A STUDY OF THE MOLECULAR VARIATION BETWEEN ORBIVIRUS PROTEINS

BY

TONI WHISTLER

Submitted in partial fulfilment of the requirements for the degree of Master of Science in the Faculty of Science, Rhodes University, Grahamstown.

APRIL 1984

ACKNOWLEDGEMENTS

I should like to thank Professor John Newman, the supervisor of this project, for his guidance and support. He could always spare the time, no matter when called upon. This was much appreciated.

I am grateful to Mrs Val Hodgson for her never ending supply of BHK cell monolayers and to Dr Don Hendry, who gave his time and help unselfishly.

Miss Heather Kew typed this manuscript; a task beautifully done, thanks very much.

I would also like to thank Dr H. Huismans at Onderstepoort Veterinary Research Institute for providing the BTV samples and for the time spent discussing my work with me. My thanks also go to Dr BJ Erasmus, of this same Institute, for the BTV antisera used in this study.

To my co-workers in the laboratory, Sirion Robertson and John Dewar, thanks for the many hours of amusement, support and friendship. Thanks also to Moira Pogrund for the time patiently spent proof reading this manuscript.

Last, but not least, I should like to thank the rest of the staff of the Microbiology Department for everything they did.

I acknowledge the financial support from Rhodes University, the Poliomyelitis Research Foundation and the Stella and Paul Lowenstein Research Foundation, without which I would not have been able to further my studies.

ABSTRACT

The aim of this study was to initiate a structural analysis of the capsid polypeptides from several serotypes of bluetongue virus in order to provide insight into the relatedness and possible origins of the different serotypes. Tryptic peptide mapping of ^{125}I-labelled group antigen by ion exchange chromatography was used to assess the structural relatedness of seven BTV serotypes from Southern Africa, North America and Australia. Each serotype had several tyrosine containing tryptic peptides which were unique, but approximately 35% of the peptides analyzed were found to be highly conserved between all 7 serotypes. BTV-20 appeared to be closely related to BTV-8 and these two serotypes with BTV-4 and BTV-17 appeared to form a closely knit central cluster.

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CHAPTER ONE

INTRODUCTION

A group of arthropod-borne viruses have been distinguished from the togaviruses on the basis of morphology, physico-chemical and antigenic criteria.[6; 81] Verwoerd (1969) and Borden et al (1971) suggested that these viruses were sufficiently distinct to form a new taxonomic group and Borden, in 1971, proposed the name orbiviruses.[6]

Orbiviruses form a major genus in the family Reoviridae, which according to the International Committee for Taxonomy of Viruses encompasses those viruses which:

(a) possess genomes consisting of several (generally 10 to 12) segments of double-stranded RNA with molecular weight values ranging from 0.2×10^6 to 3.0×10^6; are all segments being encapsidated within a single virus particle,

(b) possess a quasi-spherical capsid, 60 to 80 nm in diameter, which exhibits icosahedral symmetry.[32]

A total of 6 genera have been established in the family. They are: Orthoreo, Rota, Phytoreo, Fiji and Cypoviruses.[76]

The orbiviruses include agents pathogenic to man (i.e. Colorado tick fever), domestic animals (i.e. Bluetongue of sheep and African horse sickness (AHS)) and wild species (i.e. Epizootic haemorrhagic disease of deer (EHD)) and many other viruses not yet associated with disease.[32] Bluetongue virus (BTV), the type species of the orbivirus genus[81] is of international concern, firstly because of the serious disease it causes in sheep and occasionally in cattle, and secondly, because of the restrictions

it has elicited in international trade of susceptible species. Because of its economic importance a considerable amount of research has been done on the biological manifestations of disease, its transmission by biting insects and its prophylaxis by immunization.

The first description of bluetongue disease, occurring in imported European breeds of sheep in South Africa, was given by Hutcheon in 1881. Theiler suggested in 1906 that the causative agent was a virus and he immunized sheep by injection of a mild strain of virus which had been serially passaged in sheep. This strain was used as a vaccine for nearly forty years, despite increasing evidence that it failed to provide adequate immunity. This led Neitz in 1948 to recognize that a plurality of virus strains existed in nature. Since then, twenty serological types of BTV have been identified worldwide.[112]

BT has been prevalent for many years mainly in South Africa, where serotypes designated 1 to 15, and 18 and 19 have been isolated,[78; 112] but during recent years the disease has been found in other countries including Spain, Portugal, Israel, India and in the Far East.[47] Between 1953 and 1967, four serotypes of BTV were identified in the United States of America, they are types 10, 11, 13 and 17[4; 78] and in 1977 a new serotype, BTV-20, was identified in Australia.[99]

The relationship between the bluetongue virus serotypes found in Southern Africa and those found in the USA and Australia have important ecological implications for the spread of the disease and could indicate the route of introduction into other areas. During recent years a large number of non-pathogenic orbiviruses have been isolated from insects, and among them was the new isolate BTV-20. Intense interest in this virus

stems mainly from the potential hazard that BT disease constitutes for the Australian sheep industry.

The regional and international distribution of the various serotypes of BTV is of importance when considering the epizootiology and the successful prophylactic immunization of susceptible stock against the disease. Effective prophylactic immunization constitutes one of the most challenging problems in current research. Under certain circumstances, where it has been established that only a single or otherwise limited number of strains of virus are active in a region, a monovalent live attenuated vaccine developed from the local strain has proved successful.[47] In the enzootic regions of Africa, where numerous antigenic types of BTV may be active during an epizootic in any particular area, control is more difficult. As a result, a polyvalent live attenuated vaccine has to be employed, with the associated problems resulting from interference between strains, difference in immunizing potency, growth rates of modified strains and marked difference in response to individual animals to such vaccines. Furthermore, polyvalent attenuated virus vaccines provide fertile conditions for reassortment of viral RNA in vaccinated animals.[2] Precedents for RNA segment reassortment between orbiviruses have been established in recent in vitro studies by Walker et al (1981) and oligonucleotide fingerprint analysis on two U.S. serotypes of BTV (types 10 and 11) argue strongly that RNA segment reassortment occurs in nature between BTV isolates.[100]

In the last few years progress has been made and new approaches have been employed in the field of vaccine preparation. This involves the introduction of the concept of genetic engineering for the production of viral proteins for use in subunit or peptide vaccines. A prerequisite for

this research is a detailed knowledge of the structure of the viral components and the elucidation of their involvement in the process of neutralization by antibodies.

1. THE SEROLOGICAL GROUPS OF ORBIVIRUSES

Viruses within the genus orbivirus have been subdivided on the basis of serological reactions, with complement fixation (CF) being the most widely used technique for identification and subsequent classification into serogroups.[112] The CF test detects group-specific, rather than type-specific reactions and is, therefore, very useful to demonstrate wider relationships between viruses. The serum neutralization (SN) test on the other hand, is generally accepted as the most sensitive serological test to demonstrate minor antigenic differences between orbiviruses and used to detect type-specific neutralizing antibodies. The viruses within each serogroup are designated as serotypes if they are distinct by SN.

Table 1.1 shows the orbivirus serological groups, these are placed into ten clusters as proposed by Gorman (1979). However, it is now clear that these methods alone are inadequate for the characterization of these viruses.[17] For example, serological cross-reactions have been shown to occur between certain serotypes in the (a) BT and Eubenangee serogroups[6; 18] and (b) the Palyam and Eubenangee serogroups.[6; 18] This is becoming a matter of some importance, since it is essential for countries free of a particular disease causing orbivirus to be able to determine the relationship of new isolates to the known pathogens as closely as possible.

TABLE 1.1: SEROLOGICAL GROUPS IN THE GENUS ORBIVIRUS AS PROPOSED BY GORMAN (1979).

GROUP	TYPE	PROTOTYPE STRAINS
A	1-9	African horse sickness
B	1-20	Bluetongue
	21-28	Epizootic haemorrhagic disease
	29	Eubenangee
	30	Pata
	31	Tilligerry
	32	Ibaraki
C	1	Colorado tick fever
	2	Eyach
D	1	Palyam
	2	Kasba
	3	Vellore
	4	D'Aguilar
	5	Abadina
	6	Nyabira
E	1	Changuinola
	2	Irituia
F	1	Corriparta
	2	Acado
	3	Bambari
G	1	Kemerovo
	2	Baku
	3	Bauline
	4	Cape Wrath
	5	Chenuda
	6	Great Island
	7	Huacho
	8	Lipovnik
	9	Mono Lake
	10	Nugget
	11	Okhotskiy
	12	Seletar
	13	Sixgun City
	14	Tribec
	15	Yaquina Head
	16	Wad Medani
H	1	Warrego
	2	Mitchell River
I	1	Wallal
	2	Mudjinbarry
J	1-5	Equine encephalosis
Ungrouped Viruses		Lebombo
		Orungo
		Japanaut
		Umatilla

1.1 SEROLOGICAL VARIATION AMONG BLUETONGUE VIRUSES

BTV is the best studied orbivirus and it is regarded as the prototype of this genus.[81; 49] Strains of BTV from outbreaks of disease among naturally infected cattle and sheep in South Africa and other parts of the world have been classified into twenty distinct immunological groups.[4;45;99]

The multiplicity of BTV serotypes was first recognised by Neitz in 1948 and it was subsequently shown that common virus antigens were detectable in CF tests,[65] in agar gel precipitin (AGP) tests[65] and in flourescent antibody tests.[93] The specificity of the SN test in defining distinct BTV serotypes was demonstrated by Howell (1970) who found 22 isolates of BTV fell into 12 antigenic types showing no cross-reactions. However, Thomas et al (1979) using plaque neutralization methods reported a spectrum of cross-reactivity between BTV isolates from virtual identity to clear antigenic differences and they suggested that BTV isolates form an antigenic continuum.

Neutralization tests performed by Erasmus et al (1981) show cross-reactions between serotypes 20, 17 and 4. They suggest that BTV-4 is distantly related to BTV-17, but shows a close immunological relationship with BTV-20. Della Porta et al (1981), using similar tests, suggested that BTV-20 could be regarded as a subtype of type 4. Furthermore, cross-immune precipitation results have indicated a relatedness of serotype 10 to BTV-4.[50; 52]

All these are supported by the results of cross-immune precipitations done by Huismans and Bremer (1981). They summarized their results in a schematic diagram given in Fig. 1.1.

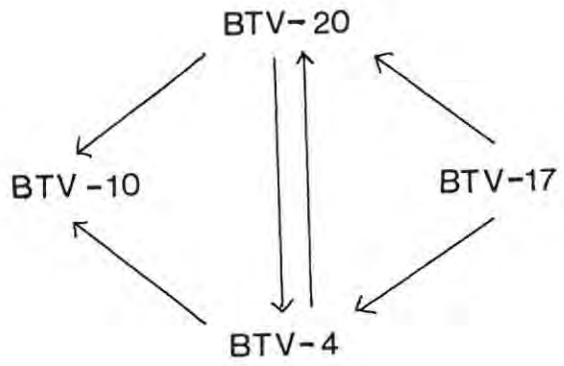


FIGURE 1.1: A schematic representation of the cross-immune precipitation of polypeptide P2 between different serotypes of BTV. The arrow between 2 serotypes indicates immune precipitation of polypeptide P2 in a reaction between soluble polypeptides from one serotype (indicated by the head of the arrow) and immune serum from another (tail of the arrow) (Huismans and Bremer 1981).

The degree of cross-immune precipitation has not been quantified, but it is evident that cross-immune precipitation of P2, the type specific antigen[50; 52] does occur between serotypes 20, 17, 10 and 4, indicating their immunological relatedness. The remaining 16 serotypes of BTV appear to show no cross-immune precipitation of the type specific antigen, P2.[50]

1.1.2 The BT-EHD-Eubenangee complex

There are some distant serological relationships that have been observed between some of the orbivirus groups. The most important of these being the BT-EHD-Eubenangee complex; where reports of serological cross-reactions between certain serotypes of each serogroup has led to confusion in classification and to difficulty in defining the disease risk of certain virus isolates. Gorman (1979) suggested an extended BT subgroup, consisting of at least 32 viruses (Table 1.1), based on the following observations:

(1) EHDV and BTV share a common group antigen as demonstrated by CF tests[6; 80] AGP tests[58; 79] and in the indirect fluorescent antibody (IFA) test;[59] but do not cross-react in neutralization tests.[3]

(2) The Eubenangee group of viruses may be related to: (i) EHDV; as the virus Pata, isolated in the Central African Republic shares CF antigens with EHDV and also with an Australian orbivirus, Eubenangee,[6] (ii) BTV through Tilligerry and Eugenangee viruses:[18] The results of CF and AGP tests suggest that the Eubenangee viruses share antigens with BTV but are not identical to members of that group.

(3) Tilligerry virus is related to Eubenangee virus by CF but they can clearly be distinguished by this test.[18]

(4) Campbell et al (1978) showed that the relationship between Ibaraki virus and EHDV appears to be more pronounced than the one between BTV and EHDV. Cross-reactions were observed between Ibaraki virus and EHDV in AGP and IFA tests for common antigens. In addition, plaque reduction neutralization tests for serotype-specific antigens revealed cross reactions between Ibaraki virus and the Alberta strain (serotype 2) of EHDV.[11]

Because of the reluctance to type viruses as BT, especially for the Eubenangee viruses which have not yet been linked with disease, the suggestion by Gorman (1979) of three groups of viruses within the BTV serogroup has been maintained. A large number of viruses make up the BTV serogroup, but the exact relationship between them at the molecular level remain to be determined.

1.1.3 Palyam group interactions

Other Australian orbiviruses showing some serological cross - reactions with Eubenangee and with BT are those related to D'Aguilar virus. These viruses are members of the Palyam serogroup (D) which were thought not to be related to the Eubenangee serogroup (B) of viruses.[6] However, D'Aguilar virus antisera reacted in AGP tests against Eubenangee virus and also gave a weak AGP reaction with BTV. Thus it would appear that D'Aguilar virus may show a distant serological relationship with BTV through the Eubenangee group.[18]

Furthermore, cross-reactions in AGP tests have been reported between Abadina virus (Palyam group) and both EHDV and BTV.[79] Abadina virus, therefore, seems to be a more distant relative of the BT and EHD viruses since cross-reactions were obtained only in AGP tests. A summary of these possible relationships is shown in Figure 1.2.

1.1.4 The ungrouped viruses

Certain orbiviruses which show no relationship to any of the established serogroups or which have not yet been adequately characterized are presently regarded as ungrouped orbiviruses (Table 1.1)

It can be seen that orbiviruses do not always fit neatly into their respective serogroups and the problem of intergroup relationships or intragroup variation is becoming more pertinent. Clear directives concerning the magnitude of the difference required to create a new serological group should be formulated. It may well be, however, that the attempt to group orbiviruses into well defined serogroups is too artificial and that an absolutely satisfactory classification on this basis may be unattainable, as the relationship between these viruses may be more spatial than linear.

2. GENETIC REASSORTMENT AS A MECHANISM FOR VARIATION.

It is becoming clear that the existence of so many closely related viruses may at least be partly due to the structure of the viral genome and the possibility of genetic reassortment between orbiviruses.[35] Gorman (1979) has reviewed evidence suggesting a high rate of concurrent circulation of

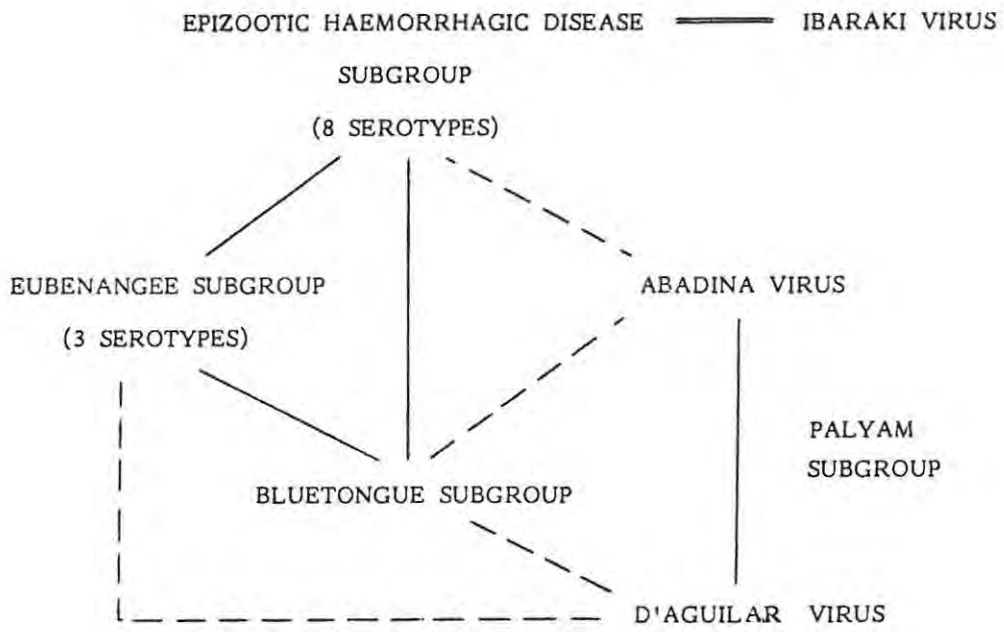


FIGURE 1.2: Summary of the known serological inter-group relationships of orbiviruses.

— — AGP

——— CF

═══ SN

related orbivirus serotypes in insect and vertebrate populations. The frequent movement of insects from host to host should provide ready opportunity for multiple infection and subsequent generation of recombinant orbiviruses.

Olligonucleotide fingerprint studies by Sugiyama et al (1981) indicated the existence of naturally occurring reassortant BTV isolates. This further suggests that the division of orbiviruses into clearly distinct serological groups may not be possible, as antigenic determinants giving rise to low level cross-reactions between two viruses in different serological groups may not be shared by all members of each group.

3. ALTERNATIVE METHODS FOR DETERMINING THE RELATIONSHIPS BETWEEN ORBIVIRUSES

3.1 SIZE HETEROGENEITY OF RNA SEGMENTS IN CLOSELY RELATED VIRUSES

It has been suggested that the patterns of separation of double-stranded RNA (dsRNA) in acrylamide gels can be used to classify viruses. Payne & Riviers (1976) showed heterogeneity in genome segment sizes of cytoplasmic polyhedrosis viruses and it was suggested that the electrophoretic profiles were more useful in grouping these viruses than were serological methods.[88]

Confusion exists in the literature as to whether RNA electrophoretic profiles are diagnostic for certain orbiviruses. Many reports have recognized distinct electropherotypes for different serotypes of BTV[34;50;68] and it has been suggested that the polyacrylamide gel electrophoresis (PAGE) profile may be diagnostic for BTV[68] and able to

classify other viruses in the orbivirus group.[112] However, other reports have indicated that due to the heterogeneity in the electrophoretic patterns of the Wallal, Eubenangee and bluetongue subgroups of orbiviruses, it was neither possible to define relationships of these viruses by the dsRNA genome profiles[32; 34] nor was it even possible to distinguish the electrophoretic pattern of the Eugenangee subgroup from bluetongue virus.[34]

Co-migration of RNA segments indicates similarities of molecular weight (MW) and does not reflect a genetic similarity. Furthermore, segments containing information coding for equivalent antigenic sites in different viruses may migrate to different positions in PAGE.[32] However, analysis of the genomes of orbiviruses by PAGE suggests a diversity which has not so far been recognised in conventional serological tests.

3.2 MOLECULAR HYBRIDIZATION

The fact that BTV contains ten segments of double-stranded RNA[107] has provided the opportunity of studying the different serological types by an alternative procedure. Nucleic acid hybridizations have been successfully applied to the study of the relationships of many other viruses.[20; 121]

Huismans and Howell (1973) performed a large number of molecular hybridizations between RNAs of different serotypes of BTV, one of the objectives being to determine whether hybridization could provide any information on the degree of relatedness between the different BTV serotypes. It was found that cross-hybridization patterns indicated a degree of relatedness between several serotypes and they suggested that serotype 4 is more closely related to serotype 11 than to serotypes 3 and

2 in that order (Figure 1.3(a)). Furthermore, the hybridization patterns of serotype 10 with types 3, 13 and 16 show that only six segments show homology, whereas a maximum of eight segments of serotype 9 hybridize with the corresponding type 10 genome segments; with serotypes 8, 6, 4 and 1 there are seven segments which show homology. These results are summarized in Figure 1.3.

In all cases segment six and either segment two or three did not show cross-hybridization with the equivalent type 10 genome segments. Segments one, four, five and eight always hybridized, although the homology was usually incomplete. The possible relationship between RNA segments and structural proteins are shown in Table 1.2.

It must be pointed out, however, that these relationships refer to the degree of homology between the total genomes of the strains and it does not necessarily refer to any degree of immunological relatedness.

This was borne out by the results of Huismans and Bremer (1981) who observed homology of only 20 to 30 per cent between the immunologically closely related BTV-4 and 20 compared with the 70 per cent homology between BTV-4 and 10. The homology normally found between BTV serotypes is at least 70%. It was also found that heterologous hybridization products of BTV-20 contained no more than 2 or 3 stable hybrid segments, as opposed to 7 out of a possible 10 found in hybrids of BTV-10 and BTV-4 (Figure 1.3). Thus the hybridization results indicate that BTV-4 and BTV-20 are neither identical nor even closely related.

Similar RNA-RNA reassociations performed by Gorman et al (1981) failed to show significant cross-hybridization between the mRNA derived from

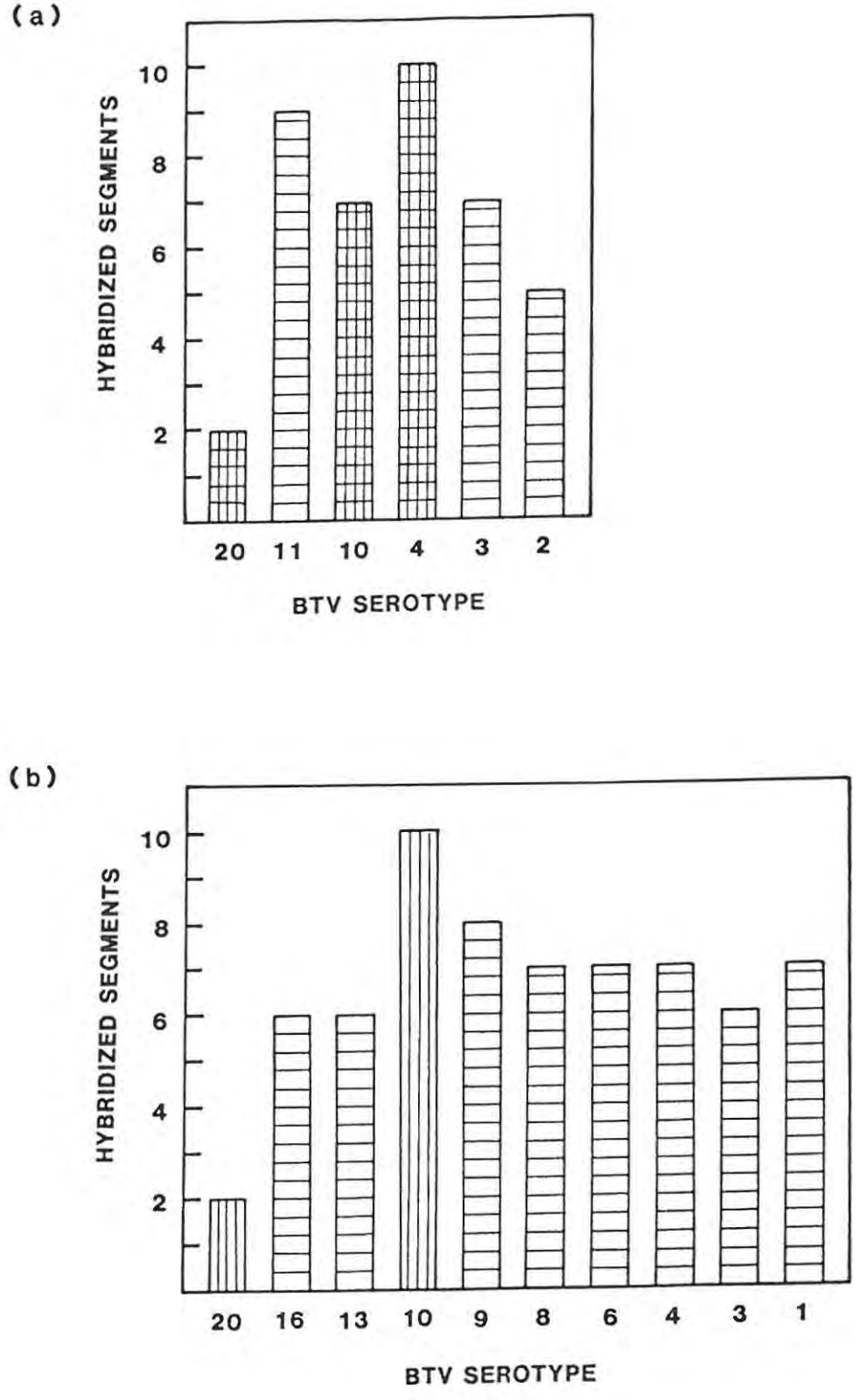


FIGURE 1.3: Diagrammatic representation of cross-hybridization results of mRNA from different BTV serotypes with ds-RNA from (a) BTV-4; (b) BTV-10

▤ Huismans and Howell (1973)

▥ Huismans and Bremer (1981)

BTV-20 with strands of opposite polarity of BTV types 1, 4, 10 and 17. The percentage homology obtained was 14, 1, 3 and 7% respectively. The reason for this discrepancy is not known.

The degree of homology existing between the different serological subgroups of the genus orbivirus was studied by Verwoerd and Huismans (1969) and Huismans et al (1979), they found it to be less than 5% in cross-hybridization tests of complete genomes of BTV with EHDV and AHSV.

3.3 MONOCLONAL ANTIBODIES

Monoclonal antibodies (MA) have been used to distinguish different types of influenza,[30] murine leukemia,[12] arena,[9] and mouse mammary tumour[75] viruses. Sometimes these antibodies have detected minor antigenic differences between viruses that were indistinguishable by conventional serological methods, but were known to be different because of some other characteristic; for example, mouse mammary tumour viruses with different host ranges were distinguished by MA but not conventional serology.[75]

Conventional serological tests are inadequate for distinguishing between closely related viruses in the orbivirus genus.[17] Analysis using MA may define these antigenic relationships more precisely than conventional serology and aid in determining the phylogenetic relationships among these viruses.[2]

Using type-specific MA, Letchworth and Appleton (1983) examined many different isolates of a single serotype (type 17) to demonstrate that type-specific antigens were conserved between isolates of the same

serotype. All isolates were neutralized by immune sera directed against BTV-17 but not by sera directed gainst BTV-10, 11 and 13. The MA detected many antigenic similarities and at least one important antigenic difference between 21 isolates of BTV-17. Three type-specific, neutralization-related antigenic determinants were found on the Wyoming isolate of BTV-17. The first (perhaps a number of epitopes) was recognized by immune sera on all BTV-17 isolates; the second, recognized by a MA on the Wyoming and eight mid-South isolates and the third recognized by another MA only on the Wyoming isolate. It appears from this study that these two MA can discriminate between BTV-17 isolates obtained from three areas in the United States. These differences may be important in serological typing of BTV isolates and in the selection of viral strains used for vaccines, particularly if subunit or peptide vaccines are to be developed.

Letchworth and Appleton (1983) present evidence which indicates that there is either a common ancestry for some of the orbiviruses or that natural reassortment occurs among them:

(1) A MA recognized an epitope carried by a non-structural viral protein, with a MW of 48 000 (probably P6a; VP7 in the terminology used by Appleton & Letchworth) which bound to all 20 BTV serotypes, to EHDV serotypes 1 and 2 and to Ibaraki virus.

(2) A serotype-restricted MA which precipitated P2 and P6 (VP2 and VP8 respectively) reacted with antigens in cells infected with BTV-17, 13 and to a lesser extent BTV-10. Thus this epitope is shared by 3 of the 4 serotypes of BTV found in

the United States, but is absent in the 17 other BTV serotypes.

Furthermore, reaction of soluble BTV-17 viral proteins with a neutralizing, serotype-specific MA precipitated P2 and P3, two structural proteins. Letchworth & Appleton (1983) suggest that these 2 proteins may be: (i) distinct but share epitopes, (ii) may be modified forms of the same protein, or (iii) associated in some way during viral replication. This still has to be determined and possibly could be done by tryptic peptide mapping.

MA were also used to map subgroup specific epitopes in BTV-17, which were found to be located on the group antigen P7 (VP9). There appear to be at least 3 antigenic domains on P7, two of which were identified by antibodies that did not bind to all BTV serotypes, indicating that there are both conserved and nonconserved epitopes on P7. This could also be elucidated by peptide mapping of these proteins.

4. THE STRUCTURE OF ORBIVIRUSES

Some of the viruses included in Table 1.1, especially those isolated from insect hosts, are difficult to cultivate and purify in sufficient quantities for biochemical studies. Morphological features have therefore, remained one of the most important, and sometimes the only one, for classification of orbiviruses.[112] In fact, the name of the genus is derived from a morphological feature, namely the characteristic ring-like capsomere structure (ORBIS = "ring") seen on the surface of the viral capsid. This feature clearly distinguishes members of the genus orbivirus from members of the Reoviridae.

4.1 MORPHOLOGY

Two phenomena have caused considerable confusion about the size of orbivirus particles, especially in the early literature. These are:

(1) OCCURRENCE OF ENVELOPED PARTICLES: Using electron microscopy Bowne and Jones (1966), Owen and Munz (1966) and Foster and Alder (1979) showed the presence of "enveloped" BTV particles. These were shown to be due to the characteristic tendency of these viruses to associate strongly with cellular membranes[108] and resulted from the release of the virus from the infected cell by extrusion across the plasma membrane.[62]

As these membranes can be removed by treatment with Tween - 80 and ether without loss of infectivity, they are not considered part of the virion and were termed "pseudo-envelopes".[24]

(2) METHOD USED FOR PURIFICATION OF ORBIVIRUSES: Electron microscopy of BTV purified on sucrose gradients showed the virus to be generally amorphous in appearance, with little or no symmetrical arrangement of morphological units. The diameter of this type of BTV particle averaged 68nm.[24; 109] Whereas BTV particles purified by isopycnic centrifugation on neutral caesium chloride (CsCl) gradients showed 32 clear morphological units arranged in a symmetrical particle with a diameter of 55 nm.[24; 109] This discrepancy was resolved when it was demonstrated that BTV possess a double-layered capsid.[74; 109]

All intact virions have a diffuse, structureless outer layer, with a diameter of 60 to 80 nm. This seems to be characteristic of orbiviruses, distinguishing them morphologically from the orthoreoviruses and the rotaviruses.[112] This outer layer, which causes the "fuzzy" appearance in electron micrographs, was first thought to be of cellular origin but Martin and Zweerink (1972) and Verwoerd et al (1972) showed it to be essential

for infectivity and coded for by the viral genome. This diffuse protein layer surrounds the nucleocapsid, obscuring its arrangement of structural units. It is removed following contact with neutral CsCl, exposing the structured subviral particle or core.

Regarding the structure of the core particle, there now seems to be general agreement that the inner capsid layer consists of 32 large ring-shaped capsomeres arranged in icosahedral symmetry, with triangulation number T = 3. The capsomeres are tube-like hollow structures 10 to 12 nm wide with an axial hole approximately 4 nm in diameter. They are 8 nm in length and consist of smaller structural units arranged in regular hexagonal and pentagonal patterns.[24] The inner capsid of BTV is 56 nm in diameter and surrounds a 38 nm icosahedral core.[87] The particles have a central cavity some 35 to 40 nm in diameter. This is the same as that in reoviruses and is in agreement with the fact that the reovirus and orbivirus genomes are similar in size.[62]

A diagrammatic representation of the possible structure of the BT virion is given in Figure 1.4.

4.2 POLYPEPTIDE COMPOSITION

Verwoerd (1969) showed that the BT virion is composed of approximately 20.0% RNA and 80.0% protein. A small amount of phospholipid (1 to 2%) was found, but was considered to be due to contamination with cellular material. The BTV particles comprise seven polypeptide species, there are four major polypeptides, namely P2, P3, P5 and P7, and three minor polypeptides, P1, P4 and P6. The MW of the polypeptides range from 30,000 to 140,000.[109] Furthermore, two non-capsid proteins have been identified in the infected cell.[49]

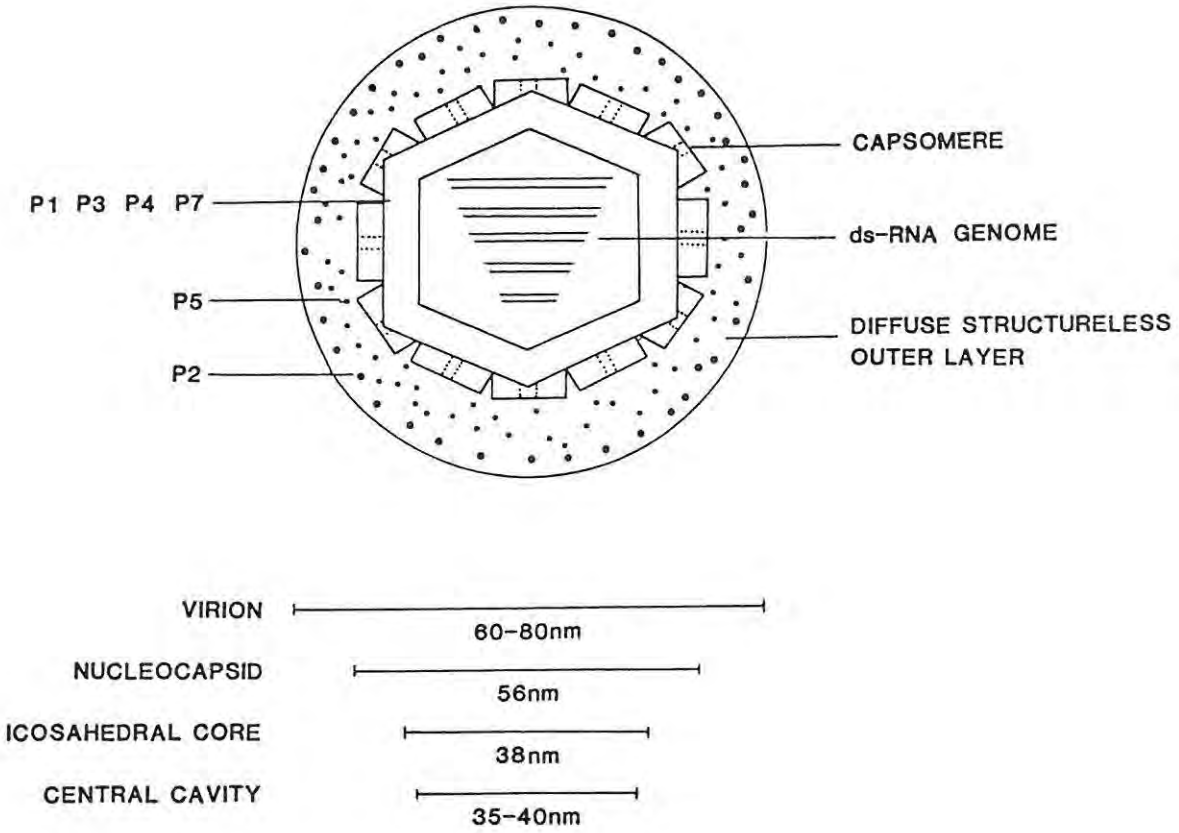


FIGURE 1.4: A diagrammatic representation of the possible structure of the BTV virion.

Verwoerd et al (1972) suggested that the capsid proteins of BTV exhibit a primary gene product relationship with RNA genome segments. The relationship between RNA segments and structural proteins are shown in Table 1.2.

Using the degrading effect of unbuffered CsCl on BTV, Verwoerd et al (1972) were able to show that two major polypeptides, P2 and P5, constitute the diffuse layer surrounding the nucleocapsid. In this layer P2 is probably on the outside, because an intermediate form, in which P2 is lost, but P5 remains attached to the core particle can also be found after CsCl treatment. This conclusion is supported by the fact that P2 and P5 are the most intensely labelled virus particle proteins when radio-iodinated using lactoperoxidase.[73]

De Villiers (1974) using PAGE, showed that P2 is the most variable of all major orbivirus capsid polypeptides with regard to size. The accommodation of polypeptides of various sizes in the loosely structured layer on the outside of these virions may be responsible for the divergence among different members of the orbivirus group.

Removal of these two outer polypeptides results in activation of an RNA dependent RNA polymerase. Verwoerd et al (1972) suggest that this masking of the viral transcriptase is a non-specific effect. It has also been shown that loss of either one or both of the outer polypeptides results in a loss of infectivity.[109]

The core particles of BTV contain two major and three minor polypeptides. The major polypeptides, P3 and P7 show very little size variation[19] and these two polypeptides are likely to be the main

TABLE 1.2: LOCATION OF POLYPEPTIDES IN THE BLUETONGUE VIRUS CAPSID AND POSSIBLE RELATIONSHIPS WITH RNA GENOME SEGMENTS.

RNA SEGMENT		POLYPEPTIDE			
No.	MW * ($x10^{-6}$)	No.	MW * ($x10^{-3}$)	Number per * Virion	Location *+
1	2.50	1	140	7	Surface Nucleocapsid
2	1.99	2	110	98	Outer Layer
3	1.82	3	101	76	Surface Nucleocapsid
4	1.31	4	82	6	Surface Nucleocapsid
5	1.16	(5a)	67 ≠	-	Non-virion
6	1.08	5	61	156	Outer Layer
7	0.60	6	42	32	Surface Nucleocapsid
8	0.54	(6a)	30 ≠	-	Non-virion
9	0.50	7	29	570	Inner Nucleocapsid
10	0.30	-	17 ≠	-	Non-virion

* Verwoerd et al 1972.

\+ Martin and Zweerink 1972, Martin et al 1973.

≠ Estimated from MW of RNA segment.

structural components of the characteristic core capsomeres. The symmetry of these structures might require that only polypeptides of a very specific size can be used in their construction.[112]

Removal of these two outer polypeptides results in activation of an RNA dependent RNA polymerase. Verwoerd et al (1972) suggest that this masking of the viral transcriptase is a non-specific effect. It has also been shown that loss of either one or both of the outer polypeptides results in a loss of infectivity.[109]

The core particles of BTV contain two major and three minor polypeptides. The major polypeptides, P3 and P7 show very little size variation[19] and these two polypeptides are likely to be the main structural components of the characteristic core capsomeres. The symmetry of these structures might require that only polypeptides of a very specific size can be used in their construction.[112]

The location of the polypeptides within the BTV core particle was studied by Martin et al (1973) using an iodination technique. They found that the major polypeptide P3 and minor polypeptides P1, P4 and P6 are easily iodinated and therefore located close to the surface of the particle. P7 was found to be less reactive and therefore probably located internally. However, recent investigations (Huismans, unpublished results) indicate that the characteristic capsomeres on the surface of the BTV core particles are composed of polypeptide P7.[52]

It is not known which, or how many, of the core polypeptides are involved in the transcriptase activity associated with intact BTV core as further dissociation of the particle completely eliminates all activity.[111]

4.3 RNA COMPOSITION

Orbiviruses are known to possess a double-stranded RNA genome, the MW of which vary between 11.0×10^6 and 12.0×10^6. [36;51;95;102;107] This RNA is present as 10 segments, which like those of reovirus, fall into 3 size classes. Verwoerd et al (1970) showed that the MW of the segments, for BTV, ranged from 0.3×10^6 to 2.50×10^6 (Table 1.2).

Oligonucleotide fingerprints of the genome segments of BTV type 20 showed that each segment was unique indicating that each contained different sequence information.[34]

Foster et al (1978) extracted the RNA genome of BTV under acidic conditions with sodium dodecyl sulphate (SDS) and phenol. Electron microscopic analysis showed the genome as an unfragmented, continuous structure. The genomes appeared to have a rosette configuration with ten loops (genes) of varying lengths emanating from a central area, generally of higher density, resembling a doughnut or crescent, due to twisting of the strands. They propose that:

(1) The segments of the BTV genome in the natural state are linked to form a continuous structure.

(2) The genome is supercoiled into a package small enough to fit inside the BTV capsid.

If the segments are linked inside the virion, linkage by means of normal covalent phosphodiester bonds is unlikely because of the reproducible fragmentation pattern obtained after extraction of the RNA.[112] However, the fact that BTV segments are transcribed individually[54] would suggest that the segments are functionally distinct inside the virion.

5. PROTEINS INVOLVED IN ANTIGENIC VARIATION

Antigenic variation in viruses reflect differences in the protein coat and it is possible to relate observed antigenic variation in orbiviruses to specific polypeptides in the virus capsid.

De Villiers (1974) compared the molecular sizes of capsid polypeptides of different serotypes of BTV by PAGE. The results indicate that the largest variation in polypeptide size amongst the different serotypes was found in polypeptide P2. This is a significant result in view of the fact that P2 is a main component of the diffuse outercapsid layer of the BT virion and the surface polypeptides of orbiviruses are likely to be involved in neutralization reactions with antibodies. The loosely structured outer capsid protein layer can apparently accommodate such P2 size variations. However, these experiments provide only indirect evidence for the implication of P2 in antigenic variation, because the results reflect differences in the size of the polypeptides and not differences between the antigenic determinants actually responsible for variation.

A more direct approach was taken by Huismans and Erasmus (1981) who studied antigenic variation among the different serotypes of BTV by precipitating soluble ^{14}C -labelled BTV proteins with homologous and heterologous antisera and comparing the immune precipitates by means of electrophoresis and autoradiography. The results showed that polypeptide P2 was precipitated only with homologous serum, suggesting that this polypeptide contains the type-specific antigen in BTV. P2 was also identified as serotype-specific when a similar experiment was carried out using the Alberta and New Jersey strains of EHDV. The same result will probably be obtained for the other orbiviruses.

These results substantiated previous conclusions drawn from hybridization experiments of Huismans and Howell (1973). They found the largest variation in the nucleic acid of different BTV serotypes to be associated with genome segment 2, the segment that presumably codes for polypeptide P2. Even though at least some antigenic determinants of polypeptide P2 appear to be serotype-specific, it is by no means certain that all the P2 precipitating antibodies should be serotype-specific.

The results of Appleton and Letchworth (1983) confirm that BTV serotype-specific and neutralizing epitopes are associated with polypeptide P2. A serotype-specific MA neutralized infectivity, inhibited haemagglutination by BTV-17 and precipitated P2 and P3. A second MA, with restricted serotype-specificity, precipitated P2 and P6. These MA show the serotype restricted nature of polypeptide P2.

In a very recent publication, Kahlon et al (1983) using oligonucleotide fingerprinting and serological analysis of BTV serotypes 10 and 11 and their intertype reassortants, were able to show that BTV RNA segment 2 does indeed code for the serotype-specific antigen. Furthermore, their peptide mapping results confirm that VP2 is the serotype-specific antigen for BTV.

The other outer capsid polypeptide, P5, also shows considerable size variation amongst different serotypes.[19] However, no evidence was obtained by immune precipitation that P5 contributes to serotype - specificity. This does not rule out the possibility that P5 antibodies can still be involved in virus neutralization.

The occurrence of neutralizing and complement fixing reactions with different specificities among orbiviruses suggests that there are at least two different antigens normally expressed by these viruses.

A group antigen detectable by CF and AGP tests was shown by Wang et al (1972) to be present in the virus particle and also to occur in large amounts in soluble form when BTV was grown in tissue culture. In order to identify the antigen(s) in the group-specific CF reaction in the case of BTV, attempts were made to correlate CF titres in sera with the ability to precipitate soluble virus proteins. In one study by Huismans and Erasmus (1981) ascitic fluid from BTV-infected mice was used for the immune precipitation. This fluid is used routinely in CF tests because it contains high titres of CF antibodies but hardly any neutralizing antibodies. Analysis of the immune precipitates indicated that polypeptide P7 was the only virus protein present.

In a second study reported by Verwoerd et al (1972) correlation was found between the known kinetics of synthesis and disappearance of CF and neutralizing antibodies in BTV-infected sheep and the ability of these sera to precipitate specific polypeptides. Antibodies against the serotype-specific polypeptide P2 remained high for at least a 5-month period. The decline in CF antibodies correlated with the decline in the amounts of P7 that could be precipitated.

From these results, which are supported by the findings of Gumm and Newman (1982), it seems reasonable to conclude that P7 is the main determinant of group specificity. Other polypeptides may however, also be involved.

All BTV serotypes investigated so far show cross-immune precipitation of P7. Huismans, Bremer and Barber (1979) studied the reported immunological relationship between BTV and EHDV, to determine if any of the corresponding capsid polypeptides of the two viruses have common antigenic determinants. It was found that an antiserum against BTV can precipitate EHDV polypeptides P3 and P7. While EHDV antiserum precipitates mainly BTV polypeptide P7, thus suggesting that the major core polypeptides of the two viruses (P3 and P7) have common antigenic determinants. This result could explain the observed reaction in serological tests and substantiates the finding that P7 is the main CF antigen of BTV.[52]

It is impossible at this stage, however, to exclude a contributory role for other virus polypeptides such as P3 or the minor polypeptide components in the group-specific responses.

6. SCOPE OF THIS STUDY

The establishment of serological groups has been important in the assessment of the relationships between viruses and in the development of concepts of virus evolution. However, the usefulness of the results of serological tests in estimating diversity in virus populations is limited.

Orbiviruses are presently grouped on the basis of serological relationships and viruses that are related by CF are grouped together. However, as with other arboviruses, orbiviruses do not always fit neatly into their respective serogroups and the problems of intergroup relationships or intragroup variation is becoming more pertinent. Clear directives concerning the magnitude of the differences required to create a new

serogroup should be formulated. It may well be, however, that the attempt to group orbiviruses into well defined serogroups is too artificial and that an absolutely satisfactory classification on this basis is unattainable, as the relationships between these viruses may be more spatial than linear.

A large number of viruses make up the BTV serological complex, but the exact relationship between them at the molecular level remains to be determined. This research project was undertaken to examine the relationships between structural components of selected viruses of the BT serogroup in an attempt to define the molecular basis for the serological reactions between them and also to detect possible relationships between the viruses which may not be detected in conventional serological tests. Comparative peptide mapping techniques have been used to investigate these relationships, enabling the antigenic variation to be defined in biochemical terms.

The certification of animals as free of BTV is based on negative reactions in group-reactive serological tests, so that the relationships between these viruses have economic as well as taxonomic significance. For those countries free of disease it is important to be able to determine the relationship of new isolates to the known pathogens as closely as possible. This has stimulated the exploration of other means of studying the relationship between these viruses, for example, comparing the RNA genomes,[34;53;100] and detection of type-specific protein epitopes.[2; 72] This research uses comparative tryptic peptide mapping of the BTV capsid proteins as a means of assessing the degree of relatedness of different BTV types.

It is hoped that the results should lead to:

1. Confirmation (or otherwise) of the Gorman classification of BTV types.
2. Determination of the relatedness of orbiviruses, both the BT viruses and to serologically related viruses.
3. A greater understanding of the serological results.
4. A significant contribution to the basic knowledge of the structure of the orbiviruses.

It is also of considerable academic interest to extend this study to look at the evolution of the Reoviridae.

CHAPTER TWO

IMMUNE PRECIPITATION

2.1 INTRODUCTION

It has been shown that the outer capsid polypeptide of BTV, P2, determines serotype specificity as:

(i) It is not precipitated by most heterologous BTV antisera.[50; 52]

(ii) The genome segment that presumably codes for it does not hybridize with the corresponding segment from other BTV serotypes.[53]

(iii) Neutralizing antibody titres were correlated with precipitation of P2 in BTV convalescent sheep sera,[52] and

(iv) Monoclonal antibodies (MA) show that the serotype specific and neutralizing epitopes are associated with P2.[2]

Experiments by Huismans and Bremer (1981) demonstrate that there are common antigenic determinants on the P2 polypeptides of BTV-4 and BTV-20; these viruses also show a certain amount of cross - neutralization.[50] However, cross-immune precipitation of P2 is not enough to indicate cross-neutralization: Protein 2 from BTV-10 was precipitated by immune serum to BTV-4 and BTV-20 (Fig. 1.1), even though there is no evidence of cross-neutralization between these strains. Huismans and Bremer (1981) suggest that it is possible that cross-neutralization between two serotypes is only found between strains that have several antigenic determinants in common, whereas cross-immune precipitation of P2 might only require one such determinant.

However, more recent studies performed by Letchworth and Appleton (1983) show that a MA directed against a single antigenic determinant, both neutralizes BTV-17 (to which the MA was raised) and protects neonatal mice against BTV-17. They also found that the neutralization-related epitopes varied between BTV-17 isolates.

The core polypeptide of BTV, P7, is probably the major determinant of group specificity as:

(i) Cross-immune precipitation of P7 occurs with heterologous BTV antisera.[50; 52]

(ii) P7 was the only viral protein isolated from precipitin bands in agar gel diffusion studies using heterotypic antiserum.[37]

(iii) CF antibody titres correlate with precipitation of P7 in BTV convalescent sheep sera,[52] and

(iv) MA show that subgroup specific epitopes are located on P7.[2]

The cross-immune precipitation studies of Huismans and Bremer (1981) and Huismans and Erasmus (1981) revealed that most homotypic immune precipitates showed precipitation of polypeptides P2, P3, P6a and P7. But with heterologous sera, the amount of P2 precipitated was considerably reduced (except for cross-precipitation of P2 between serotypes 4, 10, 17 and 20); whereas precipitation of polypeptides P3, P6a and P7 was unaffected by the serotype of the serum used.

These results are substantiated by Appleton and Letchworth (1983) who found that a MA recognised an epitope carried by a non-structural viral protein, probably P6a (VP7 in their terminology) which bound to all BTV serotypes, to EHDV-1 and 2 and to Ibaraki virus.

In the light of these results it can be seen that a number of viral proteins, including P2 and P7, appear to share antigenic determinants. It was reasoned that by using the more direct approach of investigating the immune response against single proteins, BTV sub-core particles and whole virus particles, one could examine the degree of antigenic similarity between the viruses making up the orbivirus genus. From this one might be able to determine the relationships between these viruses and see if other viral proteins, such as P3 or other minor polypeptide components, have a contributory role in the group specific response.

A similar approach was taken by Newman et al (1979) in their serological study on the virus-infection associated (VIA) antigen of the seven serotypes of Foot-and-Mouth Disease Virus (FMDV). They examined the extent of cross-reaction in a radioimmune precipitation experiment designed to measure the amount of antibody reacting with preparations of the virus particle, its 12S protein subunit and the free VIA antigen. Their results showed that the VIA antigen of the seven serotypes of FMDV are closely related.

2.2 MATERIALS AND METHODS

2.2.1 PROPOGATION OF VIRUSES

CELLS:

Baby hamster kidney (BHK-21) cells were used in all experiments and grown in Glasgow's modification of Eagles medium supplemented with 10% foetal calf serum.

VIRUSES:

Bluetongue virus types 3, 4, 8, 10, 13, 17 and 20 and Epizootic haemorrhagic disease of deer virus (EHDV), Alberta strain (serotype 2) were obtained from the Veterinary Research Institute, Onderstepoort as freeze-dried egg passage 1, BHK 2 or 3.

PRODUCTION OF VIRUS STOCK AND UNLABELLED VIRUS HARVESTS:

Once a confluent monolayer of BHK-21 cells had formed in $75cm^3$ Falcon flasks, the growth medium was discarded and the monolayer rinsed twice with Hanks balanced salt solution to remove serum globulins. Each flask was inoculated with about 10^7 plaque-forming units of tissue-culture adapted virus and allowed to absorb for 30 minutes at room temperature. Twenty mℓs of serum-free Eagle's medium was added and the cells incubated at 37°C on a shaking platform. Infected cultures showed a complete cytopathic effect within 48 hours of infection and at this time the virus was harvested and stored at 4°C

Viruses were assayed by the serial end-point dilution method (Struthers, personal communication) and the titre of the viral preparation calculated by the method of Reed and Muench.[16] The seed virus was never more than five serial passages away from the stock virus.

PREPARATION OF RADIOLABELLED VIRUS PROTEINS:

BHK cell monolayers were inoculated as described in the above section. At 12 to 14 hours post infection the infecting virus was removed and the cell monolayer rinsed three times with methionine-free Eagle's medium. The cells were incubated at

37°C for 1 hour with 10 mℓ of the same medium, after which 100uCi of ^{35}S-methionine (Radiochemical Centre, Amersham, England) was added to supplement it. Labelling was carried out at 37°C and a shaking platform was used to ensure even distribution of the isotope-containing medium. Cells became detached from the surface within 48 hours, and the virus harvest was stored at 4°C.

PREPARATION OF RADIOLABELLED HOST CELL PROTEINS:

Radiolabelled mock-infected cell cultures were prepared as described in "Production of virus stock and unlabelled virus harvests" and "Preparation of radiolabelled virus proteins", except that the culture flasks were not inoculated with seed virus.

2.2.2 CONCENTRATION OF RADIOLABELLED VIRUS PARTICLES

The radiolabelled virus harvest was centrifuged at 7,000g for 10 minutes to remove the cell debris. The virus particles were then pelleted from the supernatant fluid by centrifugation at 60,000g for 1 hour. The pellet was resuspended in 0,5mℓ 0,05M phosphate buffered saline (PBS) and dialysed extensively against this same buffer to remove all unincorporated radiolabel.

Ten microlitres of the virus preparation was used for radioactivity determination using the aqueous sample method (Appendix B).

2.2.3 ANTISERA

Sera prepared in guinea pigs to BTV types 4, 8, 10 and 17 were obtained from Dr H. Huismans (Veterinary Research Institute, Onderstepoort).

2.2.4 RADIOIMMUNE PRECIPITATION

Two fold dilutions of the indicated BTV antisera were titrated in microfuge tubes against equal volumes of ^{35}S-methionine labelled virus particles. The immune precipitate formed after 16 hrs at 4°C was further complexed with Staphylococcus aureus protein A (Miles-Yeda Ltd., Isreal). The resulting precipitate was collected by centrifugation and resuspended in Laemmli dissociation buffer. Half of this solution was analyzed by electrophoresis and autoradiography as described in Appendix A. The radioactivity present in the remainder of the precipitate was determined and the percentage of ^{35}S-methionine labelled proteins precipitated calculated. The titre of the serum reacting with the antigen is expressed as the reciprocal of the dilution of serum required to precipitate 50% of the antigen.

2.2.5 IMMUNE PRECIPITATION IN GEL

An unlabelled virus harvest (40 Falcons) prepared as previously described, was centrifuged at 7,000g for 10 minutes to remove cell debris. The supernatant fluid was then mixed with an equal volume of saturated ammonium sulphate and the proteins allowed to precipitate for 16 hours at 4°C. Precipitated protein was collected by centrifugation at 7,000g for 10 minutes, well drained, resuspended in 1mℓ 0.05M phosphate pH 7.2 and dialysed against this same buffer. Insoluble proteins were then removed by centrifugation in a Beckman Microfuge B for 1 minute.

Ouchterlony plates were prepared as described in Appendix B and wells were cut in the agar using a gel punch (Fig. 2.1). Serial two-fold dilutions of the crude viral protein preparation were used

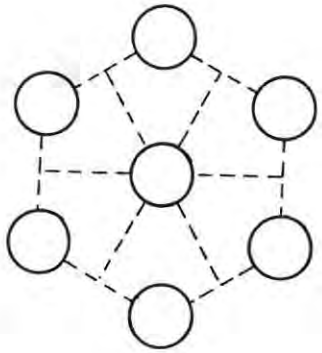


FIGURE 2.1: Well pattern used for agar gel diffusion (AGD). Outer wells containing serial doubling dilutions of crude viral proteins, with antiserum in the central well. ---- Lines along which cell was cut.

to fill the outer wells of the gel pattern and antiserum was placed in the centre well. Precipitation was at room temperature for 48 hours, after which the precipitin bands for each dilution were cut out (Fig. 2.1) and each gel slice washed with several changes of 1% NaCl in 0.05M phosphate pH 7.2 for 48 hours. After removal of all uncomplexed proteins, Laemmli dissociation buffer was added to each gel slice and heated at 100°C for 2 minutes. The molten agar from each dilution was placed in a polyacrylamide gel well and electrophoresis was at 50V until the bromophenol blue marker reached the top of the resolving gel and then at 150V. The proteins were detected using silver staining (Appendix A).

2.3 RESULTS

The serotypes used in these studies were selected for the following reasons:

(1) Serotypes 20, 17 and 4 because they cross-react in neutralization tests.[52]

(2) Serotype 10 because evidence obtained in cross-immune precipitation studies indicate a relatedness to BTV-4.[52]

(3) EHDV-2 as it is part of the BT serogroup, but is distinct from BT serotypes.[32] (Table 1.1).

(4) The remaining serotypes (BTV-3, 8 and 13) are considered to be typical non cross-reacting strains which, with regard to precipitation of P2, react very much like the majority of BTV serotypes in cross-immune precipitation experiments.[50]

Furthermore, this cross-section of serotypes covers viruses found in the USA, Australia and South Africa.

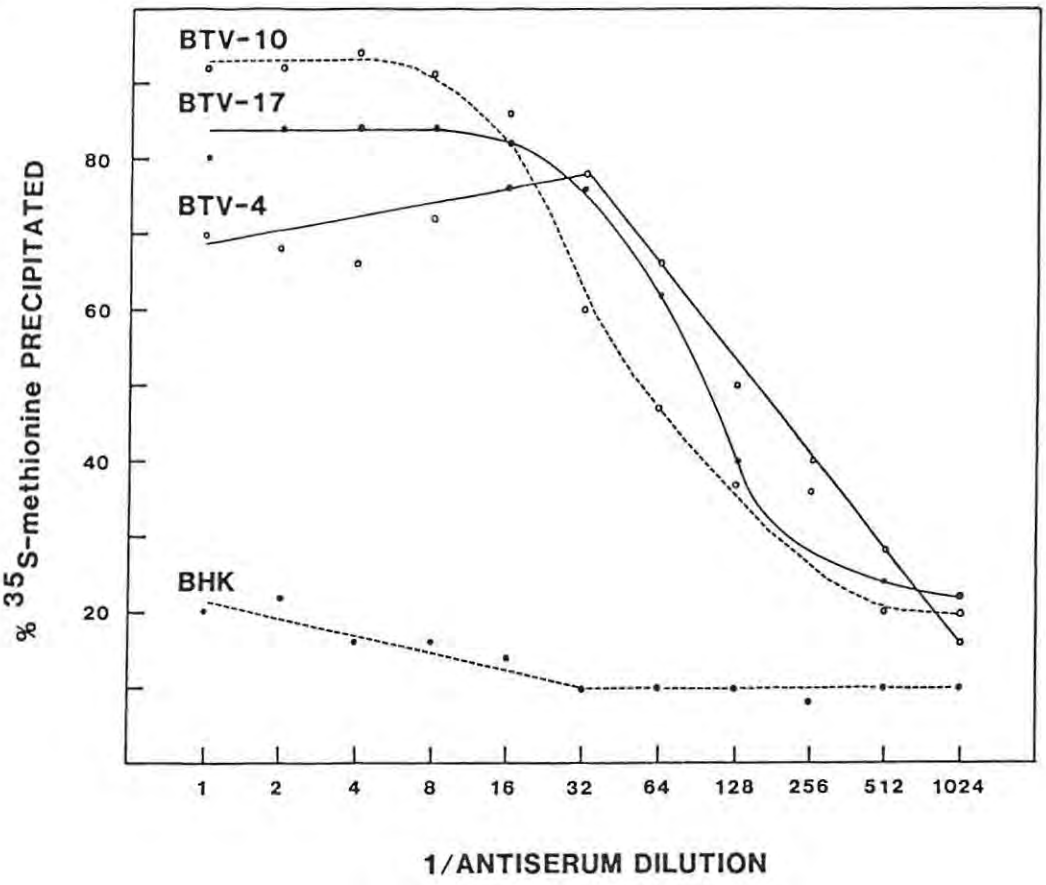
2.3.1 RADIOIMMUNE PRECIPITATION

It was hoped that these experiments would quantify the degree of cross-immune precipitation of the radiolabelled virus proteins in different reactions. It is assumed that the same weight of virus was present in each experiment. Therefore after analysis of the homotypic and heterotypic precipitates by PAGE and autoradiography, the amount of a particular protein precipitated could be determined by counting the radioactivity present in that band of gel. It was hoped that the heterotypic precipitation would give a numerical value that could apply to the study of virus interrelationships. For example, if protein x was 100% precipitated by antiserum to x; 50% precipitated by antiserum to y and 10% precipitated by antiserum to z, this could possibly allow you to suggest that x is more closely related to y than z.

Initially ^{35}S-methionine labelled virus particle preparations of EHDV-2 and BTV types 4, 8, 10 and 17 were allowed to react with dilutions of BTV type 4 antiserum. After complexation of the immune precipitates with S. aureus protein A, a portion of each precipitate was analyzed by PAGE and autoradiography. The remainder of the precipitate was used to calculate the percentage of ^{35}S-methionine labelled virus precipitated (Figure 2.2(a) and (b))

Table 2.1 gives the titre of the serum as the reciprocal of the dilution of serum required to precipitate 50% of the ^{35}S-methionine labelled virus particles. The titre of BTV antiserum reacting with BTV-4 and BTV-17 are the same, this result is not surprising since these serotypes were found to cross-react in neutralization tests.[52] However, the same titre was obtained when EHDV-2 was precipitated by BTV-4 antiserum. Unfortunately,

(a)



(b)

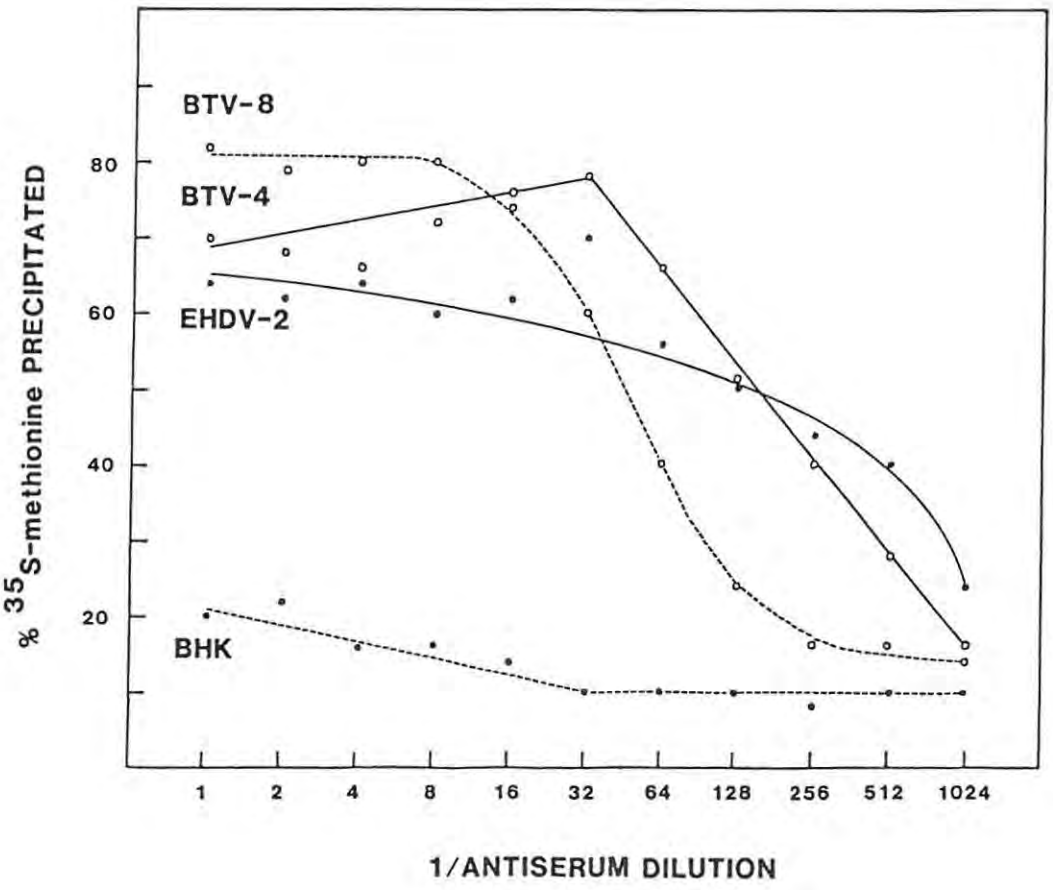


FIGURE 2.2(a) and (b): Measurements of the precipitation with BTV type 4 antiserum of the ^{35}S-methionine labelled virus particles.

TABLE 2.1 EXTENT OF CROSS-REACTION IN RADIOIMMUNE PRECIPITATION STUDIES

ANTIGEN (^{35}S-methionine labelled virus particles)	Reciprocal of serum dilution required to precipitate 50% of antigen	
	ANTISERUM	
	BTV-4	BTV-10
BTV-4	128	NT
BTV-8	32	NT
BTV-10	64	16
BTV-17	128	-
EHDV-2	128	-
BHK	-	-

NT = Not tested

analysis of immune precipitates by PAGE and autoradiography was hampered by the inability to obtain sufficient incorporation of the radiolabel into the virus particles. Because of this no conclusions could be drawn from these radioimmune precipitation results and no further experimentation was done using radiolabelled proteins.

2.3.2 IMMUNE PRECIPITATION IN GEL

Currently one of the most sensitive methods of visualizing proteins in gels are provided by autoradiography and fluorography. The disadvantages of autoradiography include the expense of the isotopes, the length of time required to complete the experiment and the inability to obtain sufficiently high specific activity of isotopically labelled proteins. However, Switzer et al (1979) have developed a highly sensitive silver staining technique which permits detection of polypeptides in polyacrylamide gels at concentrations 100-fold lower than Coomassie brilliant blue, i.e. it allows detection of proteins at concentrations which previously could be revealed only by autoradiographic methods. Several modifications of this technique have been developed[77; 84] which are simpler and much less costly than the original procedure.[84]

It was hoped that this sensitive silver staining method could be applied to immune precipitation studies, thus doing away with the need to radiolabel viral proteins. The main problem was now to ensure that only the proteins involved in the antigen-antibody (Ag-Ab) complex were analyzed. The protocol was therefore adapted from immune precipitation in liquid to immune precipitations in agar gels, thus enabling removal of uncomplexed proteins by extensive washing of the precipitin bands.

The cellular debris was removed from the virus harvest and the proteins present in the resultant supernatant fluid precipitated out with ammonium sulphate. The precipitate was resuspended in phosphate buffer and serial two-fold dilutions made. These were analyzed by double diffusion in agar against homotypic and heterotypic antisera. The resulting precipitin bands were cut out and washed in PBS to remove uncomplexed proteins. The proteins present in the immune precipitate were separated on 10% polyacrylamide gels and detected by silver staining.

Analysis of the gels after silver staining was hampered by the dark and often mottled background staining obtained. This prevented the accurate determination of the end point of precipitation of the viral proteins, and therefore, the results could not be relied upon. This problem with background staining can usually be traced to contaminants in water, or incomplete removal of gel buffer components. To insure that it was not the latter, the duration of the alcohol/acetic acid wash steps was increased. However, there was no improvement in the background obtained and the problem seems likely to be with the deionized water used or perhaps even reagent quality. Stained bands were also obtained when Laemmli dissociation buffer alone was electrophoresed (Fig. 2.3b lane 10), thus is thought to be caused by the 2-mercaptoethanol present in this buffer (Rubinstein, personal communication). Until this problem could be overcome, it was pointless pursuing this line of research.

The proteins present in the Ag-Ab complex formed between BT viral proteins and heterologous immune serum included BTV proteins P3 and P7. Precipitation of P6a as reported by Huismans and Bremer (1981) and Huismans and Erasmus (1981) was not

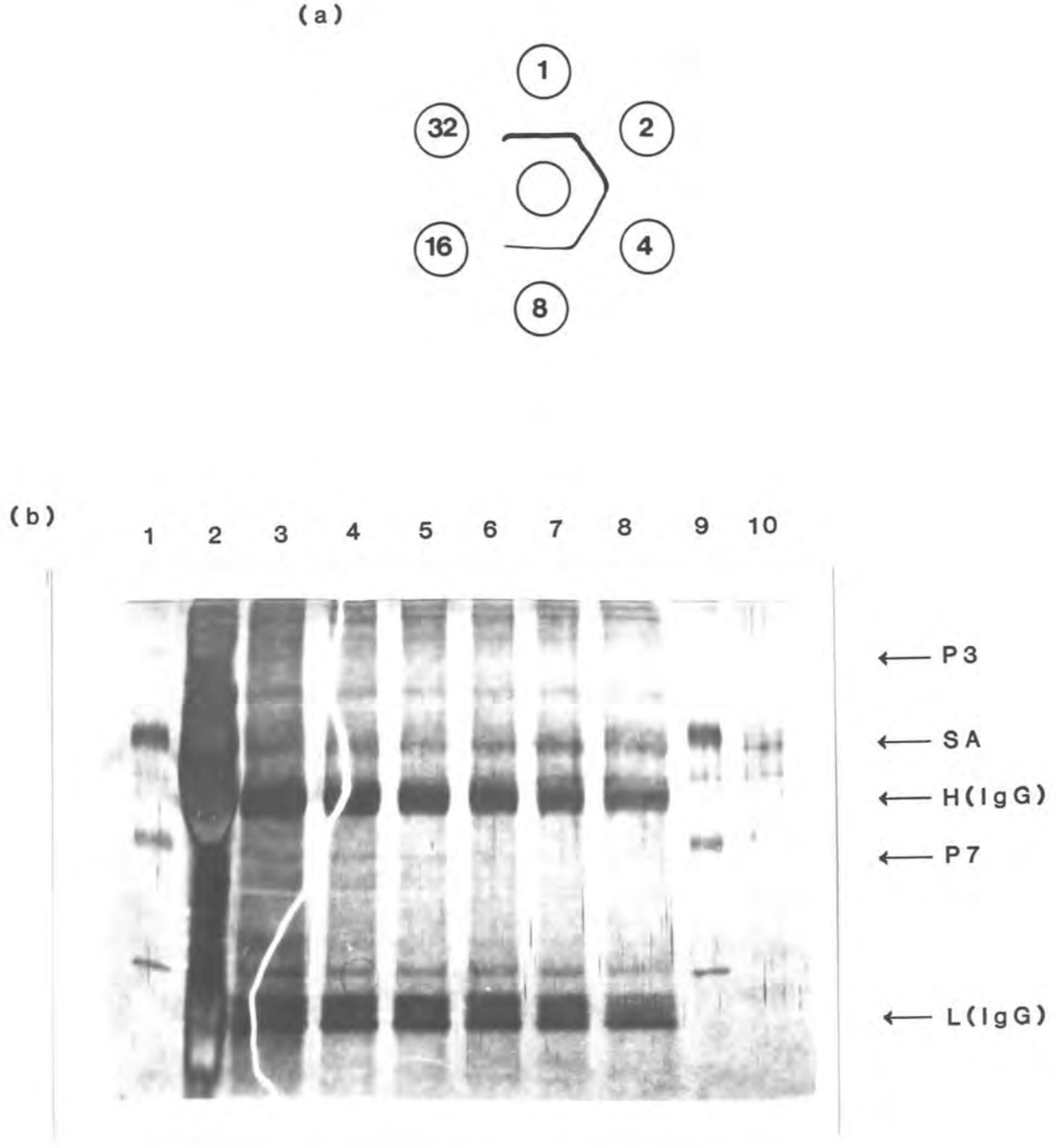


FIGURE 2.3 (a) Results of AGD on serial 2-fold dilutions of BTV-4 viral proteins against BTV-8 antiserum.

(b) Analysis of each precipitin band from (a) by PAGE and silver staining.

Lane 1 and 9	MW markers
2	BTV-8 antiserum
3 to 8	AGD precipitin bands, dilutions 1:1 to 1:32 respectively
10	Laemmli dissociation buffer.

H(IgG) - heavy chain IgG; L(IgG) - light chain IgG; SA - serum albumin.

detected in these initial experiments. Immune precipitation using homologous antiserum resulted in the precipitation of P2, the type-specific antigen, together with P3 and P7.

Figure 2.3b shows that several other proteins were also present in the Ag-Ab complex, or perhaps these proteins were not sufficiently washed from the agar gels. These include a protein identified as guinea-pig serum albumin and other proteins present in the antiserum (Fig. 2.3b lane 2). The presence of these contaminating proteins could probably be removed by using purified IgG instead of whole serum for agar gel diffusion precipitation.

An interesting feature of the precipitin reaction is seen in Figure 2.3. The optimal proportions point (seen to be a 1 : 4 dilution of the viral proteins against undiluted serum, Fig. 2.3a) lies at a higher dilution to the zone of maximum precipitation (which occurs at a 1 : 2 dilution of viral proteins as noted in Fig. 2.3b, lane 4). The reason for this characteristic feature of precipitin reaction is unknown.[114]

2.4 DISCUSSION AND CONCLUSION

The ability of polypeptides P3, P6a and the group antigen P7, to react with homotypic and heterotypic BT antiserum to about the same extent provides evidence for the antigenic similarity among different BTV types and raises the question of the contributory role of virus polypeptides in the group-specific response.

It was hoped by investigating the immune response against single proteins, BTV core particles and whole virus particles that this would be revealed and that the degree of relatedness of the BTV serotypes could be assessed.

Initially, radio-immune precipitation studies were hampered by the inability to radiolabel BTV proteins to a higher specific activity. This problem was partially overcome by using silver staining for the analysis of immune precipitates formed during agar gel diffusion. However, because of high background staining, sensitivity was poor and the results uncertain.

The fact that precipitation of polypeptides P3, P6a and P7 was not affected by the serotype of the serum also raised the question as to what extent these serological relationships would be reflected in the nucleic acid and polypeptide components of BTV. Possibly the best way to answer these questions is to correlate the antigenic differences occurring between orbiviruses with molecular structure. Several attempts have been made to do this, for example:

(1) examining the size heterogeneity of RNA segments in closely related orbiviruses,[34;36;68;95;98;101]

(2) molecular hybridization studies between the RNA of BTV strains,[34;50;53]

(3) oligonucleotide fingerprint analysis of the individual RNA species,[34;63;100;101] and

(4) comparison of the capsid polypeptides of various BTV serotypes by PAGE.[19]

However, no comparative structural analyses at the molecular level have been done on the different orbivirus proteins. It is felt that this could answer many of the questions raised and therefore such a study was initiated.

CHAPTER THREE

ISOLATION AND PURIFICATION OF VIRAL PROTEINS FOR COMPARATIVE PEPTIDE MAPPING STUDIES

3.1 INTRODUCTION

Purification of antigens synthesized during viral infection is generally hampered because viral antigens are present in small amounts relative to cell proteins and are usually associated with cellular membranes. In attempts by Verwoerd (1969) to purify BTV, two main problems were encountered: (i) the strong tendancy of the virus to adhere to cellular material, and (ii) the increasing instability of the virus particles on removal of cellular material.

Jochim et al (1969) demonstrated the presence of a free group-specific antigen in the supernatant culture fluid of BTV infected cells. Early attempts to isolate this antigen met with questionable results. Wang et al (1972) used ion exchange chromatography, dialysis, centrifugation and gel filtration to purify group antigen. However, the molecular mass of this purified protein was such that it could not have been coded for by one of the BTV genome segments.[37;109] It therefore appeared possible that this preparation was heavily contaminated with non-viral material. However, Gumm and Newman (1982) proposed that it is possible that the soluble antigen preparations, examined by these workers, were complex structures made up of a small number of protein subunits and that the soluble group antigen in its native state, may be a polymeric structure.

Huismans (1979) showed that BTV P7 (molecular mass approximately 30,000) was produced in excess in infected cell cultures and then released into the supernatant phase. The group-specific character of this virus protein was demonstrated by the immuno-precipitation studies of Huismans and Erasmus (1981).

Radiolabelled group antigen preparations for comparative peptide mapping need to be in a concentrated form and free of other radiolabelled virus-specific proteins. Antigen obtained purely by concentration of tissue culture supernate, has other radiolabelled virus proteins present. Therefore, purification steps had to be developed for the isolation of relatively pure group antigen from the infectious supernatant before any molecular studies could be performed.

3.2 MATERIALS AND METHODS

3.2.1 PROPAGATION OF VIRUSES

Cells and Viruses: BHK-21 cells were used to grow BTV serotypes 3, 4, 8, 10, 17 and 20 and EHDV-2. (Details of which are given in Chapter 2, Materials and Methods.)

Preparation of Stock Virus or Unlabelled Virus Particles: The same protocol was followed as given in Chapter 2, Materials and Methods.

Preparation of ^{35}S-methionine labelled Viral Proteins: The details of this protocol are given in Chapter 2, Materials and Methods.

Preparation of ^{35}S-methionine labelled Virus-induced Proteins: To produce radiolabelled virus-induced proteins, BHK cell monolayers were inoculated as described in Chapter 2, Materials and Methods and 12 hours post infection (p.i.) 100 uCi of ^{35}S-methionine was added (the

details of this are given in Chapter 2, Materials and Methods). At 24 hours p.i., the medium was poured off and the cell monolayer washed with Hanks balanced salt solution to remove any free ^{35}S-methionine. One mℓ of dissociation buffer (1% sodium dodecyl sulphate (SDS), 1% 2-mercaptoethanol (2-ME) in 0.15M Tris-HCl pH 6.8) was used to lyse the cells and complete disruption was achieved by heating at 100°C for 2 minutes. The cell lysate was then pressure dialysed against several changes of dissociation buffer for 17 hours at room temperature, and stored at 4°C until required.

3.2.2 PURIFICATION OF GROUP ANTIGEN

Preparation of the soluble fraction of viral polypeptides: To release virus and group antigen from the cells, the virus harvest was subjected to three freeze-thaw cycles and then centrifuged at 7000g for 10 minutes to remove the cell debris. The virus particles were then pelleted from the supernatant fluid by centrifugation at 60,000g for 1 hour. The pellet was resuspended in the appropriate buffer and stored at 4°C until required. The soluble group antigen present in the supernate was precipitated by addition of an equal volume of saturated ammonium sulphate in 2mM Tris pH 7.2 for 5 hours at 0°C. Precipitated protein was collected by centrifugation at 7000g for 10 minutes.

A flow diagram of this protocol is given in Figure 3.1.

Purification of Group Antigen Labelled in vivo

Exclusion Chromatography: Separation of the proteins present in the soluble fraction was attempted by gel filtration through Sepharose 4B (Pharmacia Fine Chemicals).[37]

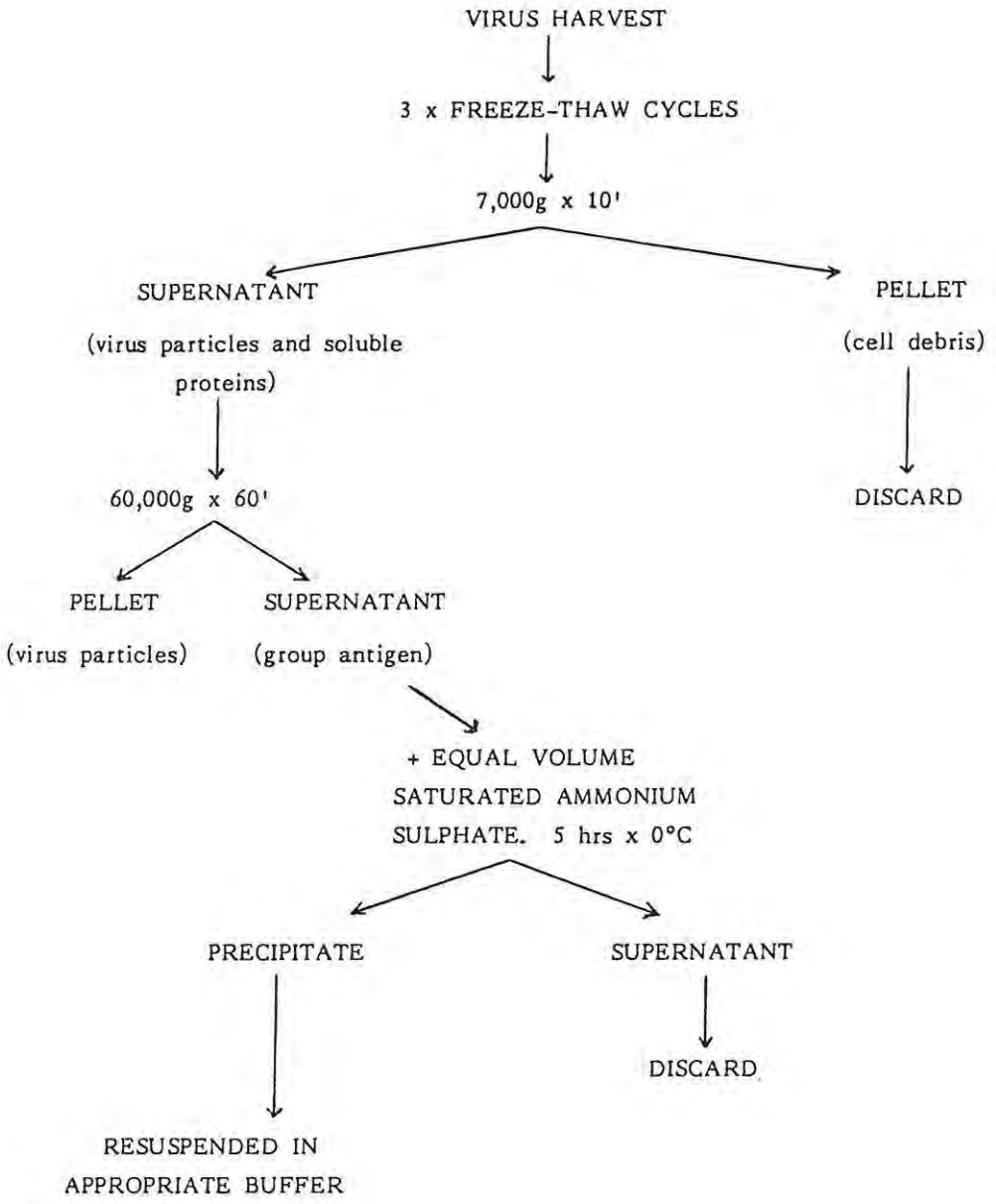


FIGURE 3.1: Preparation of the soluble fraction containing group antigen.

The ammonium sulphate precipitate of the soluble group antigen was resuspended in 1 mℓ 0.01M Tris-HCl, 0.15M NaCl pH 7.0, loaded onto a Sepharose 4B column (1.5 x 50 cm) and eluted with this same buffer. One mℓ fractions were collected and the absorbence at 260 nm determined. Radioactivity present in each fraction was determined using the aqueous sample method (Appendix B) and the fractions containing the ^{35}S-methionine labelled proteins pooled and precipitated with 3 volumes of cold acetone for 16 hours at -20°C. The precipitate was collected at 7000g for 10 minutes, resuspended in Laemmli dissociation buffer and analyzed by PAGE and autoradiography (Appendix A).

Electro-elution of group antigen from polyacrylamide gel slices: The ^{35}S-methionine labelled proteins separated by PAGE, either before or after gel filtration, were identified by autoradiography and the group antigen (P7) band cut out. The gel slice was re-swollen in Laemmli electrode buffer (0.025M Tris, 0.192M Glycine, 0.1% SDS, pH 8.3) in preparation for electro-elution of the protein.

The ISCO Model 1750 electrophorectic concentrator was used with Laemmli electrode buffer present in all chambers and the sample cup (Fig. 3.2). The re-swollen gel slice was placed on a polyester screen directly above the concentration well and electro-eluted at a constant power setting of 3 watts for 4 hours. On completion of elution the sample cup fractions were removed in the following order: 2, 1, 3 and 4, in order to prevent mixing.

The radioactivity present in the 0,2 mℓ concentrated fraction and that remaining in the gel slice was determined by liquid scintillation counting (Appendix B).

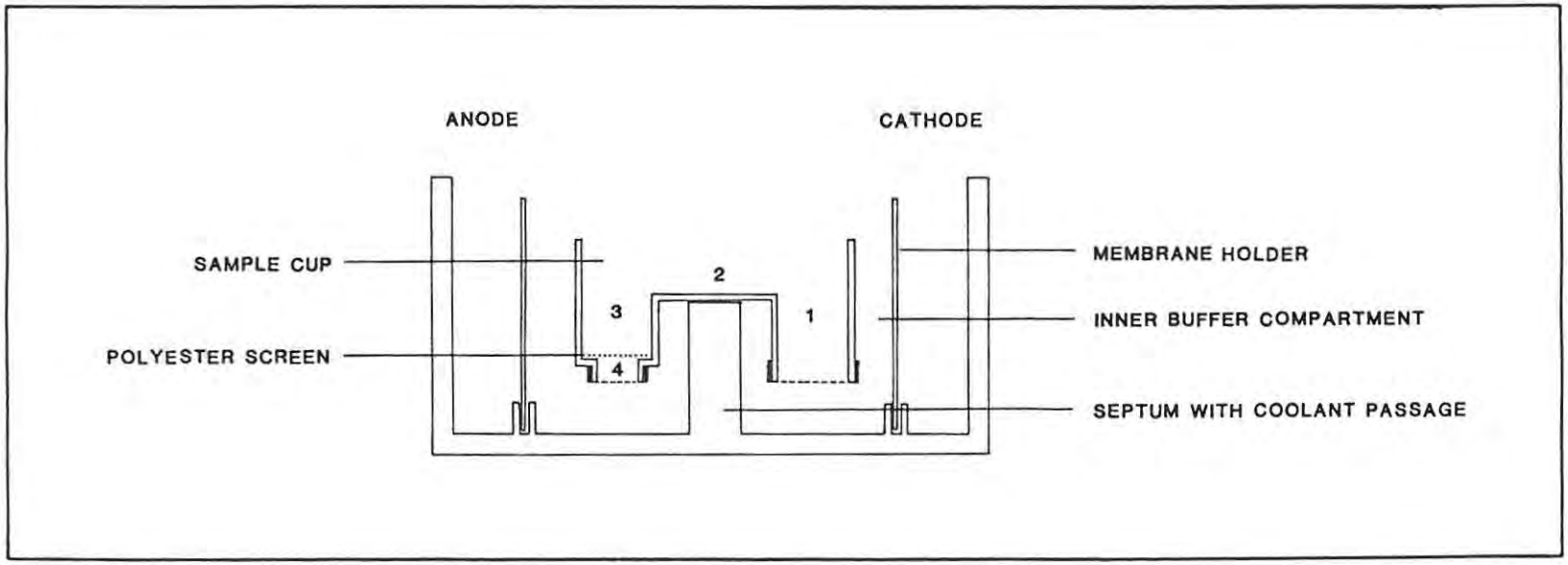


FIGURE 3.2: Cross section of ISCO Model 1750 Electrophoretic concentrator.

1. "Sample" well (3.0 mℓ)
2. Buffer bridge (5 mℓ)
3. Polyester screen placed at bottom of this fraction (1.7 mℓ)
4. Concentration well (0.2 mℓ)

The electro-eluted ^{35}S-methionine labelled group antigen was then used as a substrate for trypsin digestion and subsequent analysis.

Radio-iodination of Group Antigen using Bolton and Hunter Reagent:
Group antigen from gel slices were electro-eluted and 50 uℓ of the resulting concentrated solution drawn off by placing a micropipette on the membrane surface. (This solution was found to have the highest concentration of group antigen).

The method of Fietelson et al (1981) was used to radioacylate the group antigen with Bolton and Hunter reagent. A 100uCi aliquot of Bolton and Hunter reagent (Radiochemical Centre, Amersham, England) was dispensed into a 1 mℓ microfuge tube and dried under a gentle stream of nitrogen. The 50 uℓ of group antigen was added to this and left at 0°C for 1 hour, then at 4°C overnight. The pH was adjusted to pH 9.0, by the addition of 1uℓ 1.0M sodium hydroxide, for reductive methylation. Cold, freshly prepared reagents in 0.1M sodium borate buffer, pH 9.0 were added at 0°C as follows:

(i) 20uℓ 10M guanidine-hydrochloride,

(ii) 20uℓ sodium borohydride (10mg/mℓ),

(iii) 5 additions of 20uℓ each of a formaldehyde solution (4uℓ, 37% formaldehyde in 300 uℓ 0.1M borate buffer, pH 9.0) spaced at 5 minute intervals.

The sequence of borohydride and formaldehyde addition was repeated (ii and iii) using a new batch of borohydride. A further 20 uℓ of freshly prepared sodium borohydride was added to ensure complete reduction of all formaldehyde.

The radiolabelled protein was separated from reagents by gel filtration on a Sephadex G-25 column (1 x 25 cm) in 0.05M ammonium bicarbonate pH 8.0. One mℓ fractions were collected and the radioactivity determined by the aqueous sample method (Appendix B).

To explain the results obtained for radioacylation of group antigen the iodination was repeated using 250 ng of Bovine serum albumin (BSA) in 50uℓ of 0.025M Tris, 0.192M glycine pH 8.3, i.e. no SDS was present in the sample buffer.

Purification of Group Antigen Labelled in vitro:

Isolation of viral proteins by immunodiffusion:

Preparation of Antigen: Unlabelled virus harvests were centrifuged at 7,000g for 10 minutes, and an equal volume of saturated ammonium sulphate added to the supernatant fluid. Precipitation was at 4°C overnight, after which the precipitate was collected by centrifugation at 7,000g for 10 minutes. The precipitate was taken up in 1 mℓ 0.05M phosphate buffer pH 7.2 and dialysed extensively. Insoluble material was removed by centrifugation in a Beckman Microfuge B for 1 minute. This preparation was then used for immunodiffusion.

Antisera: Sheep sera immune to BTV types 1, 3 and 4 were obtained from the Animal Virus Research Institute, Pirbright, England and sera prepared in guinea pigs to BTV types 4, 8, 10 and 17 were obtained from Dr H. Huismans (Veterinary Research Institute, Onderstepoort, South Africa).

Immunodiffusion using the Ouchterlony Method: Ouchterlony plates were prepared as described in Appendix B. Initially, serial two-fold dilutions were made of the viral antigens (prepared as described above) and these were titrated against undiluted BTV antiserum. Optimal proportions point (OPP) was judged by the sharpness of the precipitin band, and the antigen concentration one step above OPP was used for preparative agar gel diffusion.[114] The overlapping well pattern used for isolation of viral proteins by immune precipitation is shown in Figure 3.10. Both homotypic and heterotypic reactions were used against BTV proteins. Plates were incubated for 48 hours at room temperature and precipitin bands visualized using oblique light and a dark background.

Separation of proteins in the immune complex: The precipitin bands were cut from the agar and washed for 48 hours in 0.05M phosphate buffer pH 7.2, 1% NaCl, to remove uncomplexed host cell and viral proteins. The gel slices were then heated at 100°C for 2 minutes with one tenth volume 10 times concentrated Laemmli dissociation buffer and 5 $\mu\ell$ Bromophenol blue. The molten agar was then poured into the wells of a 12% SDS-polyacrylamide slab gel and electrophoresed (Appendix A). Proteins were detected with Coomassie blue stain and the viral proteins identified.

Radio-iodination of proteins in polyacrylamide gel slices: The polyacrylamide gel slice containing the viral protein to be iodinated was washed in 10% methanol overnight and then dried down. The dehydrated gel slice was placed in a microfuge tube ready for radio-iodination. The method of Elder et al (1977) for radio-iodination of proteins in polyacrylamide gel slices was used; this involved the sequential addition of the following solutions to the dehydrated gel:

(i) 40uℓ, 0.5M phosphate buffer, pH 7.5,

(ii) 100uCi Na ^{125}I (Radiochemical Centre, Amersham, England)

(iii) 10 uℓ Chloramine-T (1mg/mℓ).

The gel slices were allowed to absorb these solutions for 1 hour at which time 1 mℓ of sodium metabisulphite (1mg/mℓ) was added to stop the reaction. After 15 minutes, the metabisulphite solution was removed and the gel slices washed with several changes of 10% methanol until the radioactivity (in counts per minute) being eluted out was about 0.5 to 2.0% of the input ^{125}I radioactivity.

3.3 RESULTS

3.3.1 ANALYSIS OF POLYPEPTIDES INDUCED IN CELLS INFECTED WITH BTV-10.

BTV infected BHK cell monolayers and mock-infected cells were labelled at 12 hours post-infection with ^{35}S-methionine. Cells were disrupted at 24 hours post-infection and the virus-induced proteins of BTV-10 were analyzed by electrophoresis and autoradiography. The results are shown in Fig. 3.3.

At 12 hours post-infection the synthesis of polypeptides is predominantly virus-specified, however, there is some incorporation of the radioisotope into cellular proteins; as host cell protein synthesis is not completely suppressed, even late in the infection cycle.[49]

The polypeptides are numbered P1 to P7 in order of decreasing molecular mass and the two non-capsid polypeptides are numbered P5a and P6a.

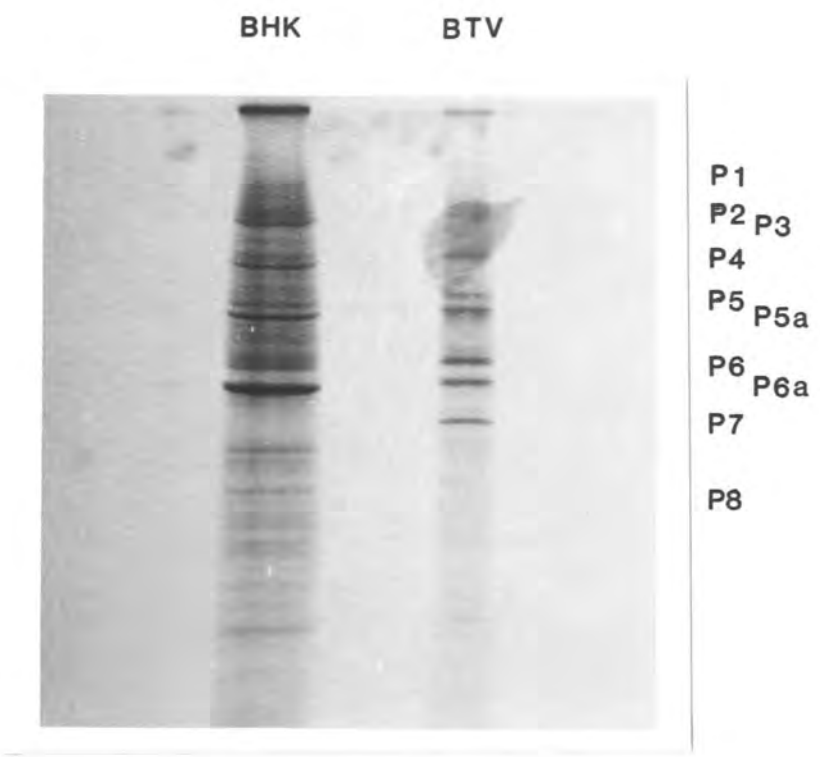


FIGURE 3.3: Autoradiogram of the electrophoretic fraction of BT type 10 virus-induced proteins and mock-infected BHK cell proteins. Electrophoresis on 12% PAG using the discontinuous Laemmli system.

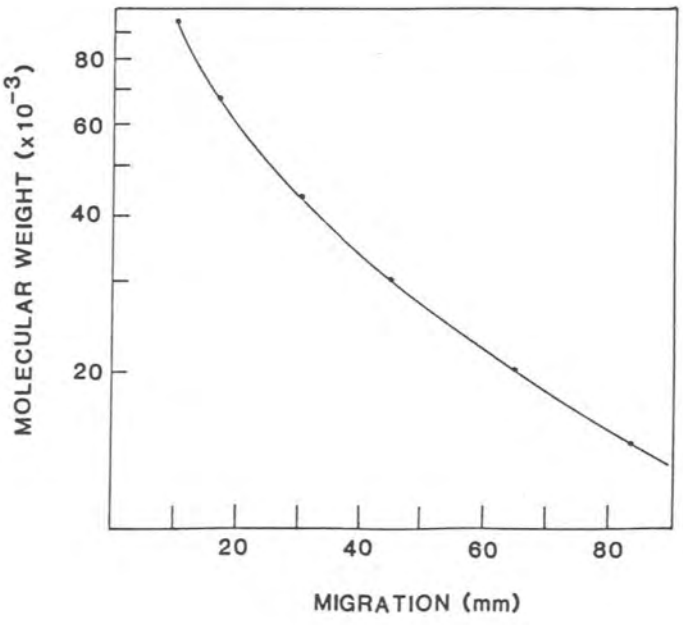


FIGURE 3.4: The relationship of log MW and mobility of marker proteins electrophoresed on 12% PAG.

In a very recent publication Kahlon et al (1983) detected 10 virus-induced polypeptides in cell extracts; previously only 9 viral proteins had been identified. Huismans (personal communication) has also detected this small viral protein, presumably coded for by RNA segment 10. By comparing the non-infected and infected cell extracts in Fig. 3.3 this protein, P8, can be seen to be present only in the latter at a very low concentration.

3.3.2 MOLECULAR WEIGHT DETERMINATION OF THE BT POLYPEPTIDES.

Molecular weight marker proteins, enumerated in Appendix A, were electrophoresed on the same slab gels as the BT virus-induced proteins, and detected by staining of the gel with Coomassie brilliant blue. Their mobilities were used to construct a calibration curve from which the molecular weights of the viral polypeptides were determined (Fig. 3.4).

It must be noted that for any given gel concentration the relationship between $\log_{10}$ molecular weight and relative mobility is linear over only a limited range of molecular weight. Hames (1981) states that for the SDS-discontinuous buffer system, the linear relationship holds true over the following ranges: 15% acrylamide, MW 12,000 - 45,000; 10% acrylamide, MW 16,000 - 70,000. The linear relationship appears to hold over the 14,000 - 45,000 MW range for 12% acrylamide (Fig. 3.4).

The MW markers were used in all PAGE analyses as they provide a measure of the reproducibility between different gel runs.

The molecular weights obtained for the BTV-10 viral proteins are shown in Table 3.1 and are compared to those published by Verwoerd et al (1972) and Huismans (1979). The slightly higher MW values can probably be ascribed to the different gel and buffer systems used.

3.3.3 PURIFICATION OF GROUP ANTIGEN RADIOLABELLED IN VIVO.

Centrifugation: Verwoerd (1969) reported that under the conditions used for the production of a BTV harvest, 80% of the infective virus consistently remained attached to the cellular material. In an attempt to disrupt the cells and liberate virus, the ^{35}S-methionine labelled BTV harvests were subjected to 3 cycles of freezing and thawing. Freezing and thawing, together with sonication and treatment with trypsin are known to inactivate BTV,[107] however, for the purposes of this study, inactivation of the virus was not an important consideration. The cell debris was removed from the BTV harvest by centrifugation at 7,000g for 10 minutes, followed by centrifugation of the supernatant fluid at 60,000g for 1 hour to separate the virus particles from the soluble proteins (Fig. 3.1).

Analysis of the 7,000g pellet by PAGE (Fig. 3.5, lane 2) shows that a large proportion of the radiolabelled virus particles were not liberated from the cells, even after the 3 cycles of freezing and thawing.

PAGE of the 60,000g supernatant fluid showed the presence of high concentrations of group antigen (P7) remaining in solution. However, after concentration of the supernatant with 50% saturated ammonium sulphate (Fig. 3.5, lane 5) all radiolabelled viral proteins are seen to be present. Therefore, group antigen cannot be obtained purely by concentration of the tissue culture supernate. Furthermore, staining of

TABLE 3.1: MOLECULAR WEIGHTS OF THE BTV POLYPEPTIDES

POLYPEPTIDE	MOLECULAR WEIGHT x 10^{-3}	
	HUISMANS (1979) AND VERWOERD et al (1972)	CALCULATED FROM FIG. 3.4
P1	140	101
P2	110	80
P3	101	78
P4	82	69
P5	61	61
P5a	54	58
P6	42	50
P6a	40	47
P7	29	42
P8	-	28

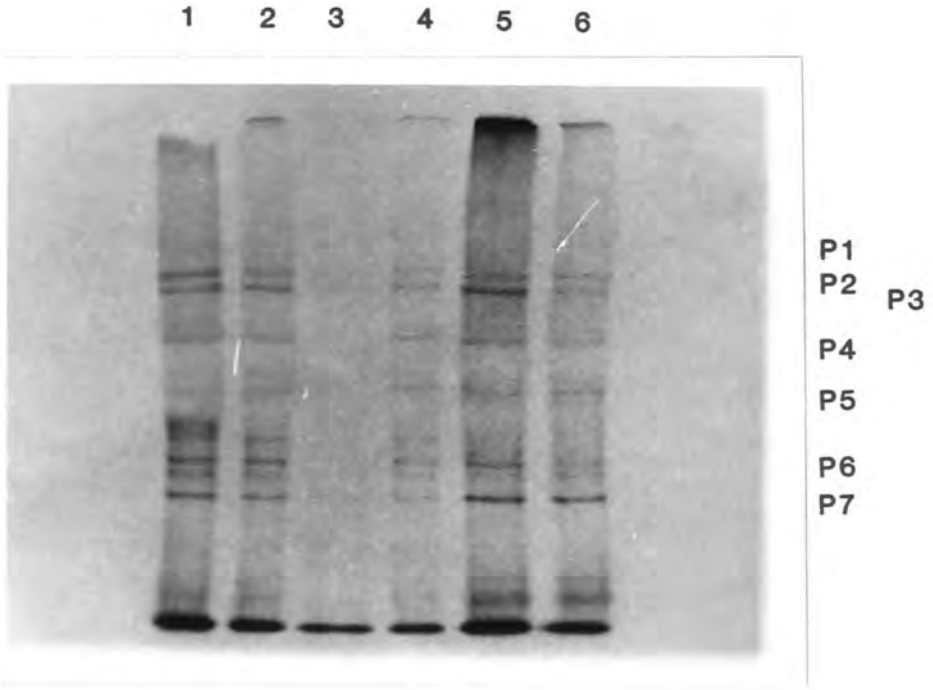


FIGURE 3.5 Autoradiogram of the electrophoretically separated ^{35}S-methionine viral proteins at different stages in the purification of group antigen.

1. BTV harvest
2. 7,000g pelleted cell debris
3. 60,000g supernate, containing BTV soluble proteins
4. 60,000g pellet of virus particles
5. Ammonium sulphate precipitate of 60,000g supernate
6. Acetone precipitate of 60,000g supernate after exclusion chromatography on Sepharose 4B.

the polyacrylamide gels with Coomassie brilliant blue revealed the presence of "contaminating" host cell proteins in this same fraction.

Exclusion chromatography: Gumm and Newman (1982) were able to remove large amounts of contaminating protein from ^{35}S-methionine labelled crude group antigen preparations by exclusion chromatography on Sepharose 4B. In an attempt to reduce the amount of contaminating protein, the ammonium sulphate precipitate of the 60,000g supernatant fluid was resuspended in Tris,NaCl buffer and eluted through a Sepharose 4B column.

Sepharose 4B has a fractionation range for proteins of MW from 6×10^4 to 20×10^6, therefore it was hoped that the larger MW proteins would easily be separated from the group antigen (MW 43,000).

Figure 3.6 shows that some separation of unlabelled proteins has occurred and that the ^{35}S-methionine labelled proteins eluted as a single peak. This peak contained approximately 70% of the total counts per minute (cpm) applied to the column. These fractions were pooled and concentrated by precipitation with 3 volumes of acetone.

Analysis of the precipitate by PAGE (Fig. 3.5, lane 6) revealed the presence of all the ^{35}S-methionine labelled viral proteins, however, the group antigen is present in a higher concentration than the other viral proteins, relative to their respective concentrations in the untreated ^{35}S-methionine labelled BTV harvests (Fig. 3.5, lane 1).

The elution of the concentrated 60,000g supernate through Sepharose 4B has effectively removed large amounts of contaminating protein from the crude group antigen preparation.

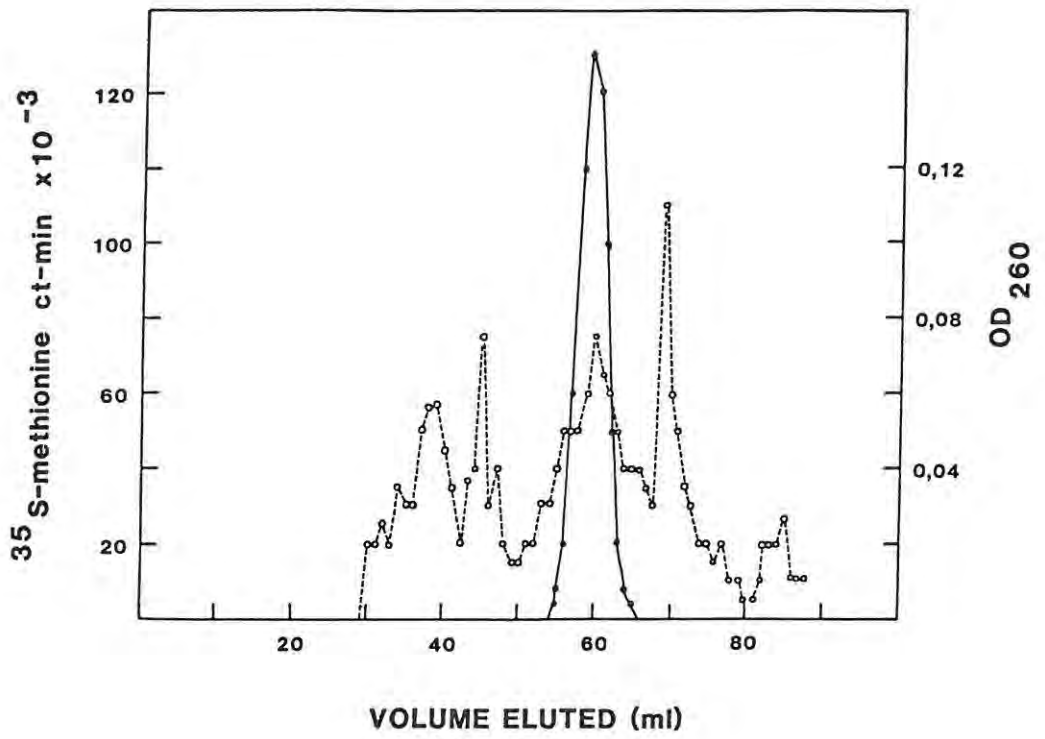


FIGURE 3.6: Exclusion chromatography of a ^{35}S-methionine labelled crude antigen preparation on Sepharose 4B.
o----o ^{35}S counts; o——o OD_{260}.

Elution of group antigen from PAG slices: The acetone precipitate of the partially purified ^{35}S-methionine labelled group antigen preparation was taken up in Laemmli dissociation buffer and the proteins separated on 12% SDS-polyacrylamide slab gels. The group antigen band was located by autoradiography and that section of the gel cut out and the protein eluted.

In early experiments, elution of proteins from polyacrylamide gels was by diffusion; the gel slice was placed in 0.5 mℓ Elution buffer (10mM phosphate buffer, 0.1% SDS, 0.05% 2-ME, 1mM EDTA, pH 7.2) and left at 37°C for 24 hours. The supernatant was removed and a second volume of Elution buffer added and incubated for a further 24 hours. Recovery of the group antigen in the pooled supernatant fractions using this procedure was approximately 60%.

To try and increase the recovery of group antigen from the acrylamide gel slice, electro-elution was tried. Several buffer systems were tested, the one finally used was 0.025M Tris, 0.192M Glycine, 0.1% SDS, pH 8.3 (i.e. Laemmli electrode buffer). Better recoveries were obtained and only 20% of the radioactivity was found to remain in the gel slice.

This method of eluting group antigen from PAG slices has several other advantages over elution by diffusion:

1. Elution was complete in 4 hours as opposed to the 48 hours required for the simple diffusion method.

2. The final volume of eluted group antigen was 0.2 mℓ instead of the 1 mℓ in the diffusion method.

Masceration of the gel slice prior to electro-elution was found not to increase the efficiency of recovery or decrease the time of electro-elution.

The advantage of using biosynthetically labelled viral proteins for peptide analysis is that host cell protein labelling is minimal, as host cell protein synthesis is supressed early in the infection cycle. If any host cell proteins were biosynthetically radiolabelled and co-purified with the viral proteins, it was felt that the viral proteins would be in such excess that contaminating labelled proteins could possibly be ignored.

In my experience, the major drawback using ^{35}S-methionine labelled BTV proteins was that very rarely were purified group antigen preparations biosynthetically labelled to high enough specific activities. A total of $5x10^5$ cpm was found to be required for peptide analysis. In order to obtain this activity several purified preparations of the eluted group antigen had to be pooled. Effectively large amounts of ^{35}S-methionine were being used to produce a single peptide map. As ^{35}S-methionine is an expensive radiolabel, iodination protocols were introduced to try and increase the activity of radiolabelled purified group antigen preparations and hopefully reduce the costs incurred as iodine is a cheaper radio-isotope. Smaller quantities of ^{35}S-methionine could be used as its only function would be to facilitate detection of the viral proteins.

Iodination of group antigen using Bolton and Hunter Reagent: The most important consideration for radio-iodination of the electro-eluted viral proteins is that the protein to be analyzed must be pure. All of the purification procedures employed to this stage, (i.e. centrifugation, exclusion chromatography, PAGE) involve separation of components on the basis of their molecular weight. Therefore, there was a strong possibility that there would be host cell protein present with the ^{35}S-methionine labelled viral proteins. PAGE on BT viral proteins (Fig. 3.3) shows that the only viral protein that does not appear to migrate at the same rate as a host cell protein, was the group antigen (P7). It

was therefore felt that, with stringent controls, it might be possible to successfully iodinate this protein, increase the radiolabel specific activity and allow unambiguous peptide mapping.

The method of radioacylation of proteins using Bolton and Hunter reagent was chosen over direct iodination using the Chloramine-T protocol, because the Bolton and Hunter reagent condenses with free amino groups of proteins (for example, the epsilon amino side chains of lysine residues or the N-terminal amino group), which are more frequent in most proteins than the tyrosine residues most commonly iodinated using the Chloramine-T method. This results in a larger number of peptides being labelled by acylation rather than direct iodination, which could increase the sensitivity of peptide mapping by giving rise to more structural information.

The protocol of Fietelson et al (1981) was used for radioacylation of the group antigen. Their results show that only 5 to 25 picomoles of protein is needed for the reaction. Because the reaction is concentration-dependent, only the most concentrated fraction of the group antigen after electro-elution was used. This protein was then radioacylated and reductively methylated as described in Materials and Methods. The protein was then separated from the unreacted hydrolysis product on Sephadex G-25.

Sephadex G-25 has a MW range of 1000 to 5000 over which proteins can be fractionated. Proteins with MW above the exclusion limit, (e.g. BTV group antigen, BSA) are totally excluded from the gel and eluted in the void volume; whereas molecules smaller than the lower limit, (e.g. the hydrolysed iodinated propionate molecule of the Bolton and Hunter reagent) are usually eluted at an elution volume approximately equal to the bed volume.

Elution of the group antigen iodination reaction mixture on Sephadex G-25 (Fig. 3.7a) gives a single peak of radioactivity, identified as the hydrolysed iodinated propionate molecule. No radioacylated group antigen appeared to elute out in the void volume. This result shows that the group antigen was not successfully radioacylated. To confirm that the peak of radioactivity eluted was not group antigen, a portion of the pooled peak fractions was analyzed by PAGE (Fig. 3.7b, lane 2). The fractions corresponding to the void volume were also pooled, carrier protein added, precipitated with an equal volume of saturated ammonium sulphate and run on the same gel (Fig. 3.7b, lane 1). The autoradiogram confirmed that no radioacylated group antigen was present in either fraction.

Two explanations for this result were immediately apparent:

1. The electro-elution buffer contained SDS; therefore the intrinsic charges of the polypeptides were insignificant compared to the negative charges provided by the bound detergent. This alteration of charge on the polypeptide chain could interfere with the iodination reaction.
2. The concentration of protein to Bolton and Hunter reagent may not have been optimal.

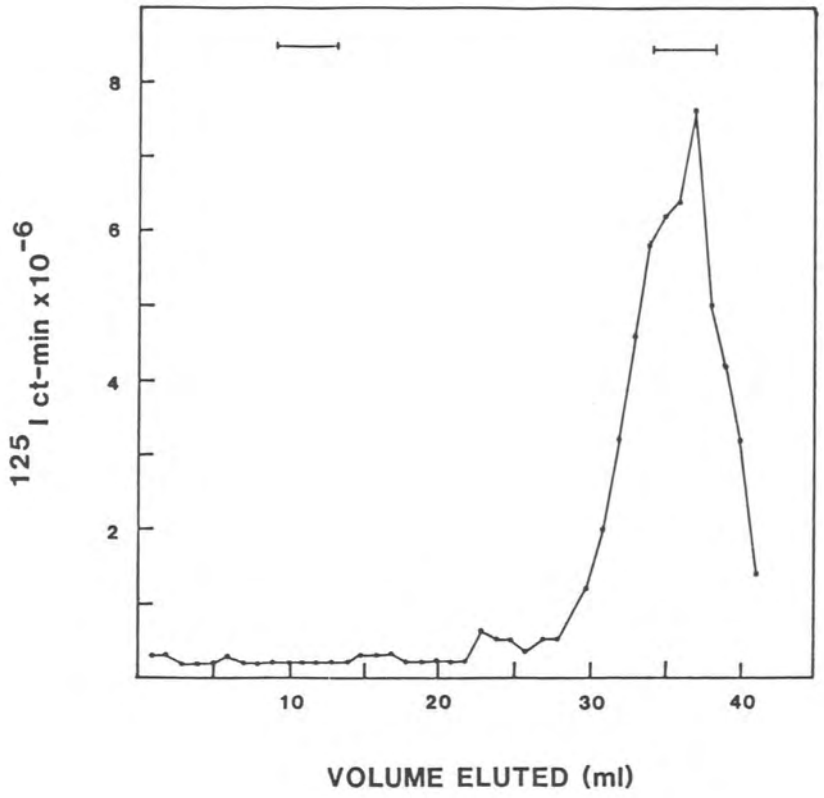
To determine whether radioacylation was affected by the presence of SDS on the protein, this experiment was repeated using 250 ng of BSA dissolved in 50uℓ of Electro-elution buffer which contained no SDS.

The elution profile on Sephadex G-25 showed the presence of radioacylated material eluting in the void volume (Fig. 3.8a). Analysis of this peak by PAGE and autoradiography confirmed that the BSA had

FIGURE 3.7(a): Separation of BT group antigen from low molecular weight radio-iodination products on Sephadex G-25. ⊢——⊣ pooled fractions.

(b): Autoradiogram of PAG for detection of radioacylated BT group antigen in void volume (lane 1) and ^{125}iodide peak (lane 2).

(a)



(b) 1 2

FIGURE 3.8(a): Separation of ^{125}I-labelled BSA from low molecular weight radio-iodination products on Sephadex G-25. ⟷ pooled fractions.

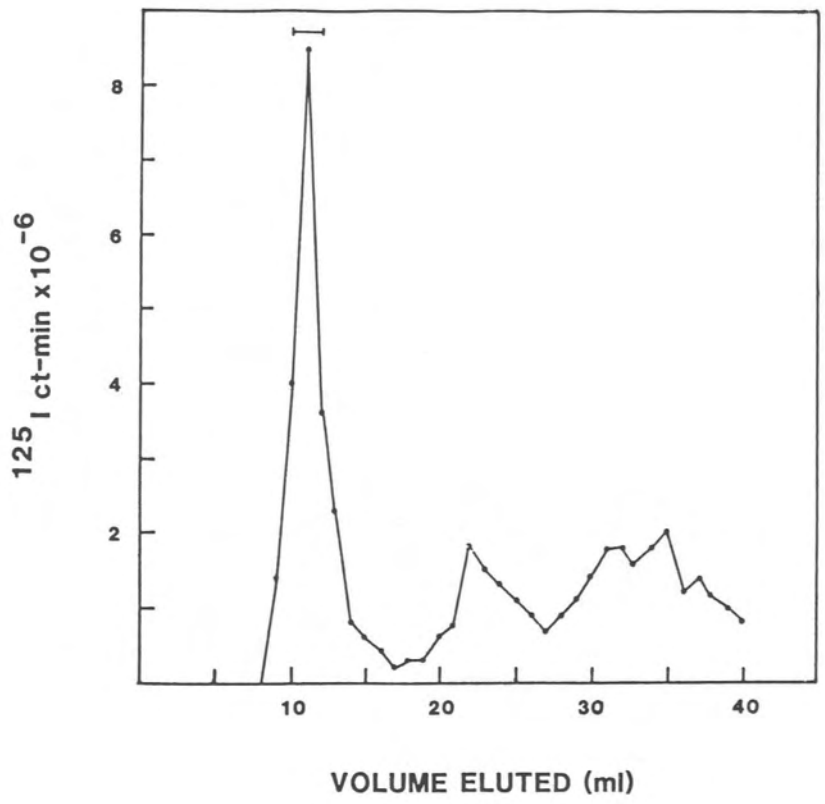
(b): Stained PAG showing analysis of BSA used for radio-acylation. Lane 1 and 5 - Pharmacia molecular weight marker proteins:

Phosphorylase B	MW	94,000
BSA		67,000
Ovalbumin		43,000
Carbonic Anhydrase		30,000
Trypsin inhibitor		20,100
∝-Lactalbumin		14,400

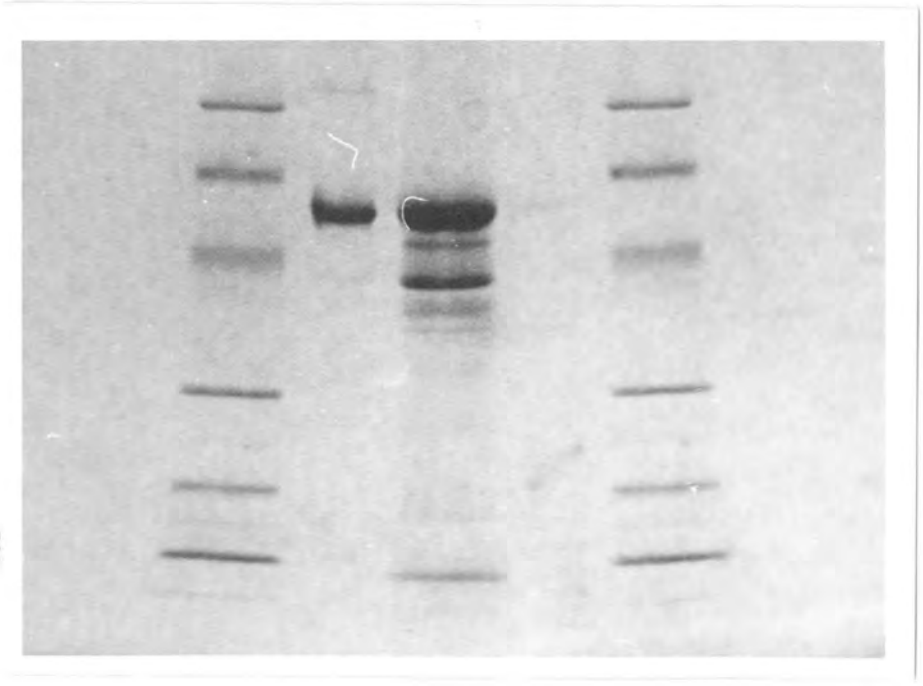
Lane 2 - BSA control; Lane 3 - higher concentrations of BSA showing presence of smaller proteins, contaminants and/or breakdown products. Lane 4 - radioacylated BSA eluting in void volume from Sephadex G-25.

(c): Autoradiogram of PAG (Fig. 3.8(b), lane 4), showing presence of ^{125}I-labelled BSA.

(a)



(b) 1 2 3 4 5



(c) 4

been radioacylated (Fig. 3.8c). The autoradiograph also showed the presence of smaller radioacylated proteins, however, these were shown not to be caused by the radioacylation reaction as these bands were also present in the BSA control (Fig. 3.8b, lane 3).

It was noted that the BSA used in the radioacylation control experiment migrated further into the PAG than the BSA component of the MW markers (Fig. 3.8b) indicating a smaller MW. By increasing the concentration of BSA analyzed on the gel, smaller proteins, present at lower concentrations, were detected (Fig. 3.8b, lane 3). These proteins were the same as those detected on the autoradiogram of the radioacylated BSA. It appears that some breakdown of the BSA has occurred resulting in the lower MW; but it is also possible that the BSA was contaminated, as the molecular weights of these smaller proteins are too high to be only breakdown products.

The radio-iodination of BSA in the absence of SDS indicates that SDS has a marked effect on the radioacylation reaction. However, the second possibility cannot be ruled out completely as the concentration of group antigen used in the experiment was unknown and therefore could not be simulated in the control experiment.

In the protocol given by Fietelson et al (1981) the 250ng of protein was present in 2 to 2.5uℓ of buffer, whereas in this experiment the same amount of BSA was present in 50uℓ. Radioacylation of the protein still occurred with incorporation of 70% of the ^{125}I into the protein. Therefore, even if the group antigen was present in a lower concentration some radioacylation should have occurred.

3.3.4 PURIFICATION OF GROUP ANTIGEN LABELLED IN VITRO.

Structural analysis of BTV proteins has so far been hampered by the inability to obtain sufficient quantities of homogeneous proteins radiolabelled to high levels of specific activity. Gumm and Newman (1982) demonstrated that ^{35}S-methionine labelled BTV proteins could be extracted from immune complexes in agar; all uncomplexed proteins could be washed from the agar gel, leaving only the antigen-antibody complex. This was used as the principle behind purification of the group antigen and other viral proteins.

Immunodiffusion: A forty Falcon harvest of unlabelled BTV, with the cell debris removed, was concentrated by ammonium sulphate precipitation. After dialysis of the resuspended precipitate, to remove the salts, insoluble material was pelleted leaving a crude preparation of virus in the supernatant fluid.

Initially serial two-fold dilutions were made with a portion of each harvest, and analyzed by agar gel diffusion against a particular BTV antiserum in order to determine the optimal proportions point. It was found that precipitation occurred over a broad range of ratios of antigen to antibody (Fig. 3.9).

It is known that maximum precipitation of antigen lies at a concentration of antigen above that giving OPP.[114] The reason for this characteristic feature of precipitin reaction is unknown. Analysis of the precipitin bands from each dilution of viral antigen (against undiluted BTV antiserum) by PAGE confirmed this phenomenon. (Chapter Two), and the results showed that it was the concentration of antigen just above OPP that gave maximal precipitation of the BT proteins. Therefore, it was this dilution of the viral antigens that was used in all the preparative agar gel diffusions.

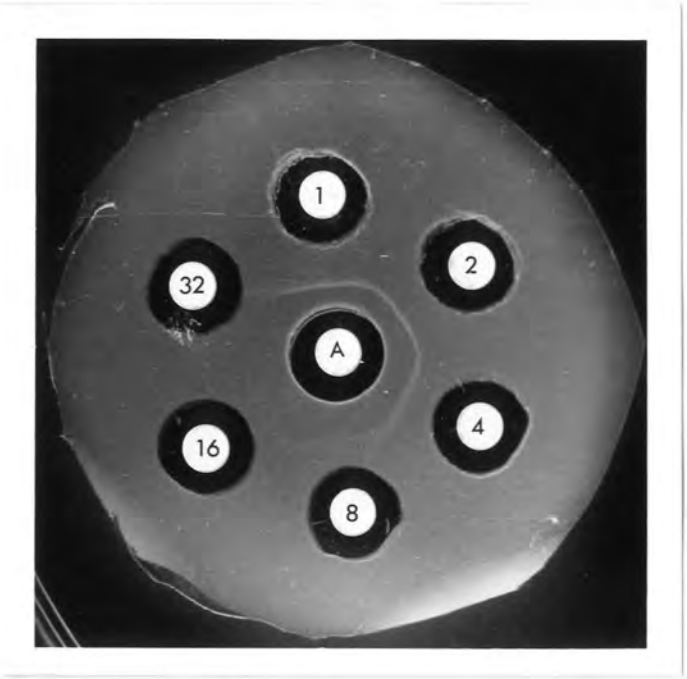


FIGURE 3.9: Immunodiffusion of dilutions of BTV proteins (BTV-10) for OPP determination. A = BTV type 1 antiserum.

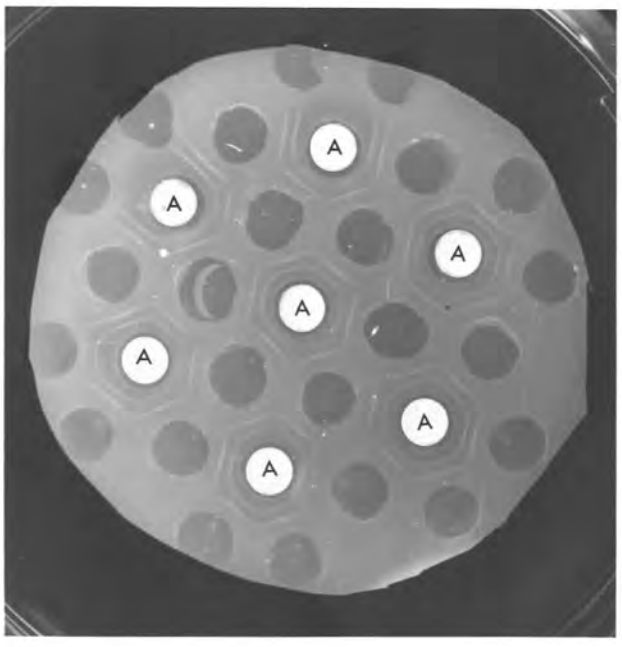


FIGURE 3.10: Overlapping well pattern used in preparative agar gel diffusion. A = BTV antiserum (in this case BTV type 10 antiserum) Remaining wells, appropriate dilution of viral proteins (BTV-10).

Heterotypic precipitation of the viral proteins produced a single precipitin band, whereas precipitation using homotypic antiserum produced a second precipitin band (Fig. 3.11).

To ensure that the precipitin bands produced were due to reactions with viral proteins only, agar gel diffusion tests were performed using BHK host cell proteins. The BHK cell monolayer was detached and processed in the same manner as the viral proteins. Agar gel diffusion against the different BTV antisera produced no precipitin bands, indicating that the precipitin bands formed are not due to non-specific reactions.

Radio-iodination of proteins: Initially radio-iodination was performed on the precipitin bands formed during agar gel diffusion. The agar slices containing the precipitin bands were washed extensively to remove the uncomplexed proteins, dried onto the hydrophilic side of Gel-Bond (FMC Corporation, Maine, USA), and then iodinated using Elder et al (1977) adaption of the Chloramine-T method. The ^{125}I-labelled proteins making up the Ag-Ab complex were then separated on 12% polyacrylamide gels, and detected by autoradiography (Fig. 3.12(a) and (b)). The proteins were labelled to high specific activities, as only 2 to 10 hour exposures of the X-ray film were required. However, a large proportion of the radiolabel was associated with the heavy and light protein chains of IgG. The specific activity of the viral components was relatively low due to competition with the antibody proteins for radioactive iodine. It was noted that the immune complex proteins separated on the polyacrylamide gel could be detected by staining with Coomassie blue (Fig. 3.12(a)), and the protocol was altered: the washed Ag-Ab complex was separated on 12% gels before iodination, the stained viral protein band cut out and then radio-iodinated. With this modification to the initial protocol only the viral proteins to be analyzed were radio-iodinated.

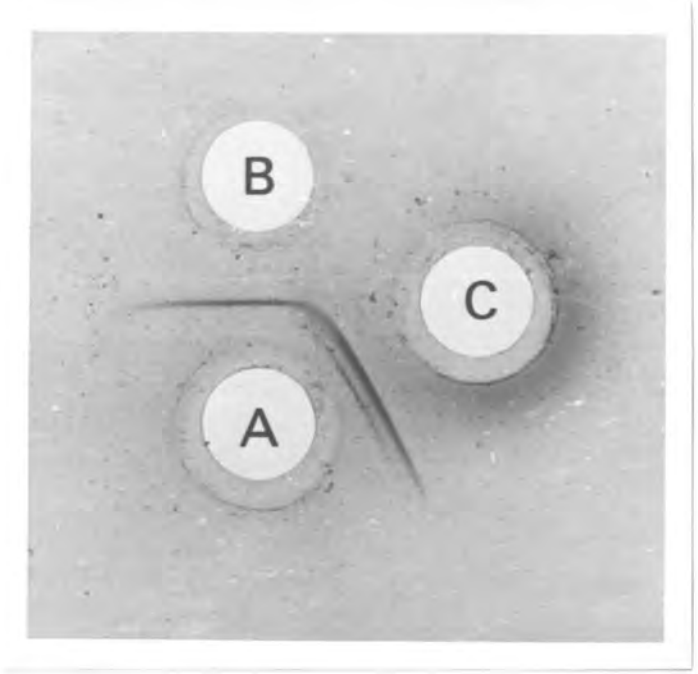
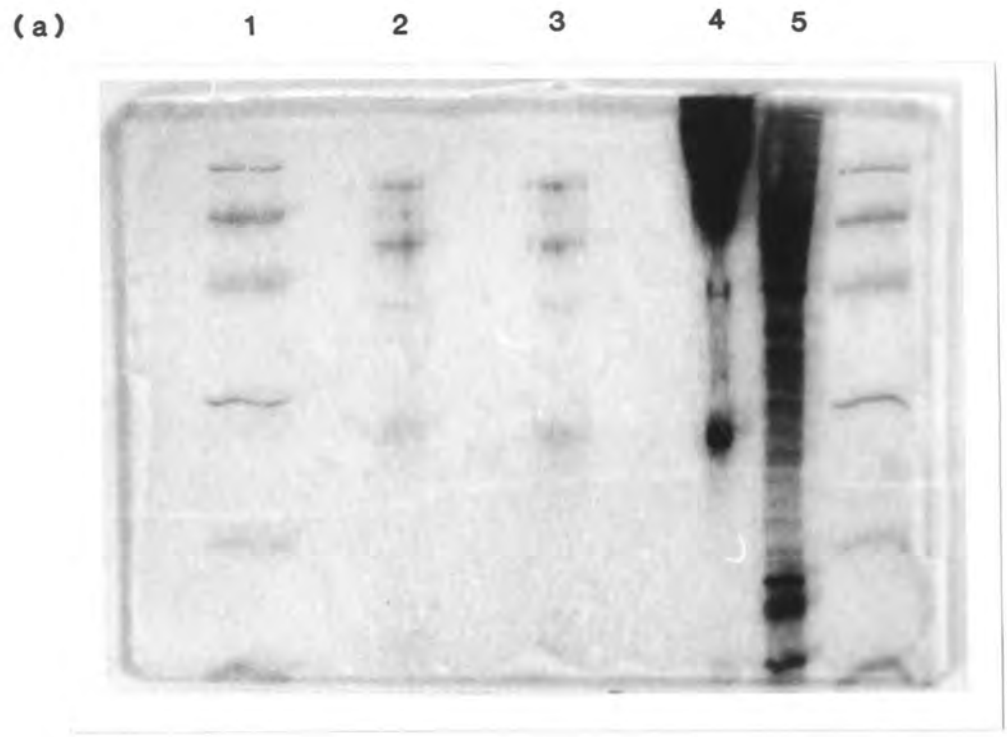


FIGURE 3.11: Immunodiffusion of BTV against homologous and heterologous immune sera showing precipitin bands formed.

A = BTV type 1 virus;
B = BTV type 4 antiserum;
C = BTV type 1 antiserum

(Reproduced by permission of J.F.E. Newman from Gumm and Newman, 1982).



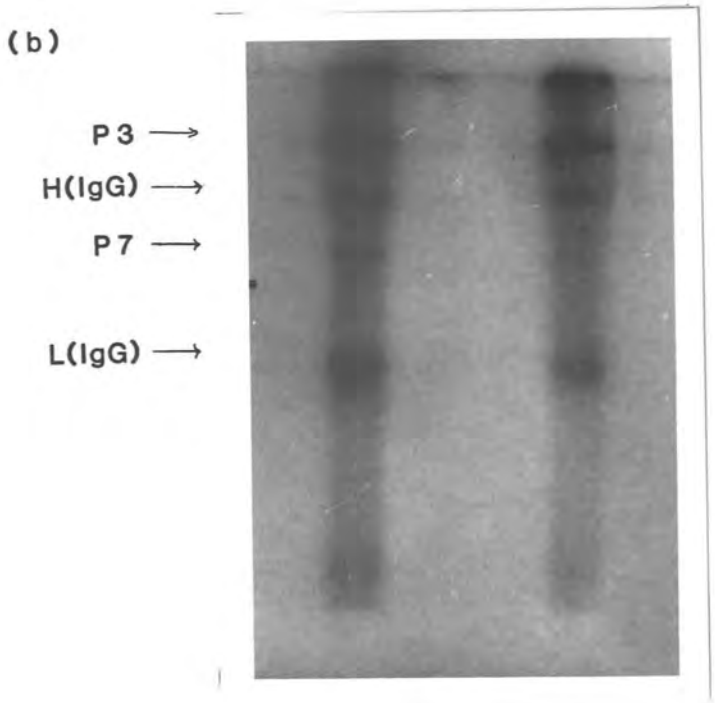


FIGURE 3.12(a): Radio-iodinated immune complex proteins separated on 12% PAG and stained with Coomassie blue.

Lane 1: Molecular weight markers (Appendix A).
Lane 2: BTV-8 viral proteins precipitated by BTV type 1 antiserum.
Lane 3: BTV-10 viral proteins precipitated by BTV-1 antiserum.
Lane 4: BTV type 1 antiserum.
Lane 5: BHK host cell proteins.

H(IgG) - heavy chain IgG; L(IgG) - light chain IgG; SA - serum albumin.

(b): Autoradiogram of the above gel.

A problem previously encountered in radio-iodinating proteins was the presence of SDS; the differences then were (i) the protein was in a solution containing SDS, and (ii) Bolton and Hunter reagent was used for the iodination. However, with this protocol SDS and other possible contaminants can be removed from the gel prior to iodination by washing with 10% methanol. SDS is very soluble in this solvent and easily removed.

The radio-iodination of proteins in dehydrated gel slices involves the addition of phosphate buffer pH 7.5, $Na^{125}I$ and Chloramine-T. The total volume of labelling solution being approximately the volume required for complete rehydration. Because the rate-limiting process in the radio-iodination of proteins within a gel matrix is diffusion, rather than the actual radio-iodination, the time of incubation of the gel slice during radio-iodination is based on the time required for complete rehydration of the gel. Elder et al (1977) found that the time of incubation, protein amount, or amount of ^{125}I added to the reaction affect only the total incorporation of ^{125}I into the protein and not the uniformity of labelling. Radio-iodination by the Chloramine-T method results in the incorporation of radio-iodine into the amino acid tyrosine; some iodine may also react with histidine, tryptophan or sulphydryl groups.[5] However, the Chloramine-T reaction has a pH optimum of 7.5 and at this pH, tyrosine is the principal amino acid involved. Iodination was allowed to proceed for 1 hour after which the Chloramine-T was reduced by the addition of sodium metabisulphite and free iodine was reduced to iodide. Unincorporated label was removed from the gels by washing in 10% methanol until a low background count was achieved.

Proteins present in the immune complex: Analysis of the precipitin bands by PAGE revealed that the major viral proteins precipitated with heterologous antisera were P3 and P7. Occasionally, precipitation of the non-structural polypeptide, P6a, was noted, however, this was never extensive and could not be detected by Coomassie blue staining. Homologous antisera precipitated P2, which is serotype specific. Similar results have been obtained in immune precipitation studies by Huismans and Bremer (1981) and Huismans and Erasmus (1981).

Besides the heavy and light chains of IgG (MW calculated as 56,000 and 27,000 respectively), one other protein was detected by staining with Coomassie blue. This was identified as serum albumin (probably from the antiserum) and in the majority of cases was only present in trace amounts. This was shown as the only contaminating protein in the autoradiograms of iodinated precipitin bands (Fig. 3.12b). However, analysis of the proteins in the washed precipitin bands by silver staining (Fig. 2.3b) revealed the presence of several other contaminating proteins. These were all identified as proteins present in the serum preparation, which for some unknown reason were not completely removed by washing of the agar gel. It was felt that even if these contaminating proteins were consistently present, but remained undetected because of the insensitivity of the Coomassie blue stain, they would not interfere with the isolation of the viral proteins, as they differed sufficiently in MW to be easily separated from them.

3.4 DISCUSSION

In an attempt to determine the degree of relatedness of different BTV serotypes, structural analysis of the group antigen seemed to offer a reasonable starting point. However, before this could be attempted,

procedures for the purification of these antigens had to be developed. Generally, purification of antigens synthesized during infection is difficult, because viral antigens are present in low concentrations and usually associated with cellular material. However, Huismans (1979) showed that BTV group antigen was synthesized in excess in infected cell cultures and released into the supernatant fluid. This led me to try and attempt the isolation of group antigen from the infectious culture fluid, as had several other workers.[37;48]

Numerous different procedures were tried for obtaining purified group antigen radiolabelled to high specific activity. The protocol finally adopted is outlined in Fig. 3.13. Using this protocol, the group antigen, P7, could be purified and radiolabelled to high specific activity, thus facilitating its analysis. Furthermore, P3, another protein of the BTV core particle, could also be isolated and iodinated using the same procedure. Homologous antisera precipitated P2, P3 and P7; for analysis of P2 further separation of P2 and P3 would be required as they migrate very closely together on 12% PAGs. This could be achieved by re-electrophoresing these proteins for longer times.

The advantages of this purification procedure are that:

1. microgram quantities of purified protein can be obtained and radiolabelled to high specific activity; (especially when compared to the biosynthetic incorporation obtained with ^{35}S-methionine into the viral proteins);
2. the technique is simple, fast and relatively inexpensive.

Structural analysis of the viral proteins can now be undertaken. Peptide mapping is considered a stringent test of protein identity and other than amino acid sequencing, is probably the best technique available at present.

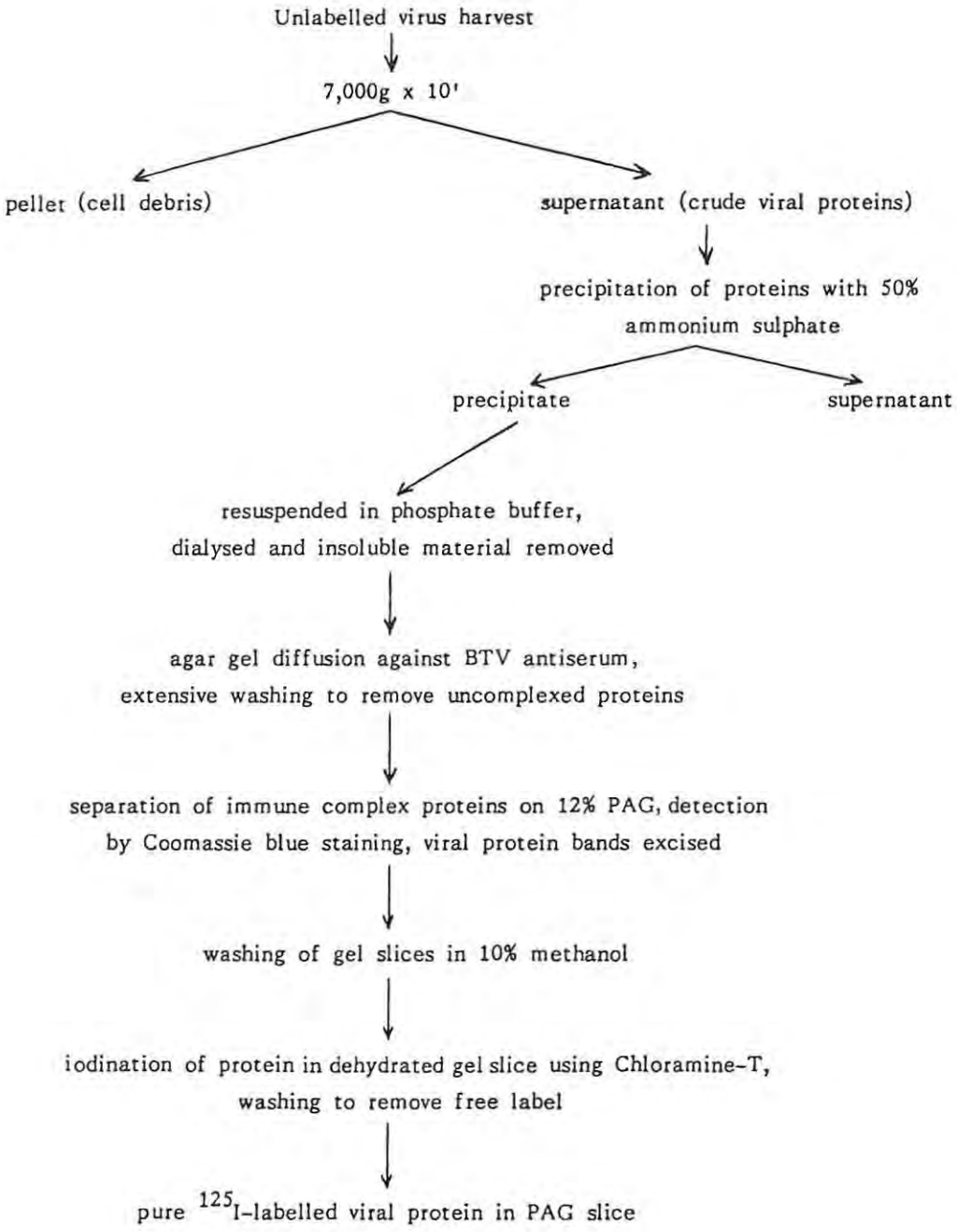


FIGURE 3.13: Flow diagram of the protocol for the purification of BTV proteins.

There is one obvious limitation to this type of analysis. The structural information obtained by "fingerprinting" will only be of the tyrosine-containing peptides. However, this protocol has been used to compare all the major protein components of Moloney and Rauscher leukemia viruses[22] and was sensitive enough to identify the precursor-product relationships of the proteins.

CHAPTER FOUR

COMPARATIVE PEPTIDE ANALYSIS OF THE CAPSID POLYPEPTIDES OF BLUETONGUE VIRUS SEROTYPES

4.1 INTRODUCTION

Fingerprinting of peptides, obtained from a purified protein by proteolytic digestion, was originally used for sequence investigations but soon gained importance as a possible way to characterize proteins, to compare sequence similarities or to detect small changes in primary structure.[8]

Structural analyses of viral proteins can provide important biological insights. For example: Antigenic shifts among human influenza A viruses involve the appearance of new epidemic strains of virus posssessing different haemagglutinin (HA) genes.[118] The active form of the influenza HA molecule consists of two subunits (HA1 and HA2) connected by a disulphide bridge.[70] Peptide mapping of both subunits of the HA from these viruses has indicated that they are structurally quite different.[118] It has been shown that HA1 is the immunologically important part of the molecule in that it is the antigen against which neutralizing antibody is directed.[56; 57] On the other hand, HA2 is apparently not involved in the immunological response of the host to viral HA. Nucleotide and amino acid sequencing studies on the HAs of several influenza viruses have shown that HA1 is considerably more conserved than HA2.[31; 116] Similarly, HA1 shows many more nucleotide and amino acid changes than does HA2 during antigenic drift of a given pandemic strain.[31; 116] Gentsch and Fields (1981) used tryptic peptide mapping to analyze directly the structural basis for the biological properties of the reoviruses. Their

results suggested that the mammalian reovirus genome segments encoding each of the viral outer capsid polypeptides were derived from common ancestral segments which diverged to different degrees.

Over the past few years, there has been spectacular growth in the acquisition of new separation methods for peptide mapping. In the classical peptide mapping technique developed by Ingram (1956) the enzyme digest is subjected to a two-dimensional analysis involving a combination of electrophoresis and chromatography. It may be carried out on sheets of paper or on thin layers. The former has been widely used in the past, but due to increased demand for greater sensitivity thin layer peptide mapping is now in vogue. The technique is rapid and simple to perform.

Cleveland et al (1977) have developed a convenient method of peptide mapping in one dimension. In their procedure, the protein is subjected to limited proteolysis in the presence of SDS and the partial digestion products are analyzed by polyacrylamide gel electrophoresis. This technique is quite versatile and simple to use. Several fingerprints can be obtained simultaneously in a matter of hours and can be displayed on the same slab gel, thereby allowing easy, unambiguous comparisons. A further advantage is that no special equipment beyond the standard slab gel electrophoresis apparatus is required. However, peptide mapping by two-dimensional methods is a more stringent test of polypeptide identity.[39]

High performance liquid chromatography (HPLC) may employ the principles of adsorption, partition, ion exchange, exclusion and affinity

chromatography. This makes it an extremely versatile technique and helps to explain its recent emergence as the most powerful single chromatographic procedure. The technique has the advantages of high efficiency, rapid resolution, it may be used with pico and femto gram amounts of material and it is suitable for preparative and analytical, qualitative and quantitative analysis. In its reverse-phase and ion exchange form, HPLC is the most powerful tool available for the analysis of peptides. Because of the excellent resolving powers of these procedures, very subtle molecular changes can easily be revealed, for example, deamidation of asparagine or glutamine, amino acid deletion or replacement, post-translational phosphorylation of serine, tyrosine, etc.[42] The only drawback of this technique is that the equipment required is highly specialized and therefore very expensive.

4.2 MATERIALS AND METHODS

4.2.1 PROTEOLYTIC DIGESTION OF PROTEINS

Tryptic digestion of ^{35}S-methionine labelled group antigen:

The electro-eluted ^{35}S-methonine labelled group antigen preparations were subjected to digestion with trypsin according to the method of Shih et al (1978). The protein samples were diluted to 2.2 mℓ with distilled water and adjusted to pH 8 by the addition of 0.2 mℓ 0.5M Tris-HCl pH 8.0. Bovine serum albumin (BSA) was added to a concentration of 1mg/mℓ as carrier. After reduction in the presence of dithiothrietol at 3.3mg/mℓ (37°C, 30 minutes) and alkylation with iodoacetamide at a final concentration of 9mg/mℓ (0°C, 60 minutes, in the dark), the proteins were precipitated by adding an equal volume of 50% (w/v) trichloroacetic acid (O°C, 30 minutes). The precipitate was collected by centrifugation at

3000rpm for 30 minutes in an MSE bench-top centrifuge. The precipitate was washed three times with 5 mℓ of ether to remove residual acid and then dispersed in 1 mℓ 0.1M ammonium bicarbonate buffer pH 8.0. One drop of toluene was added and digestion was carried out at 37°C for 16 hours with 0.1mg/mℓ diphenyl carbamyl chloride (DPCC)-treated trypsin (Sigma). The mass ratio of trypsin to BSA was 1 : 25. to ensure digestion was complete a second, equivalent sample of DPCC-trypsin was added and incubated for a further 8 hours. The digest was then evaporated to dryness and the residue dissolved in 0.4 mℓ 0.04M pyridine-acetate buffer pH 2.45. Recovery of radioactivity was determined after filtration through a glass fibre disc to remove insoluble material.

Elution and digestion of ^{125}I labelled viral proteins:

The dried gel slices containing the ^{125}I labelled viral protein were placed in a siliconized plastic tube and 1 mℓ of 50ug/mℓ DPCC-trypsin or Staphylococcus aureus V8 protease (Miles) in 0.05M ammonium bicarbonate buffer pH 8.0 was added to each tube. The samples were then incubated at 37°C overnight, after which the supernatants were removed and dried under a gentle stream of air. The samples were dissolved in the appropriate starting buffer for analysis and filtered through a glass fibre disc to remove insoluble material.

4.2.2 PEPTIDE MAPPING

Two-dimensional thin layer electrophoresis and chromatography:

BUFFERS:

Buffer I (for electrophoresis): Glacial acetic acid : formic acid : water, 15 : 5 : 80 (v/v)

Buffer II (for ascending chromatography) Butanol : pyridine : glacial acetic acid : water, 32.5 : 25 : 5 : 20 (v/v)

ELECTROPHORESIS:

The ^{125}I-labelled peptides were dissolved in 20uℓ of Buffer I and applied to a 20 x 20 cm cellulose-coated thin layer plate (Merck) with a microsyringe. Repeated applications were made, with intermittent drying in a stream of dry air, until 0.5 x 10^6 cpm had been applied.

To ensure the peptides of each sample migrated a consistent distance, progress of electrophoresis was monitored using a mixture containing 2% Orange G (w/v) and 1% acid fuschin (w/v) in Buffer I. The dye was spotted at the opposite end from the sample and each plate was removed when the lead dye component reached a preset mark.[22]

Prior to electrophoresis the plate was sprayed as uniformly as possible with Buffer I, so that the whole plate was damp but the surface remained dull and not shining with surplus liquid. The plate was placed in the electrophoresis tank and electrical contact made with the aid of moistened Whatmann No. 3MM paper wicks. A moistened glass plate was placed above the thin layer plate to reduce evaporation from its surface (Fig. 4.1).

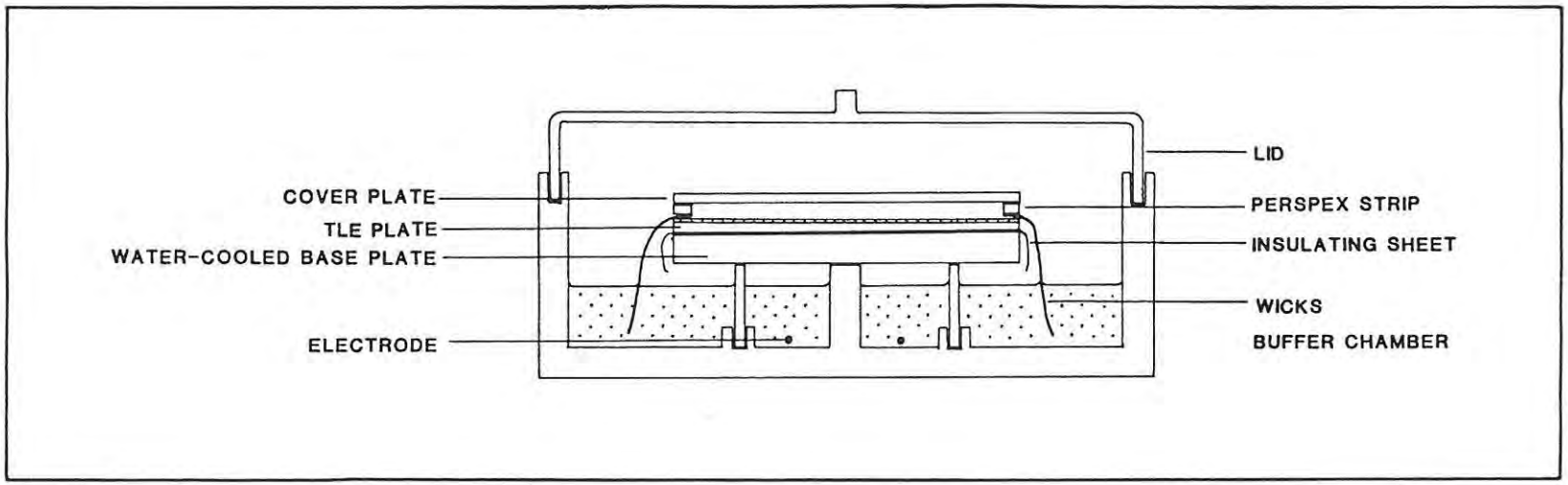


FIGURE 4.1: Cross section of the thin layer electrophoresis apparatus.

Electrophoresis was at 500 volts until the lead dye component reached the sample origin (approximately 90 minutes). The plate was then dried in a fumehood overnight.

ASCENDING CHROMATOGRAPHY:

The atmosphere within the chromatography tank was saturated with vapours of the solvent mixture (Buffer II) by hanging strips of moistened filter paper down the sides of the tank. After 1 hour of equilibration the thin layer plate was placed vertically in the tank, chromatography being at right angles to the direction of electrophoresis. Peptides were chromatographed until the solvent front reached a preset mark, the plates dried and then analyzed by autoradiography (Appendix A) for 5 days.

Ion Exchange Chromatography

BUFFERS:

The buffers used for the elution of the ion-exchange column were:

(1) 0.04M pyridine acetate, pH 2.45;

(2) 2.00M pyridine acetate, pH 4.90.

Column performance is greatly affected by the quality of the solvent used as the mobile phase, therefore a highly pure pyridine, Aristar pyridine (BDH chemicals) and Analar grade glacial acetic acid (Merck) were used to make up these buffers. The buffers were filtered through a 0.2 micron millipore membrane to remove particulate matter.

RESIN WASHING AND EQUILIBRATION:

Prior to packing the column it was necessary to wash and equilibrate the resin.

10 mℓ of Chromobead type P cation exchange resin (Technicon Instruments, New York) was filtered through a few layers of gauze or cheese cloth, thoroughly rinsed down with distilled water and recovered in a scintered glass filter. All insoluble material collected on the cloth. The resin was then washed with the following solutions:

(i) 500 mℓ distilled water;

(ii) 500 mℓ 10% sodium hydroxide (until filtrate becomes clear and colourless);

(iii) 500 mℓ distilled water;

(iv) 500 mℓ 10% nitric acid (until filtrate becomes clear and colourless).

The filtrate must have a pH of 7 before continuing

(v) 500 mℓ distilled water.

The resin was left overnight, agitating gently in 500 mℓ 0.2M sodium hydroxide. Finally the resin was equilibrated in 500 mℓ 0.04M pyridine acetate pH 2.45 for 5 hours, allowing it to swell fully to this ionic strength.

PEPTIDE ANALYSIS:

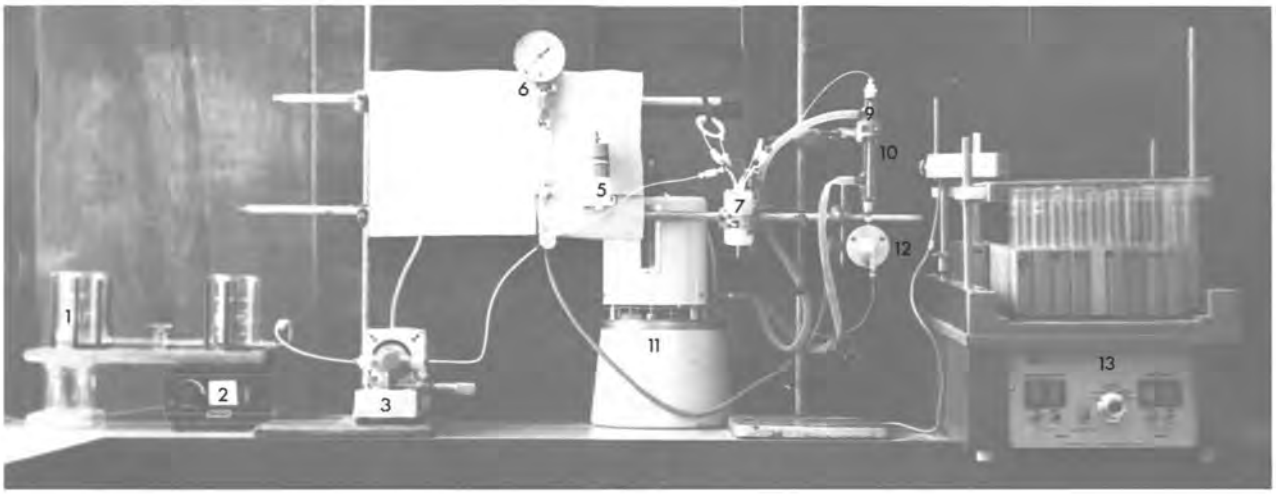
The ion exchange chromatography system used is shown in Fig. 4.2.

The peptides to be analyzed (0.5 - 1.0 x 10^6 cpm) were taken up in 0.4 mℓ 0.04M pyridine acetate buffer pH 2.45 and applied to the column (3 x 150mm) of Chromobead type P cation exchange resin through a six-port rotary injection valve. This was done whilst the system was at its operating pressure of 80 to 100 p.s.i. The chromatogram was developed at 54°C with a convex gradient increasing in pH and pyridine acetate concentration (from 0.04M

KEY TO FIGURE 4.2

1. Solvent reservoir and gradient marker.
2. Magnetic stirrer platform.
3. FMI laboratory pump (Model RP-SY-1CSC) fitted with low flow adaptors and micrometer screw gauge for flow settings.
4. Tee-connector.
5. Pressure-relief valve (Model PRV-500, Cheminert) set at 120 p.s.i.
6. Pressure gauge.
7. Six-port rotary loop injector valve (Cheminert).
8. Hamilton gas-tight syringe (No. 1010).
9. Glass column (Model MB-3-150, Cheminert) with low dead volume fittings, containing Chromobead P resin.
10. Water jacket.
11. Circulating water bath with temperature control.
12. Flow stopper connected to fraction collector.
13. LKB Ultrorac fraction collector (Model 7004).
14. Teflon tubing 1/8 inch diameter.
15. Teflon tubing 1/16 inch diameter.

(a)



(b)

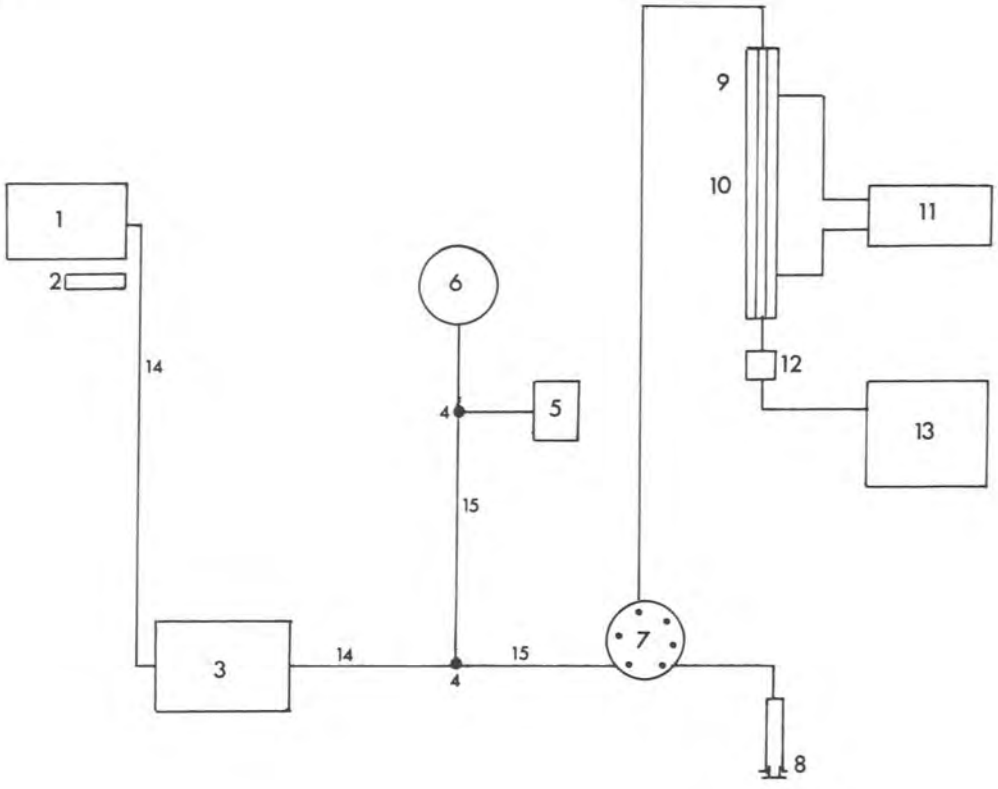


FIGURE 4.2 (a): Photograph of ion-exchange chromatography system used for peptide mapping.

(b): Schematic diagram showing layout of apparatus.

pyridine-acetate pH 2.45 to 2.00M pyridine-acetate pH 4.90). 150 fractions of 1.8 mℓ were collected and assayed for radioactivity.

The column was regenerated after each run; reversibly bound components being removed by flushing the column with a minimum of ten bed volumes of 0.04M pyridine-acetate pH 2.45.

ASSAY FOR RADIOLABELLED PEPTIDES

Liquid Scintillation Counting: The ^{35}S-methionine labelled peptide fractions were transferred to scintillation vials containing several glass fibre discs, and the pyridine-acetate buffer evaporated off by heating the vials in a fumehood. (Pyridine acts as a quencher of radioactivity thus necessitating its removal). 10 mℓ of non-aqueous scintillant (5 g 2.5-diphenyloxazol; 0.5 g Bis-MSB made to 1 ℓ with toluene) was added to each vial, the samples mixed and counted in a Beckman liquid scintillation counter (Model LS 3150 T) using the full ^{14}C-window.

Gamma Counting: The ^{125}I-labelled peptide fractions were transferred to scintillation vials and 10 mℓ of distilled water was added. Radioactivity was measured in a Beckman Model 310 gamma counter using the ^{125}I isoset.

^{125}I-labelled peptides could also be counted in the full ^{3}H-window of the liquid scintillation counter with toluene-based scintillation fluid.

4.3 RESULTS

4.3.1 TRYPTIC DIGESTION OF ^{35}S-METHIONINE LABELLED GROUP ANTIGEN

Proteolytic enzymes have been extensively used to cleave proteins into smaller peptides. The high degree of specificity of trypsin (EC 3.4.21.4) makes it very effective for preparation of appropriately sized peptides which are used in tryptic peptide mapping. Trypsin is a serine protease of the pancreas which selectively catalyzes the hydrolysis of peptide bonds on the carboxyl side of the basic amino acids, lysine and arginine. Trypsin is not an enzyme of first attack and in several instances it possesses only feeble action upon native proteins.[41] It does not, for example, readily digest collagen, ovalbumin, serum globulins and haemoglobin. These proteins are readily digested by trypsin once they have been denatured.

The protocol of Shih et al (1978) was used for tryptic digestion. The ^{35}S-methionine labelled group antigen sample was adjusted to pH 8 by the addition of 0.5M Tris-HCl pH 8.0 and BSA was added as carrier. The proteins were reduced by the addition of dithiothrietol, as a high specificity for reduction of disulphide bridges is obtained by reaction with thiol reagents:

$$R\text{-}S\text{-}S\text{-}R^1 \xrightarrow{HS\text{-}C(HO)\text{-}C\text{-}C(OH)\text{-}SH} RSH + R^1SH$$

The sulphydryl groups in the protein were then preferentially alkylated by iodoacetamide:

$$RSH + ICH_2\overset{O}{\overset{\|}{C}}NH_2 \longrightarrow RSCH_2\overset{O}{\overset{\|}{C}}NH_2 + H^+ + I^-$$

The resulting amide derivative of cysteine is very stable. Since cysteinyl residues alkylated by iodoacetamide, but not iodoacetic acid, are susceptible substrates for trypsin,[25] use of the former reagent is advantageous in creating another site for trypsinization. The presence of this additional cleavage site increases the number of labelled peptides generated and this could provide more structural information. Alkylation is also important in preventing reformation of disulphide bonds, thereby facilitating complete tryptic activity.

After reduction and alkylation the proteins were precipitated by adding an equal volume of 50% trichloroacetic acid (TCA). The precipitate was washed with ether to remove residual acid and resuspended in a volatile buffer, 0.1M ammonium bicarbonate pH 8.0. Digestion was at 37°C for a total of 24 hours. Tryptic digestion over long periods of time may produce unwanted peptides due to the presence of slight chymotryptic activity in the enzyme preparation. Such contamination reduces the effectiveness of trypsin, therefore preparations of trypsin are used in which the chymotryptic contaminant is selectively inactivated with diphenyl carbamyl chloride (DPCC). The product of this treatment is "DPCC-treated" trypsin, a highly pure form of the enzyme in the catalytic sense.

Approximately 90% of the radioactivity was recovered after reduction, alkylation and digestion. The loss of material may be due to: (i) generation of peptides too small to be precipitated by TCA, or (ii) the insolubility of a fraction of the tryptic digest, which would have been removed by filtration.

4.3.2 ELUTION AND DIGESTION OF RADIO-IODINATED PROTEINS

Following radio-iodination of the protein in polyacrylamide gel slices the protein can be eluted by protease treatment and the peptides analyzed.

Iodination using the Chloramine-T method results in radiolabelling of the generally less common residue, tyrosine, in the polypeptide chain. As fewer residues are radiolabelled with ^{125}I than for example with ^{35}S-methionine, less structural information would be obtained using tryptic digestion alone, as several unlabelled peptides may be generated. In order to obtain different peptides and therefore more structural information, digestion using Staphylococcus aureus V8 protease was also introduced.

The most striking difference between S. aureus V8 protease and other extracellular proteolytic enzymes of microbial origin resides in its high degree of substrate specificity. Houmard and Drapeau (1972) found this protease could cleave on the C-terminal side of glutamyl and aspartyl residues when hydrolysis is performed in phosphate buffer (pH 7.8). If, however, the buffer is changed to either ammonium bicarbonate (pH 7.8) or ammonium acetate (pH 4.0) cleavage occurs only at glutamyl residues.

Reduction and alkylation of the proteins prior to tryptic digestion, (as required for ^{35}S-methionine labelled group antigen) was not necessary as the secondary and tertiary structure of the protein was destroyed prior to PAGE by heating the proteins in the presence of 2-mercaptoethanol and SDS. This denatured state of the proteins is maintained after electrophoresis, facilitating the action of trypsin.

Proteolytic digestion of the radio-iodinated protein in the gel slice was limited by diffusion rather than by the time required for enzymatic cleavage.[22] Approximately 18 hours was required for 80% of the peptides to diffuse from the gel, the remaining peptides could be removed by incubation for an additional 6 hours in fresh solution. However, 80% recovery was more than sufficient for analysis and thus it was unnecessary to recover the remaining peptides.

After digestion the eluted peptides were dried down and resuspended in the appropriate starting buffer for peptide mapping.

4.3.3 PEPTIDE MAPPING

In view of the extensive evidence for the immunological relatedness of the capsid polypeptide P7 of the viruses in the Bluetongue serogroup,[11;17;37;50;52] it was felt that the chemical relatedness of this protein should be determined using peptide mapping techniques. Because of the considerable degree of homology amongst genome segments of different BTV serotypes presumably coding for the group antigen (as determined by hybridization studies[34;50;53]) this protein is likely to be highly conserved. I therefore expected a high degree of similarity in the peptide maps of the different group antigens. Furthermore, as the peptides mapped would represent only a fraction of the protein (as only tyrosine-containing peptides are detected), I chose to use two methods to analyze the tryptic peptides. The two-dimensional mapping has the advantage of resolving more peptides, while analysis of peptides on a Chromobead column shows clearly the identity or difference of peptide peaks when two proteins are compared.[40]

Two-dimensional thin layer electrophoresis and chromatography:

The ^{125}I-labelled proteins were eluted from the gel slices by digestion with trypsin and the peptides analyzed on cellulose thin-layer plates by electrophoresis and chromatography as described under Material and Methods.

Tryptic peptide maps of P7, obtained from BTV-3 and BTV-13 are shown in Figure 4.3. Examination of the maps revealed that there are only three groups of peptides common in both serotypes. These account for 30 to 40% of the total number of peptides detected. It is difficult to assess the similarities or differences between the peptides present in the central areas of the two maps because of the differences in the migration rates and the number of peptide spots in these areas.

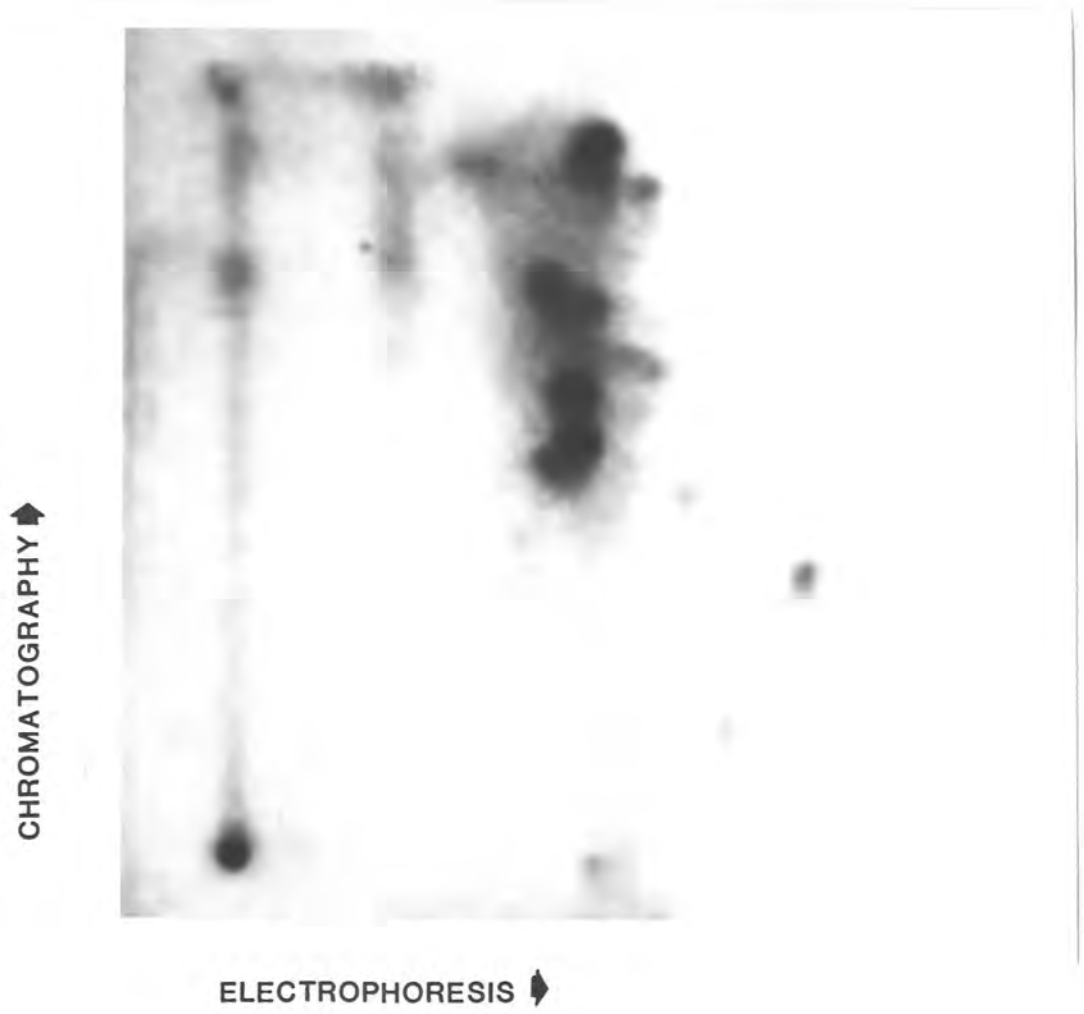
The exact number of peptides that could be detected varied according to the intensity of labelling, even so, a surprisingly large number of peptides were detected. This does not necessarily mean that the group antigen has a high tyrosine content as, although tyrosine is the principal amino acid involved in the iodination reaction, some iodine may also react with histidine, tryptophan or sulphydryl groups.[5; 22]

The polypeptide P3 of BTV-8 and BTV-10 appear to have only two radio-iodinated tryptic peptides in common (Fig. 4.4). Direct comparison of the tryptic peptide maps does not reveal this, but by comparing the values obtained for the distance electrophoresed by the peptides (relative to the marker dyes) and their Rf values this becomes apparent.

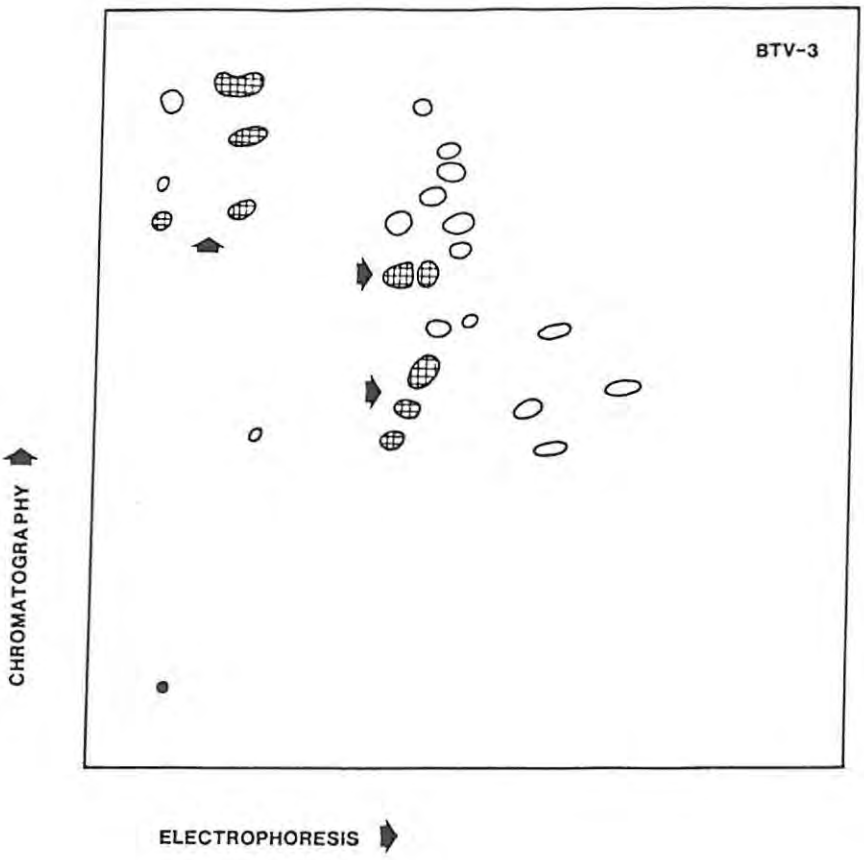
FIGURE 4.3 (a) Autoradiogram of a two-dimensional tryptic peptide map of ^{125}I-labelled group antigen from BTV-13.

(b) Diagrammatic representation of two-dimensional tryptic peptide maps of ^{125}I-labelled group antigen from BTV-3 and BTV-13. The three groups of common peptides are cross-hatched for easy identification.

(a)



(b)


BTV-3
CHROMATOGRAPHY
ELECTROPHORESIS

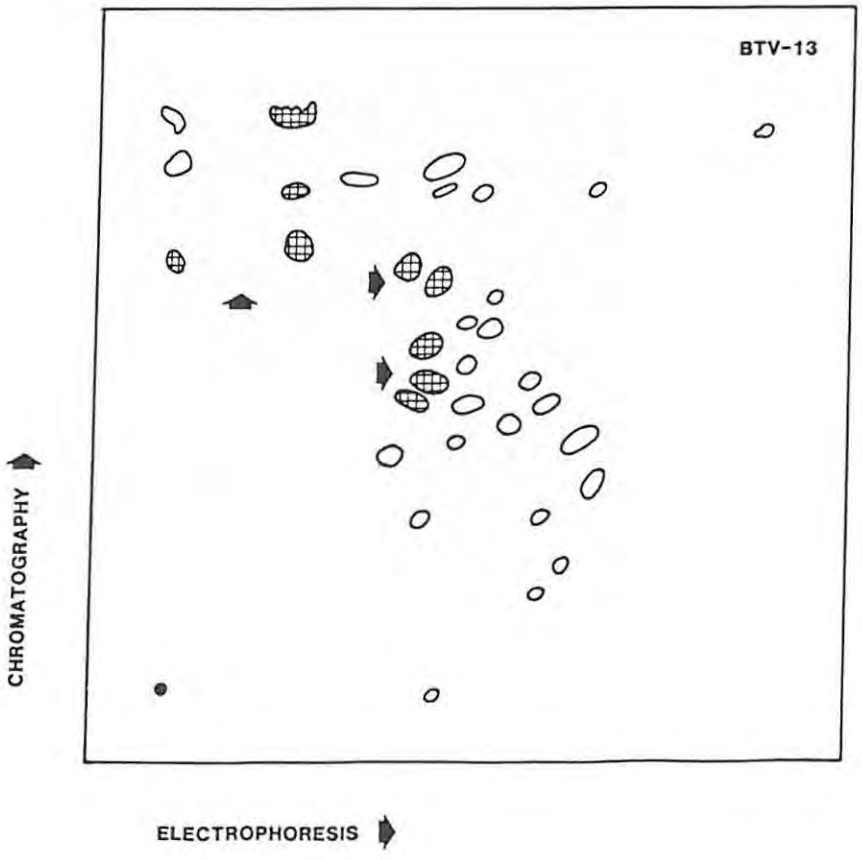
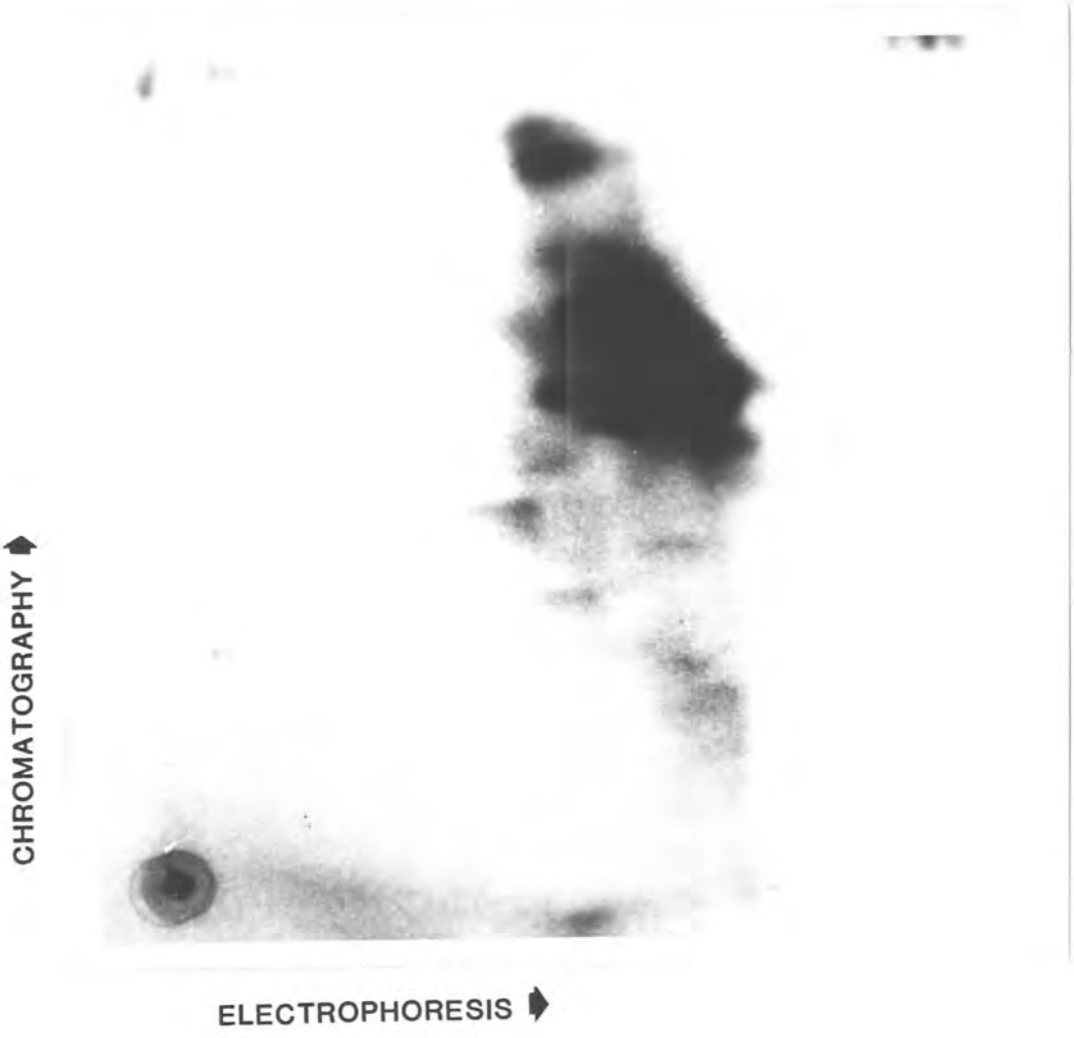

BTV-13
CHROMATOGRAPHY
ELECTROPHORESIS

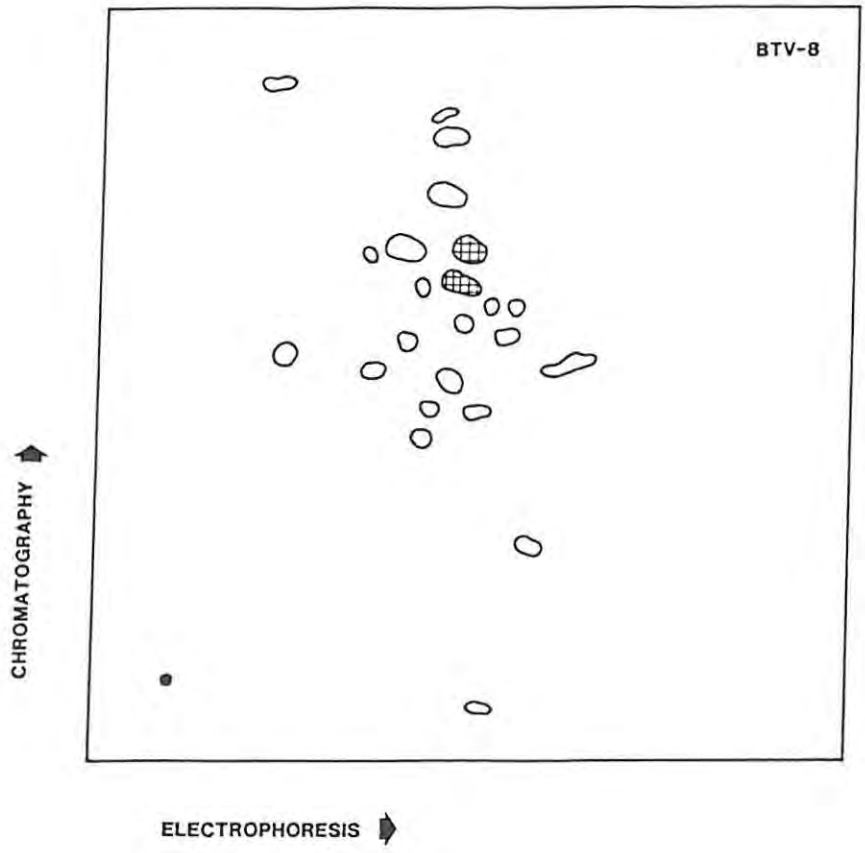
FIGURE 4.4 (a) Autoradiogram of a two-dimensional tryptic-peptide map of ^{125}I-labelled P3 from BTV type 10.

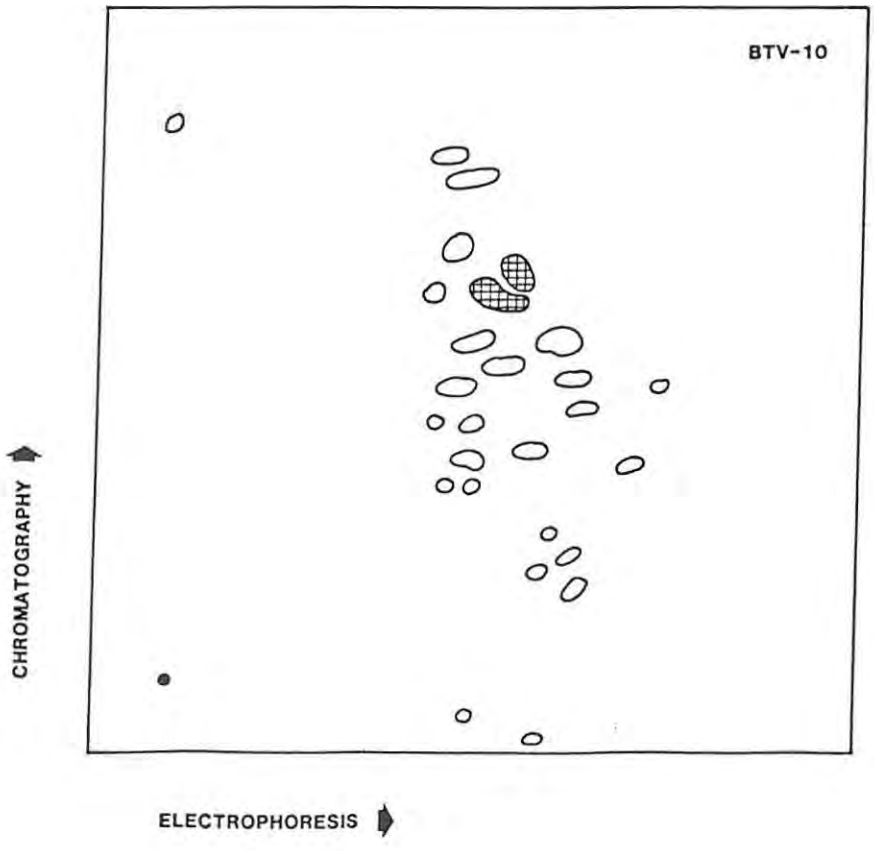
(b) Diagrammatic representation of two-dimensional tryptic peptide maps of ^{125}I-labelled P3 from BTV-8 and BTV-10. Common peptides are cross-hatched.

(a)



(b)


BTV-8
CHROMATOGRAPHY
ELECTROPHORESIS


BTV-10
CHROMATOGRAPHY
ELECTROPHORESIS

As the maps were not prepared using a constant power supply for electrophoresis and not prepared in parallel, it was difficult to superimpose the results because of variation in the extent of migration. This made it difficult to make detailed comparisons of the results and it was felt that it would be far easier and more informative to determine the relatedness of the polypeptides using the more sensitive method of peptide mapping by ion exchange chromatography, even though fewer peptides are involved in the analysis.

Ion exchange chromatography

Ion exchange chromatography using Chromobead type P cation exchange resin has been used extensively for the comparison of viral proteins by peptide mapping.[10;28;64;92;97] An ion exchange chromatography system using this resin was established for the structural analysis of BTV proteins by peptide mapping (Fig. 4.2). It has the advantages of a high performance system in that it can be used on picogram amounts of material and it has a high efficiency, however, the operating pressure of 150 p.s.i. (maximum) does not allow for a fast speed of resolution: a chromatographic run takes approximately 4 days to complete.

The Chromobead type P resin consists of spherical polystyrene beads with 4.5% divinyl benzene cross-linkage. This gives the organic matrix volume stability when the ionic strength of the mobile phase changes during elution. Sulphonic groups are bonded onto the beads facilitating separation of peptides and iodo-peptides, between 1 and 30 residues of amino acids, when ionized at low pH:

$$RSO_3^- \ldots H^+ + NH_3^+ - R^1 \longrightarrow RSO_3^- \ldots NH_3^+ - R^1 + H^+$$

Exchanger — Counter ion — Peptide to be exchanged

Once the apparatus had been set up, the performance of the system was tested. A ^{35}S-methionine labelled BTV-10 group antigen preparation was reduced, alkylated and digested with trypsin as described under Materials and Methods. The resulting digest was dried down, resuspended in 0.04M pyridine-acetate buffer pH 2.45 and filtered to remove all particulate matter. The digest was divided into two equal volumes of approximately 500, 000 c.p.m. and the individual preparations applied to the column and eluted using a gradient of pyridine-acetate buffer from 0.04M pyridine pH 2.45 to 2.00M pyridine pH 4.90. The fractions collected were assayed for radioactivity using liquid scintillation counting and the chromatographic profiles plotted (Fig. 4.5).

It is seen from these maps that the same number of peptides were eluted in the same positions relative to each other, however, the peptide maps were not directly comparable as identical peptides did not elute at the same fraction number. These minor variations are caused by the gradients not being absolutely identical. Although conditions for each run were carefully reproduced, variations in flow rate due to fluctuations in pressure could not be controlled as finely as is required to form highly reproducible elution gradients. Fortunately, this was easily overcome by measuring the pH (or ionic strength) of each fraction. The pH at which a peptide elutes from the cation exchange column is characteristic of that peptide and is related to its isoionic point.

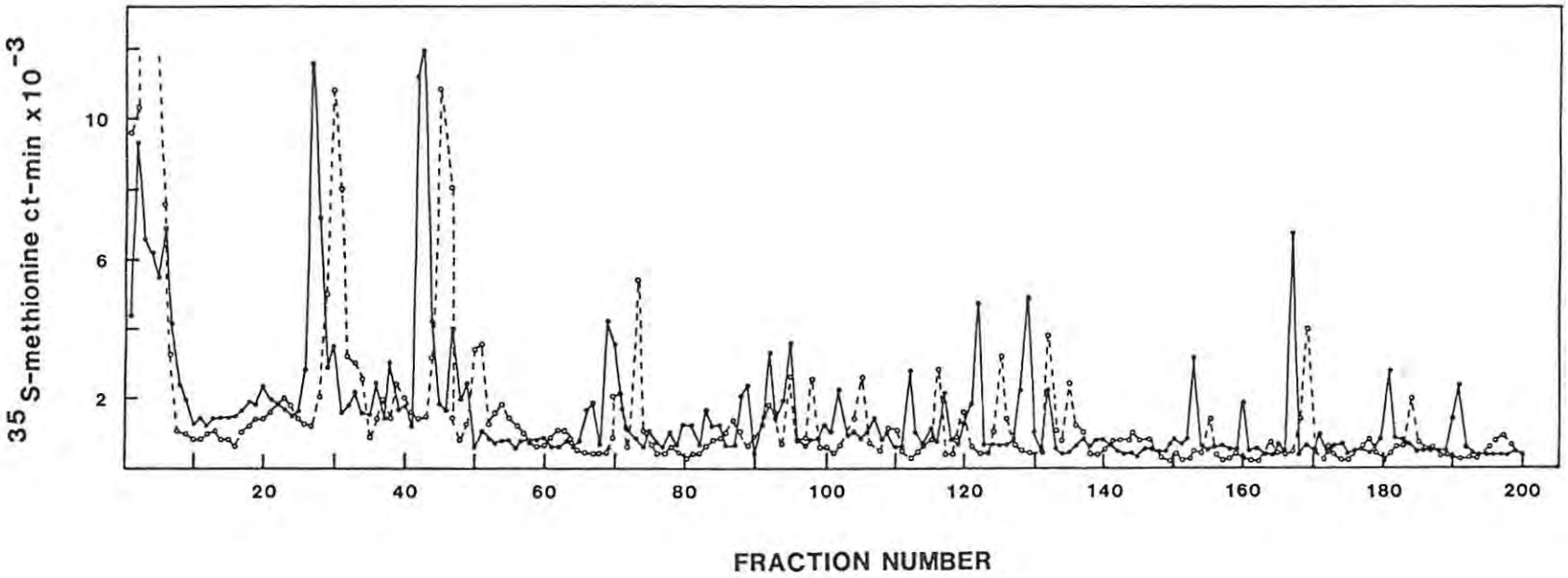


FIGURE 4.5: Replicate maps of ^{35}S-methionine labelled tryptic peptides of the group antigen (P7) from BTV-10 to show the degree of reproducibility of the ion-exchange chromatography system.

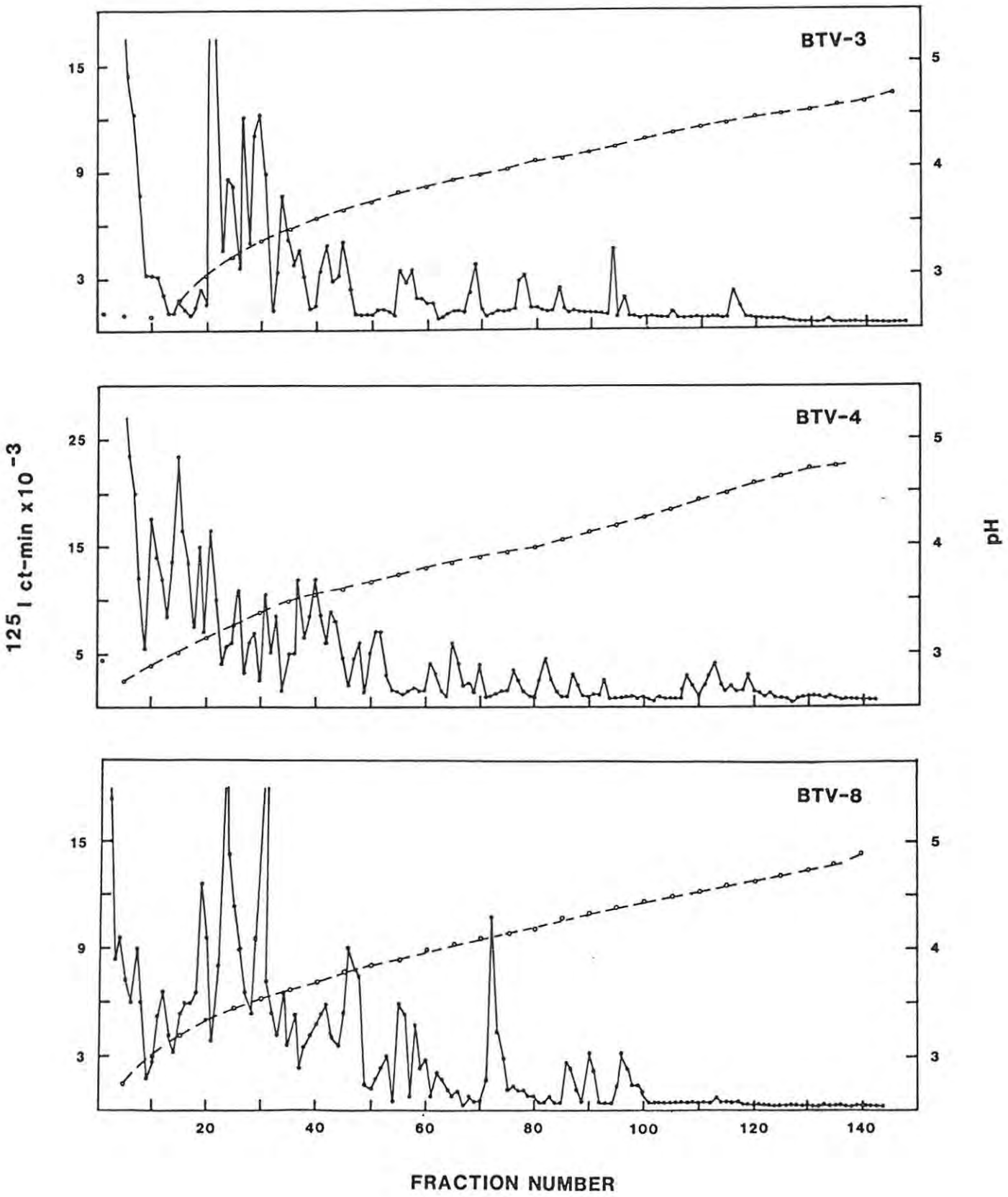
There are a large number of ^{35}S-methionine labelled peptides separated (Fig. 4.5). This would enable a fairly detailed comparison of the structures of the group antigens to be made, however, because of the difficulties encountered in obtaining group antigen preparations radio-labelled to high specific activities with ^{35}S-methionine this was not possible. Therefore radio-iodination of the viral proteins was introduced and information concerning the structural relationship among the group antigens of BTV serotypes was obtained by comparing the tyrosine-containing peptides separated by tryptic peptide mapping.

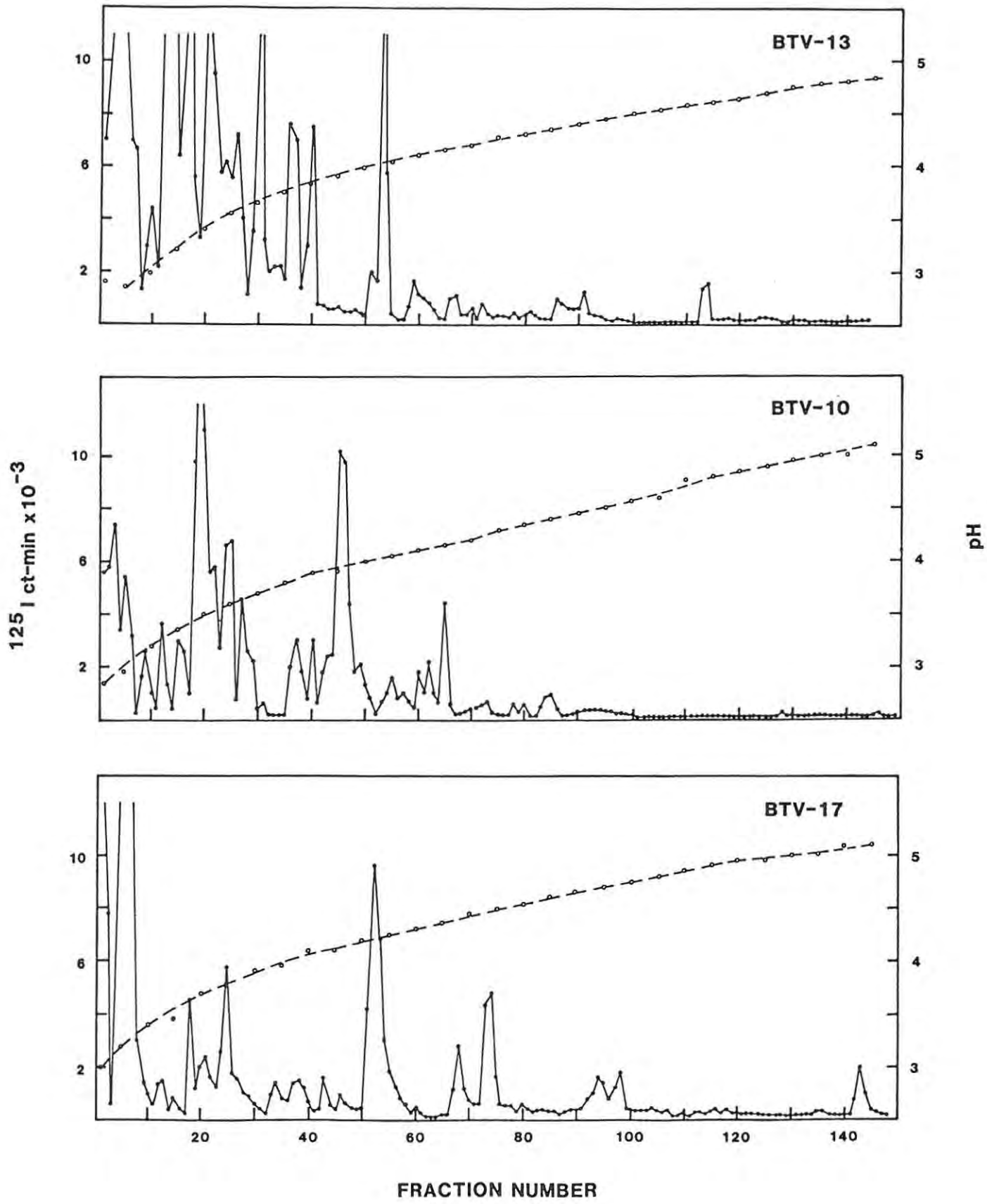
Maps of P7 obtained from BTV serotypes 3, 4, 8, 10, 13, 17 and 20 are shown in Figure 4.6. They have a similar overall pattern showing, for example, a high peak of radioactivity eluting from the column in the first few fractions. This peak is assumed to be due to the anionic peptide fragments which pass through the column unretarded. The remainder of the peptides elute from the column in the first 100 fractions.

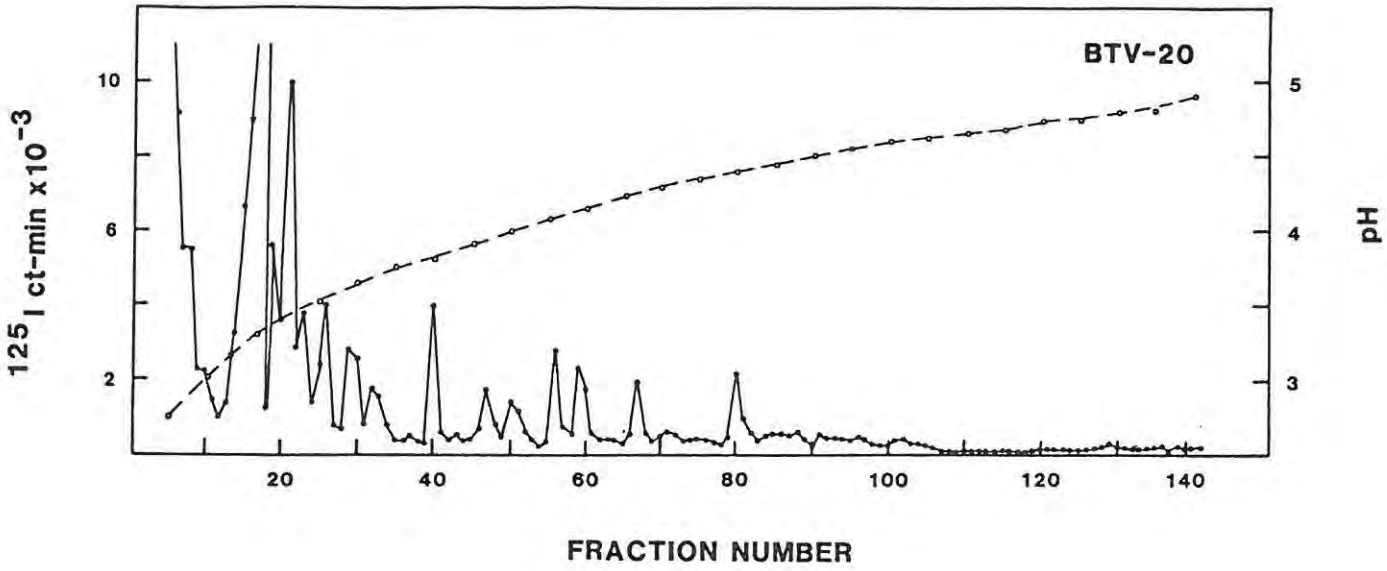
To facilitate a more detailed description of the structural relationships among the peptides from the different serotypes, the pH at which each peptide was eluted was determined. These values were used to construct a histogram for each serotype, thus enabling a direct comparison of the ^{125}I-labelled peptides to be made (Fig. 4.7).

Comparisons made between the group antigens of the seven serotypes, using Fig. 4.7, show that there are a few unique tyrosine-containing peptides in each serotype. There are several peptides shared between two or more serotypes and there are 5 peptides common to all seven serotypes. These peptides obviously

FIGURE 4.6: Ion exchange chromatography profiles of ^{125}I-labelled group antigen (P7) tryptic peptides from seven different BTV serotypes.


BTV-3
BTV-4
BTV-8
125 I ct-min x10 -3
pH
FRACTION NUMBER
15
9
3
25
5
20
40
60
80
100
120
140


BTV-13
BTV-10
BTV-17
^{125}I ct-min x10^{-3}
pH
FRACTION NUMBER
2
6
10
3
4
5
20
40
60
80
100
120
140


BTV-20
^{125}I ct-min x10^{-3}
10
6
2
5
4
3
pH
20
40
60
80
100
120
140
FRACTION NUMBER

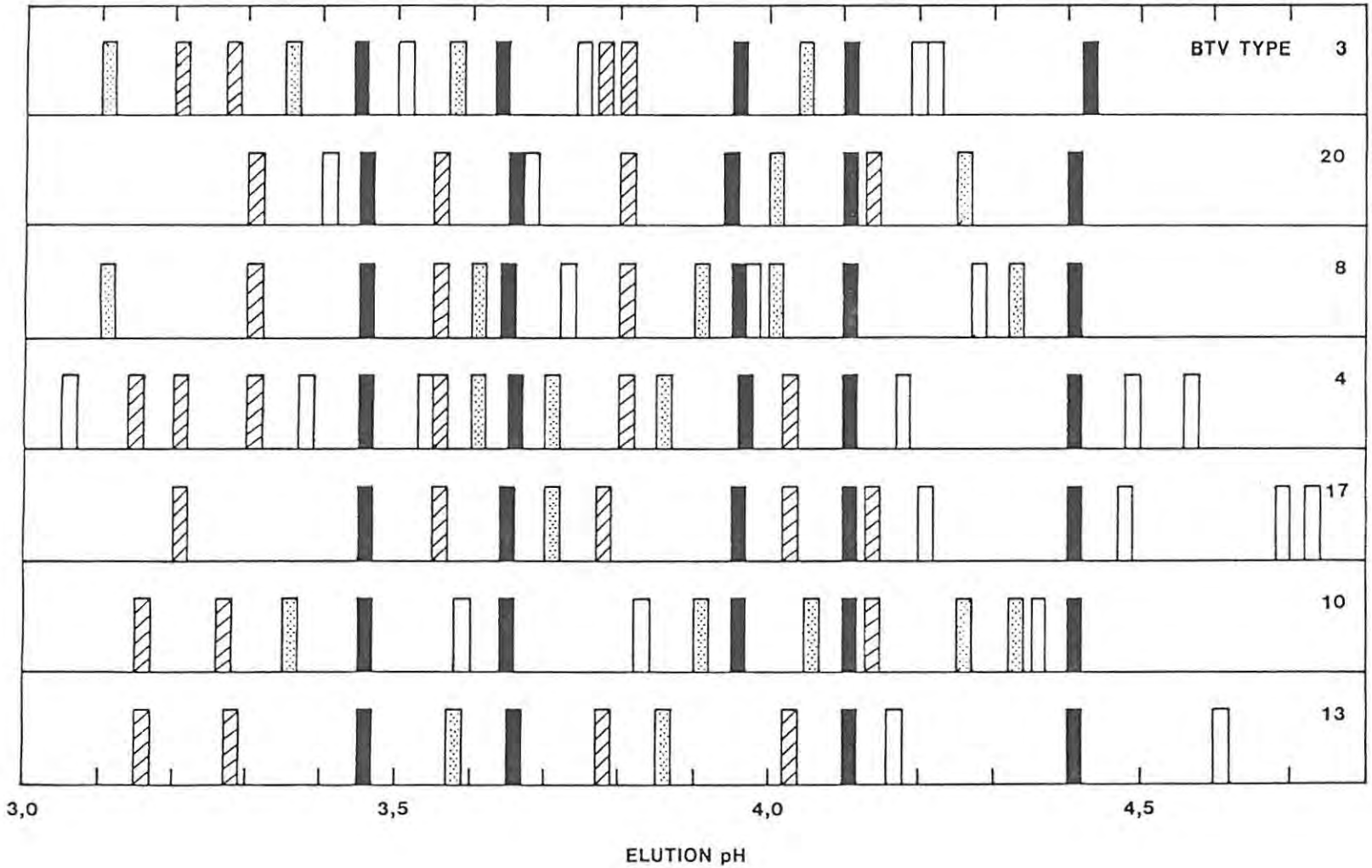


FIGURE 4.7: Diagrammatic representation of the tryptic peptides from 7 different BTV group antigens separated by ion exchange chromatography.

- ■ peptides common to all serotypes
- ▨ peptides common to 3 or 4 serotypes
- ▩ peptides common to 2 serotypes
- □ unique peptides.

represent highly conserved areas in the protein chain and it is likely that some or all of them may carry the antigenic domains for group specificity. These conserved areas of P7 are probably crucial for protein function, whereas the nonconserved areas may be lost to mutation without affecting the function of the protein. At this stage it is difficult to assess the degree to which the primary structure of this protein has been conserved between the serotypes. This would be possible if more structural information was obtained from peptide maps using different proteolytic enzymes.

The presence of common peptides between some of the serotypes suggests a common ancestry. The relatedness of the group antigens of the different BTV serotypes was assessed by comparing the number of identical peptides shared between each serotype. The inter-relationships obtained between the BTV serotypes are summarized diagrammatically in Fig. 4.8.

Of the seven serotypes used for this analysis the most closely related (as determined by structural analysis of the group antigens) are BTV-4 and 17 and BTV-8 and 20. These serotypes appear to form a central core, with BTV-4, 8 and 17 forming the predominant links with the remaining serotypes.

Probably the most controversial area of the relationships among the BTV serotypes lies with the inter-relationships of BTV-4, 10, 17 and 20. One of the aims of this investigation was to determine how closely the Australian serotype, BTV-20 is related to existing BTV serotypes such as BTV-4 and BTV-10.

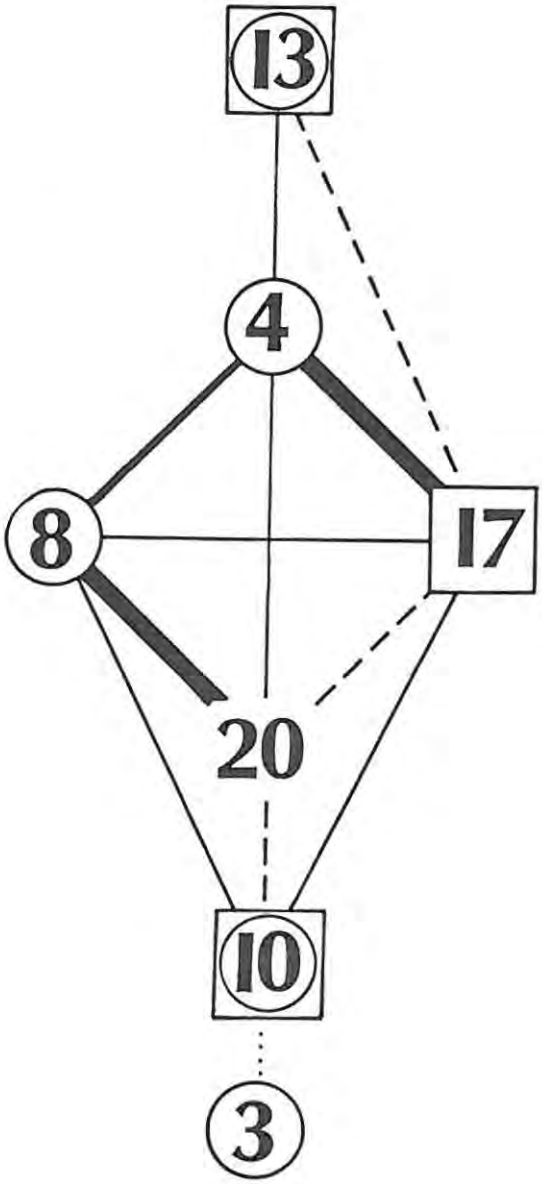


FIGURE 4.8: Diagrammatic representation of the interrelationships of different BTV serotypes assessed through the structural relatedness of their group antigens.

3 BTV serotype

○ South African serotypes

□ North American serotypes

20 Australian serotypes

The stronger the intensity of the connecting line the closer the structural relatedness of the group antigen.

Structurally the BTV-20 group antigen is most similar to that of BTV-8. Not surprisingly it also shares several peptides (over and above the 5 peptides common to all serotypes) with BTV-10 and BTV-4; the two other serotypes with which BTV-8 group antigen has close structural similarity. However, the peptides common to BTV-4 and 20 are identical to some of those shared by BTV-8 and BTV-20, (i.e. BTV-4, 8 and 20 have common peptides). But the peptides common to BTV-20 and BTV-10 are not present in either the BTV-4 or BTV-8 group antigens. BTV-17 appears to be the "bridging" serotype in this case as the peptides it has in common with BTV-20 are some of those of BTV-10 and others of BTV-4 and BTV-8.

The structural relationships between these serotypes are complex; although BTV-10 is related to BTV-8 and 8 is structurally the most similar to BTV-20, BTV-10 does not appear to be related to BTV-20 through BTV-8 but rather through BTV-17. Whereas BTV-4 is probably related to BTV-20 through BTV-8.

A cross-section of serotypes found in the U.S., Australia and South Africa were used in this study and it is seen in Fig. 4.8 that serotypes from each of these countries form part of the central core of closely related serotypes. This result may have important epidemiological implications in the spread of BTV to other countries. However, too little information is available for such speculations.

Two of the three American serotypes used in this study are also found in South Africa, (i.e. BTV-13 and BTV-10). Therefore, one would imagine that these South African/U.S. serotypes would be

closely related, however, the pattern of interrelationships appears to be far more complex. BTV-13 shows the strongest structural similarity to BTV-4 and as BTV-17 is very closely related to BTV-4 one would imagine that BTV-17 and BTV-13 would be structurally similar. However, this does not appear to be so. The BTV-10 and 17 group antigens show a degree of structural similarity which indicates that they are fairly closely related (Fig. 4.8). Relative to the other serotypes used both BTV-10 and BTV-17 are distantly related to BTV-13. However, the converse of this is not true, because BTV-13 is more similar to BTV-10 and BTV-17 than to the other serotypes (except BTV-4). Even so, this structural similarity is not as great as that shown for the serotypes making up the central core and it is felt that structurally BTV-13 can be considered the least related of the U.S. serotypes analyzed.

The BTV-3 group antigen appears to be the most structurally divergent, and is therefore very much on the periphery of the interrelationships of the serotypes in Fig. 4.8.

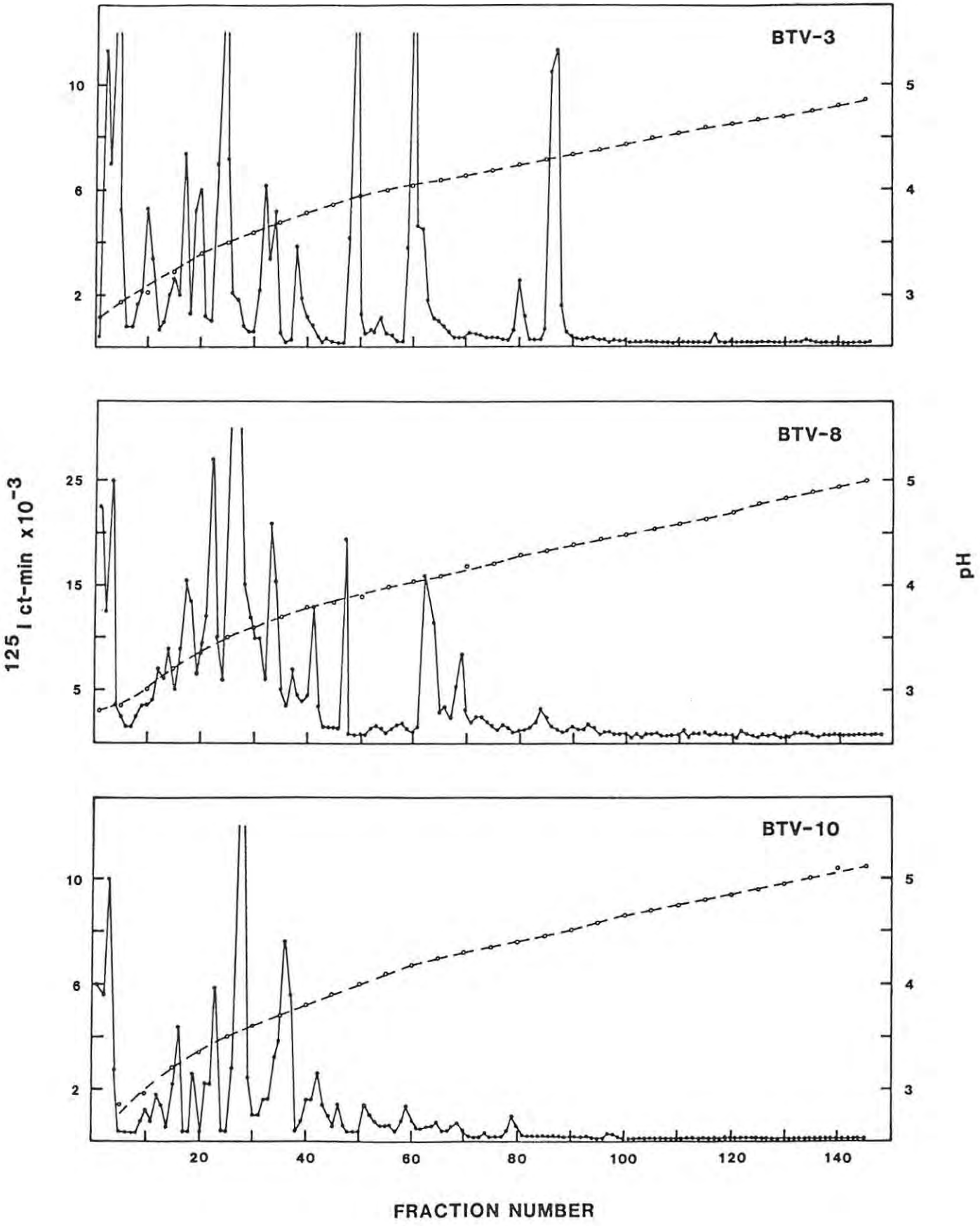
It must be emphasized that these structural comparisons are being made on the information obtained from the tyrosine-containing tryptic peptides only. A large portion of the group antigen is probably not being examined. This is obvious when one compares the number of peptides detected in the ^{35}S-methionine labelled group antigen map (Fig. 4.5) with the number detected in the ^{125}I-labelled group antigen maps (Fig. 4.6). More information could be obtained on the structure of the group antigens by using other proteolytic enzymes to generate a different set of peptides. This would enable more stringent comparisons to be made between

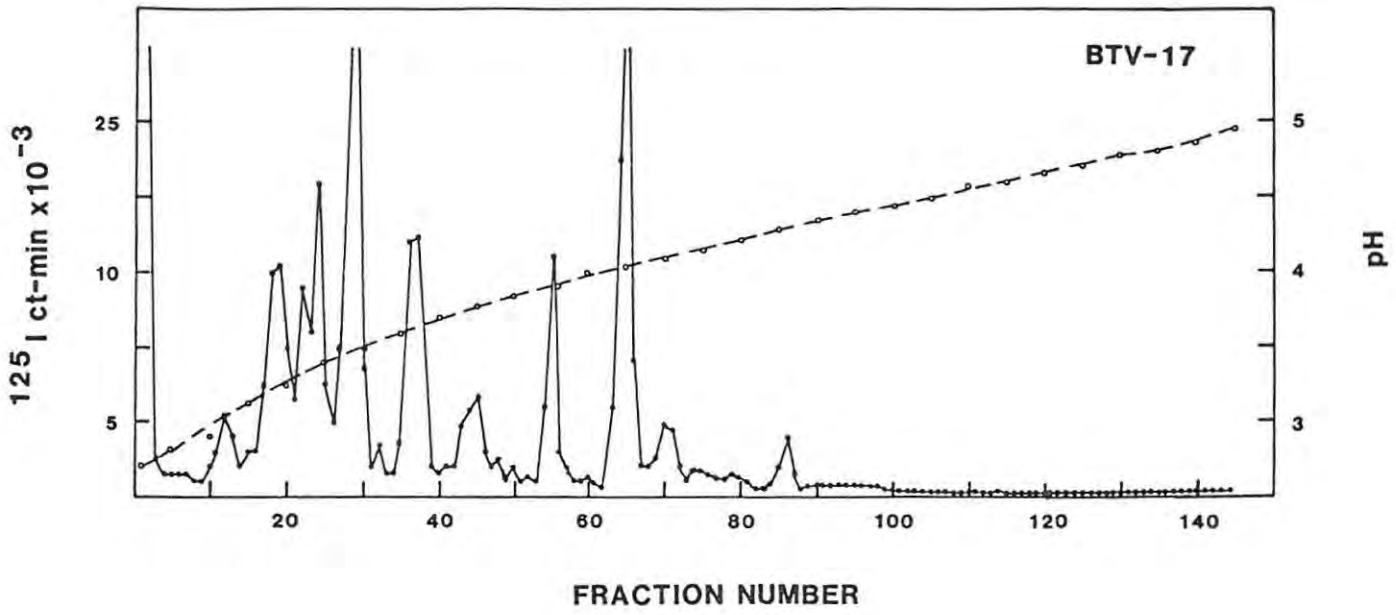
the structure of the group antigens and therefore one would be able to assess the relatedness of the serotypes far better.

BTV P3 is co-precipitated with P7 in heterotypic immune reactions and as agar gel diffusion was used for the isolation of P7, P3 was easily obtained. Because of this several peptide maps were done on P3 to see if this protein could possibly play a role in the group specific response and to examine to what extent the primary structure of this protein was conserved between the different serotypes.

The tryptic peptide maps of ^{125}I-labelled P3 from BTV-3, 8, 10 and 17 are shown in Fig. 4.9. As with P7, the results were analyzed by determining the pH at which the peptide peaks eluted and plotting these values in a histogram (Fig. 4.10). No tyrosine-containing tryptic peptides are found to be common to all four serotypes, there are two peptides common in three serotypes and several peptides shared between two serotypes. From these results it would appear that either P3 contains no group-specific antigenic determinants, as there are no peptides common to all serotypes or that the tyrosine-containing tryptic peptides detected in these maps do not reflect the structure of P3 adequately. The latter is definitely true as the number of peptides detected for P3 are similar to the numbers detected for P7. P3 is approximately three times larger than P7, therefore, we are looking at approximately one third less structural information in these maps. More structural information can be obtained using different proteases, therefore several studies were done on P3 using S. aureus V8 protease to generate a different set of peptides. These maps are shown in Fig. 4.11 and the histograms obtained given in Fig. 4.12.

FIGURE 4.9 Ion exchange chromatography profiles of ^{125}I-labelled P3 tryptic peptides from 4 different BTV serotypes.


BTV-3
BTV-8
BTV-10
^{125}I ct-min x10^{-3}
pH
FRACTION NUMBER
10
6
2
25
15
5
20
40
60
80
100
120
140
3
4
5


BTV-17
^{125}I ct-min x10^{-3}
25
10
5
5
4
3
pH
20
40
60
80
100
120
140
FRACTION NUMBER

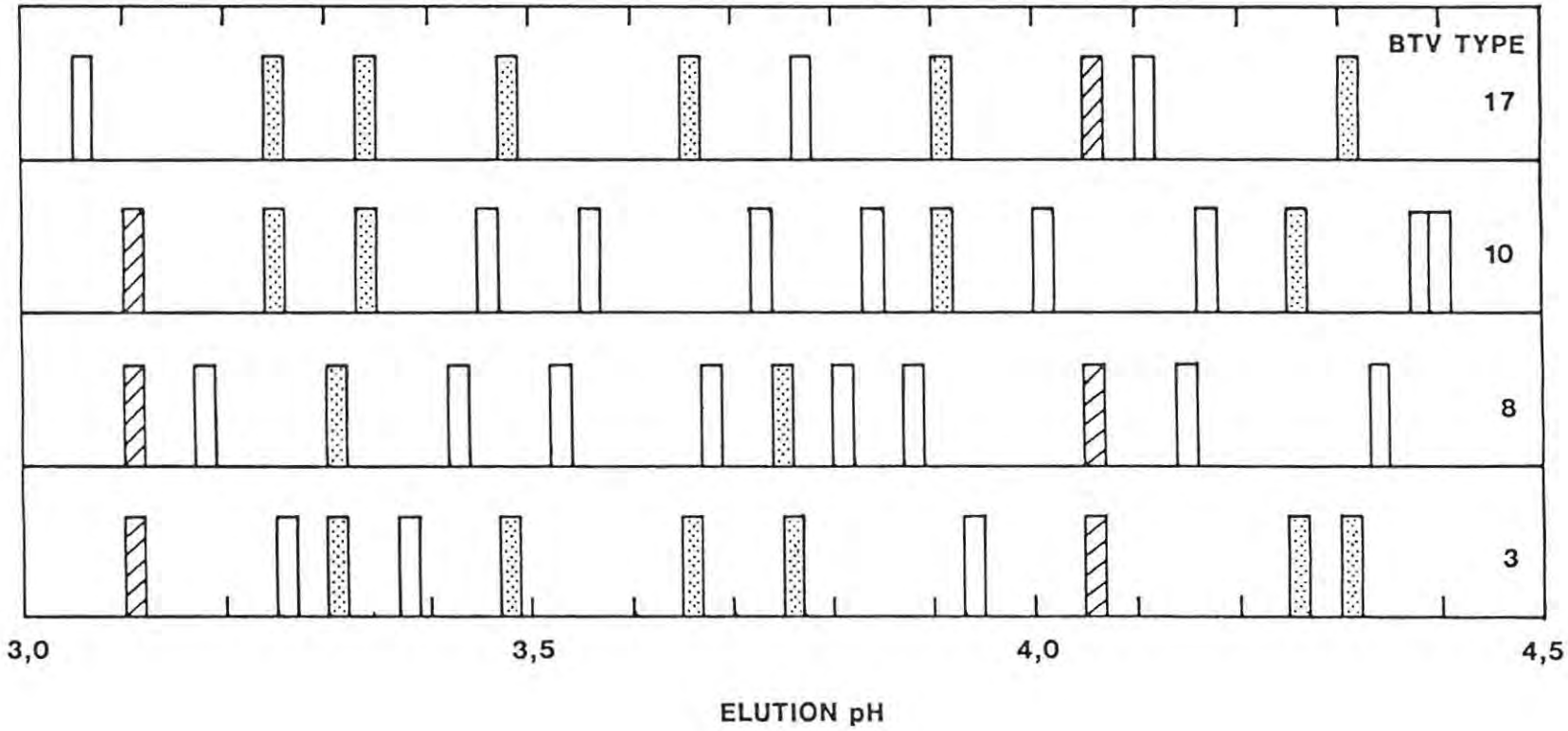
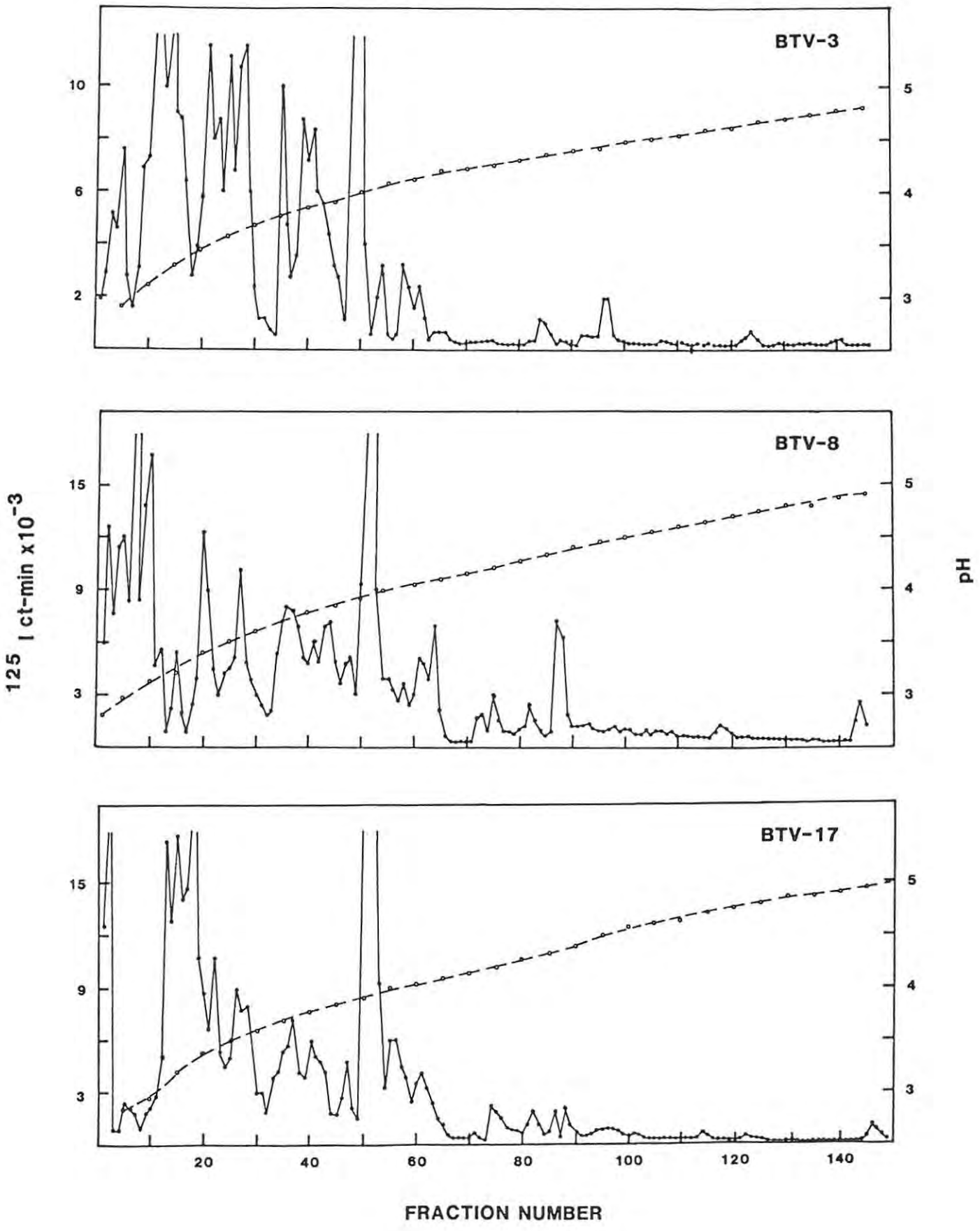


FIGURE 4.10: Diagrammatic representation of the tryptic peptides from 4 different BTV P3s separated by ion exchange chromatography.

- ▨ peptides common to 3 serotypes
- ⁘ peptides common to 2 serotypes
- □ unique peptides

FIGURE 4.11: Ion exchange chromatography profiles of ^{125}I-labelled P3 S. aureus V8 protease peptides from 3 different BTV serotypes.


BTV-3
BTV-8
BTV-17
125 I ct-min x10^-3
pH
FRACTION NUMBER
10
6
2
15
9
3
5
4
3
20
40
60
80
100
120
140

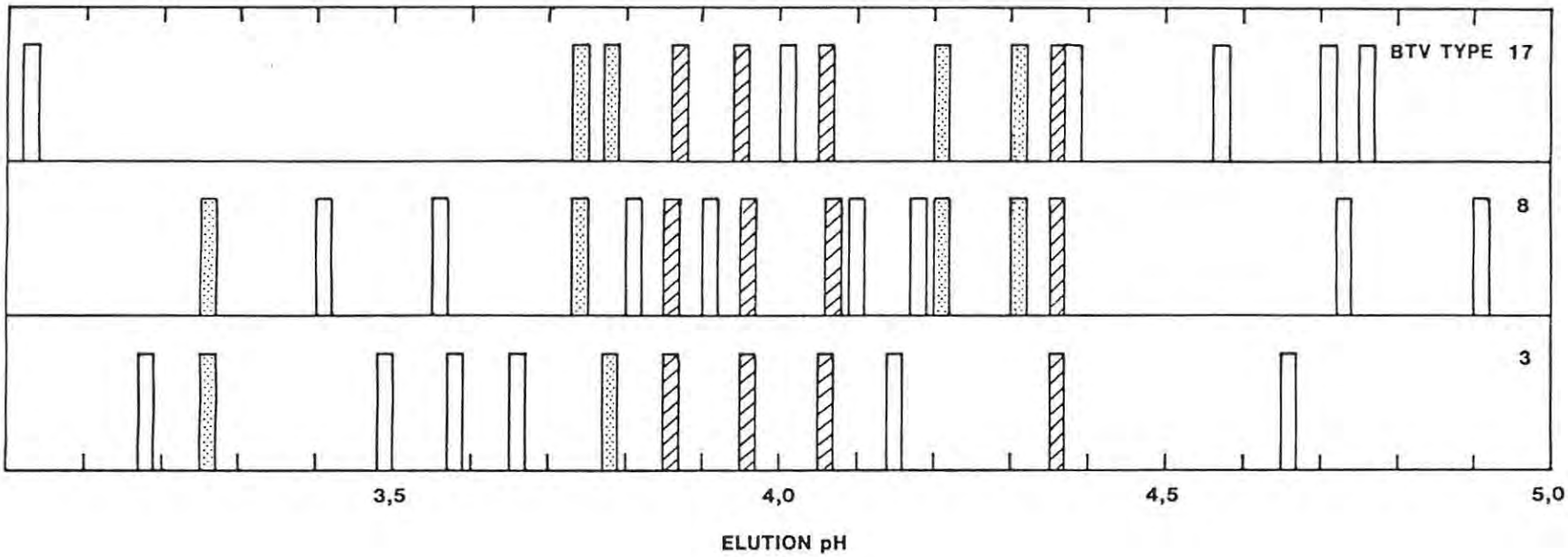


FIGURE 4.12: Diagrammatic representation of the S. aureus V8 protease peptides from 3 different BTV P3s separated by ion exchange chromatography.

- ▨ peptides common to 3 serotypes
- ⁘ peptides common to 2 serotypes
- □ unique peptides

Four tyrosine-containing peptides have been produced with this protease which are common to BTV-3, 8 and 17. It is difficult to assess the importance of these conserved areas of protein sequence as so few serotypes were mapped. Furthermore, because of the size of P3 several other proteases should be used to estimate the extent to which the protein sequence is conserved between serotypes. Only when this has been determined will it be possible to say whether the core polypeptide, P3, of BTV can be used to determine the relatedness of the BTV serotypes and whether it may have a role in the group specific response of these viruses.

4.4 DISCUSSION

Serological tests alone are inadequate for assessing the relationships between orbiviruses. Orbiviruses contain 10 segments of dsRNA each of which is the gene for a single protein.[32; 112] The group-reactive antigens in bluetongue viruses are found mainly on the core protein of purified virions and the type specificity is determined by antigens located on one of the surface proteins.[52] Thus the conventional serological tests do not allow a detailed comparison of the 10 proteins of bluetongue and related viruses.

The aim of this research was to initiate a study on the relationships between the structural components of different BTV serotypes in an attempt to define the molecular basis for the serological relationships between them and also to detect possible relationships between the viruses which may not be detected in conventional serological tests.

Several workers have tried to establish how closely the Australian bluetongue isolate, BTV-20, is related to other BTV serotypes. This has important epidemiological implications for the spread of BTV and could indicate the route of introduction of the virus into Australia.

Serological studies indicate that there is a close immunological relatedness between BTV-4, which appears to be the most widespread of all the BT serotypes, and BTV-20.[18; 99] They are the only two strains that cross-react both ways in immune precipitation studies.[50] Furthermore, serum from BTV-20 and BTV-4 both precipitate P2 from BTV-10, whereas P2 from BTV-4 and 20 are both precipitated by BTV-17 immune serum (Fig. 1.1). Della Porta et al (1979) suggested that BTV-20 is a subtype of BTV type 4 and that BTV-4 may be distantly related to BTV-17.

In contrast to the serological evidence, biochemical studies, which examined nucleic acid homology between several serotypes by cross-hybridization, indicate that BTV-20 and BTV-4 are very different at the gene level.[34; 50] Huismans and Bremer (1981) showed that BTV-10 and 4 have approximately 71-77% homology. This agrees with the previously described values for the homology between different BTV serotypes.[53;110] The homology between serotypes 20 and 4 was in the order of 20-30% and that between BTV-20 and BTV-10 about 19-22%. The homology between BTV-17 and BTV-20 was approximately 25% as against 70% homology between BTV-17 and the other serotypes.[50] These hybridization results indicate that BTV-4 and BTV-20 are neither identical nor even closely related.

Analysis of the RNA hybrids from BTV-1 to 19 by PAGE showed 6 or 7 of the 10 RNA segments were closely related,[53] whereas only two related segments between BTV-20 and BTV-4 were detected.[50] The segments that were related were RNA segments 7 and 10 possibly coding for the viral protein associated with the group CF antigen. These biochemical results would appear to agree with serological results which indicate that BTV-20 contains the group antigen involved in the CF test

for BTV;[99] but are in contrast to the close antigenic relationships between BTV-20 and BTV-4 reported by Della-Porta et al (1981) and Huismans and Bremer (1981).

These results showed that further biochemical studies were required to clearly define the position of BTV-20. The study undertaken here involved examining the degree of structural relatedness of the group antigen from different BTV serotypes by tryptic peptide mapping. This protein was the obvious choice for initial biochemical studies as it carries the determinants for group specificity and has the most conserved structure of all the viral proteins (as judged by nucleic acid hybridization results[50; 53]). The conserved primary structure probably has something to do with protein function.

For this initial study seven serotypes were chosen, including BTV-4, 10, 17 and 20. Of the seven serotypes analyzed, BTV-4 and BTV-17 have the greatest similarity in group antigen structure indicating that they are the most closely related of these BTV serotypes. BTV-4 is related to BTV-20, but this appears to be through BTV-8, the serotype with which BTV-20 shows the closest structural homology with respect to group antigen. These results do not support the suggestion of Della Porta et al (1979) that BTV-20 is a subtype of BTV-4. The interrelationship between BTV-20, 17 and 10 appears to be a little more complex. These serotypes are more distantly related to BTV-20 than either BTV-4 or BTV-8. BTV-10 shares a different, smaller group of peptides with BTV-20 than BTV-4 and BTV-8; whilst BTV-17 has peptides in common with BTV-4, 8 and 20 and BTV-10 and 20. It is possible that BTV-17 is a "bridging" strain between these serotypes with respect to their inter-relationships with BTV-20. From Fig. 4.8 it is apparent that BTV-4, 8, 17 and 20 form a cluster as they appear to be the most closely related

of the serotypes analyzed. BTV-10 appears to be linked to this cluster through its similarities with BTV-8 and BTV-17.

These results add a new perspective to the relationship of the Australian BTV type 20 to the other BTV serotypes. Nucleic acid hybridization results show BTV-20 is very different to these serotypes on the gene level[34; 50] and cross-immune precipitation of P2 indicates a strong immunological relatedness to BTV-4.[18;50;99] The structural analysis of P7 shows BTV-20 to be most closely related to BTV-8. These results do not actually conflict when one considers that the striking immunological relatedness between BTV-4 and BTV-20 does not mean that there has to be a general homology between all the genome segments; but rather that there are very specific homologies between the genome segments coding for the group antigen (probably segment 7) and the common antigenic determinants on the serotype specific antigen P2.

Serological tests cannot be used to estimate the relatedness of **each** of the genome segments of orbiviruses, and nucleic acid hybridization results refer to the degree of homology between the **total** genomes of the strains. Difficulties arise in assessing the degree of hybridization, except on a broad basis, and it is difficult to define interacting serotypes. Therefore, the simplist and probably most precise way in which to determine the relatedness of the different serotypes is by comparing the structures of the viral proteins. As stated earlier the protein most likely to reflect the relatedness of serotypes is P7.

The interrelationships between the three American BTV serotypes used in this analysis confirm the results of Appleton and Letchworth (1983). They found that BTV-13 could be distinguished from the other U.S. serotypes (BTV-10, 11 and 17) by several monoclonal antibodies which

precipitated P7. The tryptic peptide mapping results show that structurally the BTV-13 group antigen is less similar than the group antigens of BTV-10 and BTV-17.

The South African serotype, BTV-3 is the most divergent of these seven serotypes, it has the least number of peptides in common with any of the serotypes. However, it probably has a greater structural similarity to other types not included in this study.

Although steps have been made to determine the relatedness of the different BT serotypes, the results are not yet clear cut. Firstly, the relationships shown here between BTV-4, 8, 10, 17 and 20 have to be more carefully defined. A more comprehensive structural analysis needs to be done on the group antigens to ensure that the relationships are as what has been determined here. This involves further peptide mapping analyses using several different proteases to give a more stringent test of structural similarity.

Secondly, one has to define the relationships occurring between all 20 BTV serotypes. Low-level cross-reactions between serotypes have been observed by Davies and Blackburn (1971) for Kenyan BTV isolates which react strongly with anti-BTV-1 serum and weakly with anti-BTV-4 serum, whereas Howell et al (1970) found that South African BTV isolates react specifically in the plaque-inhibition test. Furthermore, Davies (1978) reports that a Kenyan BTV-11 isolate shows some relationship with BTV type 14. Therefore, these relationships also need to be defined.

It is possible that there may be intermediate or bridging types of BTV and that separation into 20 distinct serotypes, based on serum neutralization tests, may not represent the actual situation. Far too few

serotypes were examined in this study to be able to support or refute this idea. However, the concept of a continuum hypothesis has desireable attributes from a virus evolution standpoint - notably evolution under immune pressure by the host population. Classification by reference to concepts of evolutionary species would define genetically interacting groups, estimate the extent of diversity within these groups and establish phylogenetic relationships between species.

Heterologous immune precipitates show precipitation of polypeptides P3, P6a and P7.[50; 52] Polypeptide P6a is a non-capsid polypeptide[49] whereas P3 and P7 are both major components of the BTV core particle. Polypeptide P7 has been shown to be the group-specific antigen.[2;37;50;52] However, at this stage it is impossible to exclude a contributory role for other virus polypeptides, such as P3 or the minor polypeptide components, in the group-specific response.

As P3 was co-purified with P7, several peptide maps were performed to try and establish the molecular basis for the precipitation of P3 with homologous and heterologous antisera. The tyrosine-containing tryptic peptides revealed no conserved sequences between all four serotypes analyzed; several peptides were found to be common between 2 or 3 serotypes. Further analysis of P3 with S. aureus V8 protease revealed several common peptides, but it is difficult to assess if these are likely to carry group-specific antigenic determinants as so few serotypes were analyzed.

Appleton and Letchworth (1983) showed that two monoclonal antibodies, which had neutralizing activity and inhibited haemagglutination, precipitated P3 in addition to P2. They postulated that these proteins may be distinct, but share epitopes; they could be modified forms of the same protein or are associated in some way during viral replication.

It would be interesting to extend this study, not only to examine P3 for peptides which may possibly carry group-specific epitopes, but also to see if any of the above postulates are true. This could be done by comparing the structure of P2 and P3 by peptide mapping.

GENERAL DISCUSSION AND CONCLUSIONS

The aim of this research was to study the relationships between the structural components of selected viruses of the bluetongue serogroup of orbiviruses in an attempt to define the molecular basis for the serological reactions between them and also detect possible relationships between the viruses which may not be detected by conventional serological tests.

Techniques have been developed for the isolation of viral proteins using agar gel diffusion and their subsequent radio-iodination using an adaption of the Chloramine-T method. Comparative structural analysis of the viral proteins was achieved by peptide mapping on an ion exchange chromatography column.

In this initial study the interrelationships between the Australian bluetongue isolate, BTV-20, and several other BTV types from South Africa and the United States were established.

A small dent has been made in the assessment of the interrelationships of the different BTV types. This study needs to be continued so that the interrelationships of the remaining BTV serotypes can be determined. Furthermore, other viruses need to be drawn into this study, for example, members of the epizootic haemorrhagic disease of deer serogroup and the Eubenangee serogroup as these viruses have been shown to be related to the BT serogroup.[6;32;80]

Such structural analyses should provide insight into the different biological properties of the viral proteins, give a greater understanding of the serological results and help in the development of concepts of virus evolution.

APPENDIX A

SDS - SLAB GEL ELECTROPHORESIS

(Laemmli 1970)

STOCK SOLUTIONS:

Resolving Gel Buffer Stock Solution (1M Tris-HCl pH 8.8)

Tris	60.6g
Conc. HCl	7.3mℓ
Distilled water to	500mℓ

Stacking Gel Buffer Stock Solution (1M Tris-HCl pH 6.8)

Tris	60.6g
Conc. HCl	41.0mℓ
Distilled water to	500mℓ

Electrode Buffer Stock Solution (0.25M Tris, 1.92M Glycine 1% SDS pH 8.3)

Tris	30.3g
Glycine	144.1g
SDS	10.0g
Distilled water to	1ℓ

Dilute 10 fold before use

Acrylamide - Bisacrylamide Stock Solution (30 : 0.8)

Acrylamide	150.0g
N,N'-bis-methylene acrylamide	4.0g
Distilled water to	500mℓ

Dissociation Buffer Stock Solution

SDS	10.0g
2-mercaptoethanol	10.0 mℓ
1.25M Tris-HCl pH 6.8 to	100mℓ

SAMPLE TREATMENT:

A 1/5 volume of dissociation buffer stock solution is added to the sample along with 2uℓ Bromophenol blue and a few grains of sucrose to increase the sample density. Samples are then heated at 100°C for 2 minutes prior to loading into wells.

SLAB GEL FORMULATIONS:

Resolving Gel:

STOCK SOLUTION	Final Acrylamide Gel Concentration (%)		
	10	12	15
Acrylamide - bisacrylamide	26.7	32.0	40.0
Resolving gel buffer	30.0	30.0	30.0
Distilled water +	18.5	13.2	5.2
10% SDS (w/v)	0.8	0.8	0.8
1.5% fresh $NH_4S_2O_8$ (w/v)	4.0	4.0	4.0
Temed (uℓ)	20	20	20

+ Degas solution for 5 minutes before the addition of remaining solutions

Total volume : 80mℓ

Other % acrylamide calculation:

(i) $\frac{\%\ \text{required}}{30} \times 80 = m\ell$ acrylamide

(ii) $m\ell$ acrylamide + $m\ell$ distilled water = 45.2

Stacking Gel

STOCK SOLUTION	4% ACRYLAMIDE
Acrylamide - bisacrylamide	2.0
Stacking Gel Buffer	1.9
Distilled Water	9.25
10% SDS (w/v)	0.15
80% glycerol	1.0
1.5% fresh $NH_4S_2O_8$	0.7
Temed (uℓ)	20

ELECTROPHORETIC RUNS:

All gels were run on the Bio-Rad Model 220 dual vertical slab electrophoresis cell. Electrophoresis was at 50V until the Bromophenol blue (BPB) tracking dye reached the top of the resolving gel, after which electrophoresis was at 100V until the BPB reached the bottom of the resolving gel.

STAINING SOLUTIONS

1. **Coomassie Brilliant Blue:**

Stain

Methanol	45mℓ
Glacial acetic acid	10mℓ
Coomassie brilliant blue	0.2g
Distilled water	45mℓ

The dye was dissolved in a small amount of methanol and filtered. Then the remaining methanol, water and acetic acid were added. The gel was stained for 2 - 3 hours with gentle shaking to prevent the gel sticking to the container.

Destain

Methanol	450mℓ
Glacial acetic acid	70mℓ
Distilled water	480mℓ

The gel is destained until the background is clear.

2. **Silver Stain**

Stock Solutions:

Silver Reagent

Silver Nitrate	19g
Distilled/deionized water to	1ℓ

Dilute 10 fold before use.

Oxidizer

Potassium dichromate	10g
Nitric acid	2mℓ
Distilled/deionized water to	1ℓ

Dilute 10 fold before use.

Developer

Sodium hydroxide	30g
37% Formaldehyde	7.5mℓ
Sodium borohydride	100mg
Distilled/deionized water to	1ℓ

Silver Stain Protocol:

Gloves must be worn for all manipulations.

REAGENT	Volume (mℓ)	Duration for 1mm thick gels (min)
1. Fixative 40% methanol/10% acetic acid	400	30
2. Fixative 10% ethanol / 5% acetic acid	400	15
3. Fixative 10% ethanol / 5% acetic acid	400	15
4. Oxidizer	200	5
5. Deionized water	400	5
6. Deionized water	400	5
7. Silver reagent	200	20
8. Deionized water	400	1
9. Developer	200	0.5
10. Developer	200	~5
11. Developer	200	~5
12. Stop 5% acetic acid	400	5

GEL DEHYDRATION:

The gel to be preserved was placed on a piece of Whatmann No. 3MM filter paper, transferred to a vacuum drying apparatus, covered with polythene and vacuum dried for 6 - 8 hours.

AUTORADIOGRAPHY:

Radiolabelled proteins present in dehydrated polyacrylamide gels or on TLC plates were visualized by autoradiography. The gel or plate was taped into a glass sandwich frame and covered with a thin sheet of polythene. The X-ray film was placed on top of this and the sandwich frame taped closed. This was wrapped in two layers of aluminium foil. The X-ray film was exposed until the correct intensities were obtained and then developed according to the manufacturers instructions.

Three different X-ray films were tried: Kodak SB-5 Medical X-ray film, Agfa gevaert Curix X-ray film and Cronex MRF-31 Medical X-ray film. The Cronex MRF-31 X-ray film was found to give the best results with the least fogged background.

MOLECULAR MASS MARKERS:

The molecular mass markers used in all experiments were products of Pharmacia Fine Chemicals. Each vial contained the proteins listed in Table A1.

TABLE A1: PHARMACIA FINE CHEMICALS MOLECULAR MASS MARKER PROTEINS.

PROTEIN	ug/vial	Molecular Mass
Phosphorylase B	64	94 000
Bovine Serum Albumin	83	67 000
Ovalbumin	147	43 000
Carbonic anhydrase	83	30 000
Soybean trypsin inhibitor	80	20 100
∝-Lactalbumin	121	14 400

The contents of each vial were reconstituted with 200uℓ of 5 times concentrate Laemmli dissociation buffer, divided into 20uℓ alliquots and stored at -20°C. Just before use the following reagents were added:

Laemmli dissociation buffer	45uℓ
Distilled water	100uℓ
Bromophenol blue	2uℓ
Sucrose	a few grains

Samples were heated at 100°C for 2 minutes and 40uℓ used per track.

APPENDIX B

GENERAL METHODS

OUCHTERLONY GELS

The diffusion medium contained 1% purified Oxoid agar in 0.05M phosphate buffer, pH 7.2; sodium chloride and sodium azide were added to a concentration of 0.86% and 0.1% respectively. The medium was autoclaved for 10 minutes at 20 pounds per square inch. Plates were prepared by adding 15mℓ of agar medium into each 8.5cm diameter Petri dish. These were stored upside down at 4°C.

RADIOACTIVITY DETERMINATIONS USING LIQUID SCINTILLATION COUNTING

1. AQUEOUS SAMPLES: 10 to 100uℓ of sample was added to a pre-counted vial containing 10mℓ of Ready-Solv EP (Beckman Instruments), mixed and counted in the Beckman Model LS 3150T liquid scintillation counter. ^{125}I-labelled samples were counted in the open ^{3}H window and ^{35}S-methionine labelled proteins in the open ^{14}C window.

2. GEL SLICES: The gel slice was placed in the scintillation vial and 200uℓ of Soluene (Packard Instruments) and 5mℓ of Ready-Solv EP was added. The solutions were mixed and incubated overnight at 60°C. The vials were then counted in the appropriate channel of the liquid scintillation counter.

REFERENCES

1. Allington, W.B., Cordry, A.L., McCullough, G.A., Mitchell, D.E. and Nelson, J.W. (1978). Electrophoretic concentration of macromolecules. Anal. Biochem. 85: 188-196.

2. Appleton, J.A. & Letchworth, G.J. (1983). Monoclonal antibody analysis of serotype-restricted and unrestricted BTV antigenic determinants. Virology, 124: 286-299.

3. Barber, T.L. & Jochim, M.M. (1973). Serological characterization of selected Bluetongue virus strains from the United States. Proc. 77th Annu. Meet. U.S. Anim. Hlth Assoc., pp. 352-359.

4. Barber, T.L. & Jochim, M.M. (1975). Serotyping Bluetongue and Epizootic haemorrhagic disease virus strains. Proc. 18th Annu. Meet. Am. Assoc. Vet. Lab. Diag. Portland, Ore. pp. 149-162.

5. Bolton, A.E. (1977). Radio-iodination Techniques. (Review 18) Radiochemical Centre, Amersham, England.

6. Borden, E.C., Shope, R.E. & Murphy, F.A. (1971). Physiochemical and morphological relationships of some arthropod-borne viruses to Bluetongue virus - A new taxonomic group. Physiochemical and Serological Studies. J. Gen. Virol. 13: 261-267.

7. Bowne, J.G. & Jones, R.H. (1966). Observations on Bluetongue virus in the salivary glands of an insect vector Culicoides variipennis. Virology, 30: 127-133.

8. Brust, J. & Fasold, H. (1981). Microfingerprints of proteins through labelling acetylation of their proteolytic peptides. Anal. Biochem. 106: 268-272.

9. Buchmeier, M.J., Lewicki, H.A., Tomori, O. and Johnson, K.M. (1980). Monoclonal antibodies to lymphocytic choriomeningitis virus react with pathogenic arenaviruses. Nature (London), 288: 486-487.

10. Burroughs, J.N., Doel, T.R. & Brown, F. (1978). Relationship of San Miguel Sea Lion virus to other members of the Calicivirus group. Intervirology, 10: 51-59.

11. Campbell, C.H., Barber, T.L. & Jochim, M.M. (1978). Antigenic relationship of Ibaraki, Bluetongue and Epizootic haemorrhagic disease viruses. Vet. Microbiol. 3: 15.

12. Cheseboro, B., Wehlry, K., Cloyd, K., Britt, M., Portis, J., Collins, J. and Nishio, J. (1981). Characterization of mouse monoclonal antibodies specific for Friend Murine Leukemia Virus-induced erythroleukemia cells: Friend-specific and FMR-specific antigens. Virology. 112: 131-144.

13. Cleveland, P.W., Fischer, S.G., Kirschner, M.W. & Laemmli, U.K. (1977). Peptide mapping by limited proteolysis in sodium dodecyl sulphate and analysis by gel electrophoresis. J. Biol. Chem. 252: 1102-1106.

14. Davies, F.G. (1978). Bluetongue studies with sentinel cattle in Kenya. J. Hyg. Camb., 80: 197-204.

15. Davies, F.G. & Blackburn, N.K. (1971). The typing of Bluetongue virus. Res. Vet. Sci. 12: 181-183.

16. Davis, B.D., Dulbecco, R., Eisen, H.N., Ginsberg, H.S. & Wood, W.B. (1973). Computation of 50% endpoints (LD_{50} or ID_{50}) by method of Reed and Muench in Microbiology. (Second edition). pp. 663-664. Harper and Row, New York.

17. Della-Porta, A.J., Herniman, K.A.J. & Sellers, R.A.F. (1981). A serological comparison of the Australian isolate of BTV type 20 (CSIRO 19) with BT group viruses. Vet. Microbiol. 6: 9-21.

18. Della-Porte, A.J., McPhee, D.A. & Snowdon, W.A. (1979). The serological relationships of Orbiviruses. Second Arbovirus Symposium. pp. 64-71.

19. De Villiers, E.M. (1974). Comparison of the capsid polypeptides of various Bluetongue virus serotypes. Intervirology, 3: 47-53.

20. Dietzschold, B., Kaaden, O.R. & Ahl, R. (1972). Hybridization studies with subtypes and mutants of Foot-and-Mouth-Disease virus type O. J. Gen. Virol. 15: 171-174.

21. Dunker, A.K. & Rueckert, R.R. (1969). Observations on MW determinations on polyacrylamide gel. J. Biol. Chem. 244 5074-5080.

22. Elder, J.H., Pickett, R.A., Hampton, J. & Lerner, A. (1977). Radioiodination of proteins in single polyacrylamide gel slices. J. Biol. Chem. 252: 6510-6515.

23. Els, H.J. (1973). Electron Microscopy of Bluetongue virus RNA. Onderstepoort J. Vet. Res. 40: 73-76.

24. Els, H.J. & Verwoerd, D.W. (1969). Morphology of Bluetongue virus. Virology, 38: 213-219.

25. Fietelson, M.A., Wettstein, F.O. & Stevens, J.G. (1981). Tryptic peptide mapping of picomolar quantities of protein labelled with the Bolton-Hunter reagent. Anal. Biochem. 116: 473-479.

26. Foster, N.M. & Alders, M.A. (1979). Bluetongue Virus : A membraned structure. 37th Ann. Proc. Electron Microscopy Soc. Amer. San Antonio, Texas 1979. G.W. Bailey (Ed.). pp. 48-49.

27. Foster, N.M., Alders, M.A. & Walton, T.E. (1978). Continuity of the dsRNA genome of Bluetongue virus. Current Microbiology, 1: 171-174.

28. Friesen, P., Scotti, P., Longworth, J. & Rueckert, R. (1980). Black Beetle Virus : Propogation in Drosopholia Line 1 cells and an infection-resistant subline carrying endogenous Black Beetle Virus-related particles. J. Virol. 35: 741-747.

29. Gentsch, J.R. & Fields, B.N. (1981). Tryptic peptide analysis of the outer capsid polypeptides of mammalian reovirus serotypes 1, 2 and 3. J. Virol. 38: 208-218.

30. Gerhard, W., Yewdell, J., Frankel, M.E. & Webster, R. (1981). Antigenic structure of Influenza virus haemagglutinin defined by hybridoma antibodies. Nature (London). 290: 713-716.

31. Gething, M.J., Bye, J., Skekel, J. & Waterfield, M. (1980). Cloning and DNA sequence of double-stranded copies of haemagglutinin genes from H2 and H3 strains elucidates antigenic shift and drift in human influenza virus. Nature (London). 287: 301-306.

32. Gorman, B.M. (1979). Variation in Orbiviruses. J. Gen. Virol. 44: 1-16.

33. Gorman, B.M. (1983). On the Evolution of Orbiviruses. Intervirology, 20: 169-180.

34. Gorman, B.M., Taylor, J., Walker, P.J., Davidson, W. & Brown, F. (1981). Comparison of Bluetongue type 20 with certain viruses of the Bluetongue and Eubenangee serological groups of Orbiviruses. J. Gen. Virol. 57: 251-261.

35. Gorman, B.M., Taylor, J., Walker, P.J. & Young, P.R. (1978). The isolation of recombinants between related Orbiviruses. J. Gen. Virol. 41: 333-342.

36. Gorman, B.M., Walker, P.J. & Taylor, J. (1977). Electrophoretic separation of dsed RNA genome segments from Warrego and Mitchell River viruses. Archives of Virology, 54: 153-158.

37. Gumm, I. & Newman, J.F.E. (1982). The preparation of purified Bluetongue virus group antigen for use as a diagnostic reagent. Archives of Virology, 72: 83-93.

38. Hafez, S.M. & Ozawa, Y. (1974). Immunodiffusion technique with Bluetongue virus antigens prepared from infected MS cell cultures. J. Egypt. Vet. Med. Ass. 34: 469-489.

39. Hames, B.D. (1981). An introduction to polyacrylamide gel electrophoresis. In: Gel Electrophoresis of Proteins : A Practical Approach. Hames, B.D. & Rickwood, D. (Eds.). pp. 1-92. IRL Press Limited, London.

40. Harms, E., Rohde, W., Bosch, F. & Scholtissek, C. (1978). Biochemical studies on influenza viruses II. Assignment of gene functions to RNA segments 5, 7 and 8 of Fowl Plague virus and virus N. Virology, 86: 413-422.

41. Haschemeyer, R.H. & Haschemeyer, A.E.V. (1973). Primary structure of proteins (Chapt. 4) In: Proteins: A Guide to Study by Physical and Chemical Methods. Wiley-Interscience, New York.

42. Hearn, T.W., Regnier, F.E. & Wehr, C.T. (1983). HPLC of peptides and proteins. International Laboratory. Jan/Feb. 16-36.

43. Houmard, J. & Drapeau, G.R. (1972). Staphylococcal protease : a proteolytic enzyme specific for glutamoyl bonds. Proc. Natl. Acad. Sci. U.S.A. 69: 3506-3509.

44. Howell, P.G. (1960). A preliminary antigenic classification of strains of Bluetongue virus. Onderstepoort J. Vet. Res. 28: 357-363.

45. Howell, P.G. (1970). The antigenic classification and distribution of naturally occurring strains of Bluetongue virus. J. S. Afr. Med. Ass. 41: 215-223.

46. Howell, P.G., Kümm, N.A. & Botha, M.J. (1970). The application of improved techniques to the identification of strains of Bluetongue virus. Onderstepoort J. Vet. Res. 37: 59-66.

47. Howell, P.G. & Verwoerd, D.W. (1971). Bluetongue Virus. Virology Monographs, 9: 35-74.

48. Hubschle, O.J.B. & Yang, C. (1983). Purification of the group specific antigen of Bluetongue virus by chromatofocusing. J. Virol. Meth. 6: 171-178.

49. Huismans, H. (1979). Protein synthesis in Bluetongue virus-infected cells. Virology, 92: 385-396.

50. Huismans, H. & Bremer, C.W. (1981). A comparison of an Australian BTV isolate (CSIRO 19) with other BTV serotypes by cross-hybridization and cross-immune precipitation. Onderstepoort J. Vet. Res. 48: 59-67.

51. Huismans, H., Bremer, C.W. & Barber, T.L. (1979). The nucleic acid and proteins of Epizootic haemorrhagic disease virus. Onderstepoort J. Vet. Res. 46: 95-104.

52. Huismans, H. & Erasmus, B.J. (1981). Identification of the serotype-specific and group-specific antigens of BTV. Onderstepoort J. Vet. Res. 48: 51-58.

53. Huismans, H. & Howell, P.G. (1973). Molecular hybridization studies on the relationships between different serotypes of Bluetongue virus and on the difference between the virulent and attenuated strains of the same serotype. Onderstepoort J. Vet. Res. 40: 93-104.

54. Huismans, H. & Verwoerd, D.W. (1973). Control of transcription during the expression of the Bluetongue virus genome. Virology, 52: 81-88.

55. Ingram, V.M. (1956). A specific chemical difference between the globins of normal human and sickle-cell anaemia haemoglobin. Nature (London), 178: 792-794.

56. Jackson, D.C., Brown, L.E., White, D.O., Dopheide, T.A.A. & Ward, C.W. (1979a). Antigenic determinants of influenza virus. IV. Immunogenicity of fragments isolated from the haemagglutinin of A/Memphis/72. J. Immunol. 123: 2610-2617.

57. Jackson, D.C., Dopheide, T.A.A., Russell, R.J., Waive, D.O. & Ward, C.W. (1979b). Antigenic determinants of influenza virus haemagglutinin. II. Antigenic reactivity of the isolated N-terminal cyanogen bromide peptide of A/Memphis/72 haemagglutinin heavy chain. Virology, 93: 458-465.

58. Jochim, M.M. (1976). Improvement of the AGP test for Bluetongue. Proc. 19th Annu. Meet. Am. Assoc. Vet. Lab. Diagn. pp. 361-373.

59. Jochim, M.M., Barber, T.L. & Bando, B.M. (1974). Identification of Bluetongue and Epizootic haemorrhagic disease virus by indirect fluorescent antibody procedure. Proc. 17th Ann. Meet. Am. Assoc. Vet. Lab. Diagn. pp. 91-103.

60. Jochim, M.M. & Chow, T.L. (1969). Immunodiffusion of Bluetongue virus. Am. J. Vet. Res. 30: 33-41.

61. Jochim, M.M. & Jones, S.C. (1980). Evaluation of a Hemolysis-in-gel-test for detection and quantitation of antibodies to Bluetongue virus. Am. J. Vet. Res. 41: 595-599.

62. Joklik, W.K. (1974). Reproduction of Reoviridae. In: Comprehensive Virology, Vol. 2 (H. Fraenkel-Conrat & R.R. Wagner, Eds.). pp. 231-334. Plenum, New York.

63. Kahlon, J., Sugiyama, K., & Roy, P. (1983). Molecular basis of Bluetongue virus neutralization. J. Virol. 48: 627-632.

64. Kew, O.M., Pallansch, M.A., Omilianowski, D.R. & Rueckert, R. (1980). Changes in three of four coat proteins of oral polio vaccine derived from type 1 poliovirus. J. Virol. 33: 256-263.

65. Kipps, A. (1956). Complement fixation with antigens prepared from BTV-infected mouse brains. J. of Hygiene, 54: 79-88.

66. Kipps, A. (1958). The particle size of antigens prepared from Bluetongue virus-infected suckling mouse brains. S.A. J. Lab. Clin. Med. 4: 158-167.

67. Klontz, G.W., Svehag, S-E. & Gorham, J.R. (1962). A study by agar diffusion technique of precipitating antibody directed against BTV and its relationship to homotypic neutralizing antibody. Archiv Für die Gesamte Virusforschung. 12: 259-268.

68. Knudson, D.L., Butterfield, W.K., Shope, R.E., Walton, T.E. and Campbell, C.H. (1982). Electrophoretic comparison of the genomes of North American Bluetongue viruses, one Australian Bluetongue virus and three other related Orbiviruses. Veterinary Microbiology, 7: 285-293.

69. Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London), 227: 680-685.

70. Laver, W.G. (1971). Separation of two polypeptide chains from the haemagglutinin subunit of influenza virus. Virology, 45: 275-288.

71. Lee, H., Causey, O.R. & Moore, D.L. (1974). Bluetongue and related viruses in Ibadan : Isolation and preliminary identification of viruses. Am. J. Vet. Res. 35: 1105-1108.

72. Letchworth, G.J. & Appleton, J.A. (1983). Heterogeneity of neutralization-related epitopes within a Bluetongue virus serotype. Virology, 124: 300-307.

73. Martin, S.A., Pett, D.M. & Zweerink, H.J. (1973). Studies on the topography of Reovirus and Bluetongue virus capsid proteins. J. Virol. 12: 194-198.

74. Martin, S.A. & Zweerink, H.J. (1972). Isolation and characterization of 2 types of Bluetongue virus particles. Virology, 50: 495-506.

75. Massey, R.J., Arthur, L.O., Nowinski, R.C. and Schochetman, G. (1980). Monoclonal antibodies identify individual determinants of mouse mammary tumour virus glycoprotein gp52 with group, class or type specificity. J. Virol. 34: 635-643.

76. Mathews, R.E.F. (1979). Classification and nomenclature of viruses. Third report of the International Committee on Taxonomy of Viruses. Intervirology, 12: 199-205.

77. Merril, C.F. (1981). Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science, 211: 1437-1438.

78. Metcalf, H.E. (1977). Bluetongue and related diseases. Arthropod-borne Animal Disease Research Laboratory. Research outline.

79. Moore, D.L. (1974). Bluetongue and related viruses in Ibadan, Nigeria : Serological comparison of Bluetongue, Epizootic haemorragic disease of deer and Abadina (Palyam) viral isolates. Am. J. Vet. Res. 35: 1109-1113.

80. Moore, D.L. & Lee, V.H. (1972). Antigenic relationship between the virus of Epizootic haemorrhagic disease and Bluetongue virus. Archiv Für die Gesamte Virusforschung, 37: 282-284.

81. Murphy, F.A., Borden, E.C., Shope, R.E. & Harrison, A.K. (1971). Physicochemical and morphological relationships of some arthropod-borne viruses to Bluetongue virus - A new taxonomic group. Electron microscopic studies. J. Gen. Virol. 13: 273-288.

82. Neitz, W.O. (1948). Immunological studies on Bluetongue in sheep. Onderstepoort J. Vet. Sc. & Animal Industry, 23: 93-136.

83. Newman, J.F.E., Cartwright, B., Doel, T.R. & Brown, F. (1979). Purification and identification of the RNA-dependent RNA polymerase of Foot-and-Mouth disease virus. J. Gen. Virol. 45: 497-507.

84. Oakley, B.R., Kirsch, D.R. & Morris, N.R. A simplified ultrasensitive silver stain for detecting proteins in PAGs. Anal. Biochem. 105: 361-363.

85. O'Farrell, P.H. (1975). High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 256: 4007-4021.

86. Owen, N.C. & Munz, E.K. (1966). Observations on a strain of Bluetongue virus by electron microscopy. Onderstepoort J. Vet. Res. 31: 109.

87. Palmer, E.L. & Martin, M.L. (1982). Reoviridae. In: An Atlas of Mammalian Viruses. (First edition). Chapter 6, pp. 23-33. CRC Press Inc., Florida.

88. Payne, C.C., Piasecka-Serafin, M. & Pilley, B. (1977). The properties of two recent isolates of cytoplasmic polyhedrosis viruses. Intervirology, 8: 155-163.

89. Payne, C.C. & Rivers, C.F. (1976). A provisional classification of cytoplasmic polyhedrosis virus based on the sizes of the RNA genome segments. J. Gen. Virol. 33: 71-85.

90. Pharmacia Fine Chemicals (1981). Gel Filtration, Theory and Practise.

91. Regnier, T. (1983). HPLC of biopolymers. Science. 222: 245-252.

92. Robson, K.J.H., Doel, T.R., Gorman, B.M. & Brown, F. (1980). Biochemical aspects of variation in Foot-and-Mouth disease virus. J. Gen. Virol. 46: 179-193.

93. Ruckerbauer, G.M., Gray, P.D., Girard, A., Bannister, G.L. & Boulanger, P. (1967). Studies on Bluetongue. V. Detection of the virus in infected materials by immunofluorescence. Canadian J. of Comparative Medicine. 31: 175-181.

94. Schnagl, R.D. & Holmes, I.H. (1971). A study of Australian Arboviruses resembling Bluetongue virus. Aust. J. Biol. Sci. 24: 1151-1162.

95. Schnagl, R.D. & Holmes, I.H. (1975). Polyacrylamide gel electrophoresis of the genomes of two Orbiviruses : D'Aguilar and Eubenangee Intervirology, 5: 300-304.

96. Shapiro, A.L., Vinuela, E. & Maizel, J.V. Jr. (1967). Molecular weight estimation of polypeptide chains by electrophoresis in SDS-PAGs. Biochem & Biophys. Res. Comm. 28: 815-820.

97. Shih, D.S., Shih, C.T., Kew, O, Pallansch, M. Reuckert, R. & Kaesberg, P. (1978). Cell-free synthesis and processing of the proteins of Poliovirus. Proc. Natl. Acad. Sci. U.S.A. 75: 5807-5811.

98. Squire, K.R.E., Osburn, B.I., Chuang, R.Y. & Doi, R.H. (1983). A survey of electropherotype relationships of Bluetongue virus isolates from the Western United States. J. Gen. Virol. 64: 2103-2115

99. St. George, T.D., Standfast, H.A., Cybinski, D.H., Dyce, A.L., Muller, M.J., Doherty, R-L., Carley, J.G., Filippich, C. & Fraxier, C.L. (1978). The isolation of a Bluetongue virus from Culicoides collected in the Northern Territory of Australia. Aust. Vet. J. 54: 153-154.

100. Sugiyama, K., Bishop, D. & Roy, P. (1981) Analysis of the genomes of Bluetongue viruses recovered in the United States. I. Oligonucleotide fingerprint studies that indicate the existence of naturally occurring reassortant BTV isolates. Virology, 114: 210-217.

101. Sugiyama, K., Bishop, D.H.L. & Roy, P. (1982). Analysis of the genomes of BTVs recovered from different states of the U.S. and at different times. Am. J. Epidemiol. 115: 332-347.

102. Suzuki, Y., Saito, Y., Nakagawa, S. (1977). Double stranded RNA of Ibaraki virus. Virology, 76: 670-674.

103. Switzer, R.D., Merril, C.R. & Shifrin, S. (1979). A highly sensitive silver stain for detecting proteins and peptides in PAGs. Anal. Biochem. 98: 231-237.

104. Thomas, F.C., Morse, P.M. & Seawright, G.L. (1979). Comparison of some Bluetongue virus isolates by plaque neutralization and relatedness tests. Archives of Virology, 62: 189-199.

105. Van der Walt, N.T. (1979). Factors affecting the Bluetongue virus neutralizing antibody response and the reaction between virus and antibody. Onderstepoort J. Vet. Res. 46: 111-116.

106. Van Dijk, A.A. & Huismans, H. (1980). The in vitro activation and further characterization of the Bluetongue virus-associated transcriptase. Virology, 104: 347-356.

107. Verwoerd, D.W. (1969). Purification and characterization of Bluetongue virus. Virology, 38: 203-212.

108. Verwoerd, D.W. (1970). Diplorna viruses : A newly recognized group of double-stranded RNA viruses. Prog. Med. Virol. 12: 192-210.

109. Verwoerd, D.W., Els, H.J., De Villiers, E-M. & Huismans, H. (1972). Structure of the Bluetongue virus capsid. J. Virol. 10: 783-794.

110. Verwoerd, D.W. & Huismans, H. (1969). On the relationship between Bluetongue, African Horsesickness and Reovirus : Hybridization studies. Onderstepoort J. Vet. Res. 36: 175-180.

111. Verwoerd, D.W. & Huismans, H. (1972). Studies on in vitro and in vivo transcription of the Bluetongue virus genome. Onderstepoort J. Vet. Res. 39: 185-192.

112. Verwoerd, D.W., Huismans, H. & Erasmus, B.J. (1979). Orbiviruses in Comprehensive Virology. Vol. 14, pp. 285-345. (Fraenkel-Conrat, H. & Wagner, R.R. Eds.). Plenum Press, New York.

113. Verwoerd, D.W., Louw, H. & Oellerman, R.A. (1970). Characterization of Bluetongue virus ribonucleic acid. J. Virol. 5: 1-7.

114. Von Regenmortel (1982). Serological techniques. In: Serology and Immunochemistry of Plant Viruses. p. 77. Academic Press, New York.

115. Wang, C.S., Lueker, D.C. & Chow, T. L. (1972). Soluble antigen of Bluetongue virus. Infection and Immunity, 5: 467-473.

116. Waterfield, M., Espelie, K., Elder, K.& Skekel, J.J. (1979). Structure of the haemagglutinin of influenza virus. Br. Med. Bull. 35: 57-63.

117. Weber, K. & Osborn, M. (1969). The reliability of MW determinations by dodecyl-sulphate PAGE. J. Biol. Chem. 244: 4406-4412.

118. Webster, R.G. & Laver, W.G. (1975). Antigenic variation of influenza virus. In: The Influenza viruses and Influenza. E.D. Kilbourne (Ed.). pp. 270-314. Academic Press Inc., New York.

119. Wiktor, T.J., Flamand, A. & Koprowski, H. (1980). Use of monoclonal antibodies in diagnosis of Rabies virus infection and differentiation of Rabies and Rabies-related viruses. J. Virol. Methods, 1: 33-46.

120. Wood, H.A. (1973). Viruses with double-stranded RNA genomes. J. Gen. Virol. 20: 61-85.

121. Young, N.A., Hoyer, B.H. & Martin, M.A. (1968). Polynucleotide sequence homologies among poliovirus. Proc. Natl. Acad. Sci. U.S.A. 61: