CLEAVAGE OF THE PRECURSOR COAT PROTEIN OF BLACK BEETLE VIRUS STRAIN W17 IN RABBIT RETICULOCYTE LYSATE

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by

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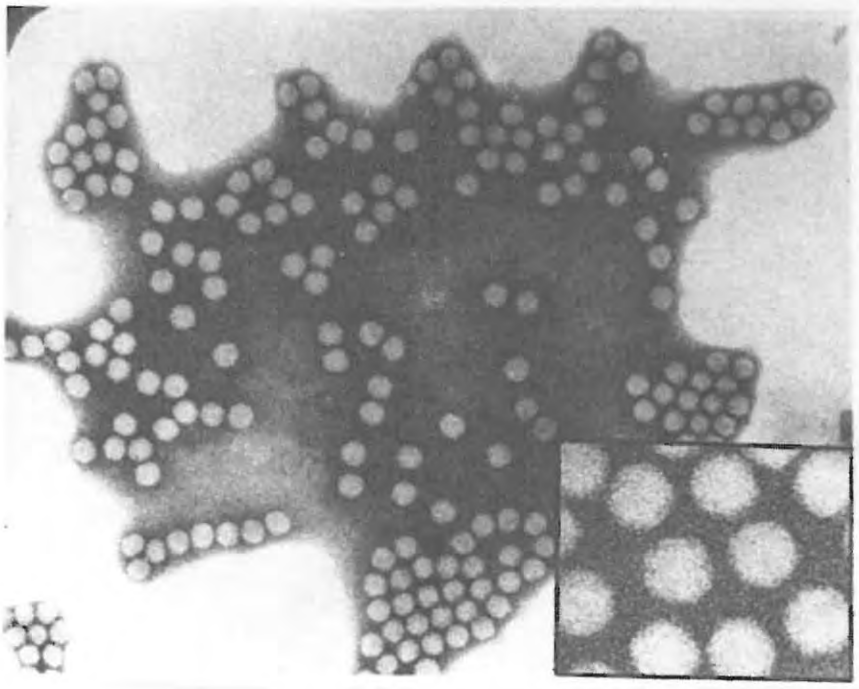

Fig. 1.1. Electron micrographs of black beetle virus x 115000 (Insert x 300000), negatively stained with 1% wt/vol uranyl acetate.

The following papers/poster papers relating to the work described in this thesis have been presented at congresses:

1. HENDRY, D.A., BLACKHURST, D. and RUECKERT, R.R.
   In vitro translational behaviour of Nodaviral RNAs.
   The First Joint Congress of the South African Biochemical Society, South African Genetics Society and the South African Society for Microbiology.
   University of the Witwatersrand, South Africa.
   29 June - 4 July 1986 (Poster paper).

2. HENDRY, D.A., BLACKHURST, D. and RUECKERT, R.R.
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   Veldhoven, The Netherlands.
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3. HENDRY, D.A., BLACKHURST, D. and RUECKERT, R.R.
   In vitro translational behaviour of black beetle virus RNA.
   Proc. 7th John Innes Symposium: Virus replication and genome interactions, Norwich, England.
   2-5 September 1986 (Poster paper).

4. BLACKHURST, D. and HENDRY, D.A.
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ABBREVIATIONS AND SYMBOLS

bis	=	bis (N,N'-methylene-bis-acrylamide)
BSA	=	bovine serum albumin
Ci	=	Curie
cm	=	centimetre
cpe	=	cytopathic effect
DMSO	=	dimethylsulphoxide
E	=	extinction coefficient
EDTA	=	ethylene diamine tetraacetic acid
FCS	=	foetal calf serum
g	=	gram
h	=	hours
kDa	=	kilodalton
ℓ	=	litre
M	=	molar
mg	=	milligram
min	=	minutes
ml	=	millilitre
mm	=	millimetre
mM	=	millimolar
m.o.i.	=	multiplicity of infection
MW	=	molecular weight
N	=	normal
nm	=	nanometre
PAGE	=	polyacrylamide gel electrophoresis
PBS	=	phosphate buffered saline
Peg	=	polyethylene glycol

pH	=	negative logarithm of the hydrogen ion concentration
PMSF	=	phenylmethansulfonylfluoride
rpm	=	revolutions per minute
RNA	=	ribonucleic acid
SCHIM	=	Schneider's insect medium
SDS	=	sodium dodecyl sulphate
s.s.	=	single stranded
STAINS - ALL	=	1-ethyl-2-|3-(1-ethylnaphtho|1,2d|-thiazolin-2-ylidene)-2-methyl-propenyl|naphtho|1,2d|-thiazalium bromide
TCA	=	trichloroacetic acid
Temed	=	N,N,N',N'-tetramethylethylenediamine
TMV	=	Tobacco mosaic virus
Tris	=	tris(hydroxymethyl) amino methane
µ (prefix)	=	micro (10^{-6} x)
µg	=	microgram
µl	=	microlitre
µM	=	micromolar
%	=	percent

ABSTRACT

Black beetle virus (BBV) is a bipartite single-stranded RNA virus belonging to the family Nodaviridae. Its host range has been found to be limited to insects.

RNA 1, the larger of the two RNA molecules, with a MW of $1,15 \times 10^6$ and the smaller RNA 2 with a MW of $0,46 \times 10^6$, are both packaged in the same virus particle. The two RNA molecules are translated separately, with RNA 1 coding for protein A of MW 105×10^3 and RNA 2 coding for protein α of MW 47×10^3. Protein α is the major capsid protein precursor, which during *in vivo* maturation is cleaved to form the coat protein β of MW 43×10^3, and protein γ of MW 5×10^3.

Cell-free translation of BBV (strain W17) mRNA was carried out in rabbit reticulocyte lysates. Protein α was detectable between 0 and 30 minutes after RNA addition. A protein 'β', which was found to co-electrophorese on polyacrylamide gels with authentic β and which was immunoprecipitated by anti-BBV antiserum, was detectable after 30 minutes.

Results of this work show that the formation of 'β' could be prevented by the addition of RNase to the lysate, indicating that intact RNA is necessary for α to β cleavage. Arresting protein synthesis by the addition of cycloheximide to the lysate mix did not inhibit the cleavage. The formation of β could also be prevented by cooling the lysate mix to 1°C.

Cleavage of α to β still occurred when RNA 2, without the presence of RNA 1, was translated. Therefore the cleavage is not dependent on a translation product of RNA 1.

Sedimentation of lysate on sucrose density gradients showed that α to β cleavage was not accompanied by assembly of BBV RNA and protein into a viral substructure as has been shown to occur with some viruses, for example certain picornaviruses.

Serial dilution of lysate containing α showed that the level of β decreased with increasing dilution, indicating that the cleavage is not mediated by autocatalysis, but by some other unknown factor.

Although much work has been carried out on black beetle virus, no work has been published to date concerning α to β cleavage as an indication of assembly in rabbit reticulocyte lysates.

Results of these cell-free translation experiments thus indicate that BBV coat protein precursor α, in association with its messenger RNA 2, undergoes a maturation cleavage in the lysate to produce BBV coat protein β. In addition, this cleavage seems to occur without assembly into any intermediate viral structure.

CHAPTER 1

INTRODUCTION

1.1. THE BROAD CLASSIFICATION AND GROUPING OF SMALL RNA VIRUSES OF INSECTS

By 1982, 54 families and groups of viruses had been approved by the International Committee on Taxonomy of Viruses, including eleven families of invertebrate viruses (Matthews, 1982). Various insect viruses were to be found in all eleven of these families.

Viruses are broadly grouped according to the host that they infect, although some viruses infect more than one host. There are bacterial viruses, fungal viruses, plant viruses and invertebrate and vertebrate viruses. Within these, the type and strandedness of the nucleic acid making up the viral genome, and the presence or absence of a viral envelope, are used to group the various viruses.

It is difficult to assign a virus to a particular family or group, because the members sometimes differ from each other in many respects and are often serologically unrelated. Therefore classification of invertebrate viruses, and in particular insect viruses, is sometimes rather tenuous.

To date, over 30 small RNA viruses of invertebrates have been described, most of these having been isolated from insects. As more viruses have been isolated and described, their classification has had to change somewhat, with the result that several new groups of

viruses have been proposed, their members often having been loosely classified under other groups. An example is the Nodaviridae.

By 1982, the small RNA viruses of insects which did not contain lipid envelopes could be broadly divided into the following groups (Moore and Tinsley, 1982)

(a) the enteroviruses;
(b) the Nudaurelia β-like viruses;
(c) the split-genome viruses with single-stranded RNA (Nodaviruses);
(d) the split genome viruses with double-stranded RNA (Drosophila X viruses) (Birnaviruses);
(e) the bee chronic paralysis virus group;
(f) undefined viruses (a large group).

The Nodamura virus group, family Nodaviridae, derived its name from the village Nodamura in Japan, where the type species was isolated (Matthews, 1982).

1.2. THE MEMBERS AND DISTINGUISHING FEATURES OF THE NODAVIRUSES

According to the fourth report of the International Committee on Taxonomy of Viruses (Matthews, 1982), there were two definite members of the Nodavirus group, namely the type species, Nodamura virus and black beetle virus. Since then two other members have been assigned to the family, namely Flock House virus (Dearing et al., 1980), (Scotti et al., 1983) and Boolarra virus (Reinganum et al., 1985). According to Matthews (1982), other possible future members could include the Arkansas bee virus and endogenous Drosophila-like virus.

However, a recent publication (Lommel et al., 1985) indicates that Arkansas bee virus has only one RNA, and is therefore not a nodavirus.

Nodaviruses are unique amongst vertebrate and invertebrate riboviruses for two reasons: firstly they are the only known animal viruses to possess a bipartite genome and secondly, the two segments of the genome are packaged in one particle. The other known virus groups with bipartite RNA genomes, namely the comoviruses, nepoviruses and tobraviruses, are all plant viruses and the two RNA molecules are packaged in different virus particles.

Nodaviruses are small non-enveloped isometric viruses, 29 nm in diameter and of molecular weight (MW) 8×10^6; $S_{20,W} = 135$ and their buoyant density in CsCl = 1,34 g/cm^3 (Longworth, 1978). Their nucleic acid consists of two single-stranded RNA molecules, one of which has a MW of $1,15 \times 10^6$ and the other $0,46 \times 10^6$, packaged in the same particle (Longworth, 1978), and comprising about 20,5% of the particle by weight. Both these RNA molecules are necessary for infection (Matthews, 1982). There is no poly (A) 3' sequence (Matthews, 1982). Individual members differ only slightly with respect to these figures.

Their lipid content is unknown, but they are not thought to contain any. Their carbohydrate content has also not been determined.

Nodaviruses are resistant to organic solvents, stable to pH 3,0 but unstable in the presence of chloride ions. The type species is serologically unrelated to the other members (Matthews, 1982).

Nodaviruses replicate in the cytoplasm of cells of several tissues.

The large and small RNA molecules are translated separately, the large RNA coding for a protein of MW 105 x 10^3 and the small RNA for a protein of MW 47 x 10^3. This latter protein is the precursor of the major capsid protein (Matthews, 1982) (Friesen and Rueckert, 1982).

The Nodaviruses were all isolated from insects: from Diptera, Coleoptera and Lepidoptera. Most are not host-specific. The type species replicates in vertebrate as well as invertebrate cell cultures (Matthews, 1982).

1.3. BLACK BEETLE VIRUS

1.3.1. NATURAL HOST AND ORIGINAL ISOLATION

Black beetle virus (BBV) (Figure 1.1) was first described by Longworth and Archibald in the New Zealand Journal of Zoology in 1975. A small RNA virus had been isolated from the larvae and adults of the New Zealand black beetle, Heteronychus arator, a soil scarab pest of New Zealand pastures, as a result of a routine survey (conducted during 1973 to 1974) for pathogens of the black beetle. Prior to that there had been reports of high adult mortality of the beetle in Northland, New Zealand, but information on pathogens was scanty. They found that the virus could be successfully transmitted to Pseudaletia separata (Lepidoptera: Noctuidae), Galleria mellonella (Lepidoptera: Galleriidae) and several other insect larvae, although when it was injected into the body cavity of H. arator adults, it was not infective.

BBV was originally included in the Picornavirus family, but it was proposed that because it, like Nodamura virus, had a divided genome, both viruses as well as several other divided-RNA viruses be put into a separate family called Nodaviridae. This was approved in 1980 by the Executive Committee of the I.C.T.V.

The host range of BBV was found to be fairly wide within the insect group, and Longworth and Carey (1976) found that, unlike Nodamura virus, it did not replicate in mice.

Longworth and Carey (1976) showed BBV to be very similar to Nodamura virus, although serologically unrelated, and by 1976 they had published the following facts about BBV:

- the cryptogram for the virus: R/1 : 1,5/28 : S/S : I/O.
- it was a spherical virus 30 nm in diameter, that contained s.s. RNA and developed in the cytoplasm of gut and fat body cells of Galleria larvae. The sedimentation coefficient was 137S and the buoyant density 1,33 g/ml.

Work done by Hosur et al. in 1984 indicated that the original estimate of molecular weight of the virus by Longworth (1978) as being 5×10^6 was probably too low, and that a more likely estimate was about 8×10^6, which required about 180 subunits in the capsid. According to Hosur et al. (1984)

- BBV seemed to display T = 3 quasisymmetry,
- BBV was stable over a pH range of 3-10,
- the virus particle consisted of 71,8% protein which comprised one

major coat protein of approximate MW = 40 000 and two minor polypeptides, and 28,2% RNA which comprised two equimolar species of RNA, a large species which sedimented at 22S corresponding to a molecular weight of $1,0 \times 10^6$ and a smaller species, which sedimented at 15S corresponding to a molecular weight of $0,5 \times 10^6$. (The figures that are now generally accepted are: coat protein MW = 43 000 (Friesen and Rueckert, 1981); large RNA MW = $1,1 \times 10^6$ and small RNA MW = $0,46 \times 10^6$ (Selling and Rueckert, 1984)). The GC content of the 22S RNA was found to be 47% and that of the 15S RNA was 49%. It was not known at this time whether the two RNA species were present in one or different particles, but because they were consistently isolated in equimolar proportions it was thought likely that they existed in the same virus particle. This is now known to be the case (Longworth and Carey, 1976; Selling and Rueckert, 1984).

BBV was found to replicate in the Drosophila 1 cell line (Schneider, 1964; Schneider, 1972; Plus, 1978; Friesen et al., 1980) without a noticeable CPE, but did not replicate in cultured cells of Trichoplusia ni, Spodoptera frugiperda, Carpocapoa pomonella, Choristoneura fumiferana or BHK or mouse L cells.

Friesen et al. (1980) studied BBV growth in Drosophila line 1 cells and discovered a subline of these cells which was resistant to infection with BBV and which carried BBV-like particles. This was a derivative of the Schneider's WR strain, called the NZ subline.

Plus (1978) tested culture medium supernates from 42 Drosophila

melanogaster cell lines and found virus in 32 of them. Drosophila X virus was the most commonly-found virus, although there were others such as the picornaviruses Drosophila C virus (DCV), Drosophila P virus (DPB) and Drosophila A virus (DAV). It was not known, however, whether these viruses had been present from time of establishment of the lines, or whether they had been introduced into the lines by external contamination.

1.3.2. PROPAGATION IN THE LABORATORY, AND PLAQUE ASSAY

BBV is usually propagated in the laboratory by using growth in Drosophila line 1 cells at 26°C. It may also be propagated by growth in Galleria mellonella (wax moth) larvae and infected dead larvae may be stored at -20°C (Friesen et al., 1980). BBV is a very high-yielding virus when grown in Drosophila line 1 cells. By the third day post-infection, about 20% of the total cell-associated protein is virus. Friesen et al. (1980) reported that they could purify 100 mg of virus per litre of culture.

Briefly, propagation of the virus in Drosophila cells entails growing the Drosophila cells to confluency, resuspending the cells in Schneider's growth medium (Schneider, 1964) containing 15% foetal calf serum and then adjusting the cell concentration to 1×10^8 cells/ml; thereafter adding BBV to obtain a multiplicity of infection of about 1 virion per cell, agitating the cells at 26°C for one hour, diluting the cells to obtain 10^6 cells/ml and then incubating the cell suspension for 24 - 48 hours at 26°C (Friesen et al., 1980; Friesen and Rueckert, 1981). BBV may then be purified from the cells (Friesen et al., 1980; Guarino et al., 1981).

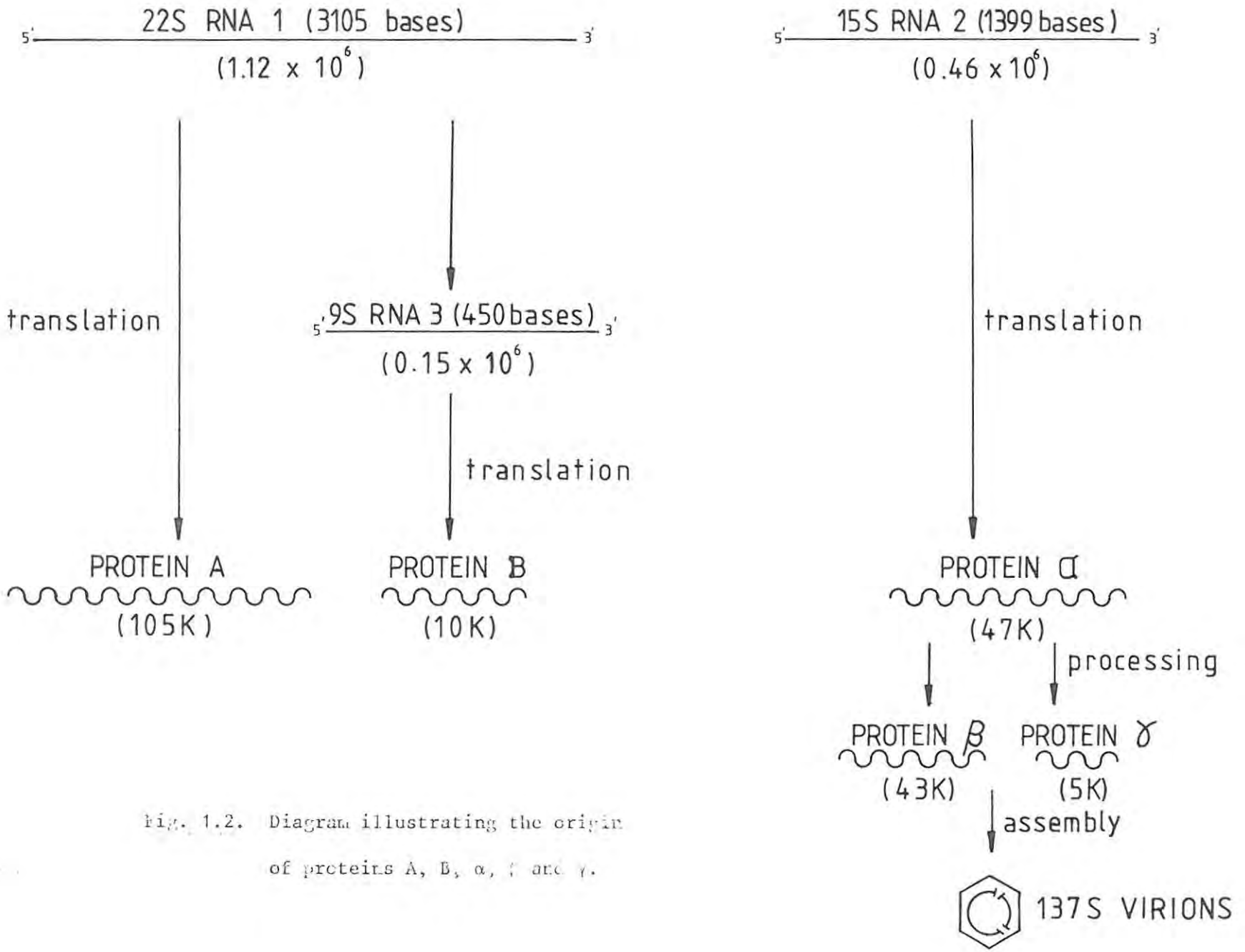

Fig. 1.2. Diagram illustrating the origin of proteins A, B, α, β and γ.

Crawford (1982) described how the first continuous coleopteran cell line (DSIR-HA-1179) was derived from primary cultures of Heteronychus arator set up in 1979. The cells grew best in Schneider's Drosophila medium, were spindle-shaped, with a mean doubling time of 6 days at 27°C, and supported the growth of black beetle virus.

They adhered well to plastic, but attempts to grow them in suspension cultures were unsuccessful. The slow cell growth rate unfortunately meant that it was impractical to use these cells in experiments with BBV. The cells were used in a comparative study (Crawford et al., 1984), where attempts were made to grow black beetle virus and Flock House virus in these and Drosophila line 1 cells. It was found that the Drosophila cells were more sensitive for detecting both viruses and that replication of Flock House virus was more rapid and more productive in Drosophila cells.

The development of a reliable plaque assay for BBV was hindered originally by the minimal cytopathic effects of the virus on Drosophila cells, and as mentioned above the only available strain of black beetle cells grew so slowly in culture as to make any work with it impractical. However, an effective method was described by Selling and Rueckert (1984) where they established that the main problem with previous attempts lay in the importance of indicator cell density. They noted that although wild-type BBV was able to grow vigorously in culture, it was not very cytolytic for Drosophila cells. Therefore they attempted to select a laboratory strain of virus that would grow more rapidly in cultured Drosophila cells. This they achieved by repeated passage of the wild-type virus through

Drosophila cells, infecting at low multiplicity to minimize formation of defective interfering particles. In this way, BBV strain W17 was first isolated. Then, once they had established the importance of indicator cell density, they were able to obtain plaques even with wild-type BBV, by simply allowing the plaques to develop for longer periods of time.

1.3.3. PURIFICATION OF BBV

Several methods have been used to purify BBV. A typical method is that described by Friesen *et al*. (1980) where the virus is first propagated in *Drosophila* cell culture monolayers at 26°C and then released from the infected cells by several freeze-thaw cycles. One can also infect cells in suspension and then lyse the *Drosophila* cells by using a non-ionic detergent such as NP-40 with β-mercaptoethanol. Cell debris is removed by centrifugation and the virus is pelleted through a sucrose cushion. The virus pellet is then resuspended in phosphate buffer containing β-mercaptoethanol and SDS and centrifuged on a sucrose gradient. Fractions are collected from the gradient and those that absorb at 260 nm are pooled and stored.

Guarino *et al*. (1981) described a similar method of purifying BBV except that polyethylene glycol (PEG) was used to precipitate the virus. They obtained a typical yield of 1 mg of pure BBV per 10^8 infected *Drosophila* cells.

1.3.4. REPLICATION STRATEGY AND GENE REGULATION

The genomic organization of black beetle virus is diagrammatically represented in Figure 1.2. RNA constitutes 28,2% by weight of the BBV virion, and the genome of this virus is divided into two segments, both of which are contained within the one virus particle in almost equimolar proportions (Longworth, 1978). Both are required for infectivity, although they have independent genetic functions. RNA 2 is not derived from RNA 1, and the two segments can be distinguished by two-dimensional oligonucleotide fingerprinting (Clewley et al., 1982).

RNA 1 is the larger of the two segments, has a MW of $1,12 \times 10^6$ (determined by gel electrophoresis) and codes for the 105-kDa protein A, the viral RNA replicase (Friesen and Rueckert, 1981; Guarino and Kaesberg, 1981). RNA 2 has a MW of $0,46 \times 10^6$ and codes for the 47-kDa protein α, precursor to the capsid protein, protein β, of MW 43 000 (Friesen and Rueckert, 1981) which is derived by cleavage of protein α. In addition to these two proteins, mature virions were found to contain a small protein of MW 5 000 called protein γ. The function of this protein is unknown.

Cells infected with BBV produce a subgenomic RNA 3 and RNA 3 codes for a 10-kDa protein, protein B (Gallagher, Friesen and Rueckert, 1983) which is somehow involved in the regulation of protein A. In 1981 Guarino and Kaesberg described how they had isolated and partially purified protein A, achieving a 43-fold purification with 84% recovery of polymerase activity.

The nucleotide sequence of RNA 1 is known (Dasmahapatra et al., 1985). It comprises 3105 bases, has a 5' non-coding region of 38 bases, a region coding for protein A and a 3' proximal region encoding RNA 3 (389 bases). The 5' end has the cap structure 7mGpppGp.

The nucleotide sequence of RNA 2 was reported by Dasgupta et al. in 1984. RNA 2 comprises 1399 bases and its 5' terminus is capped. There is a start codon 23 bases from the 5' end and this is followed by an open reading frame for a protein of the same size as that predicted by electrophoretic determinations for protein α. There is only one other large open reading frame in RNA 2, and this begins at base 1110 and codes for a putative protein of about 72 amino acids long.

RNA 2, in addition to its function as messenger for coat protein, also exerts a regulatory function on the production of subgenomic RNA 3 in infected cells (Gallagher et al., 1983). Evidence for this is the much greater amount of RNA 3 produced in cells infected with only RNA 1 compared with cells infected with RNA 1 and RNA 3 together.

The nucleotide sequence of the subgenomic RNA 3 was reported on by Guarino et al. (1984). It consists of 389 bases, and the sequence of its first 306 bases is identical to that of the 3'-terminal region of RNA 1 from base 2717 to base 3022. RNA 3 is capped at its 5' terminus and includes two large overlapping reading frames for putative proteins of size 10 kDa and 11 kDa, proteins B_1 and B_2, respectively.

Friesen and Rueckert reported in 1981 that the 3 classes of virus-induced proteins, A, B and α were produced in different amounts and at different stages in infection. Proteins A and B were present in maximum amounts early on in infection (protein A reached a maximum at 5 hours, then declined to low levels), whereas synthesis of protein α reached a maximum later. They also found that the translation products of one RNA did not seem to affect the translation or processing of the other RNA.

Crump and Moore (1981) found that 2 proteins of MW's of approximately 40 000 and 7 000 were synthesized within the first 5 minutes of cell-free translation of BBV RNA in rabbit reticulocyte lysates, and by 40 minutes, a protein of MW greater than 100 000 had been synthesized.

Friesen and Rueckert (1984) found that synthesis of protein A declined sharply by 6 hours postinfection; whereas synthesis of protein α continued for at least 14 hours. Synthesis of protein B peaked at about 8 hours.

Friesen and Rueckert (1984) found evidence of translational control by competition between RNA 1 and RNA 2 in a cell-free translational system using Drosophila lysate. The greater the ratio of RNA 2 to RNA 1 the less the ability of RNA 1 to compete with RNA 2 for a rate-limiting factor(s) required for initiation of translation; and thus the greater the 'shut-off' effect on RNA 1 and hence decline of protein A synthesis. This translational competition between mRNA's is not unique to BBV. It indicates a difference between affinities for the rate-limiting factor(s) mentioned above, of the

different mRNA's. Thus there is preferential translation of those mRNA's with the highest affinities.

They also found that when translation was carried out in both a cell-free Drosophila lysate system and rabbit reticulocyte lysate system, at concentrations of BBV RNA exceeding the saturation level (50-60 µg/ml), there was a decrease in the synthesis of protein A relative to protein α and this seemed to indicate a preferential translation of RNA 2 compared with RNA 1. It was also found that at low concentrations of RNA, reticulocyte lysates translated RNA 1 more efficiently than Drosophila extracts. Friesen and Rueckert (1984) suggested that this difference might have been due to the reticulocytes being richer in those factors that limit translation of RNA 1 in Drosophila lysates, and that a purification of these factors might prove useful for studying competition between BBV RNA 1 and RNA 2.

1.4. CELL-FREE TRANSLATION IN RETICULOCYTE LYSATES

Other cell lysate systems have been used, but rabbit reticulocyte lysate is easily available (Villa-Komaroff et al., 1974; Schimke et al., 1974; Pelham and Jackson, 1976; Eisenstein and Harper, 1984). It is intended as a general purpose reagent for protein synthesis and for the translation of most mRNA's, adjustment of potassium and magnesium concentrations not being necessary. It is also usually treated with micrococcal nuclease to remove endogenous mRNA.

As already mentioned, cell-free translation has been used to study various aspects of BBV, notably the synthesis and processing of protein products and the regulation of gene function.

In 1984 and 1985 Professor D. Hendry worked in the Biophysics Laboratory (with Professor R. Rueckert's group) at the University of Wisconsin-Madison, Wisconsin. This was one of two groups that was studying aspects of *in vitro* translation of black beetle virus RNA. Preliminary work on the *in vitro* cleavage of the precursor capsid protein into mature proteins was started there.

While investigating the translational competition between BBV RNAs 1 and 2 in Madison, it was noticed that a protein, apparently β, arose in rabbit reticulocyte lysate apparently by the cleavage of α. This had not been observed before with nodaviruses, and it was decided to investigate this. The experiment carried out was the rationale for this thesis. As putative protein β was consistently produced after extended incubation of the cell-free system, the experiment was a preliminary one to determine, so far as possible, whether it was in fact authentic protein β. Putative protein β ("β") was found to co-electrophorese with authentic BBV protein β and was also precipitated from the overnight-incubated lysate by anti-BBV antisera. This indicated that "β" was related to BBV coat protein β, and from now on therefore, "β" will be referred to as β, and not putative β.

1.5. AIMS OF PROJECT

The experiments described in this thesis were designed to investigate the aspects of the formation of protein β, demonstrated by the Madison experiment described above, listed below:

- if translation was stopped without degrading the BBV mRNA by using cycloheximide instead of ribonuclease, would the cleavage be prevented?

- if the α to β cleavage required some form of assembly, even if only incomplete, could (sub) assembly structure be detected in the lysate?

- if α was translated *in vitro* off RNA 2 alone, would cleavage still occur even if RNA 1 was absent?

- was the cleavage autocatalytic or did it involve some other factor(s)?

CHAPTER 2

PROPAGATION AND PURIFICATION OF BLACK BEETLE VIRUS, AND PREPARATION OF RNA

2.1. SUMMARY

Drosophila melanogaster line 1 cells (Fig. 2.1) were propagated in Schneider's Insect Medium (SCHIM) at 26°C. Flasks (75-cm^2 style) seeded with approximately 4 x 10^6 cells per 20 ml growth medium formed confluent monolayers within 4 days. Black beetle virus, strain W17, was purified from *Drosophila* line 1 cells by a modified version of the technique described by Guarino *et al.* (1981). A typical yield of purified virus obtained from 10^8 cells was approximately 4 mg. RNA was extracted from purified BBV by the phenol-chloroform-isoamylalcohol method |48 : 24 : 1| (Newman *et al.*, 1978). The two RNA species were separated on sucrose density gradients. A typical yield of RNA from purified virus was approximately 70-85% of the calculated total possible.

2.2. INTRODUCTION

Black beetle virus was to be propagated in cultured cells of *Drosophila melanogaster* (Friesen and Rueckert, 1981), so the first stage of this work was to establish the cell line. Once the *Drosophila* cells were growing successfully, standard procedures as described in the literature were to be used for the purification of BBV, the extraction of RNA and the separation of RNA 1 and RNA 2. These methods are described in this chapter together with certain variations that were found to increase the obtained yields of BBV and RNA.

2.3. METHODS

2.3.1. ESTABLISHMENT OF THE CELL LINE

Schneider's culture medium (see Section 2 of the Appendix) was used for propagation of the Drosophila melanogaster cells (Schneider's line 1, WR subline) (Schneider, 1972), and the medium was supplemented with 15% foetal calf serum (FCS) (Friesen et al., 1980). The cells were grown in either 850-cm^2 style roller bottles (Falcon Labware, Oxnard, CA 93030) turning at 0,5 rpm or 75-cm^2 style tissue culture flasks (Sterilin Limited, England). Bottles were seeded with approximately 2 x 10^5 cells per ml growth medium. Cells were passaged by flushing confluent monolayers into the spent medium with a pipette, and diluting the cells 75 to 100-fold in fresh culture medium.

2.3.2. POPULATION GROWTH CURVE

A growth curve experiment was set up in order to determine the population doubling time of the cells (Fig. 2.2). The doubling time was calculated as follows (MICROBIOLOGY. Third Edition. Davis, Dulbecco, Eisen and Ginsberg, pg. 65)

$$\text{growth rate} = (3{,}32 \log_{10} X_2/X_1) \; / \; t_2 - t_1$$

$$\text{doubling time} = \text{growth rate}^{-1}.$$

(X_2 = final cell count; X_1 = initial cell count; $t_2 - t_1$ = time elapsed between X_1 and X_2). A haemocytometer count was made every day for 14 days. The diluent was 0,5% Trypan blue in normal saline.

2.3.3. PURIFICATION OF BBV

The cytolytic strain of black beetle virus, BBV-W17 (Selling and Rueckert, 1984) was used throughout. The method used for purifying

the virus, a modified version of that described by Guarino et al. (1981), is described in Section 1.1.2. of the Appendix.

2.3.4. EXTRACTION OF RNA

RNA was extracted from purified virus by the phenol-chloroform-isoamylalcohol method as described by Friesen and Rueckert (1981) (see Section 1.2 of the Appendix for details).

2.3.5. SEPARATION OF THE TWO RNA SPECIES

The two RNA species were separated by means of sedimentation on sucrose density gradients as described by Friesen and Rueckert (1981) (see Section 1.3 of the Appendix for details).

2.4. RESULTS

2.4.1. ESTABLISHMENT OF THE CELL LINE

When the flasks were seeded with approximately 2×10^5 cells per ml of culture medium, confluent monolayers were formed within approximately 4 days at 26°C.

The population growth curve experiment was carried out in triplicate. Graphs of the three sets of results were very similar. Fig. 2.2. represents one such set of results. The population doubling time of the Drosophila cells was found to be approximately 13-20 hours.

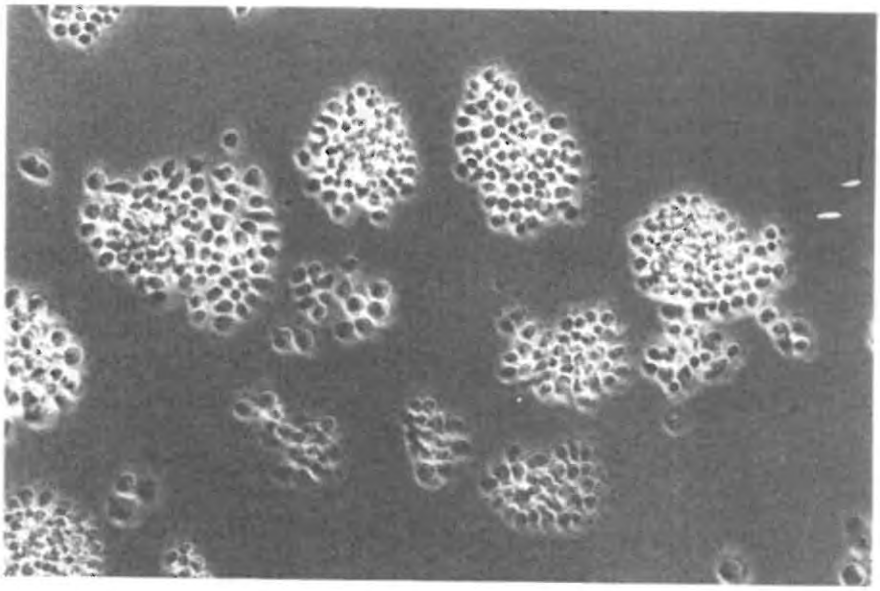

Fig. 2.1. Schneider's Drosophila melanogaster line 1 cells in the 364th passage in Schneider's Insect Medium. Phase-contrast. x 200.

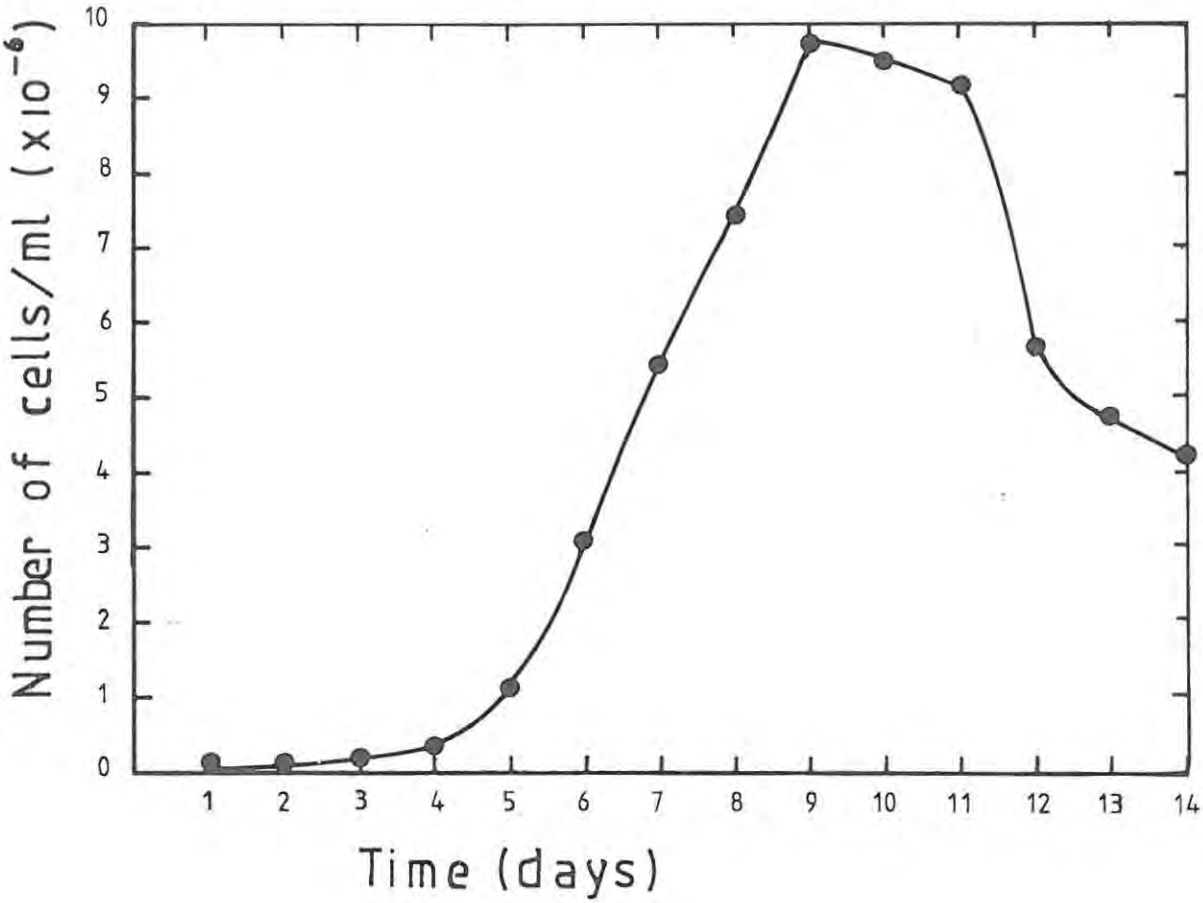

Fig. 2.2. Growth curve for D. melanogaster line 1 at 26°C in Schneider's culture medium supplemented with 15% inactivated FCS. (See text for method).

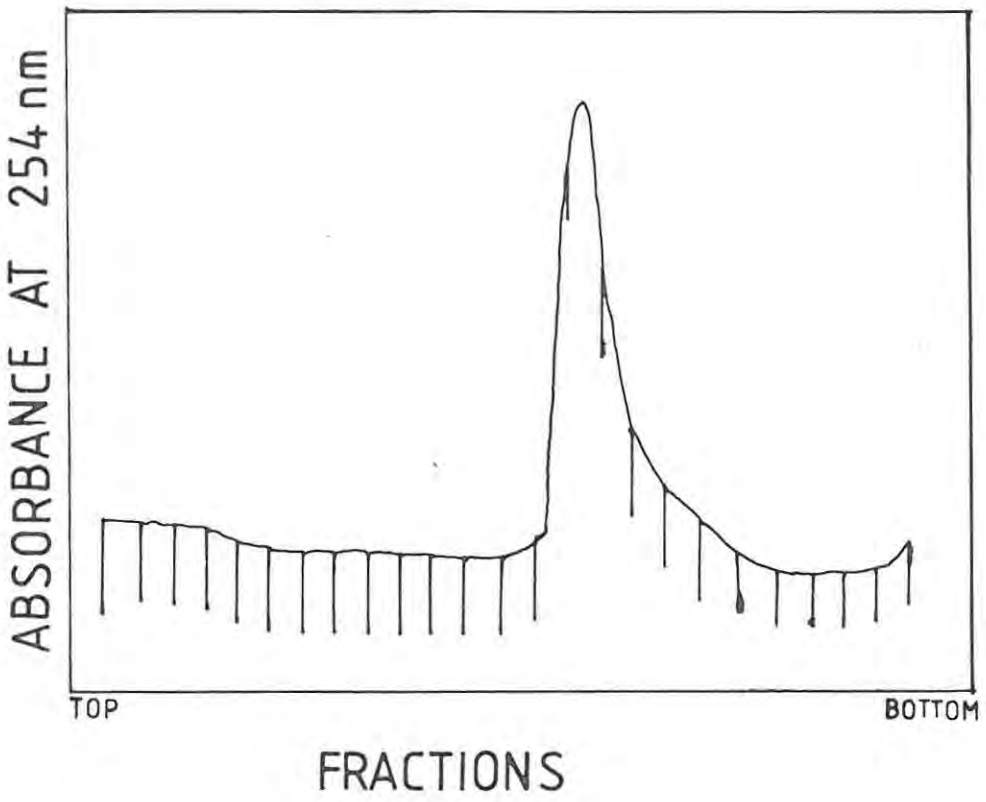

Fig. 2.3. Absorbance profile of 1,2 ml fractions of purified BBV off a 32-ml 15 to 45% (wt/vol) sucrose gradient, using an Isco density gradient fractionator model 640 with a model UA-5 absorbance monitor. (Sensitivity = 2 O.D. units full-scale; chart speed = 60 cm/h; flow rate = 3 ml/min). The top and bottom of the gradient are marked.

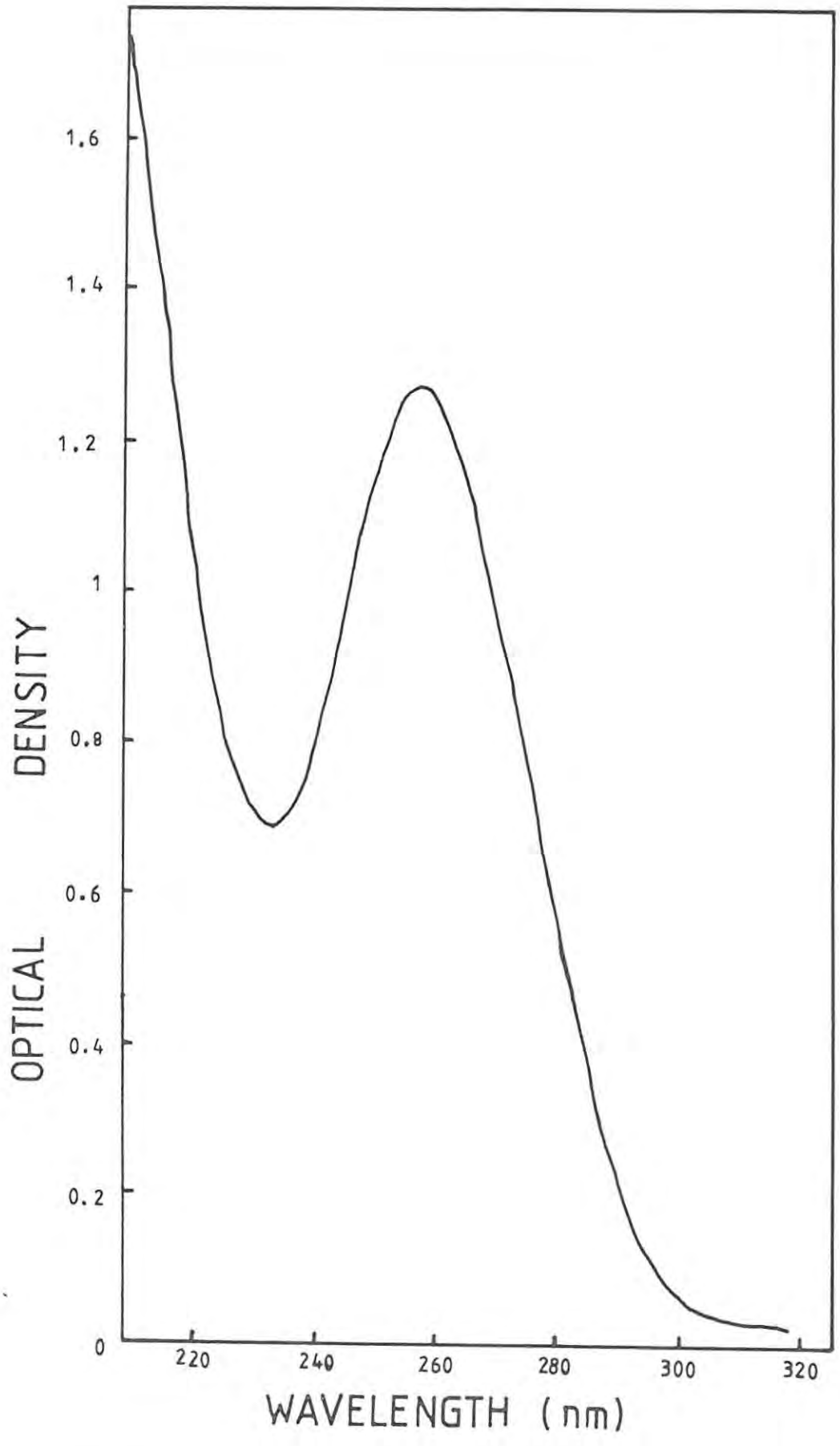

Fig. 2.4. Absorbance profile of BBV RNA.

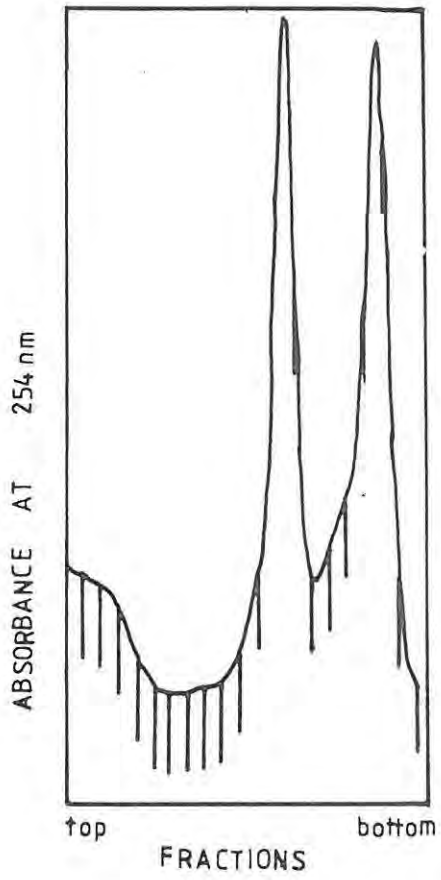

Fig. 2.5. Absorbance profile of sucrose density gradient centrifugation of BBV RNA for separation of the two RNA species. 0,4 ml fractions were collected from a 12-ml 5 to 25% (wt/vol) sucrose gradient using an Isco Density gradient fractionator model 640 with a model UA-5 absorbance monitor. (Sensitivity = 0,5 O.D. units full-scale; chart speed = 60 cm/h; flow rate = 2 ml/min). The top and bottom of the gradient are marked.

2.4.2. PURIFICATION OF BBV

Fractions of purified BBV were collected from a sucrose gradient, using an Isco density gradient fractionator with a model UA-5 absorbance monitor. The fractions were monitored for virus by absorbancy at 260 nm. A typical absorbancy profile is seen in Fig. 2.3. Fractions that absorbed at 260 nm were pooled and stored. On average, 4 mg of purified BBV were obtained from 10^8 cells.

The purification of BBV was carried out on a number of occasions. A comparison of the method of Guarino et al. (1981) was made with that described by Friesen et al. (1980) and it was noted that yields of purified BBV were consistently about 20% greater when the former method was used.

2.4.3. EXTRACTION OF RNA

It was noted that RNA yields were approximately 28% greater when extraction of RNA was performed immediately after the final stage of purification of the virus than they were when RNA was extracted from purified virus that had been stored at -20°C.

Figure 2.4. shows the absorbance profile of BBV RNA at wavelengths between 210 and 320 nm. The profile is typical for a solution of RNA, absorbing maximally at 260 nm.

2.4.4. SEPARATION OF THE TWO RNA SPECIES

BBV RNA was separated into the 2 species RNA 1 and RNA 2 by means of sedimentation on sucrose density gradients, and a typical profile

may be seen in Figure 2.5. It was found that at least two cycles of sedimentation were necessary to completely separate the two species. This was tested by translating each species in a cell-free translation experiment (Chapter 3).

2.5. DISCUSSION

The Drosophila cells grew successfully in Schneider's insect medium, as shown by the population growth curve (Fig. 2.2) and the population doubling time which was similar to that reported by Schneider (1972).

BBV was successfully purified from infected Drosophila cells on a number of occasions and the usual yield of virus was found to be approximately 4 mg from 10^8 Drosophila cells. This compared favourably with yields quoted by Guarino et al. (1981). They obtained a typical yield of 1 mg of purified virus from 10^8 infected Drosophila cells.

The phenol-chloroform-isoamylalcohol |48 : 24 : 1| method was used for extraction of RNA from purified BBV. It was noted that a typical yield of purified RNA extracted from BBV by this method was approximately 70-85% of the calculated total possible maximum. This was calculated on the basis that 28% of the BBV virion is RNA.

When the BBV RNA had been separated into RNA 1 and RNA 2 (Fig. 2.5), it was necessary to check that the separation was complete. This is described in Chapter 3.

CHAPTER 3

TRANSLATION OF BBV RNA, AND OF SEPARATED RNAs 1 AND 2

3.1. SUMMARY

In vitro dose response experiments using BBV RNA in rabbit reticulocyte lysate showed that concentrations of between 50-75 μg/ml RNA were optimal for cell-free translation. It was found that at least 2 cycles of sedimentation in sucrose density gradients were necessary to separate BBV RNA into the two species, RNA 1 and RNA 2. A dose response experiment of RNA 2 showed that between 20 and 40 μg RNA 2 was optimal for use in cell-free translation experiments. Concentrations greater than 40 μg/ml lysate saturated the system. Cycloheximide, at a final concentration of 60 μg/ml of lysate, was found to halt translation of BBV RNA in the cell-free translation system used. This was an important result since cycloheximide was to be used extensively throughout the course of this work.

3.2. INTRODUCTION

It was important to check that the rabbit reticulocyte lysate functioned efficiently as a cell-free translation system. It was also necessary to determine the optimal RNA concentration for the cell-free synthesis of BBV proteins.

The separation of BBV RNA into RNA 1 and RNA 2 has been described in Chapter 2. It was necessary to check that the separation of the RNA species was complete; to check that each species, when translated in a cell-free lysate system, produced only its respective

proteins; and also to perform a dose response experiment using RNA 2. An *in vitro* translation experiment was carried out to study the time course of incorporation of radiolabel with RNA 1 and varying concentrations of RNA 2.

It was also very important at this stage to confirm that cycloheximide, at a concentration of 60 µg/ml of lysate (personal communication from D.A. Hendry) (Pallanch *et al.*, 1980) would indeed stop translation of BBV RNA used in cell-free translation experiments.

3.3. METHODS

3.3.1. EFFICIENCY OF THE RABBIT RETICULOCYTE LYSATE

To check that the lysate was working, the ingredients for cell-free translation (see Section 1.4 of the Appendix) were added to each of 3 eppendorf tubes. To initiate translation, BBV RNA at 60 µg/ml final concentration (Friesen and Rueckert, 1984) was added to tube 2, TMV RNA (Amersham International Laboratories, England) at 60 µg/ml final concentration (the recommended concentration by Amersham) was added to tube 3, while tube 1 served as a negative control with water added in place of RNA. A time course experiment was then carried out, with 2 µl samples being removed from each of the 3 tubes at 0, 5, 10, 20, 30 and 60 minutes.

3.3.2. DOSE RESPONSE OF BBV RNA

A dose response experiment using BBV RNA was carried out using the following concentrations of RNA in an *in vitro* cell-free system as

described in Section 1.4 of the Appendix: 0, 25, 50, 75, 100 and 125 µg/ml. Translation was allowed to proceed for 60 min before it was terminated. Samples of 2 µl of each concentration mixture were spotted onto filter paper discs and treated as described in Section 1.4.3 of the Appendix. Each filter paper disc was assayed for radioactivity as described.

3.3.3. COMPLETE SEPARATION OF RNA

As described in Chapter 2, BBV RNA was separated into RNA 1 and RNA 2 by two cycles of sedimentation in sucrose density gradients. To check that the separation of the species was complete, electrophoresis of each 'separated' RNA species was carried out in a composite agarose/acrylamide gel (see Section 1.6 of the Appendix). Samples of 2 µg of each species, and 8 µg of unseparated BBV RNA as a control, were loaded onto the gel. Each species was also translated separately overnight in a cell-free translation experiment (see Section 1.4 of the Appendix). Concentrations of 40 µg/ml RNA 1 and 20 µg/ml RNA 2 were tested in the system.

3.3.4. DOSE RESPONSE OF RNA 2

The following concentrations of RNA 2 were added to cell-free lysate mixtures (described in Section 1.4 of the Appendix): 0, 20, 40, 60 µg/ml of lysate. Translation was allowed to proceed for 60 min before the reactions were terminated. Samples of 2 µl of each of the four reaction mixtures were spotted onto filter paper discs, the discs were treated as described in the Appendix and each disc was assayed for radioactivity as described.

3.3.5. TIME COURSE EXPERIMENT

Four parallel *in vitro* translation reactions were set up, and RNA was added to each reaction vial to obtain the following RNA concentrations: 40 µg/ml RNA 1, 20 µg/ml RNA 2, 40 µg/ml RNA 2 and 60 µg/ml RNA 2. Samples of 2 µl from each reaction were removed at 0, 15, 30, 45 and 60 min after addition of the RNA to the reaction, spotted onto filter paper discs, treated and assayed for radioactivity as described in the Appendix. A detailed experiment set up in order to determine the exact stage at which β was produced is described in Chapter 4.

3.3.6. THE USE OF CYCLOHEXIMIDE

To check that the cycloheximide functioned efficiently, the ingredients for cell-free translation (see Section 1.4 of the Appendix) were added to each of 2 eppendorf tubes. Translation was initiated by the addition of 60 µg BBV RNA per ml of lysate and the translation was allowed to proceed for a total of 60 minutes, with 60 µg of cycloheximide per ml of lysate being added at 15 minutes to tube 2, and at 30 minutes to tube 1. At 0, 5, 10, 15, 20, 30, 45 and 60 minutes after addition of BBV RNA, 2 µl samples of the translation mix were removed, spotted onto filter paper discs, treated and assayed for radioactivity as described in the Appendix.

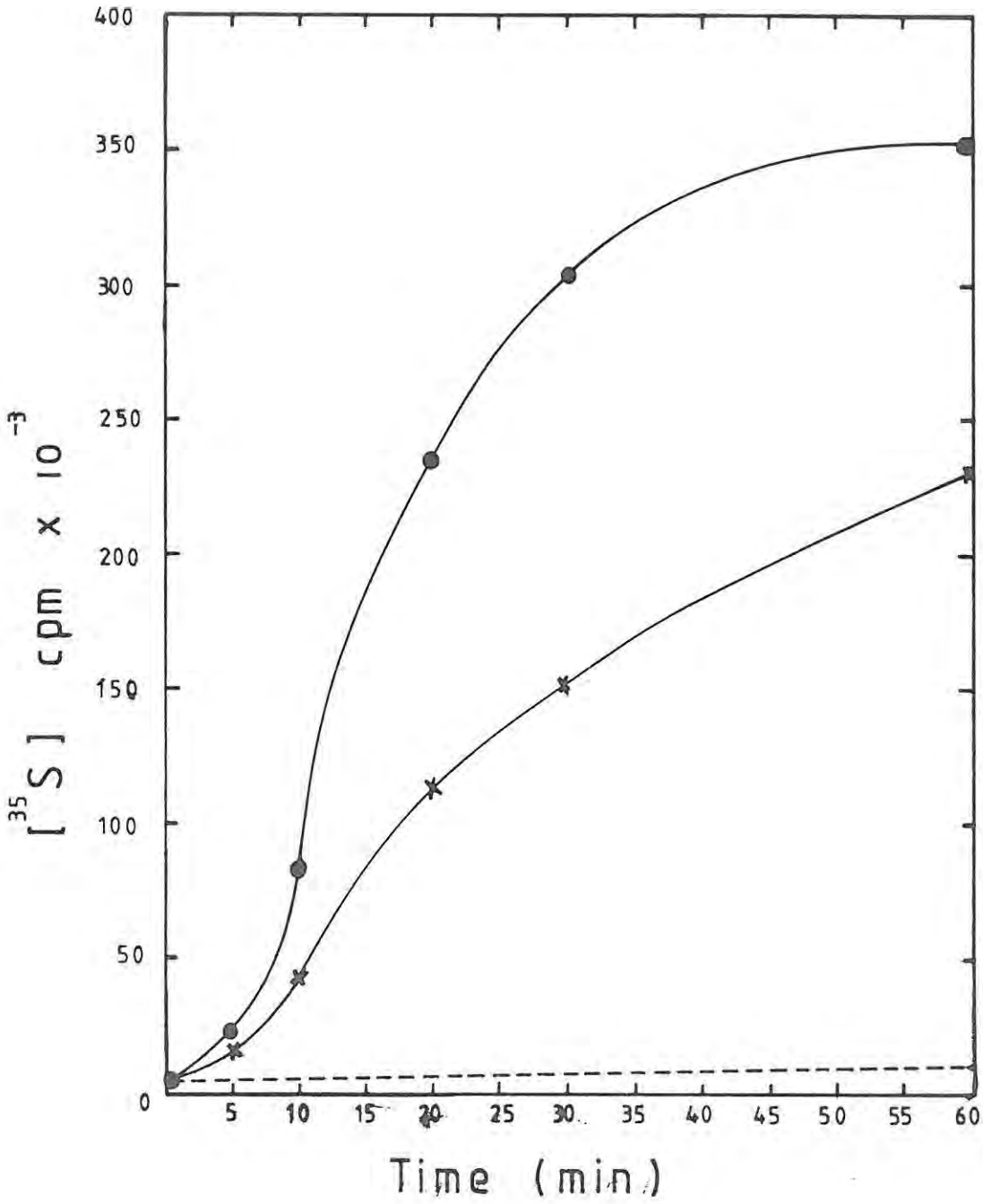

Fig. 3.1. A check on the efficiency of rabbit reticulocyte lysate as a cell-free translation system (see text for method). The figure shows the number of cpm measured per 2 µl of lysate. (---) is the result of negative control tube 1. (✕—✕) is the result of tube 3 containing TMV RNA and (●—●) is the result of tube 2, containing BBV RNA.

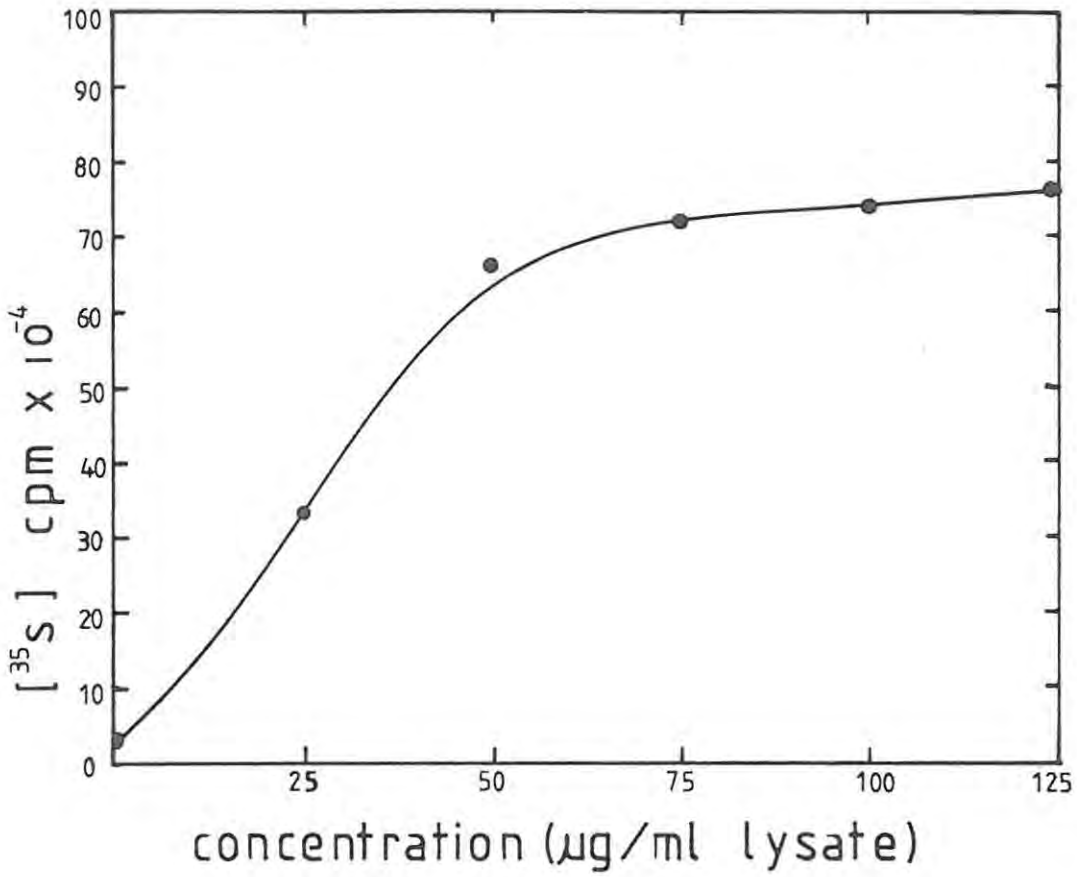

Fig. 3.2. Dose response curve of unseparated BBV RNA in rabbit reticulocyte lysate. BBV RNA was added to the lysate to achieve the indicated concentrations and, after translation had been in progress for 30 min at 30°C, 2 µl samples of lysate were removed and subjected to scintillation counting after being spotted onto treated filter paper discs. The figure shows the number of cpm measured per 2 µl of lysate.

Fig. 3.3. RNA gel showing the separation of BBV RNA into two species following two cycles of sucrose density gradient centrifugation. Electrophoresis of each species on a composite agarose/acrylamide gel shows a single band of RNA per species. Lane 1 was loaded with 8 µg BBV RNA, lane 2 was loaded with 2 µg RNA 1 and lane 3 was loaded with 2 µg RNA 2.

Fig. 3.4. Autoradiogram showing the electrophoretic separation in polyacrylamide gel of the proteins resulting from overnight translation of separated RNA 1 (40 µg/ml) (lane 1) and RNA 2 (20 µg/ml) (lane 2).

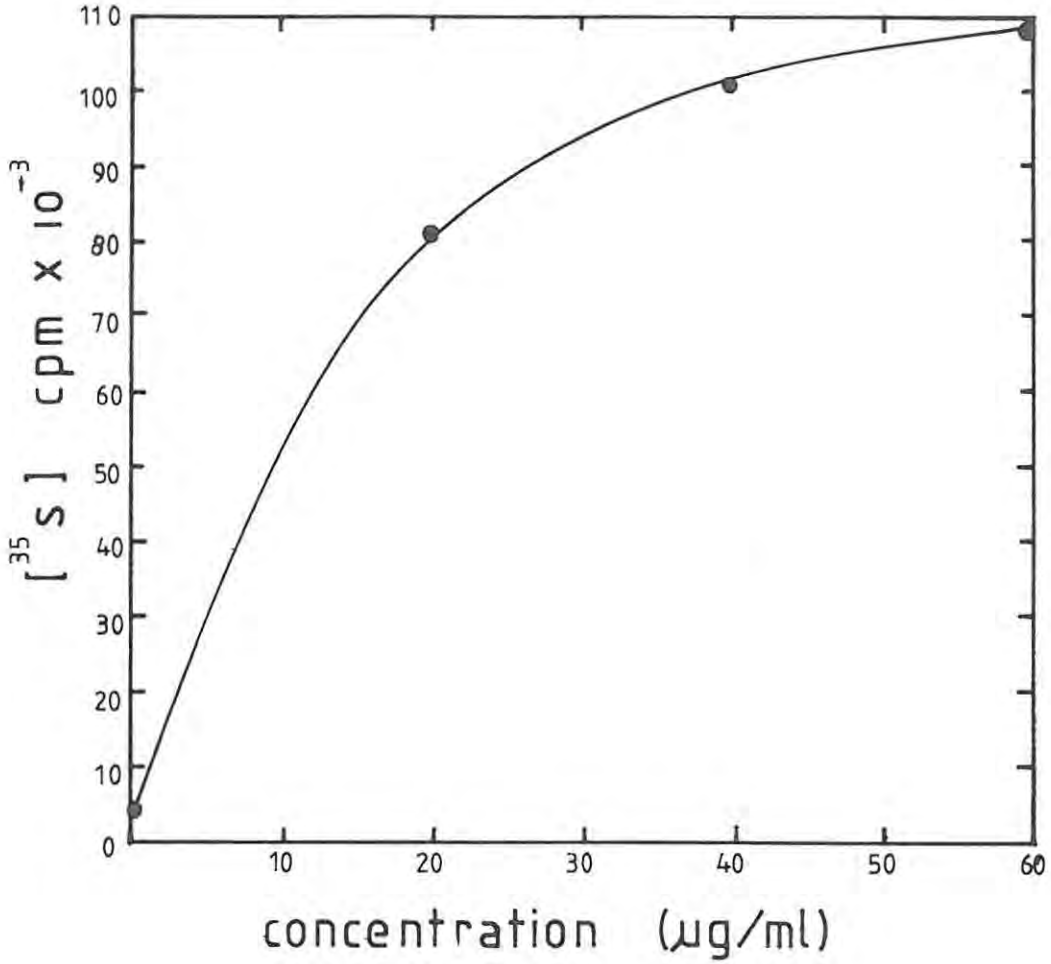

Fig. 3.5. Dose response of RNA 2 in rabbit reticulocyte lysate. BBV RNA 2 was added to the lysate to achieve the indicated concentrations and, after translation had been in progress for 30 min at 30°C, 2 µl samples of lysate were removed and subjected to scintillation counting after being spotted onto treated filter paper discs. The figure shows the number of cpm measured per 2 µl of lysate.

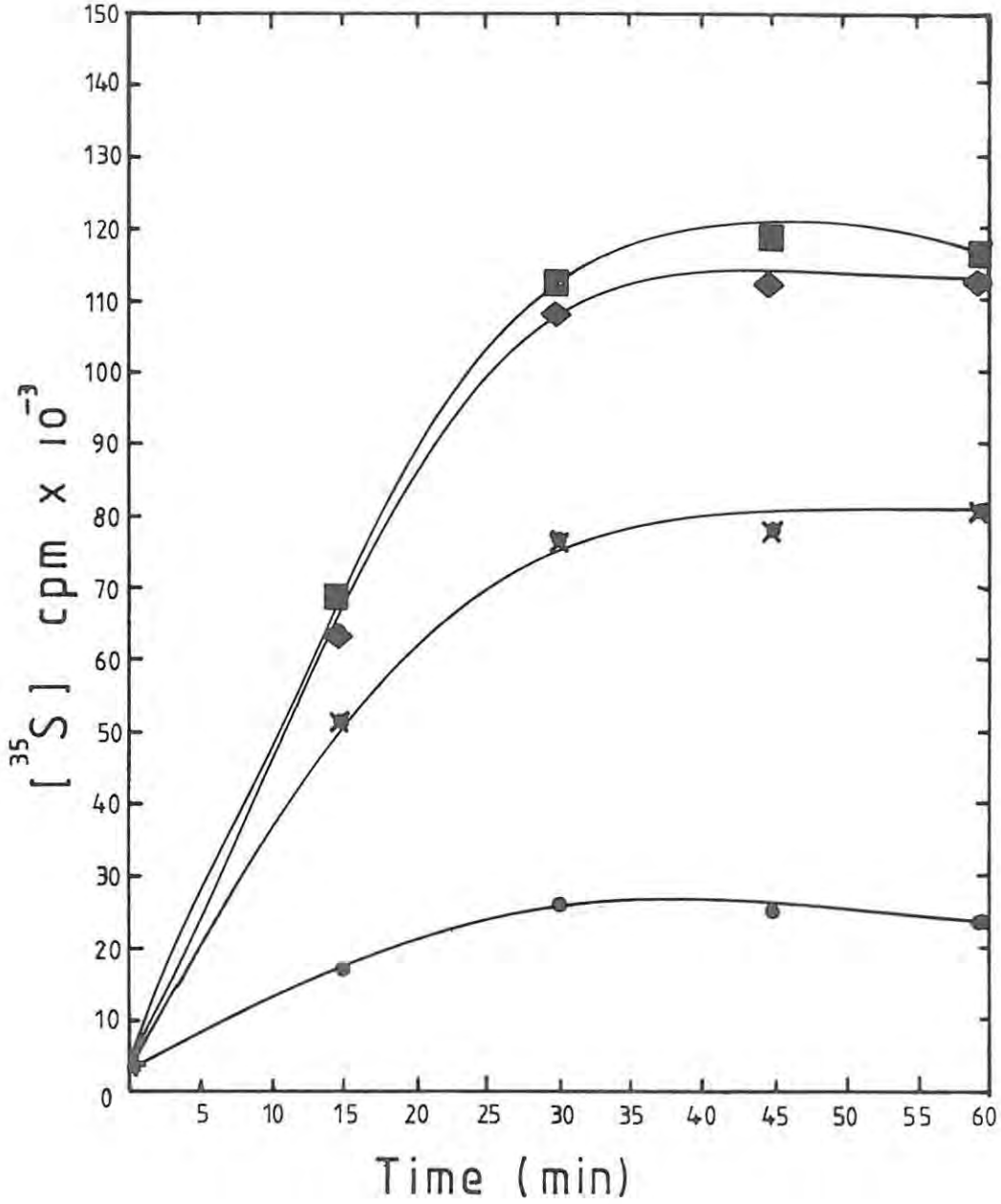

Fig. 3.6. Time course of protein synthesis in rabbit reticulocyte lysate primed with RNA 1 and 3 concentrations of RNA 2. BBV RNA 1 and RNA 2, at the indicated concentrations, were added to the lysate. Samples of 2 µl of lysate were removed at the indicated times and subjected to scintillation counting after being spotted onto treated filter paper discs. The figure shows the number of cpm measured per 2 µl of lysate. (●—● is 40 µg/ml RNA 1; ✖—✖ is 20 µg/ml RNA 2; ◆—◆ is 40 µg/ml RNA 2; ■—■ is 60 µg/ml RNA 2).

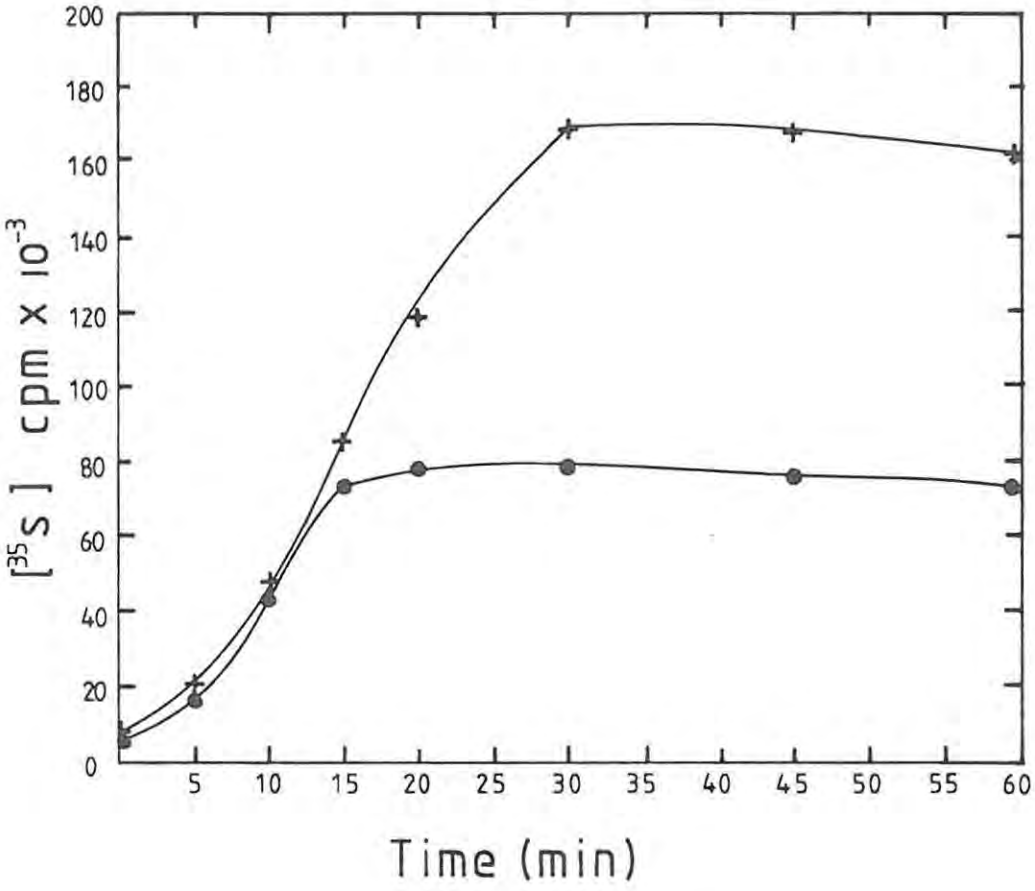

Fig. 3.7. The effect of the addition of 60 µg/ml cycloheximide to a cell-free translation mixture containing BBV RNA. The figure shows the number of cpm measured per 2 µl of lysate. (●—●) shows the results of the addition of the cycloheximide at 15 min, and (+—+) shows the results of the addition at 30 min.

3.4. RESULTS

3.4.1. EFFICIENCY OF THE RABBIT RETICULOCYTE LYSATE

Fig. 3.1 demonstrates the efficient functioning of the lysate (as indeed do Figs. 3.2, 3.5 and 3.6). The negative control, with distilled water in place of RNA, showed a negligible level of incorporation of radiolabel compared with that of tubes 2 and 3, where BBV RNA and TMV RNA, respectively, had been added. The level of incorporation of radiolabel (in other words, translation activity) in tube 3 to which TMV RNA had been added, compared very favourably with the results of the Amersham Laboratories (Batch analysis, N/211, Amersham International).

3.4.2. DOSE RESPONSE OF BBV RNA

The results of the dose response experiment using BBV RNA are seen in Fig. 3.2. The graph tended to rise steeply at concentrations of RNA of between 0 and 50 µg/ml, and tended to reach a plateau at concentrations of RNA of between 50 and 75 µg/ml.

3.4.3. COMPLETE SEPARATION OF RNA

Two cycles of sedimentation in sucrose density gradients of BBV RNA were sufficient to completely separate the RNA into RNA 1 and RNA 2. The results of the experiment may be seen in the RNA gel in Fig. 3.3 and the autoradiogram in Fig. 3.4.

Lane 1, Fig. 3.4 shows protein A translated from 40 µg/ml RNA 1, lane 2 shows the proteins translated from 20 µg/ml RNA 2, namely

protein α and, because translation was allowed to proceed overnight, also protein β. There was no evidence of cross-contamination of either RNA species, and this was confirmed in Fig. 3.3.

3.4.4. DOSE RESPONSE OF RNA 2

Fig. 3.5 shows that the graph rose steeply at concentrations of RNA 2 of between 0 and 20 μg/ml but rose less steeply at concentrations of between 20 and 40 μg/ml, and in fact tended to reach a plateau at concentrations of between 40 and 60 μg/ml.

3.4.5. TIME COURSE EXPERIMENT

Fig. 3.6 shows the results of the time course experiment using RNA 1 and increasing concentrations of RNA 2. At approximately 30 min in each case, the level of translation reached a peak and then plateaued with increasing time. It could also be seen that concentrations of RNA 2 greater than 40 μg/ml seemed to saturate the system, because the levels of incorporation of radiolabel were very similar.

3.4.6. The use of cycloheximide

Fig. 3.7 shows the effect of cycloheximide addition at two particular stages of translation, 15 and 30 minutes. As can be seen, the addition of cycloheximide at 15 min resulted in the almost immediate termination of translation, and the addition at 30 min had a similar effect, with translation stopping at 30 minutes.

3.5. DISCUSSION

The addition of both BBV RNA and TMV RNA to the rabbit reticulocyte lysate (Fig. 3.1) showed that the lysate was indeed functioning efficiently as a cell-free translation system.

The dose response curve of BBV RNA (Fig. 3.2) revealed that between 50 and 75 µg/ml of RNA was optimal for use in future experiments. This agreed with quoted results (Friesen and Rueckert, 1984).

Two cycles of sedimentation in sucrose gradients were seen to be sufficient to completely separate the two RNA species (Fig. 3.3 and 3.4). Translation of separated RNA 1 resulted in the appearance of protein A with no trace of protein α whereas translation of RNA 2 resulted in the appearance of protein α with protein β, but no protein A. The origin of the smallest protein in lane 2 is not known. It was consistently produced when RNA 2 was translated in rabbit reticulocyte lysates. Its nature is under investigation and it is thought that perhaps it might be an endogenous lysate protein.

Results of the dose response experiment of RNA 2 (Fig. 3.5) showed that concentrations of RNA 2 of between 20 and 40 µg/ml were optimal for use in future *in vitro* cell-free translation experiments but Fig. 3.6 showed that concentrations of RNA 2 greater than 40 µg/ml saturated the system.

Cycloheximide at a final concentration of 60 µg/ml of lysate was found to stop translation at the particular time at which it was added to the cell-free translation system (Fig. 3.7).

CHAPTER 4

APPEARANCE OF BBV PROTEIN β IN A CELL-FREE TRANSLATION SYSTEM

4.1. SUMMARY

Translation of BBV RNA in rabbit reticulocyte lysate showed that protein α was produced between 0 and 30 minutes of translation and protein β was detectable in the system after 30 minutes of translation. The latter increased in intensity and was maximal after 16 hours (960 minutes). At this stage the β band was equal in intensity to the α band.

4.2. INTRODUCTION

It was necessary at this stage to check when protein β was first detectable in an *in vitro* cell-free system, so that, when studying the events affecting the cleavage of α to β, the system could be halted at the stage where protein α was maximum in amount and where β was not yet evident. Subsequent experiments (Chapters 5 and 7) made use of this information.

4.3. METHODS

The standard cell-free translation reaction as described in Section 1.4 of the Appendix was set up. BBV RNA (60 µg/ml of lysate) was added to the mix to initiate translation. Ten times the normal volume of each reagent was used to allow for testing of ten different translation periods: 0, 30, 40, 45, 60, 80 min; 2, 4, 5 and 16 h. At each of these times, a 30 µl sample was removed, 2 µl of this was spotted onto a filter paper disc, and this was monitored for incor-

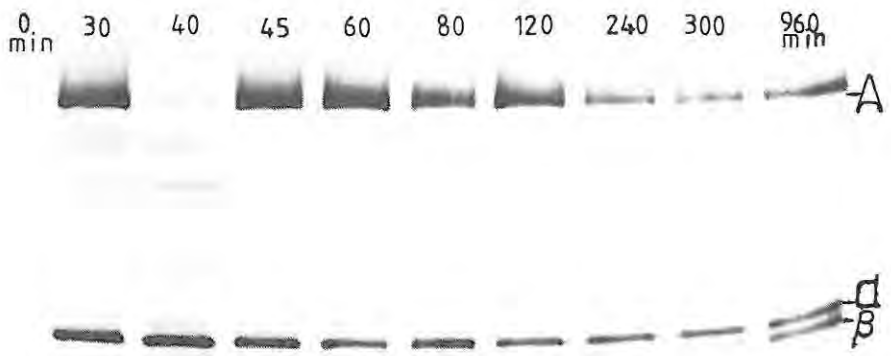

Fig. 4.1. Autoradiogram showing the beginning of the appearance of protein β after 30 min translation of BBV RNA in rabbit reticulocyte lysate. Proteins A and α are also indicated. Samples were removed for autoradiography at each of the times indicated. (See text for method).

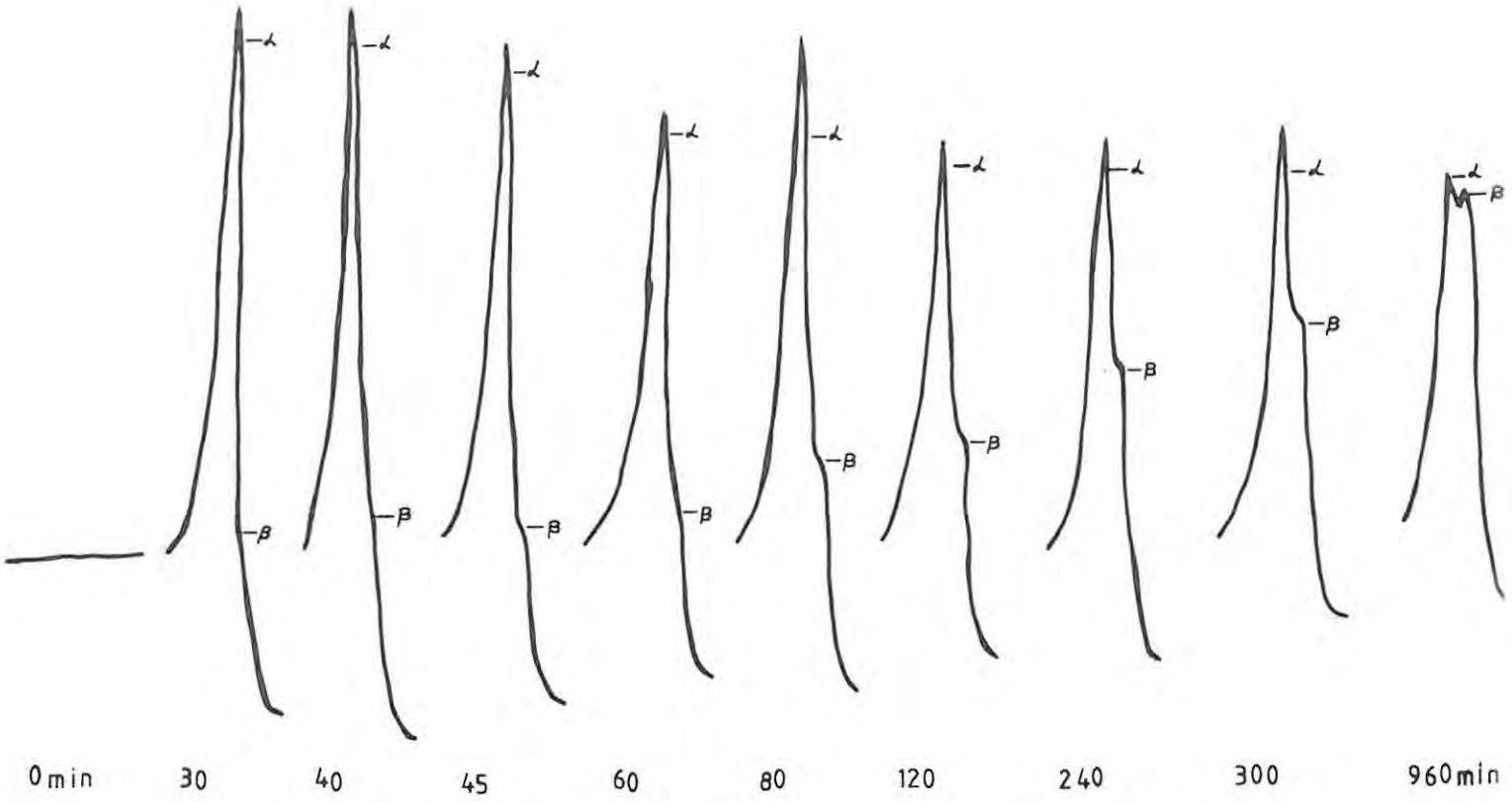

Fig. 4.2. Densitometer scan of the α and β protein regions from each lane of autoradiogram Fig. 4.1. Scanning direction is from top to bottom. Times indicated correspond with Fig. 4.1.

poration of $[^{35}S]$methionine into TCA-insoluble material. PAGE was performed on each sample, followed by autoradiography. The autoradiogram was subsequently scanned on a densitometer, to monitor the actual appearance of protein β.

4.4. RESULTS

Fig. 4.1 shows the results of the autoradiogram showing the appearance of proteins α and β. It can be seen that protein α was detectable between 0 and 30 minutes. Protein β made its appearance after 30 minutes. The densitometer scan (Fig. 4.2) showed a similar result; that protein β was first detectable in the system after 30 minutes, and steadily increased until it reached a maximum at 16 hours (960 min). The α protein band on the other hand, decreased in relative intensity from 30 minutes to 16 hours and at that stage the intensity of the α and β protein bands was very similar.

4.5. DISCUSSION

This result confirmed the observation made in Madison by D.A. Hendry that there was a corresponding decrease in the level of α accompanying the steady increase in concentration of protein β from 30 minutes to 16 hours of translation. The emergence of β in the lysate, from the cleavage of protein α, therefore seemed to be a genuine feature of strain W17.

CHAPTER 5

TRANSLATION OF BBV RNA 2, AND THE PREVENTION OF THE CLEAVAGE FROM α TO β.

5.1. SUMMARY

Translation of purified BBV RNA 2 in rabbit reticulocyte lysate resulted in the production of protein α, which, in the presence of BBV RNA 2, was cleaved to protein β. This cleavage did not require the presence of BBV RNA 1 or the presence of an RNA 1 translation product. Cycloheximide did not inhibit the cleavage.

The cleavage was prevented by the addition of RNase to the rabbit reticulocyte lysate system after 30 min. In other words, the cleavage required the presence of intact RNA. It was also found that the cleavage to β could be prevented by cooling the lysate mix to 1°C.

5.2. INTRODUCTION

It was known at this stage that translation of BBV RNA for more than 30 minutes resulted in the production of protein β (Chapter 4). It was not known whether the cleavage of protein α to protein β was dependent upon the presence of RNA 1 or RNA 2, or whether the termination of translation by the addition of cycloheximide would affect the cleavage. The experiment described in Section 5.3.1 of this chapter was designed to clarify these aspects.

It was also considered important at this stage to discover what steps

could be taken to arrest the cleavage at the precursor α stage. Once this was known, various experiments could be carried out on the precursor to determine what factors affected its cleavage. This is discussed further in Chapter 8.

5.3. METHODS

5.3.1. TRANSLATION OF BBV RNA 2

The standard cell-free translation reaction was set up (see Section 1.4 of the Appendix). BBV RNA 2 (at a final concentration of 20 μg per ml of lysate), purified by two cycles of sucrose density gradient centrifugation (Chapter 3) was added to the reaction mix to initiate translation. After 30 minutes translation at 30°C, 2 μl of cycloheximide at 1 mg/ml was added to the mix to halt translation. A sample of the reaction mixture was removed at this stage for subsequent PAGE and autoradiography. The remainder of the reaction mixture was allowed to incubate at 30°C overnight for 16 hours, after which time PAGE and autoradiography was carried out on the mixture as described in the Appendix.

5.3.2. THE EFFECT OF THE ADDITION OF RNase AND CYCLOHEXIMIDE

Four eppendorf tubes of the standard cell-free translation reaction (described in Section 1.4 of the Appendix) were set up. BBV RNA was added to each to initiate translation. After 30 minutes translation at 30°C, cycloheximide and RNase were added to the tubes as follows: cycloheximide was added to tube 2 to obtain 60 μg per ml of lysate, cycloheximide and pancreatic RNase were added to tube 3

to obtain respective concentrations of 60 µg per ml of lysate, and pancreatic RNase was added to tube 4 to obtain 60 µg per ml of lysate. Tube 1 contained no cycloheximide or RNase. At this stage, a 2 µl sample from each tube was removed and spotted onto filter paper discs. At the same time a sample was removed from each tube for PAGE. These were treated and assayed for radioactivity as described in the Appendix. The remainder of the contents of each tube was further incubated at 30°C for 16 hours, at which stage the reactions were terminated and the samples set aside for PAGE. Autoradiography was carried out on the gel.

5.3.3. THE EFFECT OF LOWERING THE TEMPERATURE TO 1°C

The standard cell-free translation reaction as described in Section 1.4 of the Appendix, was set up. BBV RNA 2 (at a final concentration of 20 µg per ml of lysate) was added to initiate translation. After 30 minutes translation at 30°C, cycloheximide was added to the mix to stop any further translation. At this stage a sample was removed to serve as the 30 minute control. The rest of the sample was divided into three aliquots, one of which was left for 16 hours at 30°C. The second and third aliquots were chilled immediately to 1°C and left at 1°C for 16 hours, after which time the third aliquot was incubated at 30°C for 4 hours. PAGE was carried out on each sample, followed by autoradiography of the gel.

5.4. RESULTS

5.4.1. TRANSLATION OF BBV RNA 2

The results of the experiment are shown in the autoradiogram Fig.

5.1. Lane 1 shows the production of protein α and very little protein β after 30 minutes translation of BBV RNA 2 at 30°C (see Chapter 4). However, after overnight incubation in the presence of cycloheximide, very little protein α remained, but the level of protein β had increased considerably. Most of protein α had been cleaved to produce protein β. Termination of translation by the addition of cycloheximide did not therefore inhibit the cleavage.

5.4.2. THE EFFECT OF RNase AND CYCLOHEXIMIDE

Autoradiogram Fig. 5.2 shows the effect of cycloheximide and RNase on the cleavage of α to β. RNase inhibited the cleavage, as is seen from lanes 6 and 8, whereas lane 4 shows the presence of β when cycloheximide was added to the lysate mix. Control lane 1 shows mostly α at 30 minutes after initiation of translation, and control lane 2 shows conversion of α to β after 16 hours of translation.

5.4.3. THE EFFECT OF LOWERING THE TEMPERATURE TO 1°C

Autoradiogram Fig. 5.3 shows the effect on the cleavage from α to β of decreasing the temperature to 1°C. The control sample (Lane 1) shows the usual predominance of protein α with just a trace of protein β after 30 minutes of translation, and lane 2 shows the cleavage of protein α to protein β after 16 hours of translation. Lane 3 shows the effect of chilling the sample to 1°C. There was a predominance of protein α after 16 hours translation, compared with lane 4 which shows again the conversion of α to β after chilling to 1°C for 16 hours, but then raising the temperature to 30°C for 4 hours.

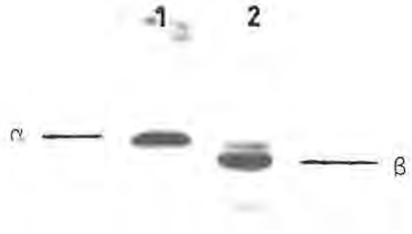

Fig. 5.1. Autoradiogram showing the dependence of the cleavage of protein α to protein β, upon the presence of RNA (see text for method).
Lane 1 shows the original 30 min translation of RNA 2 at 30°C and lane 2 shows 30 min translation of RNA 2 at 30°C, with subsequent addition of cycloheximide and a further overnight incubation at 30°C.

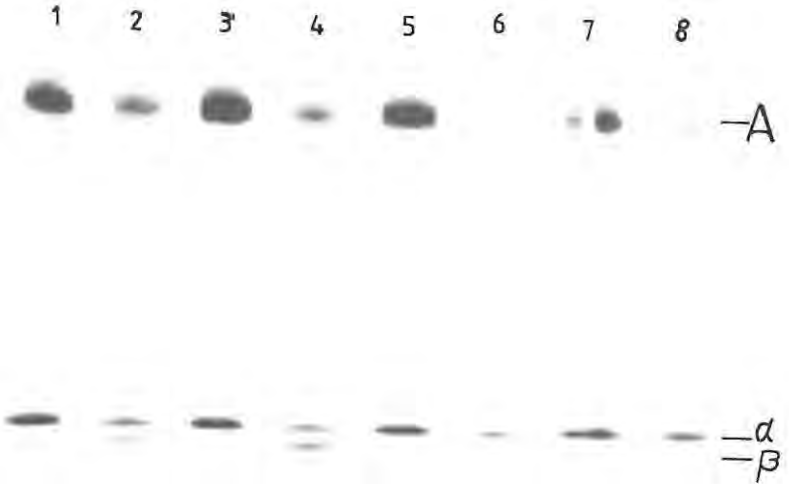

Fig. 5.2. Effect of cycloheximide and RNase on the cleavage of protein α to protein β. (See text for method). Control lanes 1 and 2 show the addition of H_2O to the lysate mix: lane 1 sampled after 30 min translation and lane 2 after 16 hours translation; lanes 3 and 4 show the addition of cycloheximide (final concentration of 60 μg per ml of lysate) to the lysate mix: lane 3 sampled after 30 min translation and lane 4 after 16 hours translation; lanes 5 and 6 show the addition of cycloheximide (final concentration of 60 μg per ml of lysate) and RNase (final concentration of 60 μg per ml of lysate) to the lysate mix: lane 5 sampled after 30 min translation and lane 6 after 16 hours translation; lanes 7 and 8 show the addition of RNase (final concentration of 60 μg per ml of lysate) to the lysate mix: lane 7 sampled after 30 min translation and lane 8 after 16 hours translation.

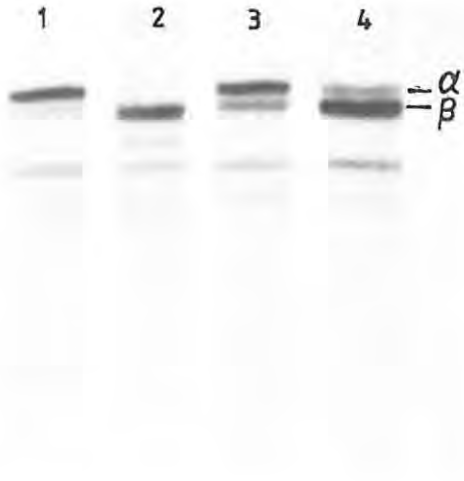

Fig. 5.3. Effect on the cleavage of α to β of lowering the temperature to 1°C. Lane 1 shows the control sample after 30 min translation at 30°C, lane 2 shows the control sample after 16 h translation at 30°C, lane 3 shows the sample chilled to 1°C for 16 h and lane 4 shows the sample chilled to 1°C for 16 h and then incubated for 4 h at 30°C (see text for method).

5.5. DISCUSSION

The cleavage of α to β occurred without the presence of any RNA 1, and the addition of cycloheximide to the lysate with subsequent incubation at 30°C for 16 hours, resulted in the formation of protein β. The formation of β from α was therefore not dependent upon the presence of RNA 1 nor was it dependent upon an RNA 1 translation product (for example, a protease).

The results seen in Fig. 5.2 showed that RNase prevented the cleavage whereas cycloheximide did not. The cleavage therefore did not depend upon continued translation, but rather upon the presence of intact RNA in the lysate mix.

The cleavage of α to β was also inhibited by lowering the temperature during translation to 1°C. Autoradiogram Fig. 5.3 shows this effect quite clearly. Translation was halted at 30 minutes by the addition of cycloheximide and a sample taken at this stage showed the usual predominance of protein α. That the addition of cycloheximide did not itself inhibit the cleavage was seen after 16 hours translation at 30°C. The direct effect of decreasing the temperature during translation was to inhibit the cleavage at that stage. The slight increase in the amount of protein β (Lane 3) compared with that in lane 1 could be due to either a difference in the amount of sample loaded onto the gel (the relative amounts of α and β in the two lanes could be scanned in a densitometer); it could be due to a time lag between the time when the sample was removed to be chilled to 1°C and the time when the sample actually reached 1°C, or it

could be due to the fact that chilling the sample to 1°C did not totally prevent the cleavage but rather inhibited it to a large extent. That this effect was reversible was seen when the temperature was once again raised to 30°C and translation was allowed to continue for 4 hours. Cleavage of α to β once again occurred.

CHAPTER 6

SEDIMENTATION ANALYSIS

6.1. SUMMARY

Evidence of *in vitro* processing of viral proteins had been found by various workers studying different viruses. Sedimentation analysis was used to study this aspect of BBV. It was found that the *in vitro* cleavage of protein α to protein β did not require assembly of BBV RNA and protein into a viral substructure.

6.2. INTRODUCTION

The morphogenesis of BBV has not been as extensively studied as that of some other viruses. At this stage, a study of possible subassembly of RNA and protein toward the elucidation of *in vitro* morphogenesis of the capsid seemed necessary. Grubman (1984) had found evidence of subassembly of mainly structural proteins of foot-and-mouth disease virus in a cell-free lysate system. Palmenberg (1982), working with translation of encephalomyocarditis virus RNA, found evidence of *in vitro* formation of capsid proteins capable of assembly into virion intermediate structures. Jobling and Wood (1985) also found evidence of *in vitro* processing of tobacco ring-spot virus proteins.

6.3. METHODS

The standard 30-µl cell-free translation reaction as described in Section 1.4 of the Appendix was set up. BBV RNA was added to initiate translation. After 30 minutes translation at 30°C, a

sample was removed to serve as the 30 minute control. A sample of 2 µl was spotted onto a treated filter paper disc, treated and assayed for radioactivity as described in the Appendix. The remainder of this control sample was kept aside for PAGE and subsequent autoradiography. The rest of the lysate mix was divided into two aliquots. Cycloheximide (at a final concentration of 70 µg per ml of lysate) was added to the one aliquot, which was then incubated for 16 hours at 30°C, then treated with EDTA (3 µl of a 0,3 M solution) and pancreatic RNase A (2 µl of a 1 mg/ml solution) for 15 minutes at 30°C (RNase was added to reduce interference by RNA on the gradients and added at this stage of translation, would not interfere in the cleavage of α to β). The other aliquot was treated with the same amounts of cycloheximide, EDTA and pancreatic RNase and then incubated for 16 hours at 30°C. RNase added after only 30 minutes translation prevents the cleavage of α to β. It is known from Chapter 5 that the presence of intact RNA in the lysate is necessary for the cleavage. After this, both aliquots were diluted 8-fold in NET buffer (see Section 3.3 of the Appendix), and each was layered onto 4,6 ml of a 5 to 20% (wt/vol) sucrose gradient in NET buffer. The gradients were centrifuged at 45 000 rpm for 22 h at 4°C in a SW 50 rotor. Marker bovine serum albumin (4.2 S) and chymotrypsin (2.6 S) were centrifuged on a similar gradient. Each gradient was fractionated on an ISCO density gradient fractionator MODEL 640 with a UA-5 absorbance monitor. Samples from each fraction were monitored for acid-insoluble radioactivity (Section 1.4 of the Appendix). Alternate fractions were precipitated with 5 volumes of acetone:H_2O (3:1) mixture after the addition of 10 µg of carrier BSA. Precipitation was allowed to take place at 37°C for 2 h and then at 4°C overnight.

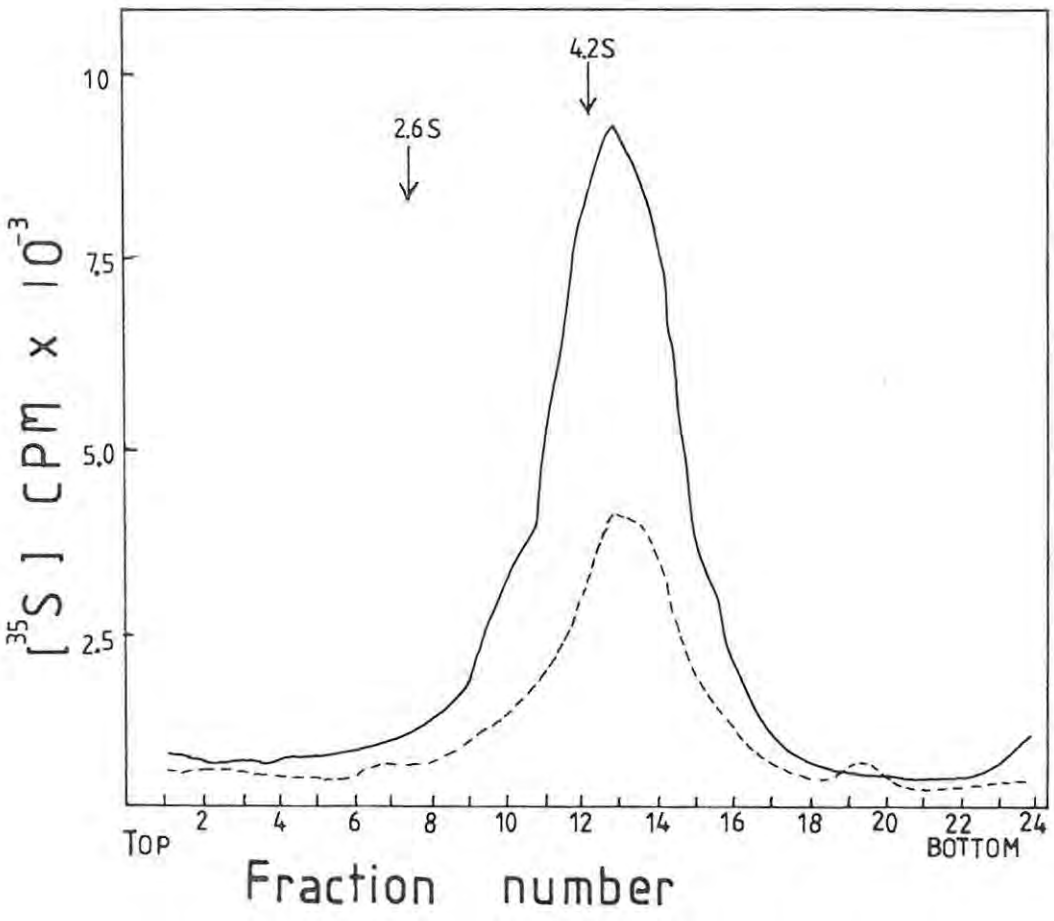

Fig. 6.1. Sucrose density gradient fractionation profile of BBV proteins synthesized in an in vitro system. (See text for methods). Centrifugation was for 22 h at 45 000 rpm. The positions of the marker BSA (4,2 S) and chymotrypsin (2,6 S) are shown. The heavy line indicates the results of the sample to which cycloheximide was added at 30 min, and the dotted line indicates the control sample to which cycloheximide, RNase and EDTA were added at 30 min. The figure shows the number of cpm measured per 100 µl of lysate.

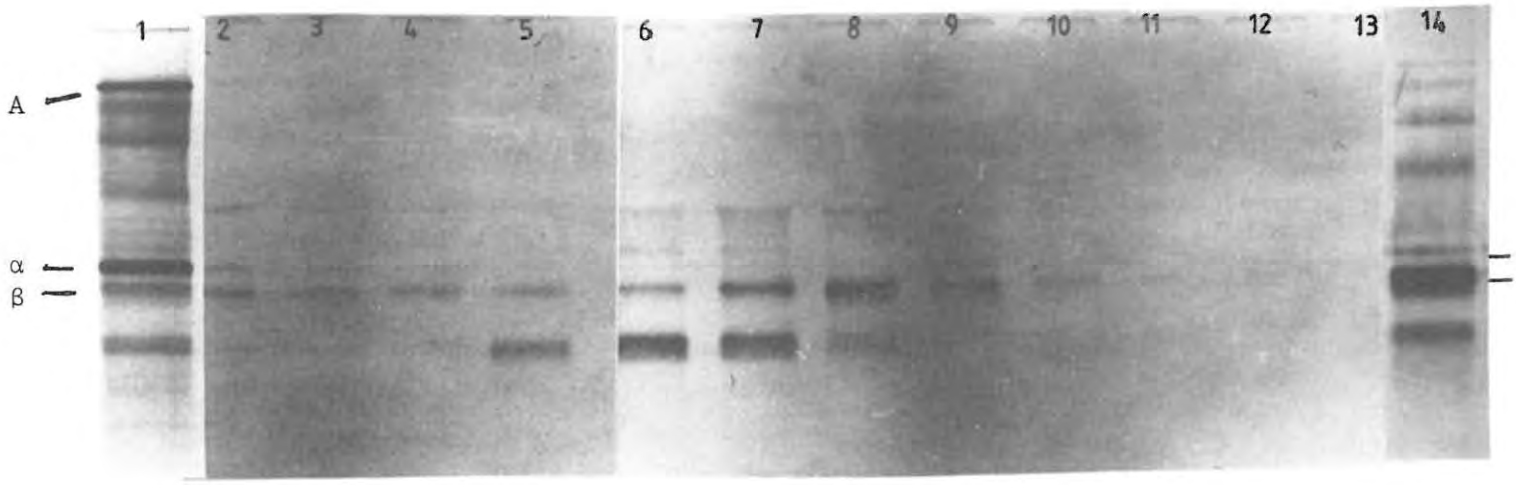

Fig. 6.2. Autoradiogram showing identification of the BBV proteins in the sucrose density gradient fractions seen in Fig. 6.1. (See text for methods).
Lane 1 shows the 30 min translation control; lanes 2 to 13 show the results of the alternate fractions seen in Fig. 6.1. : fractions 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24. Lane 14 shows the results of the 16 h translation control.

The precipitates were pelletted by microfuging for 5 min, and then resuspended in dissociation buffer (Section 3.2.5 of the Appendix). They were then analysed by PAGE and autoradiography.

6.4. RESULTS

Fig. 6.1 shows the sedimentation properties of BBV proteins produced during translation in reticulocyte lysates. The particular sucrose gradient used enabled small complexes (of 0 to approximately 10 S) to be identified. The majority of the radioactive protein sedimented very close to the BSA (4,2 S) marker (it in fact seems to be only slightly larger than this marker), at approximately 4,5 S, which represents a protein of approximately 50 000 in molecular weight. There was, however, a second smaller 'shoulder' of radioactive protein which seemed to be slightly larger than the chymotrypsin (2,6 S) marker.

PAGE and subsequent autoradiography of these fractions was used to identify the proteins. The results are seen in Fig. 6.2. Lane 1 shows the 30 min control with predominantly α and little β, while lane 14 shows the 16 h control with conversion of α to β. Lanes 2 to 13 show the proteins present in each of the alternate fractions from the sucrose gradient, corresponding with those fractions in Fig. 6.1.

Lanes 2, 3 and 4, the results of fractions 2, 4 and 6 from the sucrose gradient, showed very little radioactive protein, with just a low level of β. Lanes 5 and 6 or fractions 8 and 10, corresponding with the relatively small 'shoulder' of radioactivity in Fig. 6.1 seemed to contain a protein smaller than protein β, which is present in both

the original 30 min and 16 h controls. Lanes 7 and 8 or fractions 12 and 14, and to a lesser extent lane 9 or fraction 16, corresponding with the main peak of radioactivity in Fig. 6.1, contained a protein of the same size as protein β. Finally, lanes 10 to 13 corresponding with fractions 18, 20, 22 and 24, contained very little radioactive protein. These results correlate with Fig. 6.1.

6.5. DISCUSSION

The results showed that translation of BBV RNA in rabbit reticulocyte lysate, leading to production of protein α and subsequent cleavage into protein β, did not seem to be accompanied by assembly into intermediate viral structures, nor did β seem to remain associated with the RNA.

Although the methods describe sedimentation in sucrose gradients of the range necessary to identify only small complexes of 0 to approximately 10 S, preliminary experiments were carried out using sedimentation in sucrose gradients of different ranges and centrifugation at different speeds and for differing lengths of time in order to identify possible larger protein complexes or structures, and using markers of known sedimentation coefficients. There was no evidence of larger protein structures, however, as the peaks of radioactivity remained at the very top of the gradients.

Prevention of cleavage from α to β by addition of RNase to the lysate at 30 min resulted in a relatively low peak of radioactivity, but the peak occurred in the same fractions as those found when the cleavage

was allowed to occur. Autoradiography revealed traces of protein smaller than β, occurring only in fractions 8, 10 and 12.

Fig. 6.1 showed that the majority of the radioactive protein sedimented slightly faster than the BSA marker at 4,2 S, in fact at approximately 4,5 S, which corresponded with a protein of molecular weight approximately 50 000. PAGE analysis and autoradiography of the fractions making up the peak revealed a protein of the same size as protein β (MW 43 000).

A second relatively small 'shoulder' of radioactive protein, sedimenting slightly further towards the bottom of the gradient than the 2,6 S marker, but smaller than the 4,2 S marker, was shown on autoradiography to contain a protein smaller than protein β. It was not known at this stage whether it was a lysate or BBV protein. Immunoprecipitation using anti-BBV antiserum would need to be carried out to determine this (refer to comments on this protein in Section 3.5 of Chapter 3, and Section 7.4 of Chapter 7).

CHAPTER 7

EFFECT OF DILUTION ON α TO β CLEAVAGE

7.1. SUMMARY

The cleavage of BBV protein α to protein β took place via a mechanism that was sensitive to dilution. If lysate containing α was diluted serially and each of these dilutions was incubated under conditions where cleavage of α to β was known to occur, the amount of β decreased as the dilution factor increased. The cleavage did not therefore appear to be autocatalytic.

7.2. INTRODUCTION

Palmenberg and Rueckert (1982) published a report on encephalomyocarditis virus RNA which described how, when the RNA was translated in an *in vitro* system, a protease which was derived from cleavage of a precursor protein was synthesized. The cleavage was seen to take place via two different mechanisms, and their work showed the one mechanism to be sensitive to dilution and the other not. It was decided to study this aspect of the cleavage of BBV protein α to protein β.

7.3. METHODS

The standard cell-free translation reaction as described in Section 1.4 of the Appendix was set up. BBV RNA 2 was added to initiate translation. After 30 min translation at 30°C, cycloheximide (at a final concentration of 60 μg per ml of lysate) was added, and a

sample of the reaction mix was removed for PAGE and subsequent autoradiography. At this stage the rest of the lysate was diluted in a series of dilutions of 2- to 160-fold in NET buffer (see Section 3.3 of the Appendix) and then incubated for 16 hours at 30°C. After this incubation, NET buffer was added to the samples to bring each sample to a volume of 1600 µl. Each sample was then made 1% with SDS. The proteins in the samples were then precipitated with acetone overnight and then analysed by PAGE and autoradiography.

7.4. RESULTS

Fig. 7.1 shows the autoradiogram of the effects of dilution on α to β cleavage. Increasing dilution is shown from left to right, the dilution factor increasing from lane 2 at zero dilution to lane 9 at 160-fold dilution. It was seen that as the dilution factor increased, the amount of β decreased. At the same time, the amount of α correspondingly increased. Lane 1 shows the results of translation of RNA 2 for 30 minutes in rabbit reticulocyte lysate. It was noted that the protein smaller than β, discussed in Chapter 6, was present in the lysate mix after 30 minutes of translation of RNA 2, as shown in lane 1 of Fig. 7.1. The intensity of this protein band tended to increase with increasing dilution up to a 40-fold dilution, and then seemed to decrease with the increase of dilution from 40-fold to 160-fold. It is not known at this stage whether this is relevant or not.

7.5. DISCUSSION

BBV RNA 2 was translated in a rabbit reticulocyte lysate system for 30 min, at which stage, although the cleavage of protein α to protein β

Fig. 7.1. Autoradiogram of the effect of dilution on the cleavage of BBV protein α to protein β. (See text for methods). Lane 1 shows the results of 30 min translation of RNA 2 in rabbit reticulocyte lysate. Lanes 2 to 9 show the results of the effect of dilution: from 0- to 160-fold dilution as shown. Proteins α and β are shown as marked.

had just begun, the amount of protein α far exceeded that of β in the system. The lysate containing α was then diluted from 2- to 160-fold and incubated overnight at 30°C, conditions that are known to allow cleavage of α to β to occur. The results showed that the cleavage was sensitive to dilution: with increasing dilution of the α-containing lysate there was subsequent decrease in the production of β. This was taken as evidence that the cleavage reaction was at least bimolecular; that it was mediated by a second agent and was not due to intramolecular self-cleavage of protein α which would have shown the cleavage to be dilution-insensitive.

CHAPTER 8

SUMMARY AND CONCLUSIONS

The aim of this work was to study the *in vitro* cleavage of the precursor coat protein α of black beetle virus as a function of BBV assembly in rabbit reticulocyte lysates. To this end, BBV RNA, and then more specifically, RNA 2 (which codes for protein α) were translated for 30 min at 30°C in a rabbit reticulocyte lysate system. Results suggested that α was cleaved to produce β and it was demonstrated on a number of occasions that the rabbit reticulocyte lysate did not contain any proteins of the size of either α or β. It is also extremely unlikely that β is an entirely separate protein from α, coded for by its own gene. There are only two large open reading frames in RNA 2, one of which codes for protein α, the other codes for a protein of about 72 amino acids long which is too small for protein β (Dasgupta *et al.*, 1984).

It was known from the results of the experimental work carried out in Madison (Section 1.4) that BBV RNA was translated accurately in the rabbit reticulocyte system used because the proteins that were synthesized co-migrated with BBV-specific proteins formed in infected cells. Nevertheless, an additional experiment should be carried out to demonstrate the authenticity of the cleavage in the lysate by showing that gamma protein is generated. This could be done by co-electrophoresing $|^{35}S|$-BBV lysate proteins with $|^{3}H|$-authentic BBV virion proteins on a tube gel which could then be cut up into slices and subjected to dual-counting.

The time course experiment described in Chapter 4 which showed progressively increasing levels of β and decreasing levels of α in the lysate until the levels of the two proteins were approximately equal after about 16 hours of translation, indicated that the emergence of β was a genuine feature of strain W17 and not an artefact of the preparation of RNA.

The cleavage of α to β was found to take place in the absence of BBV RNA 1; in other words the cleavage did not require a translation product from RNA 1, but required the presence of BBV RNA 2. The addition of cycloheximide did not inhibit the cleavage, showing that continued translation was not necessary for the cleavage to occur.

It was shown that the cleavage could be prevented in two ways: one was by the addition of pancreatic RNase-A to the reticulocyte lysate system after 30 minutes (in other words the cleavage was dependent upon the presence of intact RNA); the other was by lowering the temperature of the lysate to 1°C. The significance of this was that by using either of these methods the cleavage could be arrested at the protein α stage so that protein α could be purified from the lysate. Various agents, for example BBV RNA 2, or even RNA 2 from a related virus such as Nodamura virus, could then be added back into the system to see whether the cleavage could be induced under various controlled conditions, and to determine whether the cleavage was specific for BBV RNA.

Analysis by sedimentation in sucrose gradients (Grubman, 1984) showed that the cleavage of α to β, as part of the morphogenesis of the virus,

did not seem to proceed via assembly of BBV protein and RNA into intermediate viral structures. Instead, as described in Chapter 6, all the radioactive protein sedimented at a rate expected of a protein of the size of β, and certainly not of a much larger structure of virion size. However, to clarify this, an additional experiment should be carried out in which the translation products are centrifuged soon after the 30 minute translation period. A large assembly structure might possibly be unstable after the 16 hour incubation time used or might even be unstable at the pH of the NET buffer used.

It was also shown that the cleavage of α to β was not due to the protein cleaving itself autocatalytically: as described in Chapter 7, when α-containing lysate was diluted serially in buffer (Palmenberg and Rueckert, 1982), and then incubated at 30°C overnight to enable the cleavage to occur, the level of β decreased as the dilution factor increased. Had there been autocatalytic cleavage, dilution in buffer would have made no such difference. As it was, the cleavage was seen to be at least a bimolecular reaction that involved some second agent, such as a virus-specified protease as occurs in picornaviruses (Palmenberg et al., 1979).

Thus, the aim of this work has been fulfilled in that the four questions posed in Section 1.5 have been answered. Although the results indicate that BBV coat protein precursor α is cleaved to form coat protein β, amino acid sequencing or tryptic peptide mapping would show that the cleavage was exactly the same as that which occurred during BBV morphogenesis.

It is also important to determine what factor(s) is responsible for the cleavage of α to β. It has been determined that intact RNA certainly is necessary, but this does not preclude other factors such as a protease, as mentioned above, in the system. Preliminary experiments using the protease inhibitors zinc acetate and PMSF in which the cleavage was prevented suggested the possibility of the involvement of a protease in the cleavage. However, further studies using a range of protease inhibitors would be required in order to verify this. Moreover, it has been shown in an in vivo system, using the related Flock House virus, that the cleavage occurred after assembly of the virus and therefore could not involve an external protease (Gallagher and Rueckert, 1987). A similar experiment of this nature might well reveal the same principle to hold for BBV. Thus it is clear that more work is required to elucidate this aspect.

Although cell-free translation is an important in vitro tool for studying viral morphogenesis that has been used by researchers studying various other viruses such as encephalomyocarditis virus (Palmenberg, 1982); foot-and-mouth disease virus (Grubman, 1984); tobacco ringspot virus (Jobling and Wood, 1985); potexviruses (Bendena and Mackie, 1986) and mouse hepatitis virus (Denison and Perlman, 1986), it is thought that this has been the first time that the system has been used to study the α to β cleavage of black beetle virus, indeed the first time that this particular aspect of BBV has been studied.

APPENDIX

CONTENTS

APPENDIX

1. GENERAL METHODS

1.1. PURIFICATION OF BBV

The method used was a modification of that described by Guarino et al (1981).

1.1.1. PROPAGATION OF BBV IN DROSOPHILA CELLS

Confluent cell monolayers were resuspended in the spent SCHIM, and the cells were washed twice in fresh SCHIM. Viable cells were then counted, using a haemocytometer, in 0,5% Trypan blue. Cell viability was invariably between 95-100%. The cell concentration was then adjusted to 10^8 cells/ml and 1,5 ml of this cell suspension was infected with BBV in a 500-ml sterile siliconized conical flask, with sufficient BBV particles to obtain an m.o.i. of approximately 1 virion/cell. The equations used to calculate the number of BBV particles/ml: E (1 mg/ml BBV) at 260 nm = 4,15; number of particles/ml = $\frac{6,023 \times 10^{23} \times \text{mass (g/ml)}}{\text{MW } (8 \times 10^6)}$.

The flask was agitated at 26°C for 1 hour at approximately 60 rpm. The infected culture was diluted to 10^6 cells/ml with SCHIM containing 15% FCS, and was then incubated with agitation for 24-48 hours.

1.1.2. PURIFICATION OF VIRUS FROM DROSOPHILA CELLS

24 - 48 hours after infection, virus was released from infected

cells by making the cell suspension 0,5% with NP-40 and 0,1% with β-mercaptoethanol, and agitating the flask contents on ice for approximately 15 min. The lysate was centrifuged at 10 000 rpm for 10 min in a Sorvall SS-34 rotor to remove cell debris. The supernatant fluid was made 0,1 M with NaCl and 7,5% with PEG 6 000, and the solution was then stirred for a minimum of 1 hour at 4°C. Virus was obtained by centrifugation at 8 500 rpm for 10 min in a Sorvall SS-34 rotor, and the resulting virus pellet was resuspended in 50 mM Hepes (pH = 7,0) - 0,1% β-mercaptoethanol - 0,1% SDS. This suspension was stirred overnight at 4°C, and then centrifuged at 8 000 rpm for 10 min in a Sorvall SS-34 rotor. Virus in the supernatant was pelleted through a 5 ml cushion of 30% sucrose in buffer (50 mM Hepes (pH = 7,0) - 0,1% β-mercaptoethanol - 0,1% BSA) at 22 500 rpm for 6.7 h in a Beckman SW 25.1 rotor at 6°C. Virus pellets were resuspended in 50 mM Hepes (pH = 7,0) - 0,1% β-mercaptoethanol - 0,1% SDS, and were left to dissolve at 4°C overnight. 1,5 ml samples were then centrifuged at 22 500 rpm for 4.5 h at 11°C in the Beckman SW 25.1 rotor on 32-ml 15 to 45% (wt/vol) sucrose gradients in buffer (50 mM Hepes (pH = 7,0) - 0,1% β-mercaptoethanol). Fractions (0,6 ml) were collected from each gradient, using an ISCO density gradient fractionator MODEL 640 with a model UA-5 absorbance/fluorescence monitor. Fractions were assayed for virus by spectrophotometric measurement (absorbancy at 260 nm). Fractions containing virus were pooled and stored at -20°C. Virus concentration was calculated from the optical density reading at 260 nm, assuming that 1 mg/ml has an absorbance of 4.15 per cm of light path (Longworth and Carey, 1976).

1.2. EXTRACTION OF RNA

RNA was extracted from purified virus by the phenol-choroform-isoamyl alcohol method |48:24:1| according to the method described by Friesen and Rueckert (1981).

Purified virus (approximately 1,5 mg/ml in 1% SDS-50 mM Hepes, pH 7,0) was vortexed with 1 volume of phenol (saturated with 10 mM Tris-hydrochloride, pH 8,0 - 1 mM EDTA) for 1 min at room temperature. This was followed by the addition of 0,5 volumes of chloroform-isoamyl alcohol (24:1) and vortexing for a further minute. The mixture was heated to 45°C and held at that temperature for 2 min, and was then vortexed for 2 min. Phases were separated by centrifugation at 400 g for 5 min. If separation was incomplete, the mixture was made 0,2 M with sodium chloride, and was re-centrifuged. The resulting interface and aqueous phase were re-extracted, using the same method, but omitting the 45°C incubation. The final aqueous phase was made 0,2 M with sodium acetate buffer pH 6 and RNA was precipitated overnight at -20°C by the addition of 2,5 volumes of cold absolute ethanol.

After overnight (or longer) precipitation, RNA was obtained by centrifugation at 15 000 rpm for 30 min at 0°C (in a Sorvall SS-34 rotor). The resulting RNA pellet was vacuum-dried at 37°C, and then dissolved in distilled water (to a concentration of approximately 1,5 mg/ml). The RNA was aliquoted into approximately 50-µl amounts and stored at -20°C.

1.3. SEPARATION OF THE TWO RNA SPECIES

The method described by Friesen and Rueckert (1981) was used.

RNA (approximately 1,5 mg/ml in distilled water) was made 0,1% with respect to SDS, and was heated for 5 min at 65°C to disperse possible aggregates. The solution was chilled rapidly to 0°C to minimize reannealing, and was then centrifuged at 36 000 rpm for 11 h at 10°C in the Beckman SW41 rotor on a 12-ml 5 to 25% (wt/vol) sucrose gradient in 0,1 M sodium acetate (pH = 5,0) containing 0,1% SDS. Fractions (usually 0,3 ml) were collected from the gradient, using an ISCO density gradient fractionator, MODEL 640, with a model UA-5 absorbance/fluorescence monitor. Fractions were assayed for RNA by spectrophotometric measurement (absorbancy at 258 nm) and those containing RNA 1 and RNA 2 were pooled respectively. Each RNA species was further purified by ethanol precipitation followed by sucrose gradient sedimentation. This was repeated once again. Finally, each RNA species was precipitated with ethanol, centrifuged at 15 000 rpm for 30 min at 0°C (in the Sorvall SS-34 rotor), vacuum-dried at 37°C and then dissolved in distilled water to a concentration of 1 mg/ml. Preparations were aliquoted and stored at -20°C.

1.4. PROTEIN SYNTHESIS IN RABBIT RETICULOCYTE LYSATE

1.4.1. RABBIT RETICULOCYTE LYSATE (nuclease treated, message dependent)

This was purchased from Amersham International Laboratories, Buckinghamshire, England HP7 9LL. The 1 ml ampoules were stored in 100 µl aliquots in liquid nitrogen.

1.4.2. L-[^{35}S]METHIONINE (IN AQUEOUS POTASSIUM ACETATE SOLUTION)

[^{35}S]methionine (approximately 1000 Ci/mmol) was purchased from Amersham International Laboratories, Buckinghamshire, England HP7 9LL. It was diluted to an activity of 0,5 mCi/100 µl, and divided into 50 µl aliquots and stored in liquid nitrogen.

1.4.3. CELL-FREE TRANSLATION

Translations were performed as described by Friesen and Rueckert (1981). Incubation was at 30°C. The standard 30-µl reaction mixture contained 67 µg of calf liver tRNA (Boehringer Mannheim, West Germany) per ml and 500 µCi of L-[^{35}S]methionine (approximately 1 000 Ci/mmol) per ml. Translation was initiated by the addition of the relevant BBV RNA to the reaction mixtures and was terminated by a dilution of 1 in 2 of sample in solubilizing solution (1% SDS - 2% β-mercaptoethanol). Times quoted in time course experiments were minutes or hours after addition of the BBV RNA.

Protein synthesis was monitored by measurement of incorporation of radiolabel into trichloroacetic acid-insoluble material. Samples of 2 µl of translation mixtures were spotted on to circular filter paper discs (Whatman Grade 1. Size 2,5 cm circles) that had been pretreated with 100 µl of 1% SDS - 3% Casamino Acids. The paper discs were soaked for 10 min at room temperature in 10% trichloroacetic acid - 1% SDS - 3% Casamino Acids and then boiled for 10 min in 5% trichloroacetic acid - 1,5% Casamino Acids. They were then rinsed twice in 5% trichloroacetic acid, once in 95% ethanol and then finally in diethyl ether. They were then dried and assayed for radioactivity in Scintillator 299 (a xylene cocktail - UNITED Technologies Packard), or

scintillation cocktail (see Appendix - Stock solutions and buffers) on a Beckman LS 3150T Scintillation counter.

1.5. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (LAEMMLI, 1979)

1.5.1. GENERAL

Samples (25 µl + 8,3 µl dissociation buffer (see Appendix - Stock solutions and buffers)) were analysed on 12% (wt/vol) polyacrylamide slab gels with a 4% (wt/vol) stacking gel in a discontinuous Tris-glycine buffer system (see Appendix - Stock solutions and buffers). The slab gel electrophoresis unit was a MODEL SE 600 (Hoefer Scientific Instruments, San Francisco). Electrode buffer (see Appendix - Stock solutions and buffers) was placed in the upper and lower buffer reservoirs. A voltage of 150 V for 6 h or 50 V overnight was applied. By this stage the bromophenol blue tracking dye had reached the bottom of the gel.

The gel was then removed from the unit and placed in a tank of 0,2% Coomassie brilliant blue R250 staining solution (see Appendix - Stains) for a minimum of 4 h. The gel was then destained with gentle shaking overnight or longer, in a tank of destaining solution (see Appendix - Stains). It was necessary to change the destaining solution several times, until the background of the gel was clear.

1.5.2. AUTORADIOGRAPHY

The stained gels were then dried under a vacuum and directly exposed to Gromex 4 X-ray film (Protea Electro-Medical Services (Pty) Limited) for 2 to 3 days.

1.6. ELECTROPHORESIS OF RNA ON COMPOSITE AGAROSE/ACRYLAMIDE GELS (Floyd et al, 1974).

Electrophoresis was carried out at 4°C. Samples of RNA (1-10 µg) were electrophoresed in a composite agarose/acrylamide gel (see Appendix - Stock solutions and buffers) on a MODEL SE 600 slab gel electrophoresis unit (Hoefer Scientific Instruments, San Francisco).

Gloves were worn when casting gels. A 7% gel plug was used because the resolving gel was <2.5% acrylamide. For the resolving gel, the agarose was melted in a boiling water bath and then allowed to cool to approximately 50°C. It was added to the remainder of the ingredients which had been allowed to equilibrate to 37°C. The gel was then cast, and was left overnight at 4°C to ensure complete polymerization of the acrylamide.

50 µl samples of RNA were prepared as follows:

10 M urea	25,0 µl
10 x Loening's buffer	2,5 µl
0,5% bromophenol blue (see Appendix - Stains)	5,0 µl
0,5% xylene cyanol (see Appendix - Stains)	5,0 µl
RNA sample (1-10 µg) in distilled H_2O, heated to 65°C for 10 min, then rapidly chilled	12,5 µl

The upper and lower buffer reservoirs were filled with 1 x Loening's buffer containing 0,1% SDS (see Appendix - Stock solutions and buffers). A voltage of 150 V for 5 h or 50 V overnight was applied.

After electrophoresis, the gel was removed from the unit and placed

in a tank of distilled water for approximately 1 h with at least one change of water. It was then soaked for 30 min in 50% formamide and subsequently stained overnight in 0,005% Stains-All (see Appendix - Stains). The gel was destained in water. Staining and destaining were carried out in the dark.

2. MEDIA

2.1. SCHNEIDER'S INSECT MEDIUM (SCHIM) FOR D. MELANOGASTER CULTURES (Schneider, I., 1964).

5 ℓ of double distilled water were preheated overnight to approximately 40°C to facilitate dissolving of components. 4 ℓ of the distilled water were poured into a 5 ℓ (Decon-washed) flask and to this were added 250 ml of 20 x organic acid mix and 500 ml 10 x amino acid mix (See TABLE 1). The solution was stirred at 37°C for approximately 1 hr or until it clarified. The inorganic salts (excluding the $CaCl_2$), the sugars, yeast extract, bacto-peptone, antibiotics and phenol red (see TABLE 1) were then all added and allowed to dissolve. At this stage the pH of the solution was checked, and if it was greater than pH 5, L-glutamine was added (see TABLE 1). $CaCl_2$ was then slowly added to the solution, after which the pH was adjusted to between 6,7 and 6,8 with the addition of 10 N KOH. The volume of the solution was adjusted to 5 ℓ and then filtered by membrane filtration and finally stored at 4°C. For use, 15% FCS was added.

TABLE 1

Schneider's insect medium for D. melanogaster cultures.

20 x ORGANIC ACID MIX	g/500 ml
α-KETOGLUTARIC ACID	3,5
FUMARIC ACID	0,6
MALIC ACID	6,0
SUCCINIC ACID	0,6

The above quantities were dissolved in 400 ml double distilled water. The pH was adjusted to between 5 and 6 using 10 N KOH and the volume was finally adjusted to 500 ml. The mix was stored at -20°C.

10 x AMINO ACID MIX	g/1000 ml
L-Alanine	5,0
L-Arginine-HCl	6,0
L-Aspartic acid	4,0
L-Cysteine	0,6
L-Glutamic acid	8,0
L-Glycine	2,5
L-Histidine	4,0
L-Isoleucine	1,5
L-Leucine	1,5
L-Lycine	16,5
L-Methionine	1,5
L-Proline	17,0
L-Serine	2,5
L-Threonine	3,5
L-Tryptophan	1,0
L-Valine	3,0
L-Cystine	0,2
L-Tyrosine	5,0

The above quantities of amino acids (excluding cystine and tyrosine) were dissolved in 950 ml double distilled water. The cystine and tyrosine were dissolved separately in 8 ml 10 N HCl and then added to the 950 ml solution. The volume of the solution was adjusted to 1000 ml and stored at -20°C.

INORGANIC SALTS	g/5000 ml
$CaCl_2$	3,0
$MgSO_4 \cdot 7\ H_2O$	18,0
KCl	8,0
KH_2PO_4	2,2
NaCl	10,0
$NaHCO_3$	2,0
Na_2HPO_4	3,5

$CaCl_2$ was dissolved separately in 63 ml double distilled water.

SUGARS	g/5000 ml
Glucose	10
Trehalose	10

OTHER COMPONENTS	g/5000 ml
Yeast extract	10
Bacto-peptone	25
Phenol red (dissolved in 13 ml 0,1 N KOH)	0,05
Glutamine	9

ANTIBIOTICS	g/5000 ml
Penicillin	3,1
Streptomycin sulphate	5,0

3. STOCK SOLUTIONS AND BUFFERS

3.1. SCINTILLATION COCKTAIL

Primary fluor diphenyl oxazole PPO	16,7 g
Toluene	1,0 ℓ.

The cocktail was stored in the dark and diluted 10x with toluene before use.

3.2. SDS-POLYACRYLAMIDE GEL ELECTROPHOPESIS

(Laemmli, 1970)

3.2.1. RESOLVING GEL BUFFER STOCK SOLUTION (1 M Tris-HCl pH 8,8)

Tris		60,6 g
Conc. HCl		7,3 ml
Distilled H_2O	to	500 ml

3.2.2. STACKING GEL BUFFER STOCK SOLUTION (1 M Tris-HCl pH 6,8)

Tris		60,6 g
Conc. HCl		41,0 ml
Distilled H_2O	to	500 ml

3.2.3. ELECTRODE BUFFER STOCK (10x SOLUTION) (0,25 M Tris, 1,92 M glycine, 1% SDS pH 8,3)

Tris		30,3 g
Glycine		144,1 g
SDS		10,0 g
Distilled H_2O	to	1,0 ℓ

The solution was diluted 10-fold before use.

3.2.4. ACRYLAMIDE-BISACRYLAMIDE STOCK SOLUTION (30:0,8)

Acrylamide		150,0 g
N,N'-bis-methylene acrylamide		4,0 g
Distilled H_2O	to	500 ml

The solution was stored at 4°C for up to 1 month.

ACRYLAMIDE GEL FORMULATION

3.2.5. RESOLVING GEL (12%)

Acrylamide stock solution (30:0,8)	32,0 ml
1 M Tris-HCl pH 8,8	30,0 ml
Distilled H_2O	13,2 ml
10% SDS (wt/vol)	0,80 ml
1,5% Ammonium persulphate (wt/vol) (prepared freshly)	4,00 ml
TEMED	20,0 µl
Total	80,0 ml

3.2.6. STACKING GEL (4%)

Acrylamide stock solution (30:0,8)	2,0 ml
1 M Tris-HCl pH 6,8	1,9 ml
Distilled H_2O	9,25 ml
80% glycerol (vol/vol)	1,0 ml
10% SDS (wt/vol)	0,2 ml
1,5% Ammonium persulphate (wt/vol) (prepared freshly)	0,7 ml
TEMED	20 µl
Total	15,0 ml

3.2.7. DISSOCIATION BUFFER

SDS	10,0 g
Mercaptoethanol	10,0 ml
Glycerol	15,0 ml
Bromophenol blue (0,2%)	5,0 ml
1 M Tris HCl pH 6,8	12,6 ml
Distilled H_2O	57,4 ml

8,3 µl of dissociation buffer were added to 25 µl samples in preparation for SDS-polyacrylamide electrophoresis (see Appendix - General Methods). The samples were heated at 100°C for 5 min.

3.3. NET BUFFER (Grubman, 1984)

0,15 M NaCl

0,01 M Tris-Hydrochloride (pH 7,5)

0,002 M EDTA.

3.4. ELECTROPHORESIS OF RNA ON COMPOSITE AGAROSE/ACRYLAMIDE GELS (Floyd et al, 1974)

3.4.1. ACRYLAMIDE-BISACRYLAMIDE STOCK SOLUTION (30:1,5)

Acrylamide		150,0 g
N,N-bis-methylene acrylamide		7,5 g
Distilled H_2O	to	500 ml

The solution was stored at 4°C for up to 1 month.

3.4.2. UREA (10 M)

Urea		300 g
Sterile distilled H_2O	to	500 ml

Stirring in a warm room facilitated dissolving of the urea. While warm, the solution was filtered through sterile Whatman number 1 filter paper.

3.4.3. LOENING'S BUFFER (x 10)

Tris		43,6 g
$NaH_2PO_4 \cdot H_2O$		41,4 g
Disodium-EDTA		3,7 g
Distilled water	to	1 ℓ

Ten-fold dilution in distilled water gave a pH of 7,8. The buffer was autoclaved and stored at 4°C.

3.4.4. AGAROSE (2,8%)

Agarose (Seakem LE)		2,8 g
Sterile distilled water	to	100 ml

Dissolving was brought about by heating in a boiling water bath.

3.4.5. RNA GEL PLUG (7%)

Acrylamide/bis (30:1,5)	4,67 ml
10 x Loening's buffer	2,00 ml
10% SDS	0,20 ml
Distilled water	12,9 ml
TEMED	0,02 ml
Ammonium persulfate (3,75%)	0,20 ml
Total	20 ml

3.4.6. RESOLVING GEL (2,25%)

Acrylamide/bis (30:1,5)	4,5 ml
10 M Urea	36,00 ml
10 x Loening's buffer	6,00 ml
10% SDS	0,60 ml
Distilled water	1,50 ml
TEMED	0,06 ml
2,8% agarose (hot)	10,71 ml
Ammonium persulfate (3,75%)	0,60 ml
Total	60 ml

4. STAINS

4.1. COOMASSIE BRILLIANT BLUE STAINING SOLUTION (0,2%)

Coomassie brilliant blue R250	0,2 g
Methanol	25 ml
Glacial acetic acid	10 ml
Glycerol	1,0 ml
Distilled H_2O	64 ml

The dye was dissolved in a small amount of methanol and filtered through filter paper. The remaining amounts of methanol, acetic acid, glycerol and water were then added.

4.2. DESTAINING SOLUTION

Methanol	250 ml
Glacial acetic acid	100 ml
Glycerol	10 ml
Distilled H_2O	640 ml
Total	1000 ml

4.3. TRYPAN BLUE VITAL STAIN

Trypan blue microscopical stain	0,5 g

Normal saline (0,85 g NaCl/100 ml H_2O) to 100 ml

The stain was filtered through filter paper.

4.4. STAINS-ALL (x 20)

Stains-All (1-ethyl-2-[3-(1-ethylnaphtho[1,2d]-thiazolin-2-ylidene)-2-methyl-propenyl]naphtho[1,2d]-thiazolium bromide)		100 mg
100% formamide	to	100 ml

This was diluted 20-fold in 50% formamide for use. The stock stain was stored in the dark at 4°C.

4.5. BROMOPHENOL BLUE TRACKING DYE (0,5%)

Bromophenol blue		50 mg
Distilled water	to	10 ml

This was stored at 4°C.

4.6. XYLENE CYANOL TRACKING DYE (0,5%)

Xylene cyanol		50 mg
Distilled water	to	10 ml

This was stored at 4°C.

4.7. GELSOAK

Methanol		250 ml
Glacial acetic acid		100 ml
Glycerol		10 ml
Distilled water	to	1 ℓ

5. CHEMICALS, ENZYMES, MATERIALS AND INSTRUMENTS

Chemicals, enzymes, materials and machines were obtained from the following suppliers:

Alchemist R	Liquid Paraffin B.P.
Amersham International Laboratories Buckinghamshire England HP7 9LL	Rabbit reticulocyte lysate (nuclease treated, message dependent)
BDH Chemicals, Ltd. Poole, England	Cycloheximide Acrylamide Electran Trypan blue microscopical stain.
Beckman Instruments, Fullerton, USA	Polyallomer centrifuge tubes
Bethesda Research Laboratories, Inc., USA	TEMED (N,N,N',N'-Tetra-methylethylenediamine) Acrilamide Bis(N,N'-methylene-Bis-acrylamide)
Difco Laboratories, USA	Bacteriological peptone Bacto Vitamin-free Casamino Acids
E.I. du Pont de Nemours and Co (Inc) Photo Products Dept., Wilmington, DE 19898	Gronex R MRF 31 medical recording film.

Falcon Labware, Oxnard, USA	Falcon ^{R} 3027. Tissue culture roller bottles.
Hoefer Scientific Instruments, San Francisco	Vertical slab gel electrophoresis unit: Model SE 600
	All laboratory chemicals used (analytical grade)
Merck Darmstadt, Germany	Dichlorodimethylsilane 2-mercaptoethanol Trypan blue for microscopy.
Packard Instruments, Downer's Grove, USA	Scintillator 299^{TM} (a zylene cocktail)
Protea Laboratory Services (Pty Ltd)	Glycerol (glycerine)
Serva Feinbiochemica Heidelberg W. Germany	Dodecylsulfate . Na-salt.
Sigma Chemical Company, USA	1-Ethyl-2-|3-(1-ethylnaphtho|1,2d|-thiazolin-2-ylidene)-2-methyl-propenyl|naphtho|1,2d|-thiazolium bromide. (Stains-All; 3,3'-diethyl-9-methyl-4,5,4',5'-dibenzothiacarbocyanine). Ribonuclease-A from bovine pancreas. Type 1-A. 5x crystallized (60 units/mg activity)

Protea Electro-Medical Services (Pty) Limited	Gromex 4 x-ray film. Röntgen film.
State Vaccine Institute, RSA	Foetal calf serum
Sterilin Limited, Teddington, Middlesex, England	Sterilin Tissue culture flasks, 25 cm^2 and 75 cm^2
Weber Scientific International Ltd. England	Gallenkamp haemocytometer
Whatman Ltd, Kent, England	Whatman grade 1, size 2,5 cm circle filter paper discs.

Radioactive chemical:

The Radiochemical Centre, Amersham International, England	L-$\lvert^{35}S\rvert$methionine

Machines:

Beckman Instruments, Fullerton, USA	Beckman L2-65B ultracentrifuge (Beckman SW 41 rotor; Beckman SW 25,1 rotor) Beckman microfuge B Beckman LS 315 OT Scintillation counter.
Gallenkamp, England	Orbital incubator
Hoefer Scientific Instruments, San Francisco, USA	Slab gel electrophoresis unit. Model SE 600 Slab gel dryer. Model SE 520.

Isco	Density gradient fractionator Model 640 UA-5 absorbance/fluorescence monitor
Philips, Holland	Infra-red lamp
Scientific Industries, Inc. Bohemia, New York 11716, USA	Vortex-genie™
Sorvall (Du Pont Instruments)	RC-5 Superspeed refrigerated centrifuge (SS-34 rotor).

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