply clear that any results using this ELISA format must be interpreted with utmost caution, whether it be used for epitope mapping, antigenic reactivity, screening of monoclonal antibodies or especially for estimating serological relationships between viruses.

VI References

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Appendix 3

Publications

1 - Alfalfa mosaic virus isolates from lucerne in South Australia: biological variability and antigenic similarity

M.R. Hajimorad and R.I.B. Francki

Annals of Applied Biology (1988) 113, 45-54

2 - Preparation of soluble, biologically active alfalfa mosaic virus coat protein and its $CaCl_2$-induced degradation.

M.R. Hajimorad and R.I.B. Francki

Journal of Virological Methods (1989) 25, 49-61

3 - Binding properties of alfalfa mosaic virus to polystyrene and its significance to indirect ELISA

M.R. Hajimorad and R.I.B. Francki

Journal of Virological Methods (submitted)