STUDIES ON THE DEGRADATION OF DOUBLE-STRANDED RIBONUCLEIC ACID FROM A VIRUS OF PENICILLIUM CHRYSOGENUM, AND ON THE BIOLOGICAL ACTIVITIES OF THE FRAGMENTS.

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ABSTRACT

Studies on the double-stranded RNA extracted from viruses of Penicillium chrysogenum (PcV-ds-RNA) have shown that the nucleic acid molecules are complete double helices, composed of two unnicked chains of approximately equal length. Polynucleotide kinase labelling and subsequent hydrolysis has revealed the presence of pyrimidine nucleotides at the 5'-termini of the ds-RNA molecules, instead of the purine nucleotides usually found as 5'-termini of intact natural RNAs.

Digestion of PcV-ds-RNA with RNase III, a nuclease specific for ds-RNA, revealed that some sites on the molecule are more sensitive to digestion than others, leading to the production, on limited digestion, of specific ds-RNA fragments. Determination of the 5'-ends of specific fragments show that RNase III is base-specific in its initial action on ds-RNA.

Pancreatic RNase cleavage of ds-RNA has been found to occur largely, but not completely, by the simultaneous scission of both strands of the ds-RNA, whilst ultrasonic irradiation cleaves ds-RNA in a way very similar to its mode of action on DNA.

The interferon-inducing capacity, antiviral activity and toxicity of fragmented PcV-ds-RNA in mice were investigated. Although no significant changes in the antiviral activity and toxicity were found on fragmentation when strain CD 1 mice were used, a parallel decrease of interferon-inducing capacity and molecular weight was established using mice of strain BSVS. The decrease in inducing capacity did not apparently depend on the method of fragmentation. Fragments of PcV-ds-RNA were found to be more toxic than the intact material for BSVS mice, but this toxicity was not apparently related to the extent of the fall in interferon-inducing capacity.

Thus, it seems unlikely that fragmentation will improve the prospects for the use of double-stranded RNA in antiviral therapy.

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INTRODUCTION

The induction of the antiviral protein Interferon (IF) is of importance, both from a fundamental biological standpoint, and also with a view to the possible use of IF-inducers or IF itself in the treatment of virus infection or neoplastic disease.

A great number of substances have been shown to induce IF, but interest has centred mainly around the double-stranded RNAs (ds-RNAs) and synthetic double-stranded polyribonucleotides as these are readily available, very efficient inducers, and of relatively low toxicity (at least compared to other IF inducers) and low antigenicity. Most work has concentrated on the use of the synthetic ds-polyribonucleotide complex, polyinosinic:polycytidylic acid [poly(I).poly(C)] as the inducer, and relatively little work has been done on IF-induction by a natural ds-RNA, which perhaps resembles more closely the inducer active during a virus infection. Although many experiments have been performed, our knowledge of the structural requirements for IF-inducing activity and toxicity is very limited; one of the ways in which these requirements might be investigated is by modification of the inducing ds-RNA. One possible molecular alteration is the fragmentation of the inducer, which allows a study of the effects of inducer size on its biological activities. Little is known about the fragmentation of natural ds-RNA, therefore some work on the effects of various methods of cleavage has been undertaken. Such studies on the mode of cleavage by different agencies, and the characterisation of the products of such fragments have not previously been attempted, and throw some light on the fine structure of a natural ds-RNA.

1. ds-RNA and ds-RNA Viruses

a) The in vitro production of ds-polyribonucleotides.

Warner (1956,1957) reported that mixing equimolar amounts of the synthetic polyribonucleotides polyadenylic acid [poly(A)] and polyuridylic acid [poly(U)], resulted in an immediate fall in the absorbance at the wavelength of maximum absorption (termed a hypochromic shift) of about 30 %, as compared to the sum of the absorbances of the individual homopolymers. The hypochromic shift was associated with increases in the viscosity and sedimentation coefficient of the molecules. These effects were explained by postulating an aggregation of the two polynucleotides, caused by hydrogen bonding between the purine and pyrimidine residues. X-Ray diffraction analysis (Rich & Davies, 1956) of dried fibres of the 1+1 complex of poly(A) and poly(U) [poly(A).poly(U)] revealed a regular pattern very similar in many respects to that obtained in the examination of similarly prepared DNA fibres. This similarity was interpreted as showing that the complex poly(A).poly(U) had a double-helical structure, the two chains being held together by hydrogen bonds between the adenine and uracil bases, analogues to the adenine-thymidine base pairing found in DNA. This poly(A).poly(U) was the first example of a polyribonucleotide complex with a configuration resembling the double-stranded structure of DNA, itself first demonstrated by Watson and Crick (1953,a,b).

Since these studies on poly(A).poly(U), many other synthetic ds-polyribonucleotides have been prepared, both by complexing of two complementary chains, and also by the self-complexing of various heteropolymers e.g. poly(A-U) (Chamberlin, Baldwin & Berg, 1963), poly(I).poly(C) (Davies & Rich, 1958), poly(I-C) (Krakow & Karstadt, 1967) and poly(G).poly(C) (Chamberlin, 1965).

The first RNA complex sequence shown to possess a highly ordered secondary structure, i.e. to be double-stranded, was the complementary RNA (C-RNA) studied by Geiduschek, Moohr and Weiss (1962). This C-RNA was prepared by polymerisation of ribonucleic triphosphates on a T_2 bacteriophage DNA template, using RNA polymerase purified from Micrococcus lysodeikticus to catalyse the reaction. Studies on the properties of C-RNA showed that it was not digested by pancreatic RNase (panc.RNase) at high concentrations if the salt concentration was high, but digestion proceeded fairly rapidly in solutions of lower ionic strength. Single-stranded RNA, and C-RNA following denaturation, were both rapidly digested in both high

and low salt concentration solutions. On slow heating of C-RNA solutions there was a sudden sharp increase in absorbance (hyperchromic effect), very similar to that observed for DNA under similar conditions (Thomas, 1954).

b) Naturally occurring ds-RNA.

This was first extracted and purified by Gomatos and Tamm (1963a) from the virions of Reovirus, an animal virus infecting many mammalian species. On the basis of the resistance of this RNA to digestion with panc.RNase in high salt media, coupled with its sensitivity to digestion in more dilute salt solutions, and the possession of a sharp thermal transition as measured by absorbance, the authors decided that Reovirus had ds-RNA as its genome. The same conclusion was also reached for the genome of the unrelated plant virus Wound Tumor virus (Gomatos & Tamm, 1963a,b).

ds-RNA has since been described as the genome of several viruses infecting the following groups of organisms :

(i) warm blooded animals e.g. Blue Tongue virus (Verwoerd, Louw & Oellerman, 1970), Colorado Tick Fever virus (Greene, 1970).

(ii) insects e.g. Silkworm Cytoplasmic Polyhedrosis virus (Hayashi & Kawase, 1964)

(iii) green plants e.g. Rice Dwarf virus (Miura, Kimura & Suzuki, 1966; Sato, Kyogoku, Higuchi, Mitsui, Iitaka, Tsuboi & Miura, 1966)

(iv) fungi, for examples see Section 1c.

(v) bacteria (sole example bacteriophage Ø6, Semancik, Vidaver & Van Etten (1973), from a Pseudomonad).

The subject of ds-RNA-containing viruses has recently been reviewed by Wood (1973).

ds-RNA is also found in cells infected with DNA or RNA viruses. In the latter case, when the genome of the infecting virus is in the form of single-stranded (ss) RNA, then the ds-RNA that is found has been considered to have a role in the replication of the virus, and is termed Replicative form RNA (RF-RNA) (Franklin, 1966). Such RF-RNA can be isolated from many types of virus-infected cells, e.g. from animal cells infected with Poliovirus (Baltimore, Becker & Darnell, 1964; Bishop, Summers & Levintow, 1965), from plants with Tobacco Necrosis virus infection (Klein & Reichmann, 1970), and in cells of E.coli infected with R 17 phage (Fenwick, Erikson & Franklin, 1964). The infecting virus is not restricted to one with an RNA genome. Vaccinia-infected cells, which contain the Vaccinia DNA genome,

have been shown to contain ds-RNA in their cytoplasm (Colby & Duesberg, 1969). Duesberg and Colby (1969) showed conclusively that the ds-nucleic acid was ds-RNA, and not a DNA-RNA hybrid. They also showed that the ds-RNA after denaturation hybridised with denatured Vaccinia virus DNA, and did not hybridise with the DNA of the host cells. Robertson and Zinder (1968) and Robertson (1971) have shown that during the transcription of DNA by E.coli RNA polymerase, three different types of nucleic acid were produced, i.e. ss-RNA, RNA-DNA ds-hybrids, and ds-RNA. This mechanism was shown to be similar to that of ds-RNA production in Vaccinia virus infected cells (Colby, Jurale & Kates, 1971). Similarly it has been shown that ds-RNA is produced in E.coli cells infected with the DNA phage T_4 (Jurale, Kates & Colby, 1970).

The fact that uninfected cells may contain ds-RNA was first shown by Montagnier (1968) who found such ds-RNA in cells of rat liver. This ds-RNA was shown to hybridise to a high degree with rat liver cell DNA (Harel & Montagnier, 1971), implying that it did not result from some latent or low-level viral infection. Kroneberg and Humphrey (1972) found a small quantity of rapidly-turning-over ds-RNA in the nuclei of Sea-urchin embryos, and suggested that its degradation was coupled with the processing and transport into the cytoplasm of messenger RNA. Small quantities of ds-RNA have also been found in cultured uninfected mouse spleen cells (Kisseljov, Semjenova, Irline & Shatalova, 1972).

c) ds-RNA viruses in fungi.

ds-RNA was first purified from a fungal virus by Banks, Buck, Chain, Himmelweit, Markes, Tyler, Hollings, Last and Stone (1968), but prior to this it had been postulated that fungi might contain viral particles (Lampson, Tytell, Field, Nemes & Hilleman, 1967) to explain their finding that Helenine, an extract of P.funiculosum with antiviral properties, contained ds-RNA. A similar fungal extract Statolon, from P.stoloniferum, was examined under the electron microscope, and found to contain hexagonal virus-like particles (VLPs) about 30 nm in diameter (Ellis & Kleinschmidt, 1967; Kleinschmidt & Ellis, 1967). The existence of these virus-like particles was confirmed by Banks et al. (1968), and these authors were also able to detect similar particles in a strain of P.funiculosum, the organism from which Helenine was produced. VLPs have also been found in penicillin-producing strains of P.chrysogenum, and in P.cyaneo-fulvum

(Banks, Buck, Chain, Darbyshire & Himmelweit, 1969a,b). Other mycelial fungi in which viral or virus-like particles have been demonstrated are : Aspergillus foetidus and A.niger (Banks, Buck, Chain, Darbyshire, Himmelweit, Ratti, Sharpe & Planterose, 1970), Stemphylium sp. (Planterose, Birch, Pilch & Sharpe, 1970), P.brevi-compactum (closely related to P.chrysogenum) (Wood, Bozarth & Mislivec, 1971), P.citrum and P.variabile (Borré, Morgantini, Ortali & Tonolo, 1971), Cephalosporium acremonium (Day & Ellis, 1971), P.claviforme (Metitiri & Zachariah, 1972), Thraustochytrium sp. (Kazama & Schornstein, 1972) and Neurospora crassa (Tuveson & Peterson, 1972; Kuntzel, Barath, Ali, Kind & Althaus, 1973).

An extract of the non-filamentous Japanese edible mushroom, Cornellis shiitake, was found to be an active IF inducer on injection into mice, and this was ascribed to infection with a ds-RNA virus (Tsunoda & Ishida, 1970). In all cases examined, except Thraustochytrium and Neurospora, it has been shown that the genome of these viruses is ds-RNA. The estuarine fungus Thraustochytrium, is thought to be infected with a DNA-containing Herpes-like virus particle (Kazama & Schornstein, 1972), whilst the VLPs of Neurospora crassa contain ss-RNA (Kuntzel et al., 1973).

ds-RNA-containing VLPs were initially found difficult to infect into uninfected strains of fungi, but this has since been achieved using the techniques of hyphal anastomosis (Lhoas, 1970,1971a) and the preparation of protoplasts (Lhoas, 1971b,1972; Border, 1972) of certain Penicillium and Aspergillus species. It is therefore correct to refer to the particles found in these species as viruses.

d) Multicomponent ds-RNA genomes.

Whenever the genome of a ds-RNA virus has been examined it has been found to consist of several pieces (Kalmakoff, Lewandowski & Black, 1969; Fujii-Kawate, Miura & Fuke, 1970; Border, Buck, Chain, Kempson-Jones, Lhoas & Ratti, 1972). This segmentation of the genome has also been found in several plant and animal viruses carrying their genetic material as ss-RNA. The multiple ss-RNA pieces are either all packaged together in one virion particle, as in e.g. Influenza virus (Duesberg, 1968; Pons & Hirst, 1968; Blair & Duesberg, 1970) (other examples are given in a recent review by Simon, 1972), or each segment is packaged into a separate particle, e.g. Alfalfa Mosaic virus (Bancroft & Kaesberg, 1960; Gillaspie & Bancroft, 1965; van Vloten-Doting, Dingjan-Versteegh & Jaspars, 1970) and Cowpea Mosaic virus (Bruening & Agrawal, 1967; van Kammen, 1968). Both of the

above types of packaging are shown by the ds-RNA viruses. The non-fungal viruses so far examined (Reovirus, Wound Tumour virus, Rice Dwarf virus, Sikworm Polyhedrosis virus), have been found to contain in each particle one of each of the ds-RNA pieces that can be purified from these virus preparations (Fujii-Kawate et al., 1970). In the case of Reovirus, the molecular weight (MW.) distribution of the fragments has been found to be very different from that expected to result from cleavage of one large ds-RNA molecule, either by shear degradation or random nuclease action (Bellamy, Shapiro, August & Joklik, 1967; Shatkin, Sipe & Loh, 1968). The different classes of segments did not cross-hybridise, i.e. the classes are without long sequences in common (Bellamy & Joklik, 1967; Watanabe & Graham, 1967). (This does not preclude sequence homology within the classes).

For a time it was considered a possibility that the segments resulted from cleavage at specific weak points along the genome. However, it has since been demonstrated that intact Reovirus particles contained as much RNA chains, as shown by the number of free 3'-termini, as did the isolated ds-RNA (Millward & Graham, 1970). This indicates that the segments isolated from the virus all existed within the virus particle. This is probably also the case for Silkworm Cytoplasmic Polyhedrosis virus, Rice Dwarf virus, and Wound Tumour virus (Fujii-Kawate et al., 1970).

The situation in the ds-RNA fungal viruses so far examined is somewhat different. It seems that in these viruses, each individual ds-RNA molecule is separately packaged, i.e. each virus particle contains one, or a very few, of the total number of RNA segments that are released on rupture of the particles. In this case, the viruses themselves are often separable into several classes as well. This was first demonstrated with the particles isolated from P.stoloniferum, where two classes of small (25-30 nm diameter) particles were revealed by serological tests, together with small amounts of a third, serologically distinct, larger (40-45 nm) particle (Buck & Kempson-Jones, 1970). These size groups of virus are resolvable on gel electrophoresis, into fast (PsV-F) and slow (PsV-S) particles. Each of these virus groups release multiple ds-RNAs, PsV-S containing 2 (MW. 1.01 and 0.94 x 10^6 daltons), whilst PsV-F was shown to contain 3 components (MW. 0.99, 0.89 and 0.23 x 10^6 daltons) (Bozarth, Wood & Mandelbrot, 1971). Similar results for the MWs. of the different ds-RNA species were obtained by Border et al., 1972.

When the viruses isolated from A.foetidus were examined, two electrophoretically distinct classes of particle could again be isolated, named as before AfV-S and AfV-F (Ratti & Buck, 1972). Each class of particle released, on extraction, several sizes of ds-RNA molecules. Both AfV-S and AfV-F could be resolved into a number of components by buoyant density gradient centrifugation in potassium tartrate, and examination of the ds-RNA isolated from each component showed that each type of virus particle contained only one class of ds-RNA molecule. A very similar situation is seen in the packaging of the ds-RNA genome in P.chrysogenum virus. This virus releases 3 species of ds-RNA on extraction; all of very similar size (Buck, Chain & Himmelweit, 1971). The MW. of the individual species were determined by gel electrophoresis, as 2.17, 2.00 and 1.87 x 10^6 daltons (Border et al., 1972). Very similar results were obtained by Wood and Bozarth (1972), who were also able to show, by computation of the average particle weight of the virus, and measurement of the nucleic acid:protein ratio, coupled with a knowledge of the MW. of the ds-RNA, that each particle could contain only 1 ds-RNA molecule. Buoyant density species were not observed, and it has been shown that the virus as extracted from the mould is serologically homogenous (Wood et al., 1971). It is not known whether all the ds-RNA species of a fungal virus are necessary for infection.

Throughout this thesis it is assumed that P.chrysogenum is infected with only one virus type, which has a multi-component genome.

2. The Physico-Chemical Properties of ds-RNA

In this section, some of the properties of ds-RNAs in general are first considered, and then the ds-RNA from P.chrysogenum virus is more specifically dealt with.

a) X-ray crystallography.

Some X-ray diffraction work has been performed on ds-RNA, mainly on the nucleic acids isolated from Reovirus and Wound Tumour virus (Langridge & Gomatos, 1963; Tomita & Rich, 1964; Langridge, Billeter, Borst, Burdon & Weissmann, 1964; Arnott, Hutchinson, Spencer, Wilkins, Fuller & Langridge, 1966; Fuller, Hutchinson, Spencer & Wilkins, 1967; Arnott, Wilkins, Fuller & Langridge, 1967a,b; Arnott, Wilkins, Fuller, Venable & Langridge, 1967) and the whole field was dealt with in a general

review of the X-ray crystallography of nucleic acids (Arnott, 1970). The present view is that as a dried fibre, at low salt concentrations, ds-RNA most closely resembles the A-form of DNA, having 11 base-pairs per helix turn, with the plane of the base-pairs forming an angle of approximately 70° with the helix axis. This is referred to as A-RNA. If the salt concentration of the fibre is increased, then the A'-form appears, which still resembles A-DNA in its major aspects, but has 12 base-pairs per helix turn. Other minor forms also exist under various conditions, but they are still basically of the A-conformation.

However, in nature ds-RNA is not encountered as a dried fibre, and its configuration in solution is of more interest to this present work. To study this, Bram (1971) has used low-angle X-ray scattering, working on the synthetic double-stranded polyribonucleotide complex poly(I).poly(C). This was shown to adopt the A'-conformation in solution, and Bram concluded that natural ds-RNAs would also exist in a 12-fold helix under such conditions. This is in contrast to solutions of DNA, which is thought to adopt the B-configuration, i.e. a 10-fold helix having the base-pairs roughly perpendicular to the helix axis (Luzzati, Nicolaieff & Masson, 1961; Luzzati, Luzzati & Masson, 1962; Luzzati, 1963).

b) Hydrodynamic properties.

Not much research has been done on these properties, most authors, e.g. Shatkin et al. (1968), Hamilton (1971) being content to assume that ds-RNA behaves exactly like DNA. This assumption has been questioned by Franklin (1967), who considered that ds-RNA was more flexible than DNA, and that therefore the use of equations relating MW. and sedimentation coefficient (S) or intrinsic viscosity (η), originally derived from work on DNA would give somewhat inaccurate results if applied to ds-RNA. Franklin developed an equation relating sedimentation coefficient to the MW. for ds-RNA, based both on his measurements and those of Gomatos and Stoeckenius (1964), but this equation was not used in the work described in this thesis, for in the author's opinion it is erroneous. The calculation is partly based on the calculations of the distance between nucleotide pairs published by Langridge and Gomatos (1963) and Tomita and Rich (1964). These authors concluded that ds-RNA was probably a 10-fold helix, and based their calculations of internucleotide distance on this. It is now considered as mentioned above that an 11-fold helix model fits the X-ray crystallography data better (Arnott, 1970) whilst in solution

a 12-fold helix seems probable (Bram, 1971), and it is therefore probable that earlier calculations of internucleotide distance are incorrect.

c) Ribonuclease resistance.

One of the major criteria used to distinguish ss and ds-RNA without complicated analytical techniques is the determination of the differential sensitivity to ribonuclease digestion, most commonly done using panc.RNase. This distinction between ss- and ds-RNA was first utilised by Geiduschek et al. (1962), who found that ds-RNA was not detectably digested by panc.RNase at an enzyme : substrate ratio of 0.05 µg panc.RNase : 1.8 µg ds-RNA, in a medium containing magnesium ions (0.003M $MgCl_2$, 0.05M Tris-HCl, pH 7.8) at 37°C. Heat-denatured C-RNA was rapidly digested under these conditions.

This resistance (under certain conditions) to digestion by panc.RNase has been used for the identification or characterisation of ds-RNA by a great number of authors, including Gomatos and Tamm (1963a,b), Montagnier and Sanders (1963), Weissmann and Borst (1963), Bishop et al. (1965), Shatkin (1965), Billeter, Weissmann and Warner (1966), Bellamy et al. (1967), Banks et al. (1969b), Lemke and Ness (1970), Wood and Bozarth (1972).

This resistance, however, has been shown to be far from absolute, and its appearance depends on the assay system used. Most of the above groups of workers used either the hyperchromic effect that occurs on fragmentation, or the production of acid-soluble oligonucleotides, to assess the extent of panc.RNase digestion. Both of these techniques only give a result when degradation is already quite extensive. These insensitive assays show that apparently, in solutions of high salt concentration or containing magnesium ions, panc.RNase has no effect on ds-RNA. If, however, more sensitive assay techniques are used, e.g. the sedimentation velocity of the substrate is determined, or the infectivity of an infectious ds-RNA (Shapiro, Bellamy, August & Joklik, 1966; Bishop, Koch & Levintow, 1967) it becomes clear that the inactivity is merely apparent, and that more sensitive assay techniques reveal a slow degradation of ds-RNA by panc.RNase. Such digestion is much slower than the panc.RNase digestion of ss-RNA. The mode of digestion of ds-RNA by pancreatic RNase has been investigated.

d) P.chrysogenum virus ds-RNA.

Virus particles were found in the mycelium of P.chrysogenum by Banks et al. (1969a) as mentioned above, using strain ATCC no. 9480. These viruses were found, like all other fungal viruses so far described (except those of Thraustochytrium and Neurospora crassa) to contain ds-RNA (Buck et al., 1971) as their genome (abbreviated here as PcV-ds-RNA).

A very similar ds-RNA was isolated directly from the mycelium of P.chrysogenum ATCC no. 9480 (Lemke & Ness, 1970) and from the derived strain ATCC no. 10002 (Cox, Kanagalingam & Sutherland, 1970). Both these groups of authors have done some studies on this ds-RNA, and work has also been reported by Buck et al. (1971), and by Border et al. (1972). The viruses of P.chrysogenum have been the subject of a detailed study by Wood and Bozarth (1972), and a short review of all these authors' conclusions is given below.

That the RNA was in fact double-stranded was established on the basis of its apparent resistance to panc.RNase in high salt concentration media, its high Tm with a sharp transition, resistance to reaction with formaldehyde, spectrophotometric properties, and chemical composition (Lemke & Ness, 1970; Cox, 1970; Cox et al., 1970; Cox, Kanagalingam & Sutherland, 1971; Buck et al., 1971; Wood & Bozarth, 1972). The ds-RNA was shown to consist of 3 components of very similar MW. (Cox et al., 1970,1971; Buck et al., 1971), and the MWs. of these three species have been measured using the technique of gel electrophoresis. Border et al. (1972) obtained results of 2.17, 2.00, and 1.87 x 10^6 daltons, whilst Wood and Bozarth (1972) found closely similar results (2.18, 1.99 and 1.89 x 10^6 daltons). Cox et al. (1970,1971) found, in addition to the high MW. ds-RNA another species, still of ds-RNA, but of much lower size (around 0.14 x 10^6 daltons) and guanosine and cytosine content (38 % GC, as against 51 % for the higher MW. ds-RNA). This low MW. ds-RNA species has not been described by other groups of workers, nor has it been encountered in this present study, so it will not be considered further. As mentioned above, the base composition of PcV-ds-RNA has been determined, showing that the proportions of A = U, and G = C. The mole % GC content was estimated as just over 50 % (56 % GC, Lemke & Ness, 1970; 51 % GC, Cox et al., 1970; 51 % GC, Buck et al., 1971).

Finally, the Tm of PcV-ds-RNA has been determined in various solvents, but in the standard solvent SSC (0.15M sodium chloride, 0.015M tri-sodium citrate, pH 7.2) has been variously found to be in excess of 100°C (Lemke & Ness, 1970), approximately 100°C (Cox et al., 1970) and 103°C (Buck et al., 1971). In 0.1 x SSC values of 92°C, and 88°C have been quoted, by Lemke and Ness (1970) and by Buck et al. (1971) respectively.

Taken as a whole then, these physicochemical studies on PcV-ds-RNA have shown that it is double-stranded overall, and have determined its size and chemical composition. Nothing has been revealed about its fine structure, e.g. the possibilities of ss-tails, or of loops or nicks in the individual strands have not been examined. For a clear view of the biological activities of the molecule, and also to understand certain features of the mode of operation of various nucleases in acting on the ds-RNA, it was important that these possibilities were investigated.

3. Biological Properties of ds-RNA

ds-RNA has been found to have a great number of biological effects, sometimes inhibitory, sometimes stimulatory, depending on the system studied. Some of the effects are non-specific, in that similar results can be obtained with other polyanions, especially bacterial endotoxins (De Clercq & Merigan, 1970a). Most work on the biological effects of ds-RNA and ds-polyribonucleotides has been done using the synthetic polyribonucleotide complex poly(I).poly(C).

a) 'Arming' and other effects on macrophages.

It has been reported that, when mice are immunised against a strain of cells, and the macrophages are then isolated from the immunised mice, these macrophages are cytotoxic for the cell type against which immunity has been raised (Evans & Alexander, 1970). There is no killing effect if a different cell type is used to test the macrophages, or if the macrophages are taken from a non-immunised animal. If macrophages from a non-immunised animal are treated with ds-RNA, and then placed in contact with some other cell type, cytotoxicity is again exhibited, and no immune specificity is involved. Endotoxin has a similar effect, and the process is referred to as the 'arming' of the macrophages (Alexander & Evans, 1971).

ds-RNA is also known to have other effects on macrophages, stimulating the synthesis of RNA and protein (Morrell, 1970,1971). This stimulation is especially interesting in the light of the essential role these cells play in host defence mechanisms, including the production of IF (Smith & Wagner, 1967; Finkelstein, Bausek & Merigan, 1968).

b) Immune enhancement by ds-RNA.

This is possibly related to the results described in the previous section. By immune enhancement is meant the stimulation of the antibody response to an antigen, and this is of considerable interest, the potential of ds-RNA as an immunological adjuvant has recently been assessed (Hilleman, Lampson, Field, Tytell, Nemes, Krakoff & Young, 1971). Braun and Nakano (1967) reported that poly(A).poly(U), (and also a complex of poly(C) and methylated bovine serum albumin) enhanced the early rate of increase of the numbers of antibody forming cells in the spleens of mice injected with sheep red blood corpuscles. They appeared to act by stimulating the nucleotide kinase activity of such antibody forming cells.

The ds-polyribonucleotide poly(G).poly(C) was also shown to be active (Braun, Nakano, Jaraskova, Yajima & Jimenez, 1968) and it was shown that such ds-complexes were also active in stimulating phagocytosis in macrophages. Similar effects, although of much lesser degree, had also been found earlier by Cohn and Parks (1967), using DNA and yeast RNA, and by Freedman and Braun (1965), studying enzyme digests of DNA. Johnson, Schmidtke, Merrit and Han (1968) showed that for adjuvant activity of poly(A).poly(U), it was essential that the molecule was in the double-helical form, as the ss-polymers alone had no adjuvant activity. The suggestion was made by Johnson et al. (1968) that poly(A).poly(U) complexes acted by increasing the division rate of potential antibody-forming cells. That the terminal number of such cells was not increased, merely their rate of appearance, was shown by Braun, Ishizuka, Yajima, Webb and Winchurch (1971). The target for the action of ds-polyribonucleotides was shown to be the thymic-derived lymphocytes by Johnson and his co-workers (Johnson, Cone, Friedman, Han, Johnson, Schmidtke & Stout, 1971; Johnson & Johnson, 1971; Cone & Johnson, 1971). This stimulatory effect extends not only to the humoral antibody response, but also increases cellular immune responses (Turner, Chan & Chirigos, 1970), e.g. poly(I).poly(C) injections reduce the survival time of isografts in mice.

It seems probable that these immune-stimulating activities play a role in the in vivo antiviral and antitumour actions of ds-RNA.

c) Effect of ds-RNA on cell-free protein synthesis.

Ehrenfeld and Hunt (1971) and Hunt and Ehrenfeld (1971) have shown that ds-RNA from a variety of sources (Poliovirus RF ds-RNA, f_2 coliphage RF ds-RNA, Reovirus genome ds-RNA) and also the polynucleotides poly(A-U) and poly(I).poly(C) inhibited the synthesis of haemoglobin by rabbit reticulocyte lysates. The inhibition was not immediate upon the addition of ds-RNA, and even at maximal inhibition levels polypeptide chain synthesis continued for a period at the uninhibited rate before the abrupt cessation of amino acid incorporation. The authors suggested that a factor required for initiation of polypeptide synthesis, which is slowly depleted under normal conditions, is either no longer regenerated, or not synthesised, in the presence of ds-RNA. Confirmatory evidence has been obtained recently by Kaempfer and Kaufman (1973), who studied the inhibition of polypeptide synthesis in rabbit reticulocyte lysates by added PcV-ds-RNA. They were able to show that an inactive complex was formed between the ds-RNA and an initiation factor (IF-3) necessary for ribosome recycling and m-RNA binding. Addition of exogenous IF-3 overcame the inhibition by PcV-ds-RNA.

A similar inhibition of protein synthesis in an E.coli-f_2 phage RNA system by poly(I).poly(C) was demonstrated by Chao, Chao and Speyer (1971).

d) Toxicity of ds-RNA for cultured cells.

During the infection of cells with Bovine Enterovirus (BEV) large quantities of ds-RNA accumulate in the cytoplasm just before cell death. The relationship between the ds-RNA and the cytotoxic effect was investigated by Cordell-Stewart and Taylor (1971), who found that the rate of cell killing in a non-infected culture was directly proportional to the amount of ds-RNA added. BEV-RF ds-RNA was more effective than non-infectious ds-RNAs such as poly(I).poly(C) and poly(A-U), which killed only 30 % of the total treated cells when an equal weight dose of BEV-RF ds-RNA killed 100 %.

The BEV-ds-RNA appears to inhibit cell-directed polypeptide synthesis, probably at the stage of translation (Cordell-Stewart & Taylor, 1973), which is similar to the effect described in section 3 c) above. The toxic effect of BEV-ds-RNA is specific for cell-directed polypeptide synthesis,

because incorporation of amino acids into BEV or Mengo virus-specific protein in infected cells was not affected by the addition of ds-RNA.

Data on the cellular toxicity of ds-polyribonucleotides, generally poly(I).poly(C), are few and often scanty. Kawade and Ujihara (1969) found that a concentration of 0.1-1.0 µg/ml poly(I).poly(C) was sufficient to have lethal effects in Chick Embryo cells, whilst Margolis and Levy (1969) found that treatment of mouse L-cells with between 10 and 50 µg/ml did not result in any toxic manifestations. A careful study of Flikke, Kjeldsberg and Lahelle (1970), using the Detroit (human) cell line, showed that 25 µg/ml poly(I).poly(C) was toxic for cultured cells. It seems probable, as suggested by Levy, Law and Rabson (1969), that the degree of toxicity varies with the cell type or with the extent of cellular differentiation.

Cytotoxicity has been reported on repeated exposure of cells to poly(I).poly(C) (Billiau, Van den Berghe & De Somer, 1972b), when each individual dose alone was insufficient to cause toxic effects. This increased toxicity was considered to be due to the priming effect of the IF induced by the first dose of poly(I).poly(C), which makes a cell hyper-reactive with regard to a later dose of poly(I).poly(C), both in producing more IF (Rosztoczy & Mecs, 1970; Billiau, 1970; Stewart, Gosser & Lockart, 1971; Tovell & Cantell, 1971; Billiau, Joniau & De Somer, 1972a; Billiau et al., 1972b) and in increasing the toxicity of the inducer (Stewart, De Clercq, Billiau, Desmyter & De Somer, 1972).

e) Animal toxicity of ds-RNA.

Poly(I).poly(C) has been found to be lethal for mice at relatively low doses (no LD_{50} was established but 20 mg/kg killed 5/10 mice in one experiment) and to provoke a local Shwartzman reaction (Absher & Stinebring, 1969). The toxic effects resembled in some ways those observed with endotoxin, and lead acetate injection (which enhance the toxicity of endotoxin) also enhanced the toxicity of poly(I).poly(C). Similar results were noted in mice treated with actinomycin D (Pieroni, Bundeally & Levine, 1971) and in adrenalectomised rats (Zedeck, Marquardt, Sternberg, Fleischer & Hamilton, 1970). Both of these treatments increased the sensitivity of the test animal to the toxic effects of both endotoxin and poly(I).poly(C).

The lethal effects of poly(I).poly(C) are only noted with doses much greater than those necessary to protect mice against virus infection. This protective dose varies with the assay system for measuring the degree of protection, but generally doses of 1 mg/kg or less are effective. (See e.g.

Nemes, Tytell, Lampson, Field & Hilleman, 1969a; Richmond & Hamilton, 1969; Lindh, Lindsay, Mayberry & Forbes, 1969; Postic & Sather, 1970; Finter, pers. commun., 1970). The LD_{50} of poly(I).poly(C) in mice after intravenous injection was about 16.5 mg/kg (Hilleman, 1970).

Poly(I).poly(C) was shown to be highly pyrogenic (Lindsay, Trown, Brandt & Forbes, 1969), and embryotoxic (Adamson & Fabro, 1969) in rabbit. Lindh et al. (1969) noted that the pathological symptoms on administration of a lethal dose of poly(I).poly(C) resembled those produced by endotoxin, whilst Braun (1969) found that poly(I).poly(C), like endotoxin appeared to damage cell membranes, specifically those of macrophages. This would be expected to cause the release of a number of endogenous substances, notably IF, pyrogens and lymphocyte stimulators. Leonard, Eccleston & Jones (1969) found that poly(I).poly(C) caused a runt-like disease in mice and rabbits, and an acceleration in the onset of the auto-immune disease in New Zealand mice has also been described (Steinberg, Baron & Talal, 1969). Like the toxicity to the haemopoietic stem cells in mice (Julien & De Maeyer-Guignard, 1971), these effects indicate that poly(I).poly(C) has considerable activity on the reticulo-endothelial system. This is interesting in view of the stimulatory effects on this system of lower doses of polyribonucleotides (section 3 b)).

Ocular treatment with poly(I).poly(C) in rabbits was reported to cause eye damage, including opaqueness of the lens and hyperaemia of the iris (Ostler, Oh, Dawson & Burt, 1970), and cerebellar toxicity has been reported in chickens (Young, Taylor, Yu & Eyerman, 1970). In this case, oedema, haemorrhage and ataxia were found, and were considered due to the destruction of the endothelial cells in the walls of the small blood vessels, leading to their rupture. In addition poly(I).poly(C) has been reported to inhibit DNA synthesis and mitosis in epithelial cells of the mouse salivary gland, after stimulation with isoproterenol (Serota & Baserga, 1970) and in the regenerating liver of partially hepatectomised mice (Jahiel, Taylor, Rainford, Hirschberg & Kroman, 1971).

The toxicity of poly(I).poly(C) in mice, rats, dogs and other animals has been reviewed by Stinebring and Absher (1971), and by Philips, Fleischer, Hamilton, Schwarz and Sternberg (1971). Data on the toxicity of poly(I).poly(C) in man has been obtained in some clinical trials, although of course in such trials the dose administered must be severely limited. Field, Young, Krakoff, Tytell, Lampson, Nemes and Hilleman (1971) were able to give doses of up to 400 µg/kg intravenously into some patients,

and observed only some slight febrile response. Regretably, even with these dosages only very limited amounts of interferon were observed.

f) Antiviral activity and the induction of interferon.

The antiviral protein Interferon, its structure and mechanism of action, are dealt with in Section 4).

Various fungal extracts and products have been found to have antiviral activity, most notably Helenine, from P.funiculosum (Shope, 1953a,b) and Statolon, an extract of P.stoloniferum (Probst & Kleinschmidt, 1961; Kleinschmidt & Probst, 1962). An antiviral antibiotic, M-8450, had been found in extracts of P.stoloniferum several years before (Powell, Culbertson, McGuire, Hoehn & Baker, 1952; Hull & Lavelle, 1953) and M-8450 was shown to resemble Helenine in its mode of action (Cochran & Francis, 1956). Even prior to this, antiviral activity had been found in an impure Penicillin preparation by Groupé and Rake (1947).

The antiviral activity of Statolon was shown to be due to the induction of IF (Kleinschmidt, Cline & Murphy, 1964) but there was for some time controversy about the chemical nature of the inducer. The generally accepted theory was that, as the major component of Statolon was complex anionic polysaccharide, this was the inducer (Probst & Kleinschmidt, 1961; Cooke & Stevenson, 1965a,b), but one group of workers were able to isolate an active substance with the chemical and spectral properties of a ribonucleoprotein (Lewis, Rickes, McClelland & Brink, 1959).

In 1967, in a classic series of papers, Hilleman and his coworkers showed that ds-RNA was a most efficient IF inducer, whereas ss-RNA and DNA had no detectable activity (Field, Tytell, Lampson & Hilleman, 1967a; Tytell, Lampson, Field & Hilleman, 1967; Field, Lampson, Tytell, Nemes & Hilleman, 1967b). They found that the active component of an IF-inducing extract from P.funiculosum was ds-RNA (Lampson et al., 1967). Since then, as mentioned in section 1 c), ds-RNA has been found in antiviral extracts of many fungal species, and has generally been shown to be the result of viral infection of the fungus.

g) Requirements for a polynucleotide to have interferon-inducing activity.

There have been numerous studies on the structural requirements for IF-inducing activity in polynucleotides, often yielding conflicting results.

For reasons of commercial availability, and apparent chemical simplicity, almost all work on the structural requirements for activity has been done on synthetic polynucleotides. One of the more important requirements is that a polynucleotide must have a stable double-stranded structure, formed by hydrogen-bonding between complementary bases. The stability referred to is generally considered as the thermal stability (the temperature range over which the helical structure of the double helical complex is disrupted, resulting in the production of two random coil single-stranded polynucleotides. The mid-point of the thermal transition is called the melting temperature or Tm). The Tm and antiviral activity (the relationship between the antiviral activity and IF induction is considered in the discussion) of a number of polyribonucleotide complexes, both homopolymer pairs and alternating copolymers, were determined by De Clercq and Merigan (1969a) and by Colby and Chamberlin (1969). The general conclusion was that the antiviral activity depended to some extent on the Tm, in that :

(1) complexes with a Tm below 50°C are inactive,

(2) complexes melting between 50°C and 60°C are moderately active, and

(3) complexes with Tm above 60°C are highly active, but the extent of antiviral activity is not related to Tm.

Since Doty, Boedtker, Fresco, Haselkorn and Litt (1959) have shown that Tm is a valuable measurement of the overall stability of a ds-polynucleotide, it is reasonable to consider that the polyribonucleotides with Tm values higher than 60°C may also be most stable at 37°C. It seems as though both strands of the complex must stay together in a highly ordered secondary structure in order to stimulate IF production and antiviral activity. Sequential administration of first one ss-polyribonucleotide, and then the complementary strand, also results in good IF yield, in fact perhaps better than administration of an equivalent amount of the ds-polyribonucleotide complex (De Clercq & De Somer, 1971,1972). It is probable that the complementary strands meet up and complex either in the circulation or on the cell surface.

Single-stranded RNA has generally been shown to be inactive at inducing IF and antiviral activity (Lampson et al., 1967; Field et al., 1967a,b; Colby & Chamberlin, 1969) although some workers have found low levels of activity in various preparations of poly(I), poly(X), poly(C) and poly(G) (De Clercq & Merigan, 1969a; Baron, Bogomolova, Billiau, Levy, Buckler, Stern & Naylor, 1969). In this latter case, the activity found varied from batch to batch of synthetic polyribonucleotide. Both groups showed that the activity stemmed from the ss-polyribonucleotides themselves,

and not from some small contamination with ds-RNA, by digesting the polypyrimidine-nucleotide samples with panc.RNase under conditions when ds-RNA is much more resistant than ss-RNA. (This proof is not absolute, however, for ds-RNA is not completely resistant to panc.RNase.) Such digestion destroyed completely the antiviral activity. The batches with higher activity were found to have higher RNase-resistance (Baron et al., 1969) and Baron's group considered that the active ss-polyribonucleotides were somehow in a semi-ordered configuration intermediate between a random coil and double-helix. De Clercq and Merigan (1969a) showed that active ss-polyribonucleotides, e.g. poly(G), poly(I) and poly(X), showed a greater hyperchromic effect on alkaline hydrolysis than the homopolymers poly(A), poly(U) and poly(C) which they found inactive. This suggested that the former group of polymers had a more stable secondary structure than the latter, inactive ones. As pointed out by De Clercq, (1971) immunological studies have shown that under the conditions used by De Clercq and Merigan (1969a), poly(I), poly(X) and poly(G) might all be expected to occur in highly ordered three- or four-stranded structures (Panijel, Souleil & Cayeux, 1966; Souleil & Panijel, 1968). Such multi-stranded complexes were shown to have very low activities as inducers of IF (Colby & Chamberlin, 1969; De Clercq & Merigan, 1969a; De Clercq, Eckstein & Merigan, 1970c). Some authors have reported that Newcastle Disease virus (NDV) and Semliki Forest virus (SFV) virions, containing ss-RNA as their genome, were able to induce IF under conditions when no RNA synthesis could occur (Gandhi & Burke, 1970; Goorha & Gifford, 1970; Dianzani, Gagnoni, Buckler & Baron, 1970); this has been taken as evidence for the activity of some factor other than ds-RNA acting as IF inducer. All these authors utilised concentrated unpurified virus preparations. Recently the existence of significant quantities of ds-RNA (up to 2 % of total nucleic acid) has been reported in the nucleic acid extracts of such highly concentrated, crude, virus preparations, using NDV, Sindbis virus and SFV, ds-RNA being detected by complement fixation with an antiserum specific for ds-RNA (Field, Lampson, Tytell & Hilleman, 1972).

If double-strandedness were the sole criterion for polynucleotides to have IF-inducing activity, then DNA would be expected to have high activity. This is not so, DNA and polydeoxyribonucleotide complexes have generally been shown to have little or no IF-inducing activity (Lampson et al., 1967; Field et al., 1967a; Colby & Chamberlin, 1969; De Clercq et al., 1970c). DNA differs physico-chemically from ds-RNA in three major ways, at least one of which may be responsible for the inactivity of the deoxy-

polymers. The substitution of uridine for thymidine is not likely to be of any importance, for although the activity of different synthetic polyribonucleotides varies, and their variation may be the result of, amongst other things, the base composition, the synthetic polydeoxyribonucleotide poly(dI).poly(dC) is inactive, whilst poly(I).poly(C) is one of the most active inducers (Vilcek, Ng, Friedman-Kien & Krawciw, 1968; Colby & Chamberlin, 1969; De Clercq et al., 1970c).

The second major difference is in the structure of the double-helices, as reviewed in section 2 a) above. As stated before, ds-RNA in solution is considered to be in the form of a 12-fold helix, with the bases tilted at 70°-80° to the helix axis, whilst DNA probably adopts the B-conformation, a 10-fold helix with the plane of the base-pairs perpendicular to the helix axis. Work on DNA-RNA hybrids shows, however, that this difference in conformation is not the cause of the inactivity of DNA. These hybrids adopt the A-conformation in solution, resembling ds-RNA (Milman, Langridge & Chamberlin, 1967; O'Brien & McEwan, 1970; Bram, 1971). DNA-RNA hybrids, however, do not induce IF (Vilcek et al., 1968; Colby & Chamberlin, 1969; Colby, Stollar & Simon, 1971). The finding of low activity in a DNA-RNA hybrid preparation (Nemes, Tytell, Lampson, Field & Hilleman, 1969b), can be explained by inadequate purification of the product, for as mentioned before, the use of RNA polymerase to make such hybrids has been shown to result also in the production of small amounts of ds-RNA (Robertson & Zinder, 1968; Robertson, 1971).

Therefore it would seem that the hydroxyl group on the 2-carbon of the ribose moiety (2'-OH) is necessary in both strands for the presence of IF-inducing activity. This is borne out by work involving the chemical reaction of the 2'-OH groups (De Clercq, Zmudzka & Shugar, 1972; Black, Eckstein, Hobbs, Sternbach & Merigan, 1972) which shows that such chemical substitutions of this group as methylation or chlorination greatly reduce the activity of the ds-polyribonucleotide complexes as IF-inducers.

However, in one case, DNA has been found to be an active IF-inducer. Kleinschmidt, Douthart & Murphy (1970) reported that although the DNA extracted from T_4 coliphage particles did not induce IF, (and neither did the protein coat ghosts), the intact phage was an efficient IF-inducer in rabbits. It was considered extremely unlikely that T_4 phage would replicate in mammalian cells, which might have given rise to the production of some ds-RNA. The authors ascribed the activity of the intact T_4 virions to the protection against nuclease digestion afforded to the DNA by the protein coat, and that rapid digestion by cellular and circulatory nucleases was the

cause of the inactivity of DNA as an inducer. As an alternative hypothesis, Kleinschmidt and his coworkers suggested that the DNA inside the phage particles might adopt some special conformation, resulting in activity as an IF-inducer. (See also Kleinschmidt, 1972). That in fact the DNA inside phage particles possesses a different conformation from its state in solution was shown by the Optical Rotary Dispersion work of Maestre and Tinoco (1967).

Perhaps more important for activity than the physical stability of a polynucleotide complex is the resistance to digestion by various nucleases. However, the published data is complicated and sometimes contradictory, and it is difficult to get a clear picture of the absolute importance of nuclease resistance.

Immediately an inducer molecule is put onto a tissue culture or injected into an animal, nuclease digestion will commence, and it is of obvious importance that the inducer should withstand this long enough to trigger the IF-induction process. As described in section 2 c), it is known that panc.RNase (under certain conditions) will digest ss-RNA rapidly to oligo- and mono-nucleotides, whilst having very little effect on ds-RNA. This has been considered to be the reason for the inactivity of ss-RNA as an inducer (Baron et al., 1969), and if this is the case, then a direct relationship between RNase-stability and IF-inducing capacity might be expected. Most work on the RNase-resistance of IF-inducing ds-polynucleotides has been done using panc.RNase to test the resistance to nuclease digestion, although there is no reason to believe that this nuclease is solely, or even at all, responsible for the breakdown of ds-polyribonucleotide IF-inducers in vivo. Indeed a nuclease specific for ds-RNA has been described in the sera of numerous animals, including rats, rabbits and man (Stern, 1970a,b).

It has been shown that polyribonucleotide complexes containing I-C and G-C base pairs are more active as inducers of antiviral activity and IF than are complexes containing A-U base pairs [i.e. poly(A).poly(U) and poly(A-U)] and that the former are more resistant to digestion with panc. RNase (Field et al., 1967a; Colby & Chamberlin, 1969; De Clercq et al., 1970c). That the difference in IF-inducing activity is probably not an uptake phenomenon was shown by Colby and Chamberlin (1969), who found that poly(I).poly(C) and poly(A-U) are taken up into chick embryo cells at very similar rates.

The importance of nuclease resistance is further supported by work on polyribonucleotide analogues. Polynucleotides containing thiophosphate groups instead of phosphate are readily prepared, and the Tm of such substituted polynucleotides does not differ significantly from that of the unsubstituted compound (Eckstein & Gindl, 1970). However, such substitution, either in half (poly(A-$_s$U) and poly(-$_s$I-C) or all the residues (poly(-$_s$A-$_s$U) and poly(-$_s$I-$_s$C) resulted in increased antiviral activity (De Clercq, Eckstein & Merigan, 1969; De Clercq, Eckstein, Sternbach & Merigan, 1970d,e). As the thiophosphate bond is resistant to cleavage by many nucleases, including panc.RNase, RNase T_1, and the RNase activity of serum (De Clercq, 1971), then it was not unexpected that poly(-$_s$A-$_s$U), poly(-$_s$I-$_s$C) and poly(-$_s$I-C) were markedly more resistant to digestion by panc.RNase than the parent compounds poly(A-U) and poly(I-C). The partially substituted derivative poly(A-$_s$U) did not differ in sensitivity to panc.RNase from the unsubstituted poly(A-U) (Matzura & Eckstein, 1968). This is, of course, because panc.RNase usually cleaves after pyrimidine residues (Markham & Smith, 1952) and in poly(A-$_s$U) the thiophosphate groups only occur after the purine residues. Poly(-$_s$I-$_s$C) and poly(-$_s$I-C) are equally resistant to digestion with panc.RNase, whilst if digested with RNase T_1 instead, the partially substituted polynucleotide poly(-$_s$I-C) was found to be as sensitive as its parent compound (poly(I-C), whilst poly(-$_s$I-$_s$C) was very much more resistant (De Clercq *et al.*, 1970c; Eckstein & Gindl, 1970). This again is to be expected, in view of the known specificity of RNase T_1, which cleaves only after guanosine and guanosine-derivative nucleosides, e.g. inosine (Sato & Egami, 1957; Sato-Asano, 1959). All these substituted compounds have increased efficiency in the induction of antiviral resistance (De Clercq *et al.*, 1970d). This suggests the importance of nuclease resistance for IF-inducing activity. Other evidence for the importance of nuclease resistance stems from the high activity of vinyl-substituted polyribonucleotide complexes, poly(I).poly(vC), which also have very high resistance to digestion with panc.RNase (Pitha & Pitha, 1971).

Finally, numerous groups of workers have found that complexing poly(I).poly(C) with the polycation diethylaminoethyl-dextran (DEAE-dx) greatly increases the antiviral activity *in vitro* of the polynucleotide (Dianzani, Cantagalli, Gagnoni & Rita, 1968; Vilcek *et al.*, 1968; Colby & Chamberlin, 1969; Billiau, Buckler, Dianzani, Uhlendorf & Baron, 1970b). The polycation could exert its stimulatory effect in either (or both) of two ways : by protecting the inducing polyribonucleotide from nuclease digestion, or by facilitating its uptake to some cellular site. Billiau and his coworkers (1970b) were unable to decide between these two mechanisms,

but experiments by Dianzani, Gagnoni & Cantagalli (1970) led them to the conclusion that the activity of DEAE-dx in mouse L-929 cell cultures is exerted largely by increasing the cell permeability to the inducer rather than by protecting against nuclease digestion. Similar conclusions were reached by Colby and Chamberlin (1969).

The above work on the action of DEAE-dx shows that nuclease resistance is perhaps not so all-important. Colby and Chamberlin (1969) studied the resistance to panc.RNase of several IF-inducing polyribonucleotides, and could find no clear correlation between antiviral activity and nuclease resistance. For example, poly(G).poly(C) and poly(I).poly(C) are almost equally active as IF-inducers, yet the former ds-polyribonucleotide is very much more resistant to RNase digestion. Similarly, of the two adenine-uridine polymers, poly(A-U) is the more efficient IF-inducer, whilst poly(A).poly(U) is much more resistant to digestion with pancreatic RNase.

These differences between poly(A-U) and poly(A).poly(U) may be explained, however, in a different way. Under normal physiological conditions, poly(A).poly(U) tends to reorganise into a three-stranded form : $2\text{poly(A).poly(U)} \rightleftharpoons \text{poly(A).2poly(U)} + \text{poly(A)}$ (Hamilton, 1971). Triple- and multi-stranded nucleic acids have been shown to be ineffective as IF inducers (De Clercq & Merigan, 1969a; Colby & Chamberlin, 1969; De Clercq et al., 1970c).

So, the situation with regard to the importance of nuclease resistance is unclear, possibly because of the reliance on panc.RNase in assessing nuclease resistance. Perhaps the use of other nucleases would clarify results.

Perhaps the most important 'other nuclease' is the activity against ds-RNA found in serum, and in mammalian cells (Stern, 1970a,b; Stern & Wilczek, pers. commun., 1972). It is not known if the serum and cellular activities are due to separate enzymes. The existence of serum RNases having high activity against ds-RNA was suspected earlier, when it was found that sera of several animal species (e.g. chickens, foetal calf, and man) were active in abolishing the pyrogenic activity of poly(I).poly(C) (Nordlund, Wolff & Levy, 1970). The pyrogenicity and IF-inducing capacity of ds-RNA are considered to be closely linked (Lindsay et al., 1969), and it has been found that injections of heterologous serum suppressed the IF-response to poly(I).poly(C) in mice, and the degree of suppression correlated well with the serum RNase activity against ds-RNA (Friedman, Barth & Stern, 1971). Thus, it seems possible that the differing activities of different polyribonucleotide IF-inducers might reflect their resistance

to digestion by serum RNases. This activity may also be responsible for the finite half-life of ds-RNA in the circulation, and for the short duration of IF production.

When this work was started in 1969, nothing was known about the effect of inducer MW. on the induction of IF, and related phenomena. Since then, several groups of authors have published results on the effects of altering the size of the ds-polyribonucleotide inducer molecule. The results obtained by different groups are not always consistent, and are sometimes contradictory.

Two distinct approaches to the problem of producing ds-RNA molecules of various sizes have been used. The first technique is to produce ss-polyribonucleotide molecules of various sizes, and then to complex the fragments to produce ds-polyribonucleotide molecules of the desired size. This technique has been used by Niblack and Walsh, pers. commun. (1970), Jameson and Grossberg (1970), Tytell, Lampson, Field, Nemes and Hilleman (1970), Niblack and McCreary (1971), Hamilton (1971), Morahan, Munson, Regelson, Commerford and Hamilton (1972), and by De Clercq and Merigan, unpublished results, quoted by De Clercq (1971). This approach has two related disadvantages. Firstly, as in all work so far published the ss-polyribonucleotide preparations have been of a wide MW. distribution, so that when complexes are prepared there is no way to prevent a long ss-chain hybridising with one, or more, shorter chains. This means that the supposedly ds-molecules may in fact have ss-tails or internal ss-regions of quite considerable length. The second disadvantage is that, even if preparations of ss-polyribonucleotides were obtained of a monodisperse MW., it is still impossible to make the two complementary chains anneal with their ends together, and unless this occurs, it is quite possible for a third chain to complex with the ss-tail, and further chains with ends of this. Thus, very long molecules, with single- and double-stranded branches could be produced. Thus, the ds-polynucleotide products of such complexing can be heterogenous, both with regard to structure and size, and the range of sizes can readily be greater than the MW. distribution of either of the component ss-polynucleotides.

A better method of preparing ds-RNA molecules of various sizes is to fragment the ds-RNA molecule itself, which is the technique used by the author of this thesis. This approach has also been used by other groups of workers (Field, Tytell, Lampson, Nemes & Hilleman, 1970; Lampson, Field, Tytell, Nemes & Hilleman, 1970; Levy, Asofsky, Riley, Garapin, Cantor & Adamson, 1970; Shiokawa & Yaoi, 1972). These groups have all used shear

forces to cleave the ds-RNA molecules (poly(I).poly(C) and poly(I,G).poly(C)), either by ultrasonic irradiation or homogenisation in a Ribi Cell Fractionator apparatus. Fragmentation of a ds-polyribonucleotide has the advantage of yielding a product of less broad MW. distribution than that produced on complexing ss-polynucleotide molecules and of complete base-paired ds-structure (in so far as the parent molecule had such a structure). In fact, if the MW. distribution of the original ds-RNA was very wide, then ultrasonication would actually narrow this distribution by fragmenting more efficiently the longer molecules, whilst lowering the average MW. (Shiokawa & Yaoi, 1972).

Both techniques of producing ds-RNA molecules of different sizes have yielded essentially similar results, however, in biological assays, which indicates either a low sensitivity of the induction process to the factors of structure and MW. distribution described above, or (more likely) the low sensitivity of the assay techniques themselves.

Most authors found that lower MW. ds-RNA molecules were less efficient as inducers of IF and/or antiviral protection (Niblack & Walsh, pers. commun., 1970; Jameson & Grossberg, 1970; Field et al., 1970; Lampson et al., 1970; Tytell et al., 1970; Niblack & McCreary, 1971; Hamilton, 1971; De Clercq, 1971; Morahan et al., 1972), although opinions varied as to whether the protective effect fell in parallel with the MW., and whether the IF-inducing activity and antiviral activity fell together. In two cases, however, apparently no alteration in IF-inducing activity was found on fragmentation (Levy et al., 1970; Shiokawa & Yaoi, 1972). Some workers (Field et al., 1970; Lampson et al., 1970; De Clercq, 1971) found an apparent threshold for activity : samples with MW. between approximately 8×10^6 and 10^5 daltons had very similar activities, but below 10^5 daltons, reduction in MW. resulted in a marked decline in the capacity to protect cell cultures against viral infection. This effect will also be considered in the Discussion Section.

The importance of the chain length of the individual strands in a ds-polyribonucleotide for IF-inducing activity is a matter of dispute. Interpretation and comparison of such work is very difficult in the absence of data on the fine structure of the complexes produced by the annealing of polymers of different sizes. Tytell et al. (1970) found that high in vitro activity was more dependent on the molecular weight of poly(I) than poly(C) in the ds-inducer. Activity was markedly diminished when the MW. of the poly(I) in a complex fell below 190,000 daltons (c. 550 residues), whilst the poly(C) could be of as few as 70 residues (MW. 23,000 daltons)

before activity fell to any great extent. However, this result was considered incorrect (due to the use of inappropriate MW.-S value relationships) by Niblack and McCreary (1971). They found that the MW. of the strands were equally important. In this they supported the results of Wacker, Singh, Svec and Lodemann (1969), who complexed poly(I) with oligo(C), and vice versa. They found that the IF titre induced by such complexes depended on the chain length of the oligomer, but the effects were similar with both oligo(I).poly(C) and poly(I).oligo(C).

All groups who measured the effect of MW. on the toxicity of ds-RNA found that lower MW. samples had lower toxicity in vivo (Niblack & Walsh, pers. commun., 1970; Levy et al., 1970; Niblack & McCreary, 1971; Hamilton, 1971; Hilleman et al., 1971 (discussing the samples prepared by Field et al., 1970; and Lampson et al., 1970;) and Shiokawa & Yaoi, 1972).

Whether or not fragmentation improves the toxicity/activity ratio (as was found by Levy et al., 1970; Hilleman et al., 1971 and Shiokawa & Yaoi, 1972) is of considerable importance, both from a practical viewpoint of the development of IF-inducers as a clinically useful broad-spectrum antiviral agency, and from the more purely scientific angle of obtaining an understanding of aspects of the IF-induction mechanism. There have been reports that IF-inducing capacity and toxicity are inseparable, being manifestations of the same phenomenon (De Clercq, Stewart & De Somer, 1972; Black, Eckstein, De Clercq & Merigan, 1973). Fragmentation of a ds-RNA IF-inducer is one way in which this close association of toxicity, antiviral activity and IF-inducing activity can be examined. This is one of the aims of the work described in this thesis. It was also very interesting to determine whether fragmentation of a natural ds-RNA gave similar results to work on synthetic polyribonucleotides. Before going on to describe this work, however, the induction, action and nature of Interferon will be briefly described.

4. Interferon, its Induction, Action and Nature

a) Early results.

The interference of an existing virus infection with the establishment of a second infection was described many years ago (Hoskins, 1935), and by the use of serologically unrelated viruses was shown to be independent of the antibody response (Findlay & MacCallum, 1937). Although other examples of viral interference were noted, no light was shed on the process until 20 years later, when in a classic paper, Isaacs and Lindenmann

(1957) described what they called the Interferon. This paper described the existence of an antiviral protein, the Interferon, which was present in the medium after treatment of portions of chick chorio-allantoic membrane with heat-inactivated Influenza virus. IF did not directly inactivate intact virus, but if added to the medium containing uninfected membrane fragments, it prevented their infection with live Influenza virus. IF was shown to be non-dialysable, sensitive to digestion with trypsin (and therefore probably a protein), but surprisingly stable in both alkaline (pH 10) and acid (pH 2) conditions, and not antigenically related to the Influenza virus used to induce its formation (Isaacs & Lindenmann, 1957; Lindenmann, Burke & Isaacs, 1957). Since then, the field of Interferon has been the subject of many reviews, some of the more recent of which are : Wolstenholme and O'Connor, 1967; Rita, 1968; Vilcek, 1969; De Clercq and Merigan, 1970a,b; Colby, 1971; Colby and Morgan, 1971; Kleinschmidt, 1972; Grossberg, 1972; Ng and Vilcek, 1972; and Finter, 1973.

b) Interferon inducers.

For some time after the discovery of Interferon, viruses were thought to be the only substances capable of stimulating IF production (Isaacs, 1963). It was considered that the nucleic acid of the virus particle was responsible, and it was suggested that any 'foreign' nucleic acid would act as an inducer (Rotem, Cox & Isaacs, 1963; Isaacs, Cox & Rotem, 1963). This theory was later retracted (Isaacs, 1965), but had attempted to explain the varying activity of different nucleic acid preparations. Bacteria and bacterial endotoxins were described as IF-inducers in 1964 (Youngner & Stinebring, 1964; Stinebring & Youngner, 1964; Ho, 1964) and since then the list of active IF-inducers has grown rapidly. Table I is a list of the main types of inducers, with some specific examples of each. A study of the activity column shows that nearly all of the inducers fall into two groups, either active *in vivo* only, or active both *in vivo* and *in vitro*. The latter group includes all the IF-inducing viruses, synthetic ds-polyribonucleotides, ds-RNA and ds-RNA-containing extracts. Almost all other IF-inducers, both synthetic and natural, are only active *in vivo*, or in leukocyte cultures. The titres obtained with the second class of inducers are generally lower than those produced by viruses or ds-RNAs.

The polyanions are the most commonly studied group of IF-inducers, and here certain features can be recognised as requirements for IF-inducing ability (De Clercq *et al.*, 1970c; De Clercq, 1971). These authors concluded that stability, high MW. and high negative charge density were all essential

TABLE 1.

A REVIEW OF THE CLASSES OF INTERFERON-INDUCING AGENTS
(modified after De Clercq, 1971)

Inducer	Activity in vivo	Activity in vitro	Reference
1) Micro-organisms			
a) Viruses.			
i) Of mammals. DNA viruses of the papova-, herpes-, adeno- and pox-virus groups	+	+*	1,2
RNA viruses containing ss-RNA of picorna-, myxo- and arbovirus groups	+	+*	1
RNA viruses containing ds-RNA e.g. Reovirus	+	+	3
ii) Non-mammalian Mycophages, e.g. from P.stoloniferum (active agent ds-RNA)	+	+	4
Bacteriophage T_4	+		5
b) Chlamydia Trachoma	+	+	6,7
c) Rickettsiae e.g. Rickettsia tsutsugamushi		+	8
d) Bacteria e.g. Brucella abortus	+	-	9,10
Bordetella pertussis	+	-	11
e) Protozoa e.g. Plasmodium berghei	+	-	12
2) Microbial and Cell Extracts			
a) Viral Extracts. ds-RNA extracted from :			
i) Mammalian viruses, e.g. Reovirus	+	+	13
ii) Insect viruses e.g. Cytoplasmic Polyhedrosis	+	+	14
iii) Plant Viruses e.g. Rice Dwarf virus	+	+	14
iv) Fungal viruses of e.g. P.funiculosum	+	+	15
v) Bacteriophages e.g. Cells infected with MS2 and R17 coliphages	+	+	16,14

d) Bacterial Extracts			
e.g. E.coli endotoxin	+	✲	10
Diphteria and tetanus toxoids	-	✲	17,18
c) Fungal Extracts			
e.g. Statolon (P.stoloniferum) (active agent ds-RNA)	+	+	19
Helenine (P.funiculosum) (active agent ds-RNA)	+	+	20
Cycloheximide (Streptomyces griseus)	+	-	21
d) Plant Extracts			
Phytohaemagglutinin	-	✲	22,23
e) Mammalian Cell Extracts			
ds-RNA from rabbit liver		+	24
ds-RNA from rat liver		+	25
3) Synthetic Compounds			
a) Of high MW. (polyanions)			
i) Polycarboxylates			
e.g. pyran copolymer (maleic anhydride/divinyl ether)	+	-	26,27
polyacrylic acid	+	-	28
ii) ds-Polynucleotides	+	+	29
b) Of low MW.			
Tilorone hydrochloride	+	-	30,31
4) Immune Recognition Mechanisms			
a) Immune lymphocytes on exposure to the specific antigen	-	✲	32
b) Antilymphocyte serum	-	✲	33,34
c) Spontaneous IF production	-	✲	35,36,37

✲ Active in leukocyte cultures.

References.

1 Finter (1973)
2 Pusztai, Beladi, Bakay & Mucsi (1969)
3 Oie & Leh (1969)
4 Ellis & Kleinschmidt (1967)
5 Kleinschmidt et al. (1970)
6 Merigan & Hanna (1966)
7 Hanna, Merigan & Jawetz (1967)
8 Hopps, Kohno, Kohno & Smadel (1964)
9 Youngner & Stinebring (1964)
10 Stinebring & Youngner (1964)
11 Lukas & Hruskova (1967)
12 Huang, Schultz & Gordon (1968)
13 Tytell et al. (1967)
14 Nemes et al. (1969b)
15 Lampson et al. (1967)
16 Field et al. (1967b)
17 Green, Cooperband & Kibrick (1969)
18 Green, Cooperband, Kleinman & Kibrick (1970)
19 Kleinschmidt et al. (1964)
20 Rytel, Shope & Kilbourne (1966)
21 Youngner, Stinebring & Taube (1965)
22 Wheelock (1965)
23 Richmond (1969)
24 Billiau & Schonne (1970)
25 Harel & Montagnier (1970)
26 Regelson (1967)
27 Merigan (1967)
28 De Somer, De Clercq, Billiau, Schonne & Claesen (1968a,b)
29 For References see Section 3 f)
30 Krueger & Mayer (1970)
31 Mayer & Krueger (1970)
32 Glasgow (1966)
33 Edelman & Wheelock (1968)
34 Falcoff, Falcoff, Eyquem, Sanceau, Gatinot & De Vomecourt (1970)
35 Nagano, Kojima, Arakawa & Kanashiro (1966)
36 Smith & Wagner (1967)
37 Northrop & Deinhardt (1967)

prerequisites for activity, but that these factors alone were insufficient for IF-induction; as some polyanions which seemingly possess all the above characters, e.g. DNA-RNA hybrids, polygalacturonic, polyaspartic and polyglutamic acids, are either inactive or of very low activity.

As the ds-polyribonucleotides are by far the best known interferon inducers, and are probably those with the highest activity, the major interest will be concentrated on them in the following descriptions.

c) The induction of interferon.

In this section, present ideas about the production of IF are discussed. Again, the bulk of the work has been performed using viruses or synthetic ds-polyribonucleotides (usually poly(I).poly(C)) as the inducers.

Colby and Chamberlin (1969) postulated that the specificity of IF-induction i.e. the relatively restricted range of substances that will induce IF, was due to the interaction between a specific intracellular receptor, probably a protein, and the inducer molecule. The degree of induction is proportional to the amount of inducer-receptor complex formed. This hypothesis has been extended in a review by Colby and Morgan (1971), but there is no direct evidence for the existence of a receptor site protein, nor is there any information on how the proposed complex induces the synthesis of IF.

Evidence that IF synthesis was under direct genetic control was obtained by De Maeyer and De Maeyer-Guignard (1970) and De Maeyer, De Maeyer-Guignard and Bailey (pers. commun., 1971), who found that different strains of mice responded to a different extent to identical doses of the same inducers (poly(I).poly(C), Newcastle Disease virus, Friend Leukaemia virus and Sindbis virus). By back-crossing experiments they could show that high and low interferon responses were alleles of the same single genetic locus.

The genetic sites governing the production of IF and of the Antiviral Protein (AVP) (see section 4 d), the Action of Interferon) have been located, using monkey/mouse hybrid cell lines (Cassingena, Chany, Vignal, Estrade & Saurez, 1970; Cassingena, Chany, Vignal, Saurez, Estrade & Lazar, 1971). They found that the cistrons governing the production of IF and AVP were at different sites, possibly even on different chromosomes, in these hybrid cells.

A model for the genetic regulation of IF synthesis and action has been proposed by Kleinschmidt (1972), and is shown schematically in figure 1.

Kleinschmidt's model proposes that normally, the i gene of the IF cistron, and the i gene of the AVP cistron, operate to produce repressor proteins, which keep the production of IF and AVP, switched off. The synthesis of these repressor proteins is probably relatively slow. An IF-inducer derepresses the IF gene by combining directly or indirectly with IF repressor protein, which is bound to the operator locus of the IF operon. When the repressor protein is removed in this way, the IF gene can be transcribed to produce an m-RNA, which in turn is translated into a protein, IF. The AVP cistron is activated by the combination of IF with the AVP repressor protein, essentially as described above for the derepression of the IF operon.

In the model IF also serves another role, as well as producing antiviral resistance in the cell by inducing synthesis of AVP. The IF molecules are postulated to aggregate into oligomers (IF)x, and these aggregates are themselves capable of combining with the IF repressor protein. Instead of de-repression, however, this results in the repressor being made more efficient, and is termed a super-repressor. This model of IF induction fits many of the data obtained using metabolic inhibitors at various stages in the induction process. Basically similar models have have been put forward by Grossberg (1972) and Ng and Vilcek (1972).

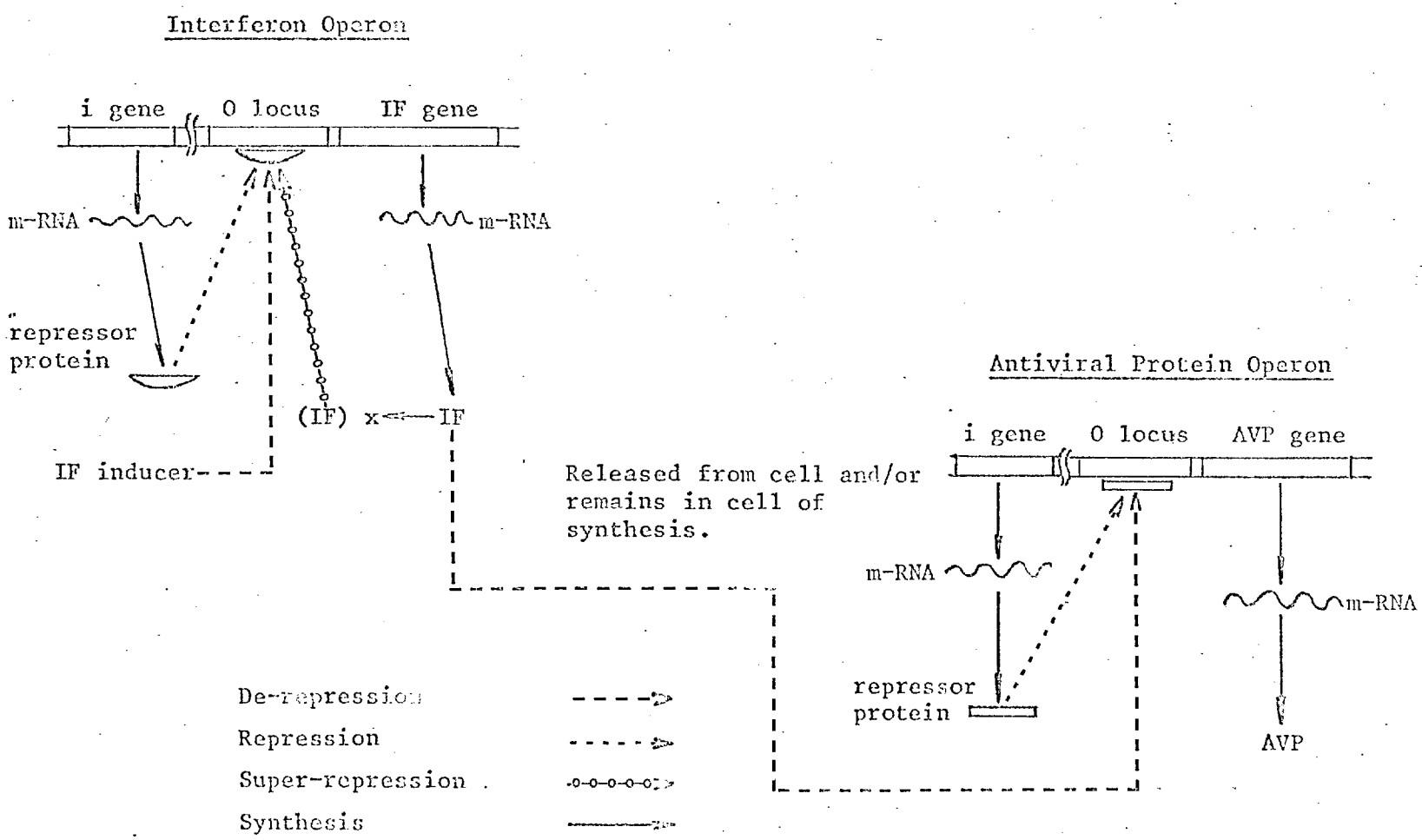
d) The action of interferon.

The work described here is, like that in the above section on IF-induction, fragmentory, and there is very little direct evidence for much of the postulated mechanism. Much of the evidence that exists is contradictory.

As IF action requires both RNA and protein synthesis (Wagner, 1961; Heller, 1963; Taylor, 1964; Lockart, 1964; Friedman & Sonnabend, 1964), it has been postulated that IF exerts its effect through an Antiviral Protein (AVP). There is no direct evidence for the existence of the AVP, although Cassingena et al. (1970,1971) have shown the existence of a genetic locus mediating the antiviral state in monkey/mouse hybrid cells. Mouse/human somatic cell hybrids have been used by Tan, Tischfield and Ruddle (1973) to locate the genes for human IF-induced AVP on chromosome G-21. AVP is thought to be synthesised in response to IF which has been produced either in the same cell, or in other cells, and it is AVP that prevents the

Figure 1.

Model for the Genetic Control of Interferon, after from Kleinschmidt (1972)



replication of infecting viruses. IF itself has no antiviral activity, either directly against viruses (Ho & Enders, 1959; Grossberg & Holland, 1962), or in IF-treated cells (Stewart & Lockart, 1970). The antiviral action occurs subsequent to the penetration and uncoating of the infecting virus, since IF treatment will protect cells against infectious viral RNA (Grossberg & Holland, 1962; De Somer, Prinzie, Denys & Schonne, 1962), and it is now thought that the synthesis of viral proteins is somehow blocked. Early evidence seemed to show that the translation of viral m-RNA into protein was blocked, possibly by some IF-mediated modification of the ribosomes (Marcus & Salb, 1966,1968; Joklik & Merigan, 1966; Carter & Levy, 1967,1968).

Kerr, Sonnabend and Martin (1970) could, however, find no evidence that the ribosomes were modified in cell-free protein synthesising extracts of IF-treated chick cells. Extracts from both treated and untreated cells were equally efficient in binding viral m-RNAs, and in incorporating amino acids in response to such m-RNAs, and also poly(U). A similar suggestion, that IF did not exert its effect via a modified ribosome, was made by Guggenheim, Friedman and Rabson (1969), in their work with human-chick heterokaryotic cells.

Since then results have been accumulated which make it seem more likely that IF exerts its activity at the level of transcription, rather than translation (Oxman & Levin, 1971; Marcus, Engelhardt, Hunt & Sekellick, 1971; Bially & Colby, 1972). However, conflicting results favouring translational control have been published (Metz & Esteban, 1972; Friedman, Esteban, Metz, Tovell, Kerr & Williamson, 1972). This contradiction between transcription and translation as the site of IF action, may be more apparent than real, for the interpretation of the results of many groups depends on assumptions as to the precise action of the metabolic inhibitors added to the systems, and on the assumption that these inhibitors do in fact completely block the critical step, and only that step. All groups used relatively impure IF preparations, which also makes for uncertain results. It is even possible that IF might act on both transcription and translation, with the degree of action on one or other step depending on the exact system under study.

e) Non-antiviral activities of interferon.

There is evidence that IF has general, as well as specifically antiviral effects. These include activity against tumours, both virus- and chemical-induced, and transformed cells both *in vivo* and *in vitro* (Orman, Rowe & Black, 1967; Wheelock & Larke, 1968; Gresser, Bourali, Lévy, Fontaine-Brouty-Boyé & Thomas, 1969; Gresser & Bourali, 1970; Gresser, Brouty-Boyé, Thomas & Macieira-Coelho, 1970; Wheelock, 1970; Pilch & Planterose, 1971; Salerno, Whitmire, Garcia & Huebner, 1972 reviewed by Gresser, 1972). The nature of this antitumour activity is unknown, but it may be due to inhibition of the growth of both normal and transformed cells (Gresser *et al.*, 1970; Gresser, Maury & Brouty-Boyé, 1972), to non-specific enhancement of macrophage activity and lymphocyte cytotoxicity (Lindahl-Magnusson, Leary & Gresser, 1971,1972), or to inhibition of a viral intermediate, such as an endogenous C-type virus, activated by chemical carcinogens (Whitmire, Salerno, Rabstein, Huebner & Turner, 1971).

Another activity of IF is the phenomenon of priming. By this is meant the enhancement of IF production on induction with poly(I).poly(C) or viruses after the cells have been pretreated with small amounts of IF (Isaacs & Burke, 1958; Rosztoczy & Mecs, 1970; Stewart *et al.*, 1971; Tovell & Cantell, 1971). The peak production of IF occurs sooner after induction in primed than in unprimed cells. Priming has been shown to increase the response of cells to a dose of poly(I).poly(C), not only in terms of increased IF production, but also in increased toxicity (Stewart *et al.*, 1972). The mechanism of priming is not understood.

The contrary action, called blocking, where a previous dose of interferon prevents or reduces subsequent interferon induction is also known (Lockart, 1963; Friedman, 1966; Golgher & Paucker, 1973). As this phenomenon usually requires larger initial doses of IF than priming, it is quite possible that both blocking and priming are manifestations of the same basic event. Blocking at least fits well with Kleinschmidt's model of IF-induction (1972), the initial dose of IF causes production of the repressor protein prior to the IF-induction by the later dose of inducer.

f) Structure of interferon.

As no pure preparation of IF from any source has yet been obtained (Kleinschmidt, 1972; Ng & Vilcek, 1972), knowledge of its structure is necessarily very vague. As mentioned above in section 4 a), because IF is inactivated by proteolytic enzymes, it is generally considered to be a protein, or to contain large amounts of protein in its molecule. The study of preparations of highly purified IF reveal a great heterogeneity of IF, in MW., electrical charge (isoelectric point determinations) and in stability to heat and acid (reviewed by Fantes, 1973). This heterogeneity of electrical charge is characteristic of glycoproteins (Spiro, 1969), and perhaps is related to the varying amounts of carbohydrate that were found associated with preparations of rabbit serum IF by Schonne, Billiau and De Somer (1970).

Recent evidence for the glycoprotein nature of rabbit IF has been obtained by Dorner, Scriba and Weil (1973). These authors showed that the rabbit IF molecule has at least one terminal sequence sialic acid-galactose-... and treatment with neuraminidase (removing the sialic acid) resulted in homogeneity of electrical charge, as also shown earlier by Schonne et al. (1970).

The very great differences in MW. of IF induced in the same animal species or cell strain by different inducers have resulted in suggestions that there are differing types of IF, released or induced in response to each inducer. (See Grossberg, 1972). However, Carter and his group have shown that it is possible to dissociate murine and human IF into active subunits, simply by reducing the ionic strength. The differing MW. species of IF could therefore simply represent differing degrees of association into oligomers (Carter, 1970,1971; Carter & Pitha, 1971).

The activity spectrum of IF is unusual, for it is host-, rather than virus-specific. This is generally referred to as species-specificity, although in fact there is a quite considerable degree of cross-activity between IFs from closely related species, e.g. human IF will protect some simian cell cultures and vice versa (Bucknall, 1967). Also a cross-reactivity has been noted between a number of avian species (Moehring & Stinebring, 1970) and it now seems that it would be better to refer to order-, rather than species-specificity (Grossberg, 1972). Even when this cross-reactivity is taken into account however, it remains that IF is most active in cells of the same type as those from which it was released

(Lockart, 1973). There is an exception to this; it has been found that an IF prepared from human foreskin fibroblasts by viral induction was much more active in rabbit kidney cells than in the human cells (Desmyter, Rawls & Melnick, 1970).

5. The Scope of this Thesis.

This thesis is a study of some structural aspects of the ds-RNA extracted from viruses of Penicillium chrysogenum, including the fragmentation of this ds-RNA by various techniques, and some of the biological activities of the fragments thus produced.

It forms part of a collaborative study between Beecham Research Laboratories, Imperial College, and the Medical Research Council, into fungal viruses and their potential uses as antiviral agents.

Methods have been developed for the production of large fragments of the ds-RNA by three techniques, and for the study of the fragments, with regard to size and secondary structure. The mechanisms of fragmentation have been examined, especially the mode of action of RNase III. The fragments produced have been studied with regard to their toxicity, the capacity to protect mice against viral infection, and the ability to induce IF in the serum of mice. The results obtained should aid the study of the structure of viral ds-RNA, and the understanding of the mechanisms of interferon induction and the toxicity of such double-stranded RNA.

MATERIALS AND METHODS

1. PcV-ds-RNA.

This was generally the gift of Beecham Research Laboratories Brockham Park, Betchworth, Surrey and was supplied as either a freeze-dried powder or an aqueous solution. The purification procedure was improved continually throughout the period of the work, so that each batch was prepared in a slightly different way. All batches contained no detectable DNA or protein, and only trace amounts of carbohydrate. No batches caused significant Shwartzman-type reactions. (All the above assays were by Beecham Research Laboratories).

Other samples of PcV-ds-RNA were very kindly supplied by Dr. K.W. Buck, of this department, and were prepared essentially as described by Buck et al. (1971) and Banks, Buck and Fleming (1971).

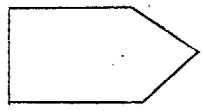
The concentration of PcV-ds-RNA solutions was determined by measurement of the A_{260}. It has been established (K.W. Buck, pers. commun.) that for PcV-ds-RNA, the A_{260} of a 1 mg/ml solution is 20, in a 1 cm pathlength cuvette.

2. Polynucleotide Kinase Labelling.

Polynucleotide kinase, prepared according to the techniques of Richardson (1965,1966,1971) was very kindly supplied by Dr. M. Szekely and T. Loviny. ATP-γ-^{32}P was purchased from the Radiochemical Centre, Amersham, and diluted with unlabelled ATP to an approximate specific activity of 6 μCi)/μ mole.

The phosphorylation mixture for the labelling of intact PcV-ds-RNA contained, in a volume of 10-20 μl of TMM buffer (0.01M Tris-HCl, pH 8.1, 0.01M $MgCl_2$ and 0.02M 2-mercaptoethanol) 25 μg of ds-RNA, 2 μl of polynucleotide kinase solution (a sufficient amount to transfer ten times the amount of ATP phosphorus under similar conditions) and 2 μl of ATP solution (4 mμ moles, 24 mμ Ci). This mixture was incubated in a capillary tube for 30 min at 37°C, and the reaction was then terminated by the addition of 0.01M ethylene diamine tetra-acetic acid (EDTA) and by freezing, which inactivates the enzyme. To remove the enzyme the reaction mixture was twice

extracted with water-saturated phenol, and the nucleic acid then precipitated with ethanol. Because of the possible contamination of the kinase preparation with a nuclease capable of cleaving the PcV-ds-RNA, and thus allowing labelling of the newly-produced 5'-termini, and because of the great excess of ATP used, it was necessary to purify the PcV-ds-RNA after kinase treatment. This was done by gel electrophoresis (see section 9 a) for details), the labelled ds-RNA being run on a 5 % polyacrylamide gel as described above, with untreated PcV-ds-RNA as a control. This process also removed the last traces of ATP, which was not entirely eliminated by the ethanol precipitation step. After electrophoresis, the gel was fixed with acetic acid and stained with toluidine blue and the position of the origin, the end of the gel, the bromophenol blue dye run as a marker and the intact PcV-ds-RNA (as shown by the control ds-RNA) were marked with radioactive ink. The gel was then wrapped in 'Saran' plastic film (Dow Chemical Co., from Transatlantic Plastics Ltd., Croydon, Surrey) and autoradiographed using a Kodak 'Kodirex' or 'Blue Brand' 35 x 43 cm X-ray film. Generally an overnight exposure was adequate at this stage, and the film was processed in Kodak D-76 developing solution. Using this film, it was quite simple to determine exactly the position of the labelled intact PcV-ds-RNA, and the appropriate portion of the gel was carefully cut out. The major band of radioactivity coincided exactly with the PcV-ds-RNA as identified from the stained gel, in every case. The ds-RNA was then extracted from the polyacrylamide gel by the technique of Adams, Jeppesen, Sanger and Barrell (1969). The small piece of polyacrylamide gel was first ground finely in a siliconised glass homogeniser, using a teflon pestle, in 0.04M Tris-acetate buffer, pH 8.2 (TA buffer). This paste was then placed in a glass tube closed at one end with a piece of DEAE-paper (Whatman DE 52, Reeve Angel & Co. Ltd., London) supported on a piece of dialysis membrane. After checking that this did not leak, the tube was filled up with TA buffer and clamped with the closed end a few millimeters below the surface of the liquid in a plastic trough containing the same buffer. Electrical contact was made from the top of the tube to a second trough of buffer by means of a bridge of glass tubing, and the nucleic acid was electrophoreted onto the DEAE-paper at 2000 V for 24 hr at 4°C. The DEAE paper was then very carefully removed (being very fragile when wet) and dried in a stream of air. The elution procedure is that described by Sanger, Brownlee and Barrell (1965). Using a monitor to check the radioactivity, the paper was carefully trimmed to an irregular pentagon shape, as shown below :



This piece of paper was washed twice in distilled water, and then, to facilitate the removal of the ds-RNA, treated with pancreatic RNase. (Smaller fragments are more readily eluted). This was done by immersing the DEAE paper in a small volume of 0.02M Tris-HCl, pH 7.2, containing 4 mg/ml bovine panc. RNase (code RAF-91B, Worthington, from Cambrian Chemicals Ltd., Croydon, Surrey) and incubating for 3-4 hr at 37°C. The paper was then removed, dried, washed twice with water to remove salts and dried again. All glassware that had been in contact with the panc. RNase was discarded.

The base of the DEAE paper was then held between two microscope slides, which were placed in a trough of distilled water. The liquid was drawn up to the piece of paper by capillary action, and then permeated along the DEAE paper. By placing a capillary tube at the very tip of the paper, water was drawn through. The paper was washed in this way for some time, each capillary being replaced when full. To reduce the radiation hazard, and also to speed the flow of liquid by reducing evaporation of the paper, the trough, paper, and capillary tubes were covered by a perspex box. The paper was then dried. The radioactive oligonucleotides were finally eluted from the DEAE paper by replacing the water in the trough with 30 % triethylamine carbonate in water, pH 10.0 (TEC); capillaries were collected until no more radioactivity was eluted from the paper (usually 2 or 3 capillaries, very approximately 100 µl).

The contents of the capillaries were pooled in a small siliconised tube, and the TEC removed by freeze-drying. To completely eliminate traces of TEC the residue on freeze-drying was redissolved in a little water and again freeze-dried. This procedure was repeated twice. The residue was then hydrolysed by dissolving in the minimum volume (5-10 µl) of 0.2N NaOH, which was then sealed in a capillary tube and incubated at 37°C overnight.

The components of the hydrolysate were resolved by high-voltage paper electrophoresis, which was performed essentially as described by Sanger et al. (1965).

The radioactive hydrolysates were spotted onto a 46 x 57 cm sheet of Whatman 3MM chromatography paper, and carefully dried. The sheet was then carefully wetted with pyridine-acetate buffer, pH 3.5 (5 % acetic acid, adjusted to pH 3.5 with pyridine, Sanger et al., 1965), and placed vertically in a tank of white spirit. Electrophoresis was at 2300-2700 V, 200 mA, for 2 to 4 hr, the progress of the run being followed by the progress of marker dyes along the edge of the paper (a mixture of 1 % Xylene cyanol F.F., 2 % Orange G and 1 % Acid Fuchsin; Sanger et al., 1965). The first pink spot (fuchsin) migrated at approximately the same speed as pUp, whilst the blue (cyanol) ran at about the same rate as pCp. Power was supplied by a Brandenburg model 501 power pack. After electrophoresis, the paper was dried, and the position of the origin, marker dyes etc. marked by radioactive ink. The paper was then autoradiographed, by placing in a lead-lined folder between two Kodak 'Kodirex' films. An exposure of a few days was allowed before the first film was developed, the development date of the second film being decided by the results of development of the first.

To allow identification of the radioactive nucleotides, known standard nucleotide diphosphates were prepared by Dr. M. Szekely, by polynucleotide kinase labelling of oligonucleotides of defined sequence, purchased from Miles-Seravac (Holyport, Maidenhead, Berks.). In each electrophoresis run, cytidine 3'-5'-diphosphate, adenosine 3'-5'-diphosphate, guanosine 3'-5'-diphosphate and uridine 3'-5'-diphosphate (pCp, pAp, pGp, pUp) were included.

Essentially the same procedure was followed for the labelling and analysis of the 5'-termini of RNase III-digested PcV-ds-RNA, except that the amount of ATP (specific activity 6 μCi/μ mole) was increased 10-fold, to compensate for the increased number of 5'-termini in the digested ds-RNA preparation. After phosphorylation, the sample was phenol-extracted and ethanol-precipitated exactly as described above. This was followed by gel electrophoresis, toluidine blue staining and autoradiography, all as previously described. The band pattern revealed by staining always corresponded well with that seen by autoradiography. Several (generally 8 per sample) of the more intensely radioactive bands were carefully cut out, and the ds-RNA fragments extracted onto DEAE paper and further processed exactly as for the intact PcV-ds-RNA.

3. Purification of RNase III.

The purification method of Robertson et al. (1968) was modified so as to be suitable for the preparation of large quantities of enzyme, sufficient to prepare digests for biological assays, where as much as several milligrams of digested ds-RNA were occasionally required.

The final technique developed involved the substitution of E.coli MRE 600 for the Su-26 strain, as the former strain is deficient in RNase I (Cammack & Wade, 1965), a possible contaminant of RNase III preparations.

A similar strain was used successfully for the isolation of RNase III by Schweitz and Ebel (1971). Robertson (pers. commun.) states that the enzyme purified from MRE 600 cells is more stable than RNase III purified from E.coli Su-26.

E.coli MRE 600 was grown for 7 hr at 34°C in a 60 litre stainless-steel fermenter in a medium containing 10 g/l tryptone digest, 1 g/l yeast extract, 1 g/l glucose and 8 g/l sodium chloride, pH 7.8 (Loeb & Zinder, 1961), until the cell density reached approximately 1.8×10^8 cells/ml, i.e. mid-log phase (Webster, Engelhardt, Zinder & Konigsberg, 1967). The medium was then chilled and the cells harvested by centrifugation at 13,200 x g in a Sharples No. 6 Super centrifuge. The cell paste was then weighed, and stored frozen at -20°C until use. From 60 litres of medium 600 - 800 g wet weight of cells was obtained. I thank Mr. G.T. Banks and the Pilot Plant Staff, Dept. of Biochemistry, for performing these fermentations.

The frozen cell paste was then broken up with a hammer into convenient pieces, and about 400 g rapidly weighed out. The cells were then thawed in an equal volume of ice-cold TM buffer (0.02M Tris-HCl, pH 7.6 + 0.01M magnesium acetate) containing 0.03M ammonium chloride, 0.006M 2-mercaptoethanol and 0.001M EDTA (Webster et al., 1967; Robertson et al., 1968). The cells were centrifuged at 8000 x g for 20 min at 4°C and then resuspended in an equal volume of cold buffer of the same composition as before. Disruption of the cells was achieved by two passages through an APV Manton-Gaulin 15M-8BA homogeniser, at 8000 pounds per square inch. The homogeniser was cooled prior to use, and between the two passages of the cell paste, by passing cold buffer through at low pressure. After disruption, the viscous homogenate was cooled in ice, and 5 µg/ml Deoxyribonuclease I (DNase, code DPFF, was obtained from Worthington) added. The mixture was then gently stirred at 4°C for 30 min, or until the viscosity, caused by the release of DNA from the cells on rupture, had

fallen sufficiently for ease of manipulation. Intact cells and cell debris were then removed by centrifugation, first at 8000 x g for 20 min, and then at 30,000 x g for 20 min. Both centrifugation steps were performed at 4°C.

Ribosomes were sedimented from this extract by centrifugation at 105,000 x g for 4 hr at 4°C in the Beckman Spinco 60-Ti rotor, and the supernatant discarded. The surface of each ribosome pellet was rinsed quickly with cold buffer, and the pellets gently resuspended in 300 ml cold TM buffer + 0.22M NH_4Cl. A teflon homogeniser was used in the resuspension, and care was taken to break up all of the pellet (which was very viscous), with a little buffer, before more buffer was added. The resuspension was carried out at 0°C.

RNase III is eluted from the ribosomes in TM buffer + 0.22M NH_4Cl (Robertson et al., 1968), and to complete this process the ribosomes were then removed by centrifugation at 105,000 x g as before, and discarded. Digestion with insolubilised RNase was used to reduce the fairly large quantities of RNA contaminating the enzyme preparation. (In Robertson et al.'s (1968) preparation this RNA was probably digested by the cellular RNase I, which is lacking in MRE-600 cells).

Enzite-RNase, code CM-AF/BR, pancreatic RNase bound to CM-cellulose, was obtained from Miles-Seravac, as a freeze-dried powder. It was reconstituted in TM buffer + 0.2M NH_4Cl; and washed twice with this buffer to remove the mannitol added to help reconstitution, and any unbound RNase. The crude RNase III solution was incubated at 37°C for 30 min with 1 mg/ml Enzite matrix-bound RNase, and then cooled to 4°C. Enzite, and also the protein precipitated during this incubation (Libonati, 1968a), was removed by centrifugation at 30,000 x g for 20 min.

Solid ammonium sulphate (22 g/100 ml) was then slowly added to the Enzite supernatant at 4°C, and the solution allowed to stand for 30 min to allow the precipitate to form completely. This precipitate was removed by centrifugation at 30,000 x g for 10 min, and further solid ammonium sulphate (14 g/100 ml) was added to the supernatant. The precipitate was allowed to form for 1 hr at 4°C, collected by centrifugation at 30,000 x g for 1 hr, and then dissolved in 10 ml TM buffer containing 0.2M NH_4Cl. The ammonium sulphate was removed by dialysis against 4 x 1 litre changes of TM buffer + 0.2M NH_4Cl, at 4°C. This dialysate was then stored in small batches at -20°C. Under these conditions the activity was generally quite stable, lasting without significant fall for at least 3 months.

To remove remaining DNase and other RNase activities, ion-exchange chromatography was used. Robertson et al. (1968) passed RNase III through a column of DEAE-Sephadex, to which the enzyme does not bind, which removed most of the RNase activity against single-stranded RNA, and then assayed the eluate from the column for RNase III activity. After passage through the DEAE-column, they found that the enzyme activity was very unstable. When their procedure was used by this author, large losses of activity were incurred. It was noted, however, that the RNase III activity eluted simultaneously with the major protein peak, as measured by the A_{280}. So, in order to avoid this loss, this absorbance peak was not assayed for enzyme activity, but instead was run directly onto the CM-Sephadex column.

The final procedure was then as follows : 2.2 ml of dialysate was thawed and diluted 1:10 with TM buffer containing no NH_4Cl, and loaded onto a 1.5 x 10 cm column of DEAE-Sephadex, (A-50, Pharmacia, Uppsala, Sweden) previously equilibrated with TM buffer + 0.02M NH_4Cl. The column was then washed with this buffer. The eluate was passed through an L.K.B. Uvicord II ultraviolet analyser, at 280 nm, and then onto a 1.5 x 6 cm column of CM-Sephadex (C-50, Pharmacia), previously equilibrated with the same buffer. When all the material absorbing at 280 nm had passed onto the CM-Sephadex column the two columns were separated, and the CM-Sephadex was washed with 2 column volumes of TM buffer + 0.02M NH_4Cl, containing 5 % sucrose to stabilise the enzyme (Robertson et al., 1968). The enzyme activity was then eluted with TM buffer + 0.2M NH_4Cl + 5 % sucrose. The fractions were assayed for activity, and the active fractions pooled and stored unfrozen at 0°C. The stability of different enzyme preparations varied considerably, some losing all activity in a few days, others remaining active for several months. This is probably a protein effect (Robertson, pers. commun.).

The results of a typical purification are shown in Table 2.

4. RNase III Assay.

Activity was assayed using the synthetic polynucleotides poly(A-U-^{3}H) and poly(G).poly(C-^{3}H) as substrates. These preparations were the kind gift of Dr. H.D. Robertson, and were separated from low molecular weight fragments and single-stranded polynucleotide by the technique of Franklin (1966), as follows :

TABLE 2.

RESULTS OF A TYPICAL RNase III PURIFICATION

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Purification factor (relative to S-30 extract)
S-30 extract	503,000	14,100	35.7	1
Ribosomes	460,000	5,000	92	2.6
0.2M NH_4Cl supernatant	240,000	1,400	171	4.8
Dialysate	120,000	91	1319	37.0
Following ion-exchange chromatography	48,700	8.3	5867	164.0

375 g of E.coli MRE 600 cells were disrupted, and the RNase III activity purified as described in the Methods, section 3. RNase III activity was assayed using poly(G).poly(C) as substrate, and protein was determined by the method of Lowry et al. (1952) (Methods, section 4. and 5.).

The polynucleotides were dialysed into STE buffer (0.1M sodium chloride, 0.001M EDTA, 0.05M Tris-HCl, pH 6.9) + 35 % ethanol, and loaded onto a 1 x 10 cm column of cellulose (Whatman CF 11 fibrous cellulose, from Whatman Biochemical Co., Maidstone, Kent) previously washed and equilibrated with this buffer. Small oligonucleotides were removed by washing with 3 column volumes of STE buffer + 35 % ethanol, and single-stranded polynucleotides eluted with 3 column volumes of STE buffer + 15 % ethanol. Finally, the column was eluted with STE buffer alone, and 1 ml fractions collected. All this was done at 4°C. 50 µl of each fraction was then removed, and the radioactivity determined by scintillation counting as described below. The fractions containing the ds-polynucleotide were pooled, reduced in volume by lyophilisation, and finally dialysed against several changes to TM buffer + 0.2M NH_4Cl. The standard reaction mixture for assaying RNase III contained, in a final volume of 0.1-0.5 ml of TM buffer + 0.2M NH_4Cl, substrate to very roughly 30 mµ moles of polymer phosphorus/ml, and enzyme. This was incubated at 35°C for 1 hr, and the reaction was terminated by mixing with an equal volume of ice-cold 10 % trichloracetic acid (TCA) in water. Precipitation was aided by the addition to each reaction tube of 0.2 mg bovine serum albumin (Fraction V, Sigma Chemical Company, London), or 0.2 mg of E.coli ribosomal RNA, and the precipitate allowed to stand for 10 min on ice. The precipitates were then collected on Whatman GF/A glass-fibre discs, diameter 25 mm, using a filtration apparatus capable of dealing with 10 filter discs simultaneously, designed by Dr. H.J. Gould and constructed in this department. The reaction tubes and filter discs were washed with several changes of ice-cold 5 % TCA, twice with ice-cold water, and the discs dried under an infra-red lamp.

Radioactivity was determined in a Beckman LS-200B Liquid Scintillation System, using a toluene based scintillation fluid (Scales, 1967) containing 50 g/litre naphthalene and 6 g/litre Butyl PBD (CIBA (A.R.L.) Ltd., Cambridge).

One unit of RNase III activity is defined (Robertson et al., 1968) as that amount of enzyme which will solubilise 1 mµ mole of TCA-precipitable polymer phosphorus per hour under the assay conditions described above.

For the counting of aqueous samples, 2-methoxyethanol was added until the aqueous phase passed into solution.

5. Other Assay Methods.

a) Protein.

Accurate final determinations were made by the method of Lowry, Rosebrough, Farr and Randall (1951), using Folin & Ciocalteu's reagent purchased from B.D.H. Ltd., Poole, Dorset. A calibration curve was prepared using Bovine Serum Albumin, Fraction V.

b) Activity against ss-RNA.

This was assayed using E.coli r-RNA as a substrate. The enzyme preparation was added to a solution of E.coli r-RNA (1 mg/ml) in TM buffer + 0.2M NH_4Cl, final volume 0.5 ml, and this was then incubated for 1 hr at 35°C. The reaction was stopped, and the undigested RNA precipitated, by the addition of an equal volume of ice-cold 12.5 % (v/v) perchloric acid (PCA). The precipitate was allowed to form fully by standing on ice for 15 min, and was then spun down at 30,000 x g for 20 min. The A_{260} of the supernatant was determined in a Unicam SP 500 spectrophotometer, using as a blank a sample to which the enzyme was added after the addition of PCA.

6. Partial RNase III Digestion.

The reaction mixture contained, in TM buffer + 0.2M NH_4Cl, PcV-ds-RNA, final concentration 1 mg/ml, and RNase III (700 U/ml). The reaction volume was generally between 1 and 10 ml. After 1 hr incubation at 36°C, the digestion was terminated by the addition of 1 % sodium dodecyl sulphate (SDS) (B.D.H. Ltd.) and the mixture then deproteinised by extraction with water-saturated phenol. The reaction mixture plus an equal volume of phenol was shaken on a Whirlimixer vortex mixer (Fisons Scientific Apparatus Ltd.) for 5-15 min, depending on the volume of solution. The layers were then separated by brief centrifugation, and the aqueous (top) layer pipetted off. The phenol layer was then back-extracted with an equal volume of water as before. The two aqueous layers were pooled, and a second phenol extraction performed. The aqueous layer from this extraction was then extracted 3 times with 3 volumes of diethyl ether to remove remaining phenol, residual ether being removed by a stream of air. The solution was then made to 0.2M with sodium acetate, and the RNA fragments precipitated by the addition of 2.5 vols. of absolute ethanol. This was stored at -20°C overnight to allow the precipitate to

form fully, and the precipitate was then centrifuged down. The precipitate was washed with absolute ethanol, spun down again and allowed to dry for a few hours at 4°C. Finally the RNA was dissolved and dialysed into phosphate buffered saline (PBS) (Oxoid Ltd., London). (8.0 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, pH 7.3, Dulbecco & Vogt, 1954).

7. Partial Pancreatic RNase Digestion.

The digestion mixture contained in 0.15 M sodium chloride, 0.015M sodium citrate, pH 7.2 (SSC), ds-RNA at a final concentration of 1 mg/ml, and between 25 and 125 µg/ml pancreatic RNase (depending on the batch of ds-RNA). The reaction volume was generally between 1 and 10 ml. This was incubated for 1 hr at 36°C, and then the reaction was stopped by the addition of 20 µl/ml diethyl pyrocarbonate (DEP) (Solymosy, Fedorcsak, Gulyas, Farkas & Ehrenburg, 1968). The RNase was then removed by phenol extraction, and the RNA ethanol-precipitated as described for the preparation of RNase III digests.

8. Ultrasonic Irradiation.

PcV-ds-RNA was dissolved in SSC or PBS, and cooled to 0°C. The nucleic acid concentration was usually 1 mg/ml, and 1.5 ml volumes were irradiated in a bijou bottle; larger volumes in an appropriate sized beaker. This was placed in a dish containing an ice-water slurry, to cool the sample during irradiation. The titanium probe (tip diameter 9 mm) of the sonicator (M.S.E. 100 watt Ultrasonic Disintegrator, Measuring and Scientific Equipment Co. Ltd., Crawley, Sussex) was placed about 2 mm below the liquid surface and the oscillator switched on. The frequency of irradiation was 20 KHz, and the instrument was tuned to maximum transducer amplitude (usually 7-8 µm peak to peak). After appropriate irradiation e.g. 200 sec for a 3 ml sample, the transducer was switched off.

The efficiency of cooling had been previously tested by irradiating a volume of water in which was inserted a copper-constantan thermocouple. This was connected to a chart recorder (Kent Mark 3 Electronic, 10 mV full scale deflection). This set-up itself had previously been standardised using a water bath and thermometer, the latter calibrated by the National Physical Laboratory, Teddington. It was found that during 5 min irradiation with the sample cooled as described, the liquid temperature did not rise

above 20°C. For prolonged irradiations, the sample was irradiated in 5 min bursts, with a 10 min cooling period between each.

After irradiation, the sample was centrifuged to remove the small flakes of metal thrown off by the probe, and then dialysed. For irradiation of volumes larger than 10 ml, a larger size probe was used (tip diameter 19 mm). This heated a 25 ml sample to 20°C in 3 minutes, and was thus used in 2 min bursts, with 10 min cooling periods.

9. Gel Electrophoresis.

a) Polyacrylamide gels.

These were prepared as follows, according to the method of Adams et al. (1969), with some small amendments. Six grams of acrylamide and 0.2 g of methylene-bis-acrylamide (both from B.D.H. Ltd.), were dissolved in 120 ml TAE buffer (0.04M Tris-acetate, pH 8.2, 0.01M EDTA), i.e. a final polyacrylamide concentration of 5 % with 1:30 cross linking. This was de-aerated under vacuum for a few minutes, and then 0.3 ml of freshly prepared 10 % ammonium persulphate and 0.3 ml of N,N,N',N',-tetramethyl-ethylenediamine (TEMED) (B.D.H. Ltd.) added. After gentle swirling to mix these in, taking care not to reintroduce air (which inhibits polymerisation) into the mixture, the solution was poured into a mould and allowed to polymerise.

The mould was composed of two 20 x 20 cm glass plates, separated by 2 strips of vaselined perspex so that the inside walls were 3 mm apart. The bottom of the mould was sealed by a strip of Sellotape clear pressure-sensitive tape, and by forcing the mould into a strip of Plasticine modelling clay. Slots were made in the top of the gel by inserting a further strip of perspex bearing 6 or 7 ridges about 5 mm high and 1 mm wide and 15 mm long.

After the acrylamide had polymerised (detected by the evolution of heat and by a change in the appearance of the meniscus, generally between 5 and 10 min after the addition of persulphate and TEMED) the slot former was removed, the whole removed from the Plasticine, and the sellotape peeled off. This tape sealing also had the advantage of producing a flat bottom to the gel, flush with the bottom of the glass plates. This made it very easy to avoid uneven current flow caused by air bubbles beneath the gel.

The gel was clamped vertically in a plastic trough containing TAE buffer and with a platinum wire serving as the anode. Connection to the cathode, another platinum wire in a similar plastic trough containing TAE buffer, was made by means of a double thickness of Whatman 3MM filter paper. A pre-run to remove persulphate was then performed at 18-20 mA, 200 V, for 2 hr, power being supplied by a Shandon VoKam power pack (Shandon Southern Instruments Ltd., Camberley, Surrey). The buffers were then discarded and replaced by fresh, and the samples loaded on. This was done by mixing each sample with a small amount of concentrated sucrose solution containing bromophenol blue dye. This mixture was then very carefully layered into the bottom of each slot under the buffer, using a glass capillary tube, and the wick then carefully replaced. Sample volume was generally between 10 and 50 µl. Electrophoresis was then carried out for 16-18 hr at 200 V, 18-20 mA, in the cold. This brought the bromophenol blue marker almost to the bottom of the gel, and intact PcV-ds-RNA about 1 cm into the gel.

After electrophoresis, the gel was separated from the glass plates, fixed for 10 min in 1N acetic acid, and then stained for 2 hr in toluidine blue (0.2 % toluidine blue, 0.4M sodium acetate-acetic acid buffer, pH 4.7). Excess stain was removed by washing gently with tap water for several hours. These gels were then stored at 4°C until photographed.

b) Agarose-acrylamide gels.

These were prepared in the same moulds as the gels described above. Agarose (B.D.H. Ltd.) (0.6 g) was mixed with 60 ml TAE buffer, and melted by heating on a boiling water bath. It was then cooled to 45°C, and mixed with the remaining 60 ml of TAE buffer, containing 2.40 g acrylamide and 0.12 g bis-acrylamide (i.e. a final 2 % polyacrylamide, 1:20 cross linking). This latter had already been de-aerated and warmed to 45°C. To this mixture were added 0.3 ml 10 % ammonium sulphate and 0.3 ml TEMED as before, and the whole poured into a mould. Loading etc. was exactly as described for the previous gels.

c) Formamide gels.

The method of Staynov, Pinder and Gratzer (1972) was used for this, as modified by J.C. Pinder (pers. commun.).

Two grams of Amberlite Monobed Resin MB-1 (B.D.H. Ltd.) was dried in vacuo, and then added to 60 ml of formamide. The suspension was stirred for about 3 hr to de-ionise the formamide. The resin was then filtered off

and the de-ionised formamide stored in the dark. Each batch was used within 3 or 4 days, and not stored any longer than this.

Four per cent gels were prepared as follows. In 20 ml of de-ionised formamide was dissoved 0.85 g acrylamide, 0.15 g bis-acrylamide, 0.104 g sodium barbiturate, and 0.06 ml TEMED. This solution was adjusted to an apparent pH of 9.0 on a pH meter, using concentrated HCl, and 0.2 ml of 18 % fresh aqueous ammonium persulphate added. The volume was then made up to 25 ml and, de-aeration being unnecessary, poured into 100 mm x 7 mm plexiglass tubes. The bottoms of these tubes had been previously sealed with parafilm. Over the gel solution in each tube was then layered, with extreme caution, water from a syringe, and the gels were allowed to polymerise (30-60 min). As the overlaying was difficult, and often did not result in a flat top to the gel, the polymerised gels were inverted and very gently pushed by air pressure down to the other end of the plexiglass tube. The gels were prevented from slipping out of the tubes by a piece of fine nylon gauze tied over the end of each tube. Each tube was placed in a Shandon Disc Electrophoresis apparatus, and overlaid with buffered formamide. (20 ml de-ionised formamide, 0.104 g sodium barbiturate, adjusted to pH 9.0, and diluted to 25 ml). The upper and lower buffer compartments contained 0.02M NaCl, which was circulated between the compartments to avoid a drift in pH in the gels. Samples for electrophoresis were dialysed into water, lyophilised, and dissolved (1 mg/ml) in buffered formamide containing 5 % sucrose and a trace of bromophenol blue. Ten microlitres of the sample was applied to the gel, and the overlay of formamide increased to 1 cm.

The gels were run at 5 mA/tube for about 2 hr, until the bromophenol blue was near the bottom of the gel. Gels were fixed and stained as described above.

10. Sucrose Gradient Centrifugation.

Five millilitres 5-20 % (w/w) linear sucrose gradients were prepared in 0.05M sodium acetate buffer, pH 5.2, containing 0.01M EDTA. The sucrose solutions had previously been treated overnight at 4°C with 5 µl/ml DEP, to inactivate any nucleases (Solymosy et al., 1968). To remove CO_2 produced by the breakdown of the DEP, the sucrose solutions were evacuated on a water pump, and then cooled to 4°C before the gradients were prepared, using a Buchler Gradient Mixer. (Buchler Instruments, Fort Lee, New Jersey, U.S.A.).

The prepared gradients, in 2" x 0.5" cellulose nitrate tubes, were allowed to stand at 4°C for a few hours, to allow any irregularities in the gradient to smooth themselves out. Twenty microgram samples of RNA were generally used, and were loaded onto the gradient in as small a volume as possible, generally 10-25 µl.

The gradients were centrifuged for 16-18 hr at 25,000 rpm at 4°C in an SW 50.1 rotor in a Beckman Spinco L2 65B centrifuge.

E.coli r-RNA (kindly supplied by T. Loviny) was run on one gradient in each experiment as S value standards. Under the run conditions described, the 4-5 S, 16 S and 23 S components were well resolved. After centrifugation, the gradients were analysed using an Isco Model D Density Gradient Fractionator, Model UA Optical Unit, which measures absorption at 254 nm, and a model 222 Ultraviolet Analyser (Instrument Specialities Company, Omaha, Nebr., U.S.A.) with a 1 cm optical pathlength flow cell. This apparatus displaces the gradient through the flow cell by pumping a very dense sucrose solution into the bottom of the centrifuge tube. Displacement of the gradients by use of a dense sucrose solution has been claimed to give improved resolution and less peak distortion than fractionation by tube puncture (McGrath & Williams, 1966; van der Schans, Aten & Blok, 1969).

11. Viscosimetry.

To follow the progress of an enzymic digestion of PcV-ds-RNA as it occurred, the digestion mixture was placed in a modified Ubbelohde Viscosimeter (minimum sample volume 3.5 ml, volume of flow 2.5 ml, flow time for water at 36°C of 56.2 sec). The viscosimeter was clamped in a thermostatically controlled water bath, previously adjusted to the desired temperature, and the flow time of the digest measured from time to time. For more accurate determination of the intrinsic viscosity $[\eta]$ of intact and fragmented PcV-ds-RNA, the measurements were made at 25°C (when room temperature was sufficiently low) or 30°C; the flow time was measured five times at each dilution. The variation in repeated determinations was less than 0.3 sec. Prior to measurement the nucleic acid samples were centrifuged at 2000 x g for a few minutes, to remove any dust particles. Solvents were filtered through 'Millipore' HAWP Filters, pore size 0.45 µm.

12. Formaldehyde Denaturation.

This was, as mentioned in the Results, section 1 a), and in the footnotes to fig. 2, derived from the method described by Freifelder and Davison (1963). A 40 % solution of 'Analar' grade formaldehyde in water ('Formalin', B.D.H. Ltd.) was neutralised with 0.1N NaOH, and was used the same day. The ds-RNA solution, 1 mg/ml in 0.1M phosphate buffer, pH 7.8, was heated to 80°C in a screw top vial, and then neutralised formaldehyde was added to a final concentration of 1.8 %. After reaction for 8 min at 80°C, the sample was rapidly cooled in a slurry of water and ice. Formaldehyde was removed by dialysis.

13. Thermal Denaturation Curves.

RNA was dialysed against three changes of 0.1 x SSC and then diluted to a concentration of roughly 25 µg/ml with 0.1 x SSC. The solution was degassed for a few minutes on a water pump, and then 2.5 ml of the solution was placed in a stoppered silica cuvette. This was weighed and then placed in the cuvette holder of a Beckman Tm Analyser, in the sample chamber of a Beckman DK2A Ratio Recording Spectrophotometer. A similar cuvette, containing 0.1 x SSC was used as a blank. The absorbance at 260 nm was determined at 25°C, and then the temperature of the cuvette holder was increased by 10 and 15°C steps, allowing 10 minutes after first reaching the desired temperature to achieve equilibration. At each temperature the A_{260} was determined. From 60°C, the temperature was increased to 65°C, and then to 70°C, allowing for equilibration before determining the absorption as before. From 70°C-100°C, the automatic programmer was used, taking 120 min for the 30°C rise, a rate of 0.25°C/min. Dove and Davidson (1962) found that the maximum rate of increase that gave good results in the thermal denaturation of DNA was 0.5°C/min. During the temperature rise, the A_{260} was recorded continuously using the Time-drive attachment of the spectrophotometer.

After reaching 100°C, the sample, still sealed in the cuvette holder was allowed to cool to room temperature, and was then removed and reweighed to check for solvent loss. Generally this was less than 1 %, and was ignored. If the loss was greater, the determination was repeated with a fresh sample of ds-RNA. Assuming that the temperature rise was linear with time, the absorption readings were then corrected for the thermal expansion of water, taking 25°C as a baseline, and then plotted in the form A_t/A_{25} against temperature, t. The ratio A_t/A_{25} is the hyperchromicity,

h. The midpoint of the transition, the Tm, was defined by Dove and Davidson (1962) as the temperature for which $h = 0.5(h_{max} + 1)$.

To test the linearity of the automatic temperature programme, from time to time the apparatus was tested as follows. Two cuvettes were filled with water and placed in the cuvette holder. Into one was placed the end of a copper-constantan thermocouple, whilst into the cuvette in the blank position was placed the platinum resistance thermometer of the Tm Analyser as usual. The thermocouple output was connected to a chart recorder, as described previously in section 8. A standard programme was then run on the Tm analyser, to yield a graph of temperature versus time. It was found that the settings on the dial of the Tm analyser apparatus showed an apparent temperature 4°C below the value established in this way, and all results were appropriately corrected.

14. Biological Activity Assays.

Toxicity and antiviral activity assays were performed by the staff of Beecham Research Laboratories, using mice of strain CD-1. I am grateful to them for allowing me to include here the results they obtained.

Assays of interferon-inducing activity were performed in BSVS mice, by Dr. F. Himmelweit of the Department of Biochemistry. Samples for assay were dissolved in PBS at 375 or 400 µg/ml. Eighty microgram samples were injected into the tail vein of 20 g (± 1 g) BSVS mice (i.e. a dose of 4 mg/kg). Generally 10 mice were used for each sample. After 8 or 10 hr, the mice were killed, bled from the heart, and the pooled sera were assayed for interferon using a modified dye uptake method (F. Himmelweit, unpublished) after Finter (1969). Fifty percent end-points were determined by probit analysis (Lindemann & Gifford, 1963).

RESULTS

Studies have been made on the intact PcV-ds-RNA, of the processes involved in the fragmentation of this ds-RNA by Ribonuclease III, bovine pancreatic RNase and ultrasonic irradiation. The double-stranded fragments produced by these three digestion methods have been compared to the intact ds-RNA for their capacity to induce IF, their antiviral activity and acute toxicity.

1. Intact PcV-ds-RNA.

As reviewed in the Introduction, section 2 d), little is known about the fine structure of this RNA, or the ds-RNA from any other fungal virus. It is known that PcV-ds-RNA is in overall terms double-stranded, but it is not known if the ds-RNA has nicks in the individual strands, or longer discontinuities in the base-pairing, and experiments have been done to test this possibility. These experiments also incidentally determined whether the double-stranded molecule results from base-pairing between two complementary chains or from hairpin formation by a long self-complementary single strand.

a) Single-strand nicks.

Also included under this heading are longer discontinuities in base-pairing. These would not be detected by digestion with panc. RNase if the ss region were composed largely of purine residues. The inactivity of RNase T_1 against this ds-RNA (M. Szekely & C. Dreyer, pers. commun.) shows that such postulated polypurine as regions would in fact have to be polyadenine.

Possible nicks etc. could not be detected by determination of the viscosity of the ds-RNA, and comparison of the MW. that could be estimated from this with the value determined by other means, to reveal whether the ds-RNA was more flexible than expected, due to the nicks. This technique is of no use for two reasons. Firstly, Hays and Zimm (1970) have shown that for DNA, a small number of nicks will have no effect on the flexibility of the molecule; and secondly, as mentioned in the Introduction, Franklin (1967) has shown that the flexibility expected of a ds-RNA molecule is already quite great compared with the flexibility of DNA. This means that

equations for the determination of MW. estimates from intrinsic viscosity measurements will give low results.

So, the basic technique used to detect ss nicks involved the denaturation of the ds-RNA molecule, and measurement of the MW. and size distribution of the resultant single strands.

If the ds-RNA was not nicked, then denaturation of each molecule would yield two single strands of half the parent MW. (Assuming that the ds-molecule in fact consists of two paired strands. If this is not the case, if, for example the molecule was formed of one large hairpin loop, then the ss-RNA produced by denaturation would have the same MW. as the parent ds-molecule). If the original ds-RNA is heterogeneous in size, either consisting of distinct size species (as PcV-ds-RNA) or a continuous size range, then the MW. distributions of intact and denatured RNA will be the same if the parent molecule is unnicked, as molecules of all sizes will have been denatured. This would be the case if PcV-ds-RNA was unnicked. Nicked ds-RNA will produce on denaturation smaller-than-predicted (1/2 parental MW.) fragments, with a polydisperse size distribution curve if the nicks are random. If nicks occur at specific points, then denaturation will yield specific fragments. This technique has been applied to the study of DNA by several groups of workers, including Davison, Freifelder and Holloway (1964), Studier (1965), Hagen (1967), and Gaudin and Yielding (1972).

As simple heat denaturation can cause hydrolytic cleavage of ds-RNA (Katz & Penman, 1966), and as denatured ds-RNA tends to renature very easily and also form non-specific aggregates on the removal of the denaturing agent, a gentle and permanent method of denaturation was required. For this, denaturation and reaction with formaldehyde was used. This has been fairly well studied in the case of ds-DNA (Haselkorn & Doty, 1961; Grossman, Lewin & Allison, 1961; Freifelder & Davison, 1963; and reviews by Grossman, 1968, and Kochetkov & Budowsky, 1969) and it has been shown that under appropriate conditions formaldehyde denatures the double-helix, and then reacts to form hydroxymethyl groups with the amino groups on the newly exposed purine and pyrimidine bases (Haselkorn & Doty, 1961). Phosphodiester backbone cleavage does not occur provided the conditions are not too extreme (Haselkorn & Doty, 1961; Freifelder & Davison, 1963). By virtue of the hydroxymethylation of the amino groups, the re-formation of hydrogen bonds on the removal of the denaturing conditions is prevented. However, two hydroxymethyl groups may sometimes react to form an inter-

strand cross-link, and this reaction is irreversible. Freifelder and Davison (1963) developed conditions for the denaturation of DNA which resulted in minimal hydrolysis and interstrand link formation. These however, are not directly applicable to ds-RNA, as this latter is known to have a greater resistance to denaturation than a DNA molecule of equivalent base composition (Chamberlin, 1965; Chamberlin & Patterson, 1965). So, the effects of variation of the time and temperature of the denaturation were ascertained. The results are shown in fig. 2, which is a plot of the relative A_{260} of the RNA sample against the time of incubation, at various temperatures. The pH, ionic strength and the concentration of formaldehyde used were as described by Freifelder and Davison (1963). Complete denaturation is indicated by the achievement of the maximal hyperchromic effect. In all further studies involving formaldehyde denaturation, the ds-RNA sample was heated under the conditions described in the Methods (section 12), for 7.5-8 min at 80°C, followed by rapid cooling.

The products of denaturation were then examined by sucrose gradient centrifugation, which showed clearly the size distribution of the intact and denatured PcV-ds-RNA, whilst specific fragments were checked for by gel electrophoresis.

On sucrose gradients, the size of the denatured products was estimated using unreacted E.coli ribosomal RNA as standards for the determination of approximate S values. This r-RNA has components with S values of 23 S, 16 S and 5 S, and was also contaminated with 4 S transfer RNA. The 4 S and 5 S peaks were not resolved on the gradients used, and this peak was described as 4-5 S. The results of a typical gradient analysis of intact and formaldehyde-denatured (ds- and fd-RNA respectively) ds-RNA are shown in fig. 3; the positions of the r-RNA peaks are marked by arrows. Plotting these r-RNA S values against the distance travelled down the gradient, it is possible to determine approximate S values for ds- and fd-RNA. The sedimentation coefficient of the intact ds-RNA was estimated by this method as 12.9 S, in good agreement with the value of 13 S published by Buck and his coworkers (1971). The molecular weight calculated from this S value using the Eigner and Doty (1965) relationship:

$$S = 0.116M^{0.325}$$

is 1.98×10^6 daltons. This value is in good agreement with estimates of the MW. of PcV-ds-RNA derived from gel electrophoresis results (Border et al., 1972; Wood & Bozarth, 1972). On

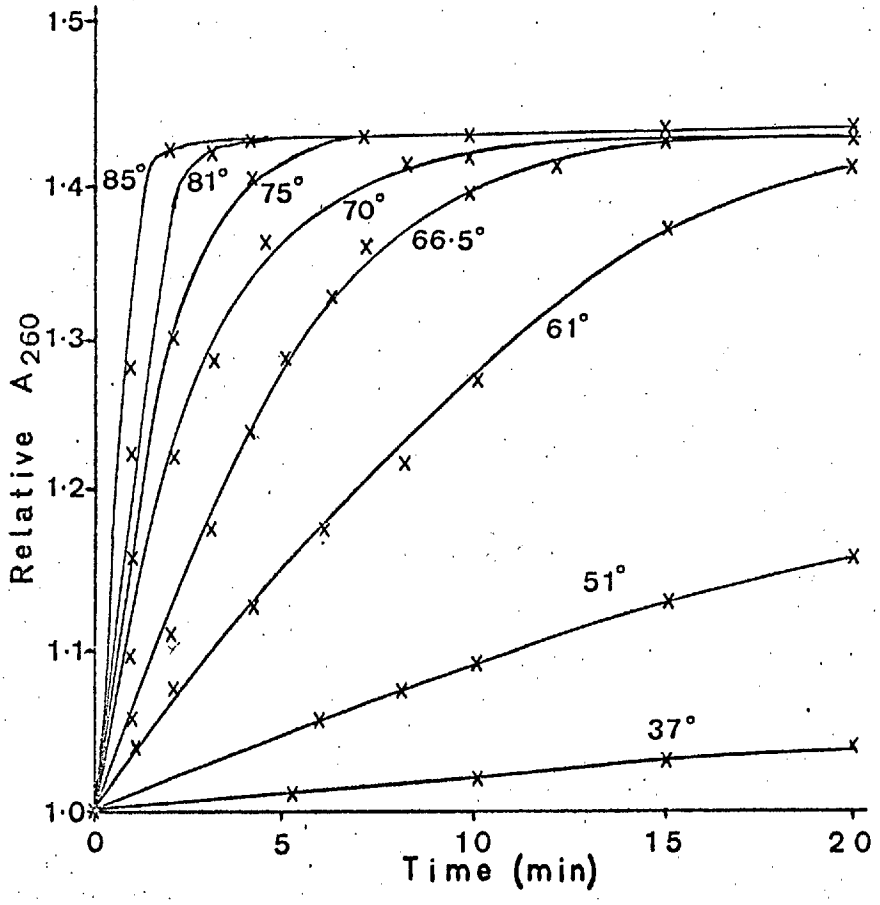


Figure 2 : Rate of Reaction of PcV-ds-RNA with Formaldehyde at Different Temperatures.

RNA batch 5/119 was dissolved in 0.1M phosphate buffer, pH 7.8, containing 1.8 % neutralised formaldehyde, at a concentration of 1 mg/ml. Samples were heated for various times at the temperatures (°C) above and then rapidly cooled in ice-water. Each samples was then diluted 1:40 with PBS (i.e. to a final concentration of 25 µg/ml) and the A_{260} determined.

denaturation, the S value increased to 19.6 S. Calculation of a molecular weight estimate from this is not easy, as reaction with formaldehyde should destroy all secondary structure resulting from hydrogen-bonding interactions, although as base-stacking is unimpaired after formaldehyde reaction (Stevens & Rosenfeld, 1966) some secondary structure will remain. Therefore, it is not correct to use the equations of Gierer (1958) or Spirin (1961), both developed for the relationship of S value with the molecular weight of native ss-RNA. Perhaps the best analogy for fd-RNA is ss-DNA following denaturation with alkali. In media of a high pH, the formation of hydrogen bonds is inhibited, due to the de-protonisation of the bases, which means that the secondary structure of the ss-DNA is not affected by hydrogen bonding. Studier (1965) found that above pH 11, denatured DNA resembled in hydrodynamic properties the behaviour of formaldehyde-reacted DNA at more nearly neutral pH levels. The increase in molecular weight of the fd-RNA due to the added hydroxymethyl groups is so small in comparison to the molecular weight that it can safely be discounted. Studier (1965) developed an empirical S value-molecular weight relationship for ss-DNA under alkaline denaturing conditions :

$$S = 0.053M^{0.4}$$

When this was used to calculate the molecular weight of fd-RNA from the sedimentation coefficient of 19.6 S, a result of 0.9×10^6 daltons was obtained. This is slightly less than half the molecular weight established for ds-RNA. More useful than the consideration of molecular weight ratios, with its inherent uncertainty due to the use of equations of unproven validity, is the comparison of the shapes of the profiles of ds- and fd-RNA. Figure 3 shows that the peak for fd-RNA is no wider than that for the PcV-ds-RNA, and that they are very similar in shape, as would be expected if the distribution of molecular weights was the same in both cases. The possibility that the ds-RNA consists of one intact single strand hydrogen-bonded to a second, heavily nicked strand was shown not to be the case on two counts. Firstly, the analysis of the gradient failed to reveal the presence of any lower-than-expected molecular weight material, and secondly, the area of the single peak obtained on the fd-RNA gradient was sufficient to account for the absorbance of all the RNA loaded onto the gradient.

As sucrose gradients are of low resolution, the products of formaldehyde reaction were also examined by electrophoresis in polyacrylamide-agarose gels. Often, quite a large proportion of the fd-RNA did not enter

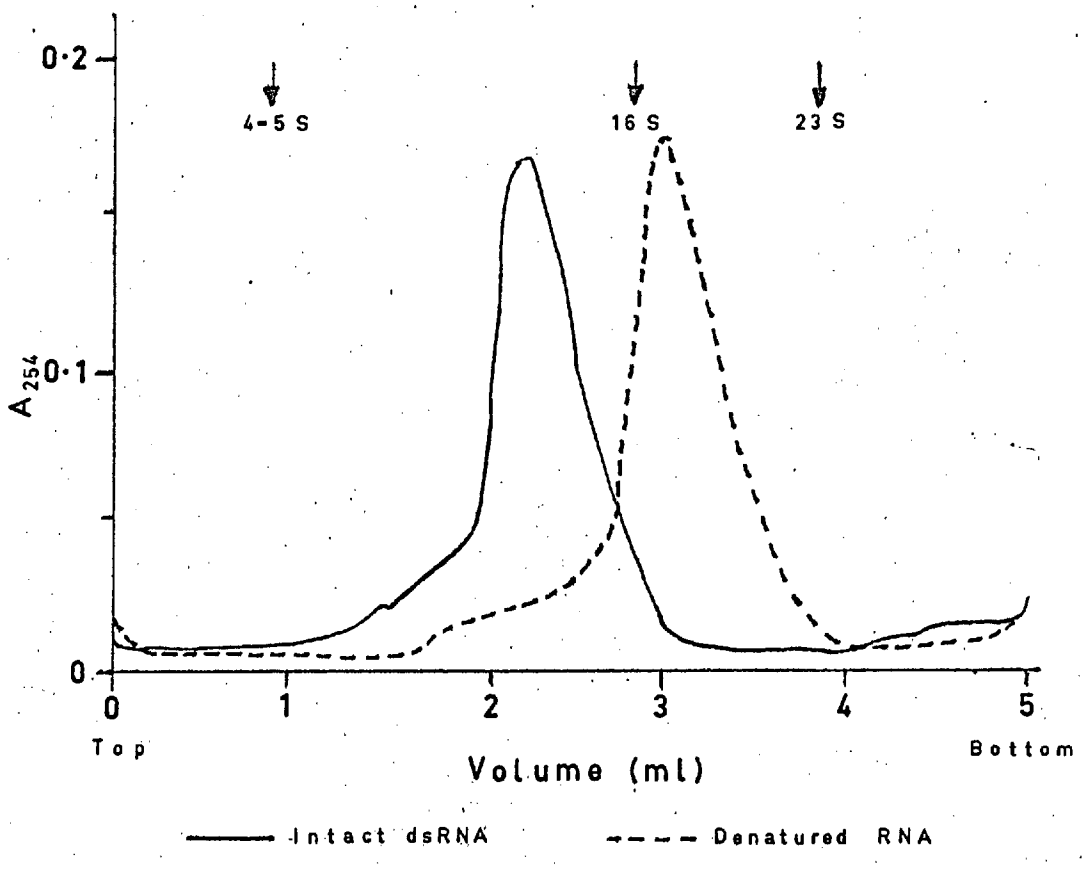


Figure 3 : Sucrose Density Gradient Profiles of Intact and Formaldehyde Denatured PcV-ds-RNA.

RNA batch MPP 63, dissolved in 0.1M phosphate buffer, pH 7.8, containing 1.8 % neutralised formaldehyde, at a concentration of 1 mg/ml was heated at 80°C for 8 min, and then cooled in ice-water. Control RNA was treated in the same way, but omitting the formaldehyde. The Tm in such a buffer is $>$ 100°C, so control RNA would not be denatured by such treatment. 20 µg samples of native and denatured RNA were run on 5 % - 20 % sucrose gradients at 25,000 rpm in an SW 50.1 rotor for 16 hr, as described in the Methods section 10), and then analysed. 30 µg samples of E.coli r-RNA were run as sedimentation coefficient markers.

a 2.4 % acrylamide : 0.5 % agarose gel, although formaldehyde denatured 23 S r-RNA did enter. This non-entry was possibly due to aggregation of the fd-RNA. Because of the fairly high pH of the gels (8.2) it is unlikely that any significant cross-linking, due to the reaction of the hydroxymethyl groups on different chains, occurred (Freifelder & Davison, 1963). It is not known why such aggregates occurred, and they were never detected on sucrose gradient analyses. The presence or absence of aggregation may well depend, of course, on the conditions, e.g. pH, ionic strength, temperature etc. under which the samples of fd-RNA were examined. The use of recrystallised acrylamide and bis-acrylamide did not result in any improvement.

However, on all these gels, at least some of the fd-RNA did enter the gel, see fig. 4. The molecular weight of the single narrow band of product, itself showing the lack of nicks in the parental ds-RNA, was estimated from the mobilities of formaldehyde-reacted 16 S and 23 S r-RNA run as standards. The 4-5 S r-RNA ran completely off these gels. The molecular weights of the two larger species have been estimated as 1.1 and 0.55 x 10^6 daltons respectively (Kurland, 1960; Stanley & Bock, 1963), and interpolation between these two values on the basis of the inverse relationship between electrophoretic mobility and the log. of the molecular weight (Bishop, Claybrook & Spiegelman, 1967; Peacock & Dingman, 1968) gives the molecular weight of the fd-RNA as 0.96 x 10^6 daltons. This is almost half the size of the ds-RNA as calculated from its sedimentation coefficient, 1.98 x 10^6 daltons, and is also a very similar size estimate to the value determined from the gradient analysis of fd-RNA. No lower molecular weight material was visible on the gel.

It was considered unsatisfactory however, that often much of the sample did not enter the gel, and so formamide was introduced as a denaturing agent (Helmkamp & T'so, 1961; Schildkraut, Marmur & Doty, 1961; T'so, Helmkamp & Sander, 1962a; Helmkamp & T'so, 1962). This has the added advantage that polyacrylamide gels can be prepared with formamide, rather than water, as the solvent (Staynov, Pinder & Gratzer, 1972), making a separate denaturation step unnecessary. In these gels, aggregation does not seem to occur, and as formamide, unlike formaldehyde, does not react chemically with RNA, estimation of molecular weights is easier. Again E.coli 23 and 16 S r-RNAs were used as molecular weight standards, and it was found that by this method the molecular weight of the single band of denatured RNA, fig. 5, could be estimated as 1.0 x 10^6 daltons. This is very similar to the values established by gradient analysis and electro-

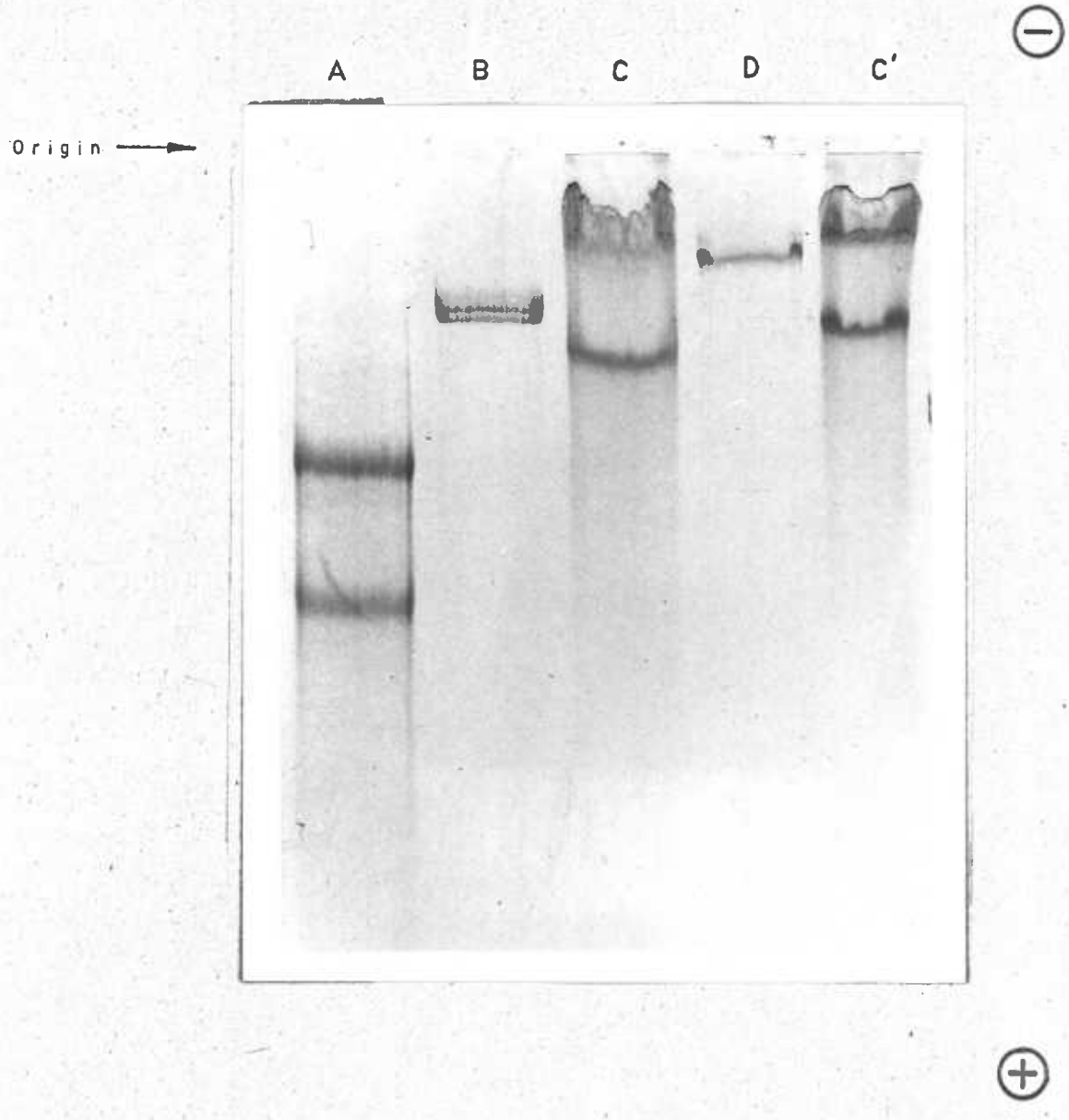


Figure 4 : Agarose-Polyacrylamide Gel Electrophoresis of Formaldehyde-reacted ds-RNA.

20 µg samples of PcV-ds-RNA and E.coli r-RNA, both untreated and following reaction with formaldehyde, were loaded onto a 0.5 % agarose-2.4 % acrylamide gel, and run overnight at 200 V, 18 mA. Preparation of the gel and samples as described in the Methods section 9 b).
A = untreated 23 S and 16 S r-RNA. B = untreated PcV-ds-RNA.
C,C' = formaldehyde-denatured r-RNA. D = formaldehyde-denatured PcV-ds-RNA.

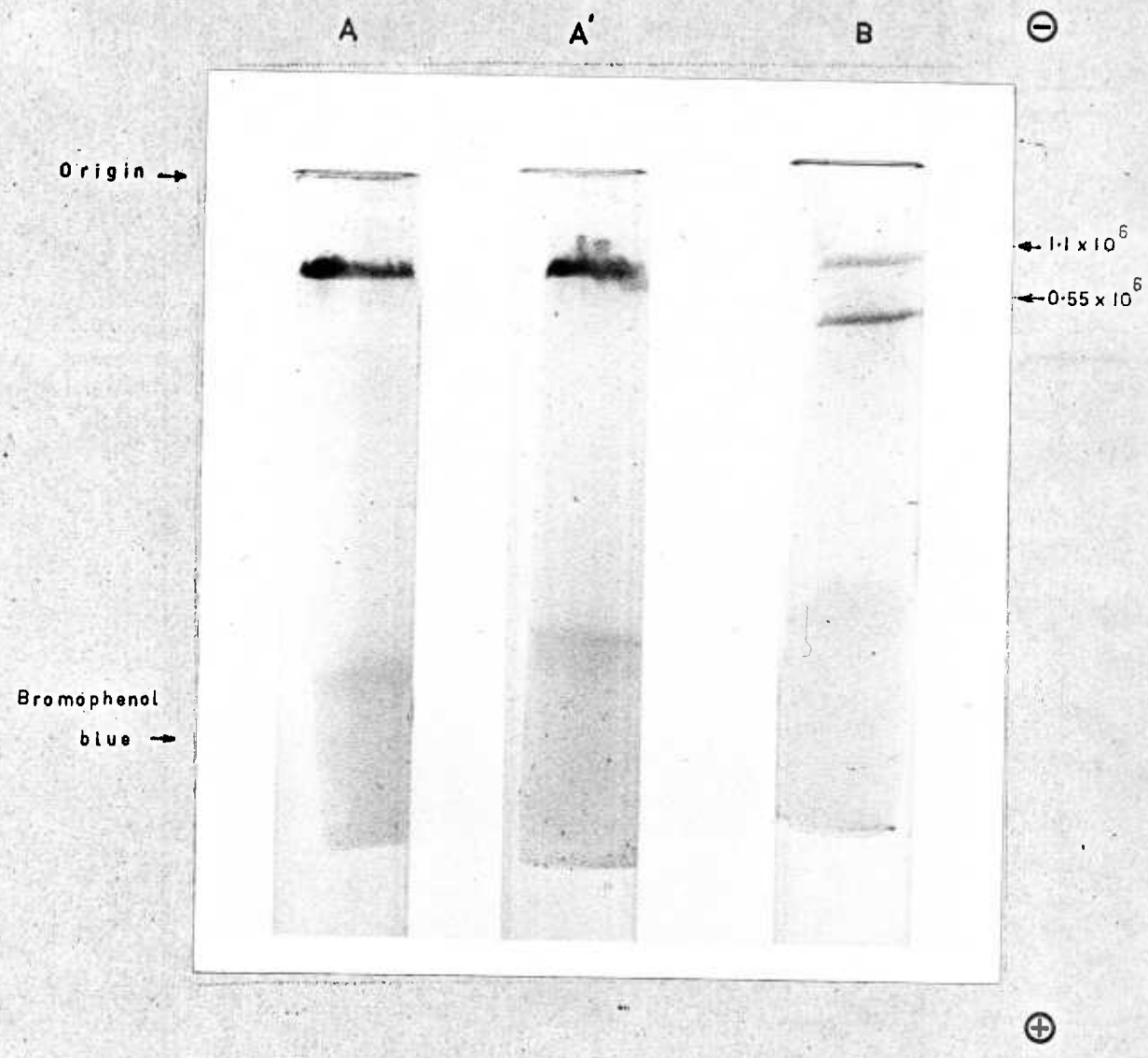


Figure 5 : Gel Electrophoresis of PcV-ds-RNA in Formamide Solution.

Gels were prepared and run as described in the Methods section 9 c), stained with toluidine blue and placed in glass tubes for photography.

A,A' = PcV-ds-RNA. B = E.coli 16 S and 23 S r-RNA, MW. 0.55 and 1.1 x 10^6 daltons.

phoresis of fd-RNA, and is also close to half the molecular weight of PcV-ds-RNA as measured by Buck et al. (1971) and Border et al. (1972).

Combining the results of gel electrophoresis and sucrose density gradient analysis of intact and denatured RNA it is possible to conclude that PcV-ds-RNA does not have any single-strand nicks, either in specific sites, or scattered at random. The size estimates of the denatured RNA also show that the RNA is in the form of a true double-stranded molecule, and is not simply a very long self-complementary hairpin-like structure.

b) Determination of the 5'-terminal nucleotide of PcV-ds-RNA.

The 5'-terminal nucleotide has been determined for several viral genome RNAs. In almost every case a purine has been found, the sole published exception being the results of Fraenkel-Conrat and Fowlks (1972), where the 5'-termini of preparations of Tobacco Mosaic virus (TMV) and Bromegrass Mosaic virus were heterogenous. In both cases, the ratio of the different nucleosides was the same, A > U > G > C. This is the sole report of such heterogeneity, however, and is in need of confirmation. In all other cases, only one major nucleoside was found at the 5'-terminal.

Wimmer (1972) has pointed out that, with some exceptions it seems that the RNA of viruses infecting eukaryote hosts, either plant or animal, have adenosine or phosphorylated adenosine as their 5'-terminal (e.g. TMV : HO-Ap...(Sugiyama & Fraenkel-Conrat, 1963); Turnip Yellow Mosaic virus : HO-Ap... (Suzuki & Haselkorn, 1968); Tobacco Necrosis virus and Satellite Tobacco Necrosis virus : ppAp... (Lesnaw & Reichmann, 1970); Influenza virus (all 6 segments): pppAp... (Young & Content, 1971); Poliovirus : ppAp... (Wimmer, 1972)), whilst the RNA bacteriophages, which by definition infect prokaryotes, by and large possess guanosine triphosphate as their 5'-terminal nucleotide (Q , R23, f2, MS2 & R17 all have pppGp... (Dahlberg, 1968; Watanabe & August, 1968; Glitz, 1968; Roblin, 1968). The differing degrees of phosphorylation of adenosine may be due to phosphatase or 5'-nucleotidase action during isolation. Plant cells are particularly rich in such enzymes (Lesnaw & Reichmann, 1970). It is perhaps noteworthy that the only ds-RNA for which the 5'-termini have been determined and published is an exception to the scheme of Wimmer (1972) described above. The Reovirus genome consists, as mentioned in the Introduction, of ten segments of ds-RNA. The 5'-termini of this RNA has been determined (Banerjee & Shatkin, 1971), and the only nucleotide found was ppGp..., meaning that this must be present at each of the twenty 5'-

termini. Some workers have suspected the presence of short (6-8 bases) purine tails at the 5'-ends (Millward & Nonoyama, 1970). However, Banerjee and Shatkin (1971) were unable to verify this, and also as the 5'-ends of the m-RNAs transcribed from the ds-RNA genome have also been shown to be ppGp... (Banerjee, Ward & Shatkin, 1971), the existence of single-strand tails was considered very unlikely (Shatkin, 1971).

The 5'-termini of PcV-ds-RNA were therefore of considerable interest, and have been determined in this study. The determinations were done by a modification (M. Szekely, unpublished) of the technique of Weiss, Live and Richardson (1968), which uses the enzyme polynucleotide kinase to transfer a labelled phosphate group from γ-^{32}P-ATP to the 5'-terminal nucleotide of the nucleic acid under study. The technique is applicable both to DNA and to RNA.

Attachment of the label to the RNA depends on the terminal nucleotide being unphosphorylated, and it is usual in such labelling experiments to remove any 5'-phosphate by phosphatase treatment before attempting to kinase-label the RNA. If phosphatase treatment leads to a great increase in the efficiency of incorporation of label, then it can be concluded that the 5'-terminal nucleotide is a 5'-phosphate. However, the reverse conclusion (i.e. that the RNA terminates with a 5'-hydroxyl group) cannot be assumed if phosphatase treatment results in no improvement. This is because polynucleotide kinase preparations have been shown to be contaminated with a phosphatase activity against 3'-phosphate groups (Szekely & Sanger, 1969). It is not known whether this phosphatase is capable of the splitting off of 5'-phosphates, but it means that it is not possible to conclude from the absence of an improvement of incorporation of label on phosphatase treatment (which was the case found in this study) whether the 5'-terminal nucleotide is phosphorylated or not. The level of incorporation of label into the ds-RNA was very low in these experiments, the reason for this is not known. Similarly, very low levels of incorporation were found during labelling experiments on other ds-RNAs of fungal viral origin (M. Szekely, T. Loviny & P. Roth, unpublished).

To purify the labelled ds-RNA, the reaction mixture was phenol-extracted to remove proteins, followed by ethanol precipitation to remove the bulk of the unreacted ATP. Finally the sample was purified by polyacrylamide gel electrophoresis, and the ds-RNA identified by autoradiography and toluidine blue staining. Unlabelled PcV-ds-RNA was run as a standard on the gels, and the labelled band corresponding in position to

this was cut out. Measurements showed that the bands of labelled and stained material coincided in every experiment. This separated the labelled PcV-ds-RNA from some other heterogenous labelled material of lower MW., possibly breakdown products, and also obviated any risks due to endonuclease cleavage at any stage prior to or during the labelling process.

The labelled PcV-ds-RNA was extracted from the slice of gel by the technique of Adams et al. (1969), as described in the Methods, section 2. After alkaline hydrolysis to separate the 5'-terminus as a labelled diphosphate, the samples, and nucleoside 3'-5'-diphosphate standards, were examined by paper electrophoresis, and the labelled diphosphates were detected by autoradiography.

The result of such an experiment is shown in figure 6, which shows the presence of one major, and three minor, spots. The major component can be identified by comparison with the standards as cytidine 3'-5'-diphosphate, but the minor components did not correspond with any of the mononucleoside diphosphate standards, and their nature remains unknown.

M. Szekely and T. Loviny (pers. commun.) found that on paper electrophoresis of alkaline hydrolysates prepared as described above, the spots were sometimes retarded. This means that what appears on fig. 6 to be identified as pCp in the hydrolysate may in fact be pAp. Both of these are of course pyrimidine nucleotides. This retardation phenomenon may also explain the unidentified nature of the other spots in the hydrolysate of PcV-ds-RNA.

This finding of a pyrimidine nucleotide as the major 5'-terminal of PcV-ds-RNA needs confirmation, although a pyrimidine has since been described at the 5'-terminal of a ds-RNA from the viruses of P.stoloniferum (M. Szekely & T. Loviny, pers. commun.).

2. The Fragmentation of PcV-ds-RNA.

Studies have been made on the fragmentation of ds-RNA by two RNases, one specific for double-strands (RNase III from E.coli), and the other with a preference for single-stranded RNA (bovine pancreatic RNase A). A physical method of fragmentation, by ultrasonic irradiation, has also been studied. The modes of action of all three agents have been investigated, and in the case of RNase III, about which little is known, the nature of the digestion products has been studied. This work was expected to yield information both on the action of the enzyme and also on the structure of PcV-ds-RNA.

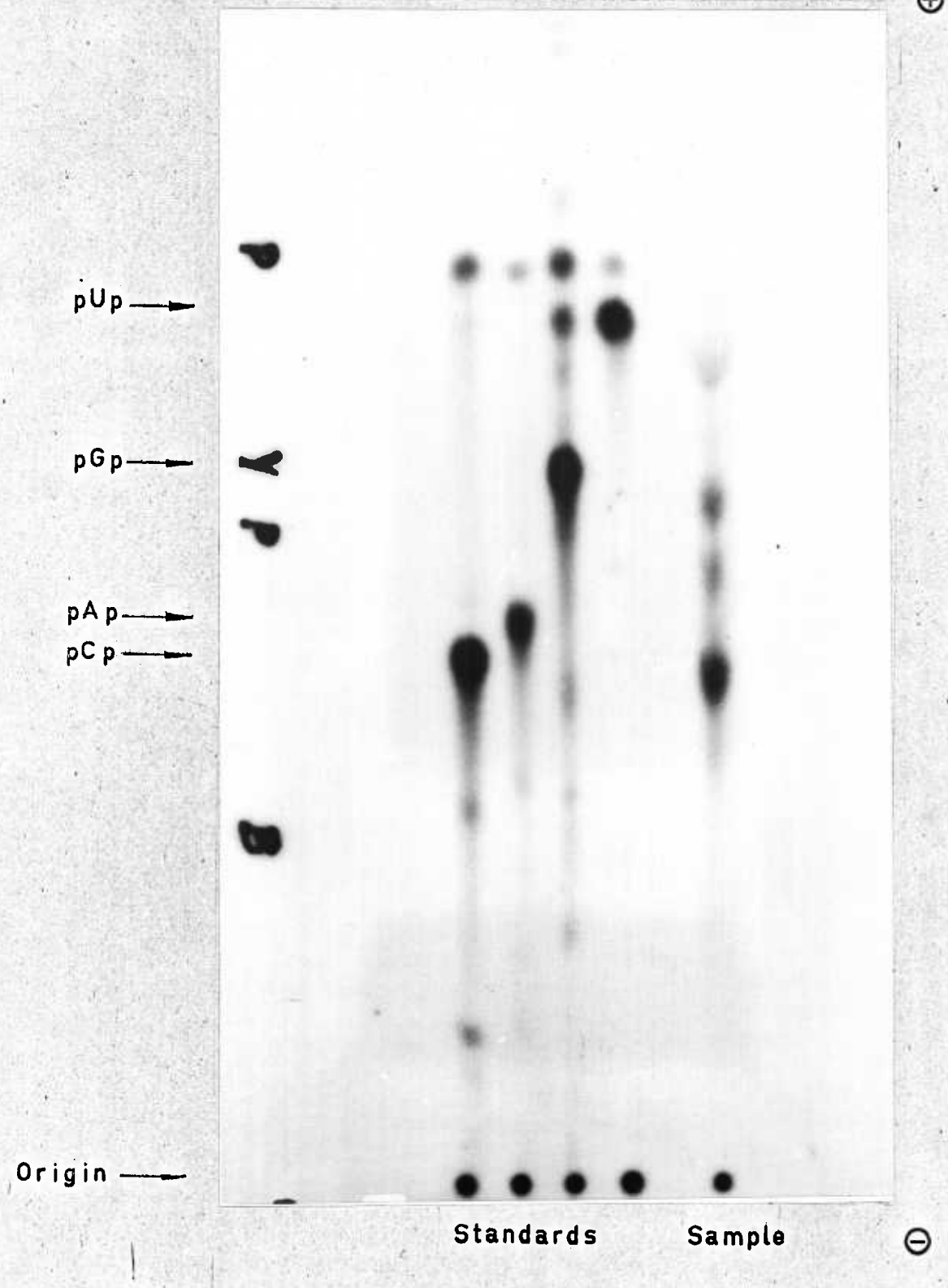


Figure 6 : 5'-End Determination of Intact PcV-ds-RNA.

80 µg of ds-RNA (batch P2) was labelled for 30 min under the conditions described in the Methods section. After ethanol precipitation and gel electrophoresis, the labelled band of intact PcV-ds-RNA was cut out, eluted and hydrolysed with alkali. The hydrolysate was analysed by high voltage paper electrophoresis, and the spots visualised by autoradiography. Two weeks exposure, Kodak Kodirex film.

a) RNase III.

This enzyme was first reported by Robertson, Webster and Zinder (1967) and Libonati, Vinuela and Weissmann (1967), in cells of E.coli, and shows an almost absolute specificity for ds-RNA as substrate. DNA and ss-RNA are not attacked at all, although the RNA strand of an RNA-DNA hybrid is hydrolysed (Robertson et al., 1968; Robertson & Zinder, 1968) leaving the DNA strand intact (Robertson, 1971).

Full purification methods were described, and some of the enzyme properties elucidated by Robertson et al. (1968) and Libonati (1968a,b). RNase III is an endonuclease, and cleaves ds-RNA to yield mainly 3'-phosphate and 5'-OH groups on the products. The enzyme appears to have no base specificity, in that poly(A-U) which forms extensive ds-hairpin loops, and the double-stranded polyribonucleotide poly(G).poly(C) are digested at very similar rates. The MW. of the enzyme was estimated by Robertson et al. (1968) to be between 45 and 55,000 daltons, and a requirement for both mono- and di-valent cations was established. The optimum pH is alkaline, lying between pH 7.6 and 9.75, but it was impossible to measure this more accurately as the activity was found to increase with increasing pH, up to levels where the ds-RNA was also undergoing alkaline hydrolysis.

The role of RNase III in cellular metabolism is unknown. Castles and Singer (1969) concluded that it was not involved in m-RNA breakdown, because of its specificity for double-helical RNA. Hehlmann and Hofschneider (1970) showed that RNase III was probably inactive in the cell, as ds-RNA appeared stable in E.coli spheroplasts; yet extracts from these spheroplasts contained active RNase III in sufficient quantity to partially digest the administered ds-RNA. Keller (1972) has suggested that ribonuclease H, or 'hybridase' functions in eukaryote cells to digest the RNA oligonucleotide used in the initial step of DNA replication by DNA-dependent DNA polymerase, and tentatively proposed that RNase III might fill a similar function in prokaryote cells. RNase H-type activity was also suggested in E.coli by the results of Miller, Gill and Riggs (1972), and its existence, as an enzyme distinct from RNase III, has been shown by Weathford, Weisberg, Achord and Apirion (1972). Weathford et al. were able to purify RNase H fractions free of activity against ds-RNA, but all RNase III fractions contained a low activity against RNA-DNA hybrids. The cellular role of RNase III therefore remains unclear.

The mechanism of action of RNase III was studied by Schweitz and Ebel (1971), after they found that it failed to digest the double-stranded regions of t-RNAs and r-RNAs. (Also noted by Robertson et al., 1968). They suggested that a double-stranded region has to be about 7 - 9 base pairs long before it can be recognised as ds by RNase III, and that the helices have to be at least 9 - 11 base pairs long for hydrolysis to occur. Robertson (pers. commun.) came to similar conclusions regarding the necessity for a ds-RNA molecule to be of a certain minimal length before RNase III would act upon it, but considered the minimum length to be greater than the estimate of Schweitz and Ebel (1971). So far however, no natural ss-RNA with a strong secondary structure has been found susceptible to RNase III, as would be expected if length of double-helical region was the only requirement for activity. All conclusions as to length requirements should therefore be regarded as tentative.

Schweitz and Ebel (1971) found that exhaustive RNase III digestion of poly(G).poly(C) or poly(I).poly(C) yielded a hydrolysate of which 80 - 90 % consisted of oligonucleotides about 10 - 25 base pairs in length, the average size being 16 nucleotide pairs. They also studied the mode of cleavage of ds-RNA by digesting poly(I).poly(C), and then comparing the electrophoresis patterns on polyacrylamide gel in the presence and absence of urea, a denaturing agent. After electrophoresis, the gels were scanned, and it was found that the pattern of fragments obtained was very similar in both cases, i.e. native fragments did not have ss nicks in them. From this, Schweitz and Ebel (1971) concluded that RNase III acts by cutting both strands of the ds-polyribonucleotide at the same level. This conclusion, however, depends on the assumption that urea denatures the ds-oligonucleotides. It has been shown (T'so, Helmkamp & Sander, 1962b) that urea probably does not rupture hydrogen bonds, which is essential if the two strands of a ds-nucleic acid are to be separated. M. Szekely (pers. commun.) has found 7 M urea insufficient to denature viral ds-RNA from P.stoloniferum. Therefore, the action of RNase III on ds-RNA has been re-investigated by different techniques.

(i) Mode of cleavage by RNase III.

During the degradation of a double-stranded polymer, cleavage can occur either by simultaneous cutting of both strands of the polymer, so that each enzyme hit results in a fall in the MW. of the substrate; or by random scission of the individual strands, so that two hits are necessary for every cleavage. The former, single-hit degradation was called

diplotomic, and the latter, involving separate scission of the two strands, haplotomic by Bernardi (1968), in his work on the mode of action of some DNases. For example, DNase I was shown by Young and Sinsheimer (1965) to cleave native DNA with haplotomic kinetics. DNase II (acid DNase) acts by a diplotomic mechanism (Oth, Fredericq & Hacha, 1958; Young & Sinsheimer, 1965; Kates & McAuslan, 1967), although Bernardi (1968) showed that some haplotomic cleavages also occurred.

In the case of haplotomic digestion of the ds-RNA (or of course of any other ds-polymer of similar hydrogen-bonded structure), each enzyme hit does not result in a molecular scission, because the two strands are held together by hydrogen bonds. It is only when a second hit occurs in the other strand very close to the first, that the hydrogen bonds between the two nicks are insufficient to bind the fragments together. Only when these weak interstrand links break does the molecule fall apart. Thus, if hits occur on the two strands at random, then a lag period can be expected after the addition of enzyme, before the MW. of the substrate starts to fall. Thus, although each cleavage of the ds-RNA only directly involves two enzyme hits, it is to be expected that many more will occur before a fall in the MW. of the substrate is detectable. Diplotomic cleavage involves the cutting of both RNA strands at the same time, and so there will be no lag period after initiation of cleavage. However, because it is possible that a very short lag period would not be detected, then although the existence of a lag period is evidence of haplotomic digestion, the absence of a detectable lag period is merely suggestive of either pure diplotomic, or mixed haplotomic/diplotomic fragmentation. Even pure haplotomic digestion could appear to commence without a detectable lag period if the conditions were so chosen.

One of the most sensitive methods for examining the very early stages of fragmentation of ds-nucleic acids is viscosimetry, first applied to the enzymic digestion of DNA by Laskowski and Seidel (1945) and by McCarty (1946) and since by several other groups of authors, e.g. Kunitz (1950), Zamenhof, Griboff and Marullo (1954), Schumaker, Richards and Schachmann (1956), Le Pecq and Bourgoin (1964), Bernardi and Cordonnier (1965) and Yasmineh, Gray and Wannamaker (1968).

The fall in viscosity of PeV-ds-RNA on RNase III digestion has been determined, and the results obtained with various amounts of enzyme are plotted in fig. 7. It can be seen that even at the lowest levels of RNase III, no lag period is visible before the viscosity starts to fall. For

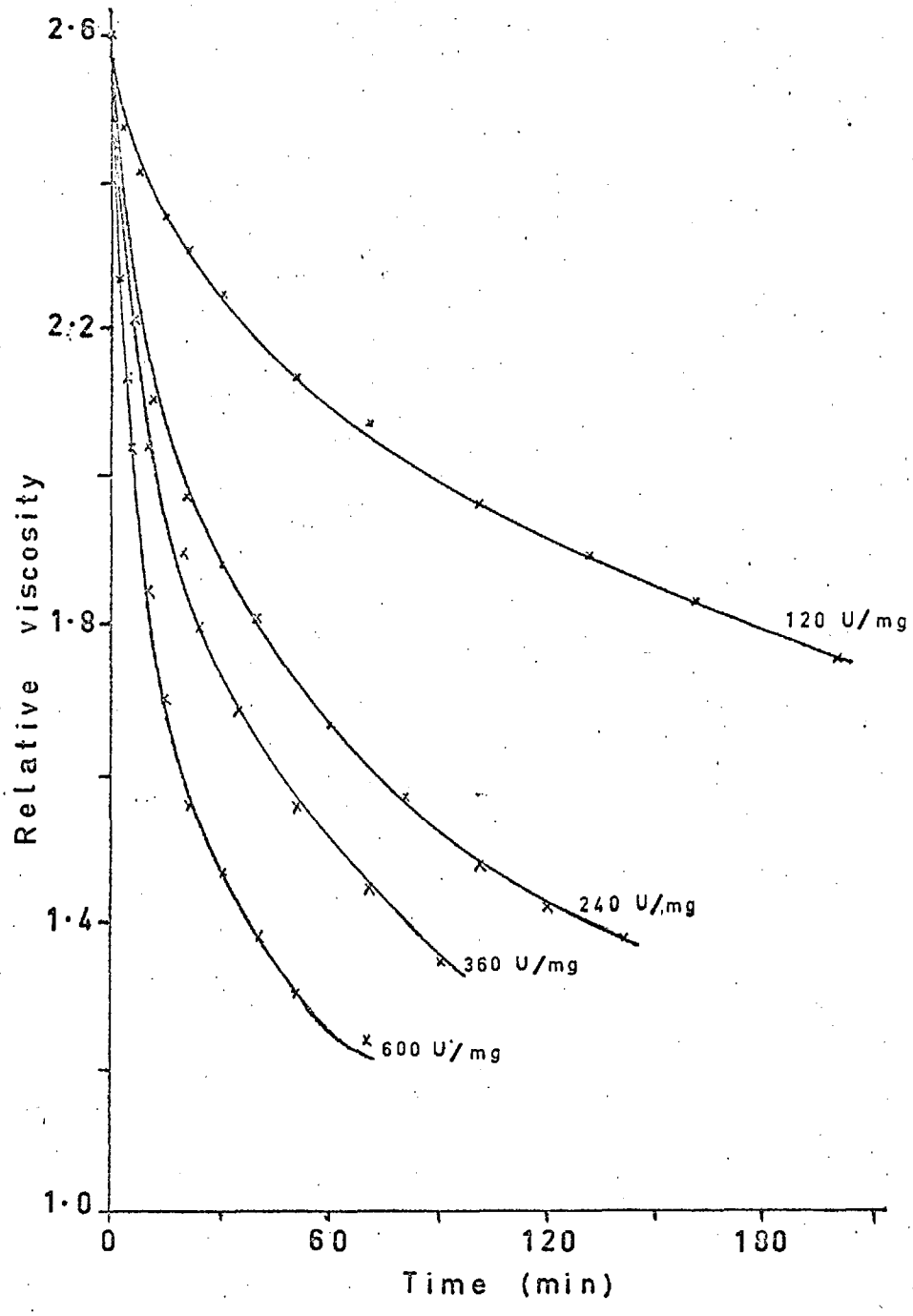


Figure 7 : Fall in Relative Viscosity on RNase III digestion of PcV-ds-RNA.

RNA batch MPP 63, at 1 mg/ml was incubated in Tm buffer + 0.1M ammonium chloride, pH 7.6, in an Ostwald viscosimeter at 36°C, with various amounts of RNase III. The relative viscosity was noted from time to time.

the reason mentioned above this merely suggests, but does not prove that RNase III acts diplotomically, and investigation by a different technique was necessary.

Viscosimetric data can be treated according to a general theory for the degradation of multi-stranded polymers of any initial distribution of molecular weights, developed by Schumaker et al. (1956). These authors showed that for the initial stages of digestion :

$$\log_{10}(1-R) = n \log_{10} p + \text{constant}$$

where R = MW.t/MW.o (MW.t being the MW. after time t, whilst MW.o is the initial MW.). The number of hits necessary to cleave the substrate molecule is expressed by n, and p is the probability that any given bond be broken at time t. When the rate of digestion is constant, then p = kt, where k is a proportionality constant. A plot of $\log_{10}(1-R)$ versus $\log_{10} t$ will have a slope equal to n. For diplotomic fragmentation, where one enzyme hit cleaves both strands of the ds-RNA, then n = 1, whilst for haplotomic digestion, involving two hits per cleavage, n = 2.

Cavalieri and Rosenberg (1961) replaced the term (1-R) in the equation above with $(\frac{1-R}{R})$; this latter function being a linear function of $\log_{10} p$ over a wider range of values of the probability than the original (1-R) term. They showed that :

$$\log_{10} (\frac{1-R}{R}) = n \log_{10} t + \text{constant}$$

and this can then be plotted in the form $\log_{10} (\frac{1-R}{R})$ versus $\log_{10} t$, to give a line of slope n.

This method of analysis has been applied to the fragmentation of DNA by several groups of workers, including both groups referred to above, and Bernardi and Sadron (1964). The difficulty in applying this technique to the study of the fragmentation of ds-RNA is the lack of any equation for the relationship between the intrinsic viscosity and MW. for ds-RNA. The work on the possibility of nicks in the intact ds-RNA showed the general inaccuracy of the equations derived for DNA when applied to ds-RNA. However, as it seems that the flexibility of DNA does not alter significantly on limited fragmentation (as one MW.-viscosity equation is applicable over the whole MW. range under study), and it seems probable that a similar situation would apply to ds-RNA, it is therefore clear that the use of equations developed for ds-DNA would not have a very great effect on the ratio MW.t/MW.o. The effect of the increased flexibility is also described below.

Most empirical relationships between the intrinsic viscosity and MW. take the form :

$$[\eta] = K.M^a$$

which is also the theoretical form of equations of long chain molecules. (See Steiner & Beers, 1961). The exponent 'a' is related to the flexibility of the molecule, having values varying in theory between 0.5 and 2.0 (for flexible random coils and rigid rods respectively). Fig. 8 is a plot according to Schumaker et al. (1956) and Cavalieri and Rosenberg (1961), using the relationship of Eigner and Doty (1965) :

$$[\eta] = 1.05 \times 10^{-7} M^{1.32}$$

to calculate the molecular weights from the values of $[\eta]$ given in Table 3. The best straight line on this has a slope of 1.29. Also on this figure is shown the effect of varying the flexibility component of the equation between the two theoretical limits of 0.5 and 2.0. In these two extreme cases, the slope values are 2.01 and 1.12 respectively.

As, from the work of Franklin (1967) is seems that ds-RNA is more flexible than DNA, the exponent used in the above equation may be somewhat too high. However, the true exponent must still be much larger than 0.5, which is the theoretical value for a perfectly flexible random coil with no rigidity at all. So, although the value of 1.29 for the number of hits necessary to cleave the ds-RNA molecule is probably to some extent an underestimate, the degree of error is such that only at the worst possible slope value (which is certain to be a considerable over-estimate, as explained before), are 2 hits apparently necessary for strand cleavage.

The non-integral value found indicates that RNase III causes both haplotomic and diplotomic cuts in the ds-RNA, and there is predominance of the latter. This does not necessarily mean that the enzyme preparation is impure, as a similar mixed mode of action was shown by Bernardi and Sadron (1964) for acid DNase, which works predominantly by a diplotomic mechanism, but with some simultaneous haplotomic cleavage.

(ii) Production of specific fragments on RNase III digestion.

It has been repeatedly found that limited digestion with RNase III produces, under certain conditions, numerous specific fragments resulting in the appearance of bands on gel electrophoresis of digests; see figs. 9 and 10 for two band patterns. On these gels, the three bands of intact ds-RNA were not well resolved due to the relatively high gel concentration (and thus short migration distance) used to obtained good resolution of the specific fragments. The fragments were not resolvable

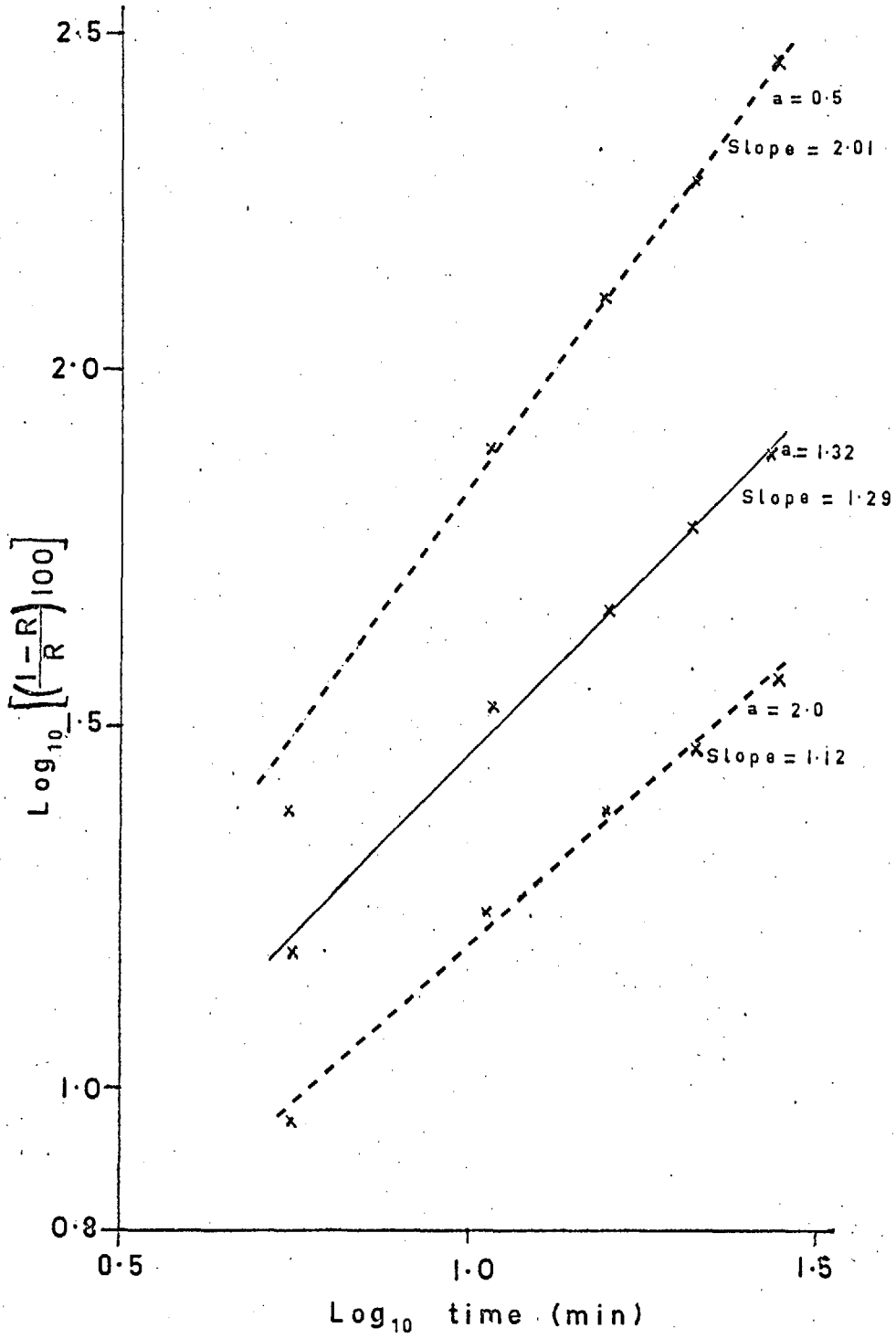


Figure 8 : Determination of the Cleavage Mode of RNase III.

A plot of log $\left[\left(\frac{1-R}{R}\right)100\right]$ vs log time (Cavalieri & Rosenberg, 1961), using the data given in Table 3. Results are plotted using the exponent (a) 1.32 in the molecular weight-viscosity relationship, as given by Eigner and Doty (1965) and also the theoretical limiting exponents 0.5 and 2.0 (Steiner & Beers, 1961) (see text, Results 2 a) (i)).

TABLE 3.

VISCOSIMETRIC DATA ON THE FRAGMENTATION OF PcV-ds-RNA BY RNase III

Time (min)	$[\eta]$	MW. $(\times 10^{-6})$	R	$\log\left[\left(\frac{1-R}{R}\right)100\right]$	log t
0	12.8	1.43	1	-	-
5.58	11.5	1.24	.867	1.184	0.747
10.58	9.6	1.07	.748	1.528	1.024
15.58	8.5	0.98	.684	1.665	1.192
20.58	7.5	0.89	.622	1.784	1.313
26.83	6.6	0.81	.566	1.885	1.429

ds-RNA, batch 5/119, at a concentration of 1 mg/ml in TM buffer + 0.1M ammonium chloride, pH 7.6, was digested at 36°C with 600 U RNase III/mg RNA. At the stated times, 5 ml samples were withdrawn and mixed with SDS to 1 % final concentration. (This was adequate to completely stop digestion, on incubation of such samples in a viscosimeter at 36°C no further fall in viscosity was detected). The viscosity of the sample was then measured at 4 dilutions, as described in the Methods, section 11. MWs. were calculated according to the empirical equation of Eigner and Doty (1965). In section a) of the Table below, the MWs. are recalculated, using modified forms of the Eigner and Doty equation.

Subtable a).

	a = 0.5			a = 2.0		
$[\eta]$	$MW_{\pm 16}$ (x 10^{-16})*	R	$\log\left[\left(\frac{1-R}{R}\right)100\right]$	$MW_{\pm 4}$ (x 10^{-4})+	R	$\log\left[\left(\frac{1-R}{R}\right)100\right]$
12.8	1.48	1	-	3.49	1	-
11.5	1.20	0.807	1.378	3.31	0.920	0.939
9.6	0.84	0.563	1.890	3.02	0.862	1.204
8.5	0.66	0.443	2.100	2.85	0.811	1.367
7.5	0.51	0.343	2.283	2.67	0.768	1.480
6.6.	0.40	0.266	2.441	2.51	0.716	1.598

* Calculated using equation $[\eta] = 1.05 \times 10^{-7} M^{0.5}$

\+ Calculated using equation $[\eta] = 1.05 \times 10^{-7} M^{2.0}$

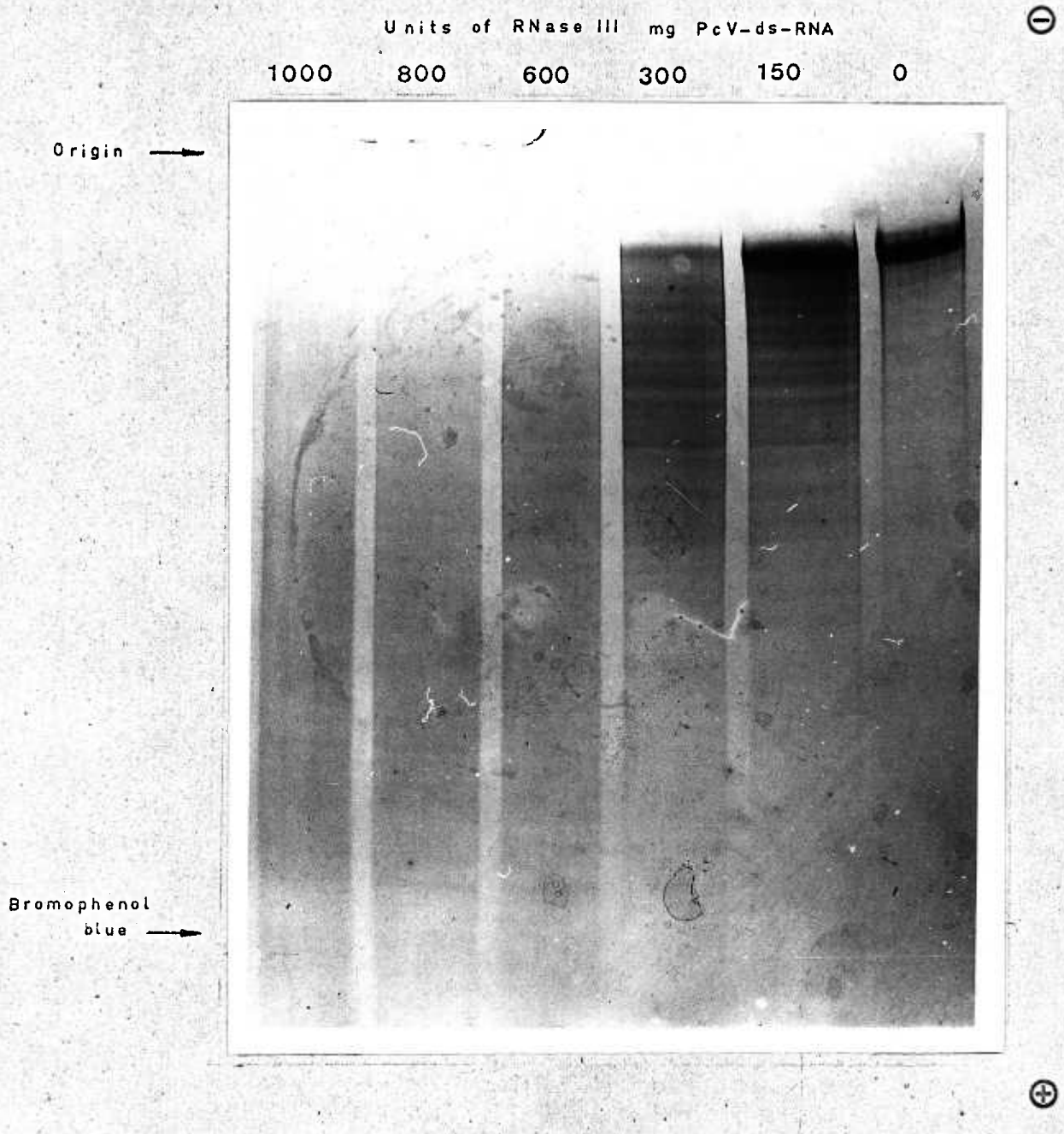


Figure 9 : Gel Electrophoresis Pattern of PcV-ds-RNA After Limited RNase III Digestion.

RNA of batch MPP 63 was digested for 1 hr at 36°C in TM buffer + 0.1M ammonium chloride, pH 7.6, with 0, 150, 300, 600, 800 and 1000 U RNase III/mg ds-RNA, concentration 1 mg/ml. After 1 hr incubation, the digestion was terminated by the addition of SDS to 1 %, after which RNase III was added to all samples to bring them to 1000 U enzyme/mg of RNA. Proteins were removed by two extractions with phenol, and the RNA precipitated by addition of 2.5 volumes of ethanol, as detailed in the Methods section 6. The precipitate, after washing, was dissolved at a concentration of 2 mg/ml in TAE buffer, and 40 µg samples run on the gel, again as described in the Methods section 9 a).

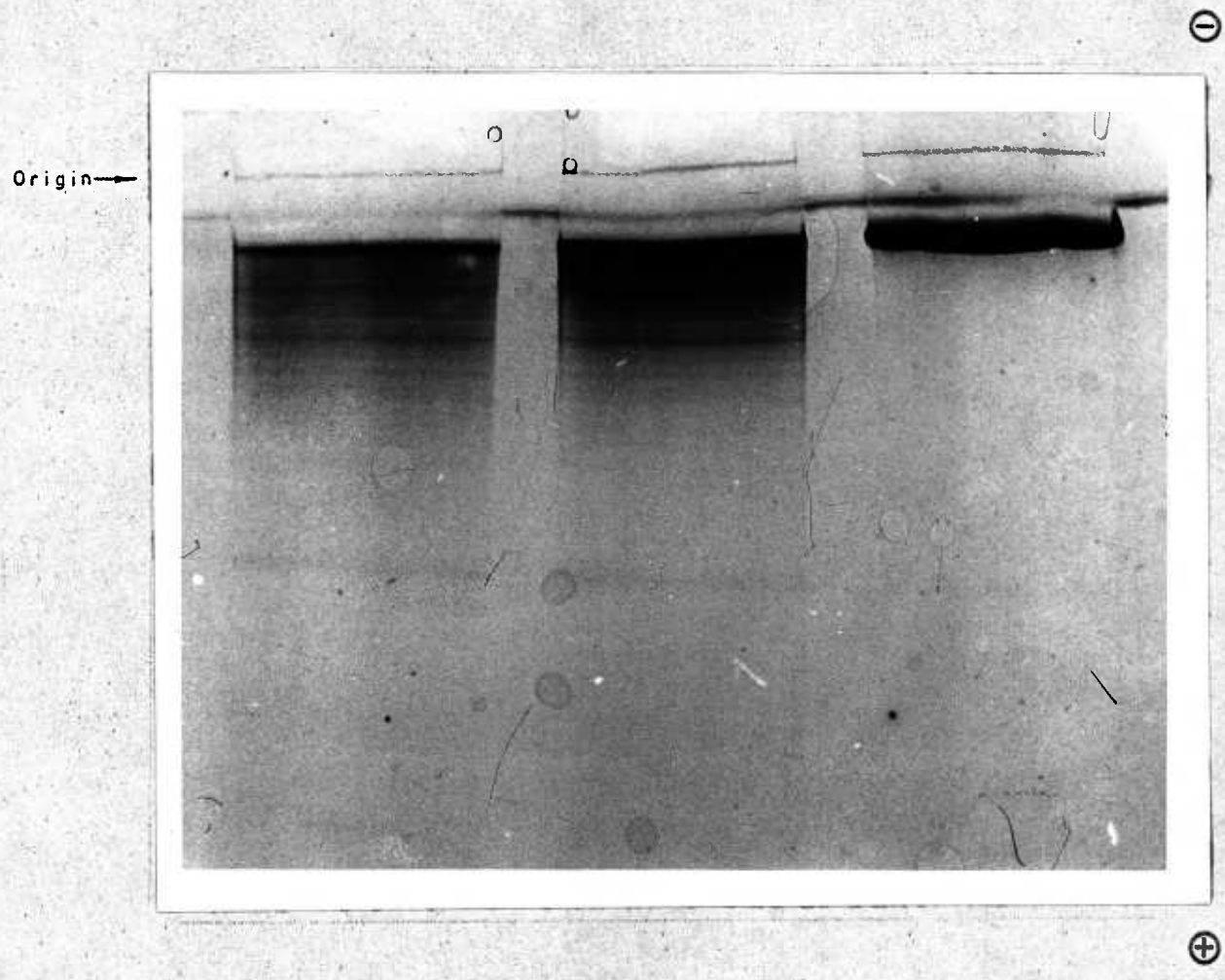


Figure 10 : Polyacrylamide Gel Electrophoresis of RNase III-digested PcV-ds-RNA.

PcV-ds-RNA, batch MPP 63, was digested with either 220 or 110 Units of RNase III/mg of nucleic acid, for 1 hr at 36°C. The reaction conditions were as described in the footnotes to fig. 9. The polyacrylamide gel electrophoresis was carried out for 6 hr only however, instead of the standard 16 hr.

on sucrose gradient centrifugation, as shown in figure 11.

Gel electrophoresis of digests shows that even on very limited digestion (so that the great bulk of the RNA was still intact), all the possible bands were present, and the relative intensity of the bands in a digest did not change with increasing digestion. This implies that RNase III cleaves one ds-RNA molecule into several specific fragments before attacking another molecule. If this were not the case, and the sensitive sites on the different molecules were cut at random, then the expected result would be that a majority of large fragments would first be produced, and significant quantities of the smaller specific fragments would not appear on the gel until the digestion was more advanced.

The pattern of bands did not change in its major aspects on prolonged digestion; it merely got stronger as more intact ds-RNA was degraded, and then faded as digestion progressed further. The product of digestion of the specific fragments was a long smear of non-specific fragments. The band pattern disappeared completely soon after the fragmentation of the remaining intact ds-RNA, as is shown on fig. 9. All the bands appeared to be digested simultaneously, indicating that between the specific sites on the intact molecule there were no other preferred sites of action. Digestion of the specific fragments did not seem to result in the production of further specific fragments, and from the distribution of non-specific fragments as assessed by the intensity of staining on the gel, it seems that these further cleavages occur at random.

The band pattern was not very reproducible from batch to batch of PcV-ds-RNA, although certain common features could be recognised. The use of different batches of RNase III had little effect with any one batch of ds-RNA. Similar band patterns were produced with RNase III both at a high level of purity, as usually used in this study, and also with the enzyme at a lower stage of purification, when the preparation still contained fairly large amounts of activity against ss-RNA.

That specific fragments of ds-RNA are produced means that RNase III has some kind of specificity in its action, since it must be able to recognise some sites to cleave on the ds-RNA molecule. Robertson et al. (1968) found that RNase III had no base specificity in its production of acid-soluble oligonucleotides, by showing that both poly(G).poly(C) and poly(A-U) were digested to acid solubility at very similar rates. In fact poly(G).poly(C) seemed to be digested slightly more rapidly than poly(A-U),

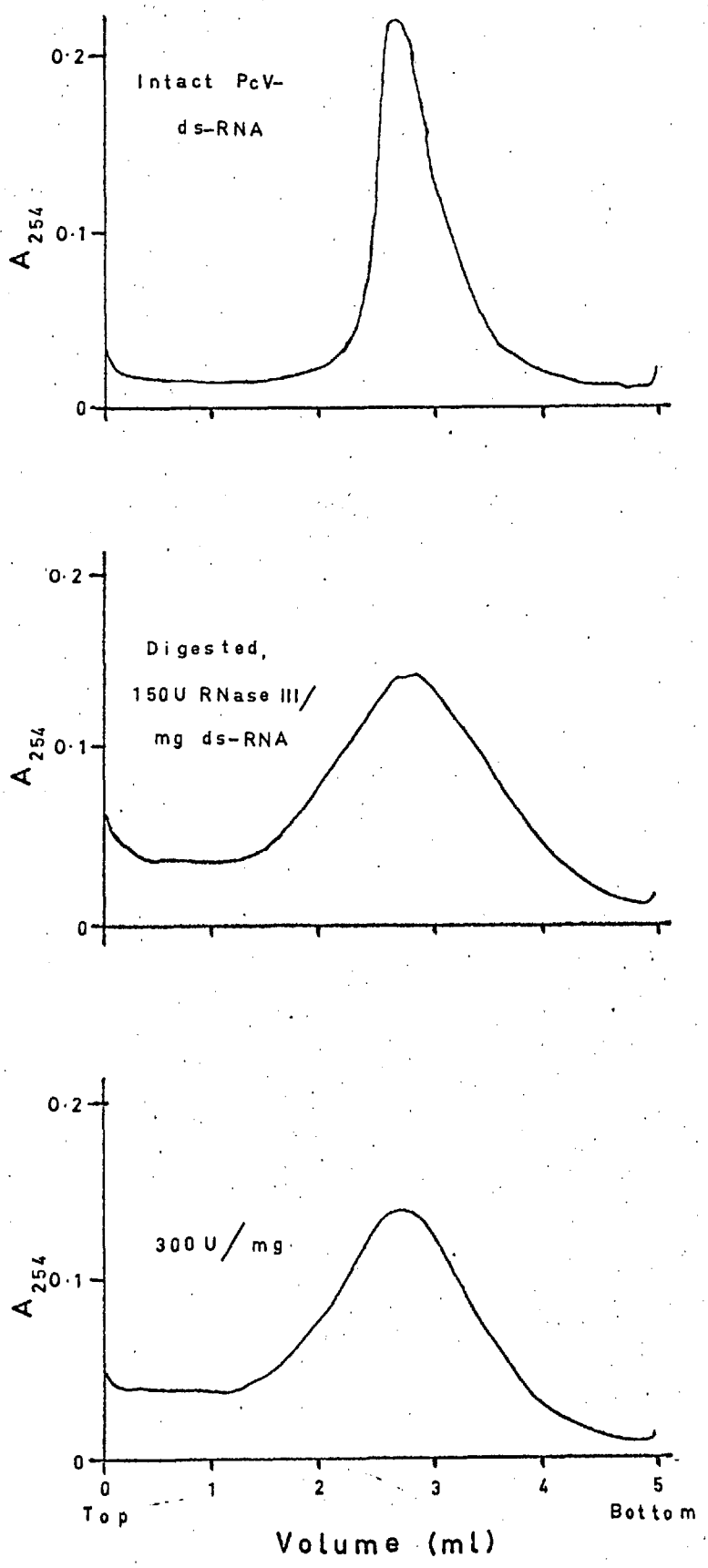


Figure 11 : continued overleaf.

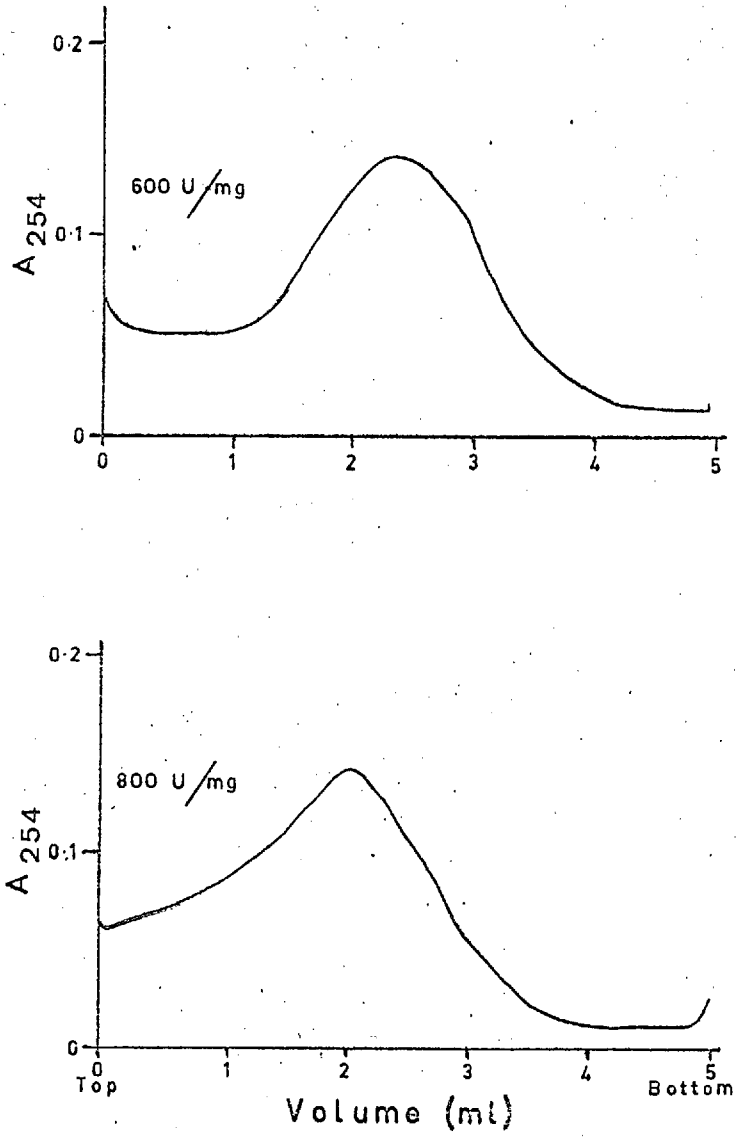


Figure 11 : Sucrose Density Gradient Analysis of RNase III Digests.

The first 5 samples described in fig. 9; digested for 1 hr with 0, 150, 300, 600 and 800 U RNase III/mg PcV-ds-RNA, were run on 5 ml 5 % - 20 % sucrose gradients for 16 hr at 25,000 rpm an an SW 50.1 rotor.

but this is probably due to the former substrate having a slightly tighter double-stranded structure than the alternating copolymer (H. Robertson, pers. commun.).

There are several possible reasons for such specificity; it could be due to inhomogeneity of the base sequence of the ds-RNA producing G:C-rich and G:C-poor areas of differing susceptibility to RNase III; it could be some unknown secondary structural feature; or it could be base specificity. To test the hypothesis that G:C-rich and G:C-poor regions might be involved in the production of specific fragments, use was made of the observation of Robertson et al. (1968) that ammonium sulphate inhibits the digestion of poly(G).poly(C) by RNase III at molarities which have little effect on the digestion of poly(A-U). These authors also found that addition of sulphate inhibited the digestion of Reovirus ds-RNA. The digestion of PcV-ds-RNA was not noticeably altered in extent by the addition of sulphate, in fact gel electrophoresis of samples digested with equal amounts of enzyme in the presence of chloride or sulphate (fig. 12) revealed that digestion was slightly stimulated in the presence of sulphate, less of the intact PcV-ds-RNA remaining. The reason for the contradiction with the results of Robertson et al. (1968) is not known. Because the purification and source of RNase III had been modified in some ways from the original procedure of Robertson, and thus the enzyme preparation might have been altered in some way, the effect of sulphate on the digestion of the two synthetic polyribonucleotides was assayed. The result is shown in Table 4, which confirms the result of Robertson et al., that sulphate acts as an inhibitor of the digestion of one of the substrates. So, as the extent of digestion of ds-RNA was not significantly inhibited by the amounts of sulphate used, the effect on the production of specific fragments was determined. The effect of replacing the ammonium chloride in the digestion buffer by an equimolar amount of sulphate is shown in fig. 12. It can be seen that although several of the original bands remain, many of them are altered in relative intensity, and some totally new bands are present. As sulphate is a divalent anion, whilst chloride is monovalent, it seemed possible that this effect might simply depend on the ionic strength. This was tested by assaying the effect of sulphate and chloride between 0 and 0.3 M, and the results are shown in fig. 13. This shows that increasing concentrations of either salt caused significant alteration in the band pattern. The effect of sulphate was more pronounced, however, but it seems that the alteration of the band pattern is due, at least in part, to the increased ionic strength, and is

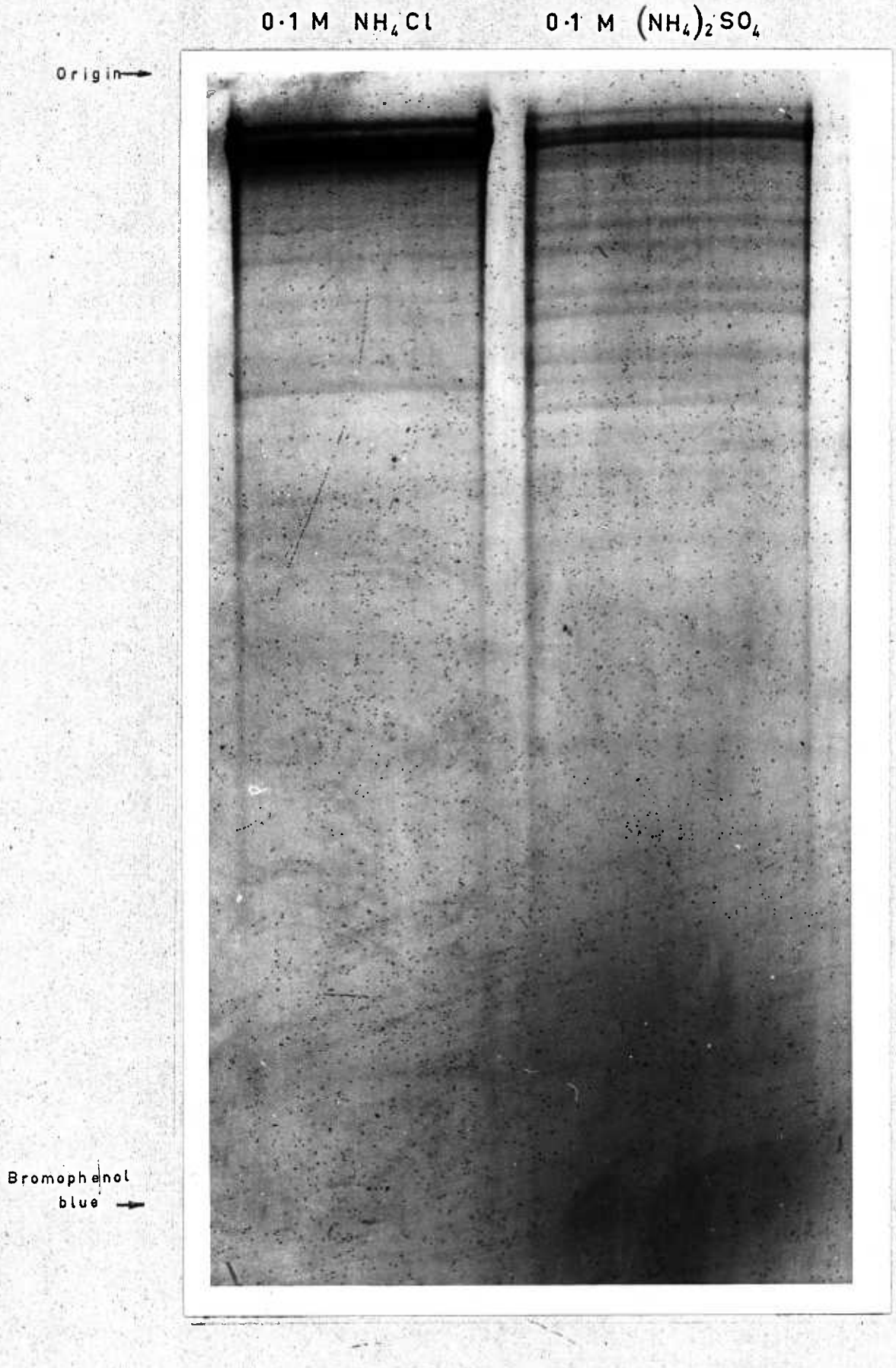


Figure 12 : The Effect of Ammonium Sulphate on the Digestion of PcV-ds-RNA by RNase III.

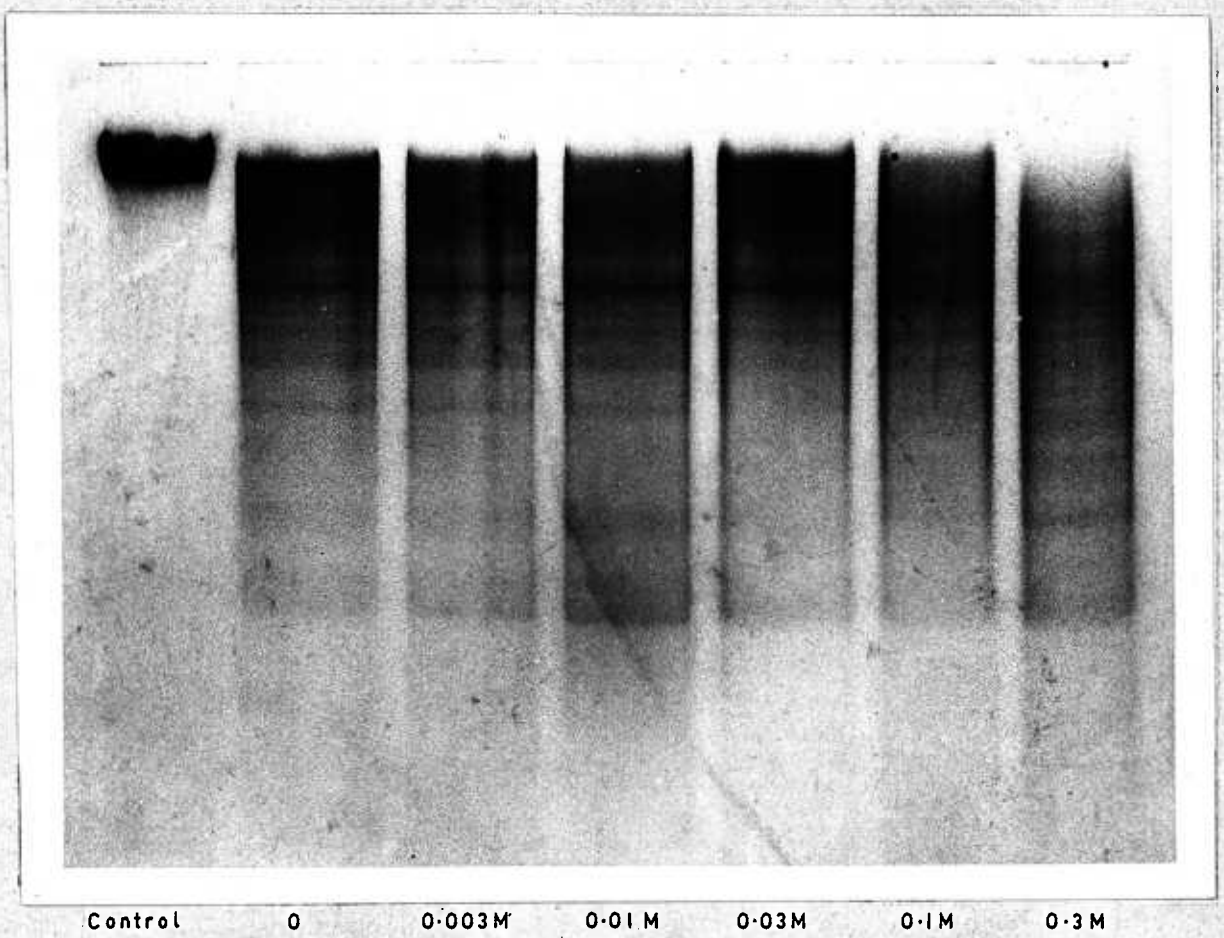
PcV-ds-RNA, batch 5/119, was digested for 1 hr at 36°C with 250 U RNase III/mg RNA. Conditions of reaction were as described in the footnotes to fig. 9, except that in one digest the buffer used contained 0.1M ammonium sulphate instead of chloride.

TABLE 4.

THE EFFECT OF AMMONIUM SULPHATE ON THE DIGESTION OF ds-POLYNUCLEOTIDES BY RNase III

Substrate	RNase III	Ammonium sulphate	c.p.m. precipitated	
Poly(A-U)	-	-	824	(100)
"	30 U	-	168	(20.4)
"	"	0.06M	204	(24.8)
"	"	0.1M	300	(36.4)
Poly(G).poly(C)	-	-	1152	(100)
"	30 U	-	384	(33.3)
"	"	0.06M	826	(71.7
"	"	0.1M	920	(79.9)

Conditions were as described in the Methods (section 4) for RNase III assay except for the addition of 1M ammonium sulphate in TM buffer to the reaction mixtures resulting in the above-mentioned final concentrations.
Figures in parentheses, are c.p.m. precipitated relative to control = 100.



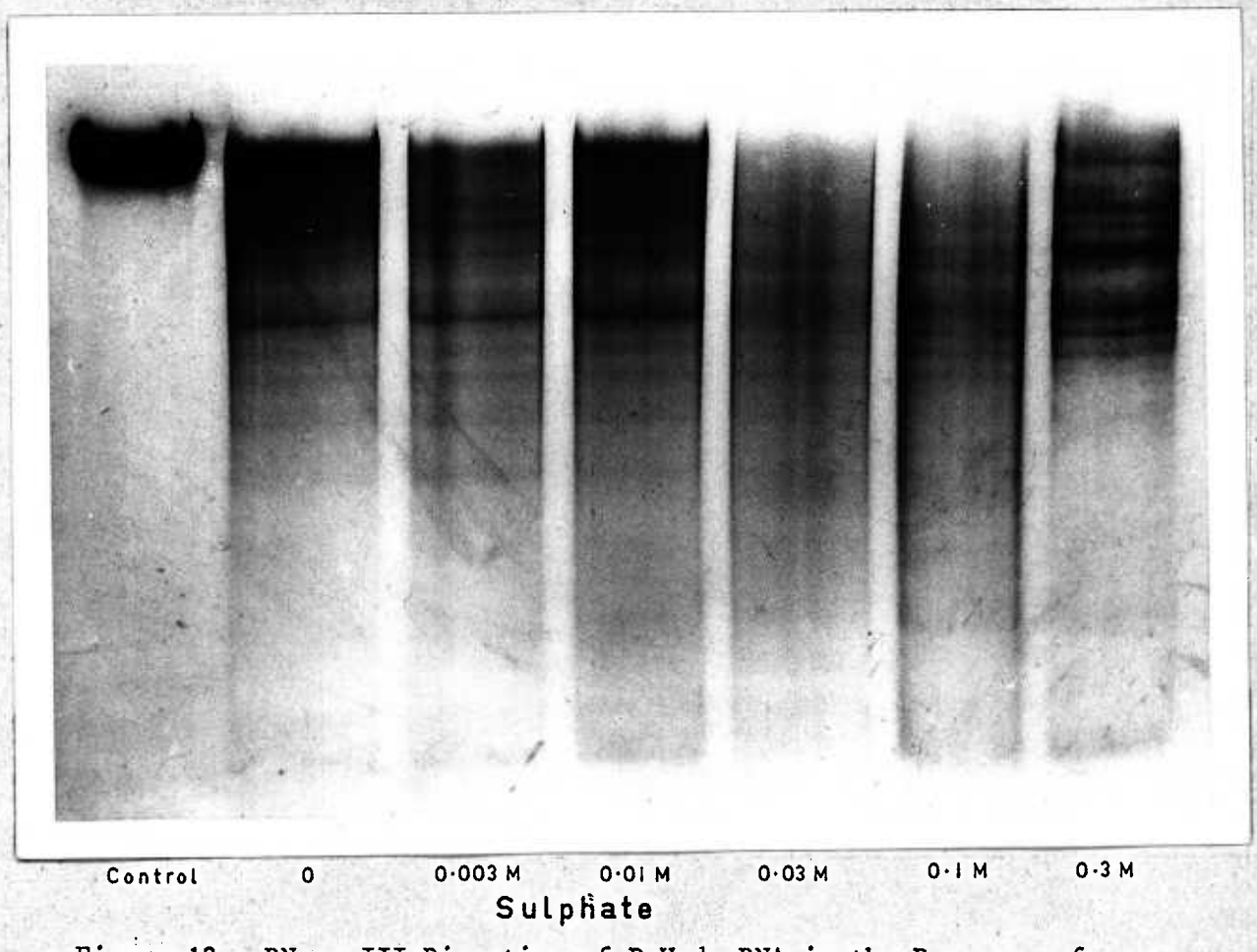


Figure 13 : RNase III Digestion of PcV-ds-RNA in the Presence of Differing Molarities of Ammonium Sulphate or Chloride.

PcV-ds-RNA, batch 5/119, was digested as described in fig. 12, except that the concentration of ammonium sulphate or chloride was varied between 0 and 0.3M.

not specific for sulphate. It must be noted that, at the higher concentration used, sulphate almost completely inhibited the digestion of poly(G).poly(C), and although the PcV-ds-RNA has a base composition of more than 50 % GC, the digestion of the natural ds-RNA was not halved in extent. This either means that the conditions of digestion of a natural ds-RNA differ considerably from those encountered by the enzyme in the digestion of synthetic polynucleotides, or that G:C-rich regions of the natural ds-RNA are not being cleaved in the production of the specific fragments, either in the presence or the absence of added sulphate. Thus it is possible to rule out G:C-rich regions as a basis for the specificity of RNase III.

(iii) 5'-termini of the specific fragments.

The fact that specific fragments are produced at all implies, as mentioned before, that RNase III is specific in its initial cleavages. This initial specificity might be reflected in the 5'-terminal nucleotides of the specific fragments. It is not important for this determination whether the enzyme is specific for the 3'- or 5'-side of its cleavage site. Specificity for the 5' nucleotide will be determined directly, specificity for the 3' by the complementary nucleotide in the opposite strand of the ds-RNA. Determination of the 5'-termini would thus throw light on the basis of this specificity. In discussing the reasons for the non-inhibition of digestion of ds-RNA by the addition of sulphate, it was suggested that G:C-rich regions might not be digested at all in the production of the specific fragments, and this implies that addition of sulphate or another anion, increasing the ionic strength, to the digestion mixture might not alter the chemical nature of the 5'-ends of the fragments, although for some reason the fragments themselves are different.

So, the 5'-terminal nucleotides of the specific fragments have been determined both for simple RNase III digests and for digests done in the presence of ammonium sulphate. The technique used was essentially similar to that described earlier for the study of the 5'-termini of the intact ds-RNA, involving the use of cold fragments labelled by the polynucleotide kinase technique. Dephosphorylation of the 5'-termini was not necessary, as RNase III was shown by Robertson *et al.* (1968) to yield mainly 3'-phosphate and 5'-hydroxyl nucleotides.

The RNase III preparation used in much of the work described in this thesis still contained small amounts of RNA contamination, although much of this was removed by the Enzite digestion step described in the

Methods section 3. This RNA was at a sufficiently low level not to interfere in other experiments, but in the case of 5'-end determination its complete removal was essential. This was because, although the level of contamination was low in comparison to the amount of PcV-ds-RNA fragments, the enzyme-associated RNA was of low molecular weight, and so had very many 5'-ends, sufficient to swamp any labelling of the specific fragments of ds-RNA. To remove this contaminating low MW. RNA a sucrose density gradient technique was used. This was based on the gradient analysis of RNase III by Robertson et al. (1968), who used the method to obtain a molecular weight estimate for the enzyme. They found that the enzyme activity could be recovered from the gradients in almost 100 % yield. This analytical technique has been scaled up so as to be suitable for purification purposes. The enzyme as recovered from the gradients was not very stable but, as shown by the A_{260} and activity analysis of the gradient, fig. 14, was essentially free of RNA. The protein content of these purified enzyme preparations was too low to show a detectable A_{280}.

Even when such highly purified enzyme was used for the digestion, autoradiography of the labelled digests revealed the presence of quite an intense background, see fig. 15. This background material was not visible on the stained gels. The nature of this material is unknown, but it is possibly related to the similar background found on autoradiography of labelled intact ds-RNA.

In this digestion, and in others using the purified enzyme, the difference between patterns produced on digestion with RNase III in the presence and in the absence of sulphate was quantitative rather than qualitative. It is not known how this was related to the further purification of the enzyme. As for the reason mentioned above it was necessary to use such enzyme, determinations of 5'-terminal nucleotides had to be done with digestions producing much less striking differences in band pattern than that shown for example in fig. 12.

To separate some of the individual specific fragments, and also to reduce the amount of background material included in the samples, eight bands were carefully cut out of the gel from the sample prepared in the presence of added sulphate, and a further eight from the sample prepared without sulphate. These fragments were then eluted as described in the Methods section 2. On fig. 15 the bands which were studied are marked with arrows. The eluate from each band was hydrolysed with alkali, and the 5'-terminal nucleotide identified by paper electrophoresis. Figs. 16 and 17 show autoradiographs obtained on examination of the fragments

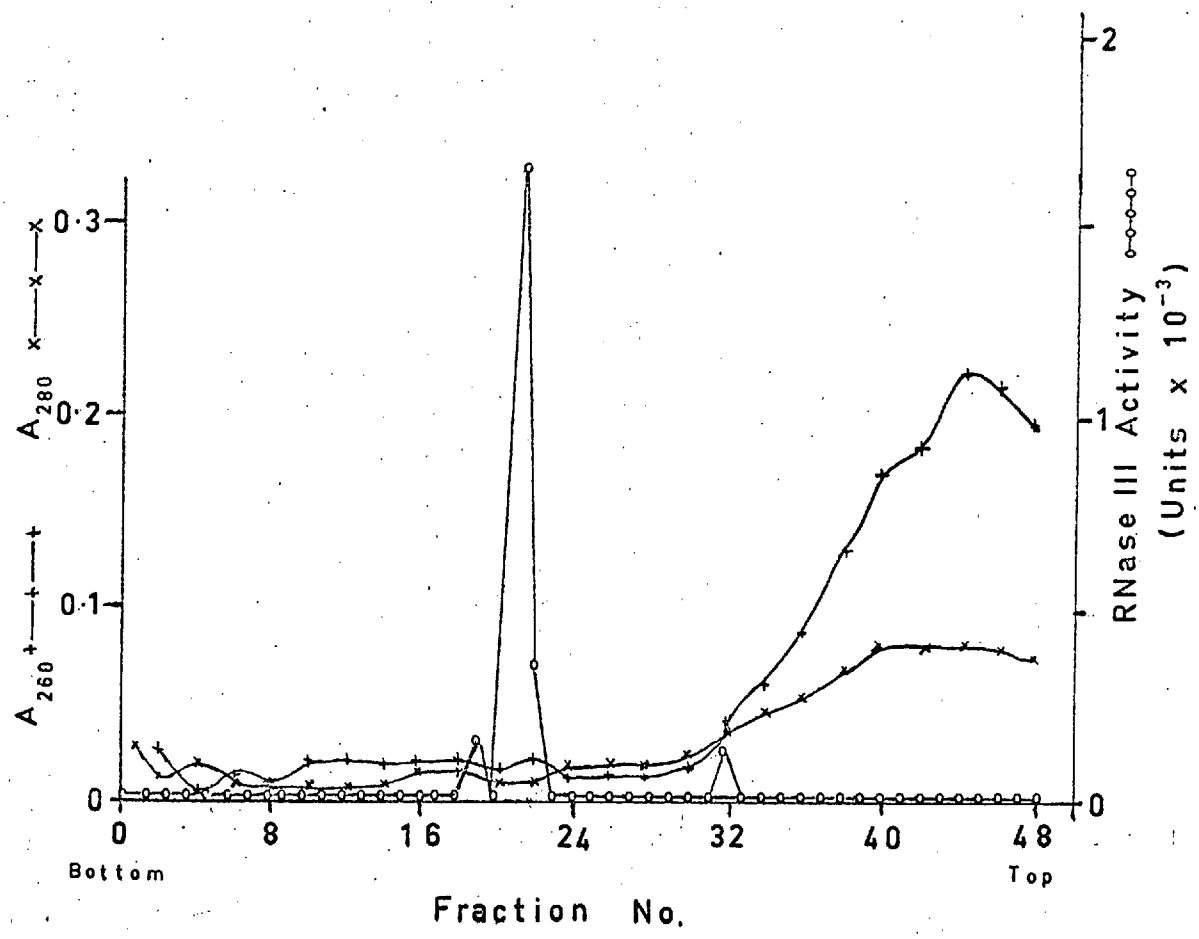


Figure 14 : Sucrose Gradient Purification of RNase III.

1 ml of RNase III, 2900 U, was loaded onto a 37.5 ml 5 % - 20 % sucrose gradient, and centrifuged for 16 hr at 26,000 rpm in an SW 27.1 rotor. 25 drop fractions were collected, and their A_{260} and A_{280} determined. 10 µl samples were assayed for RNase III activity.

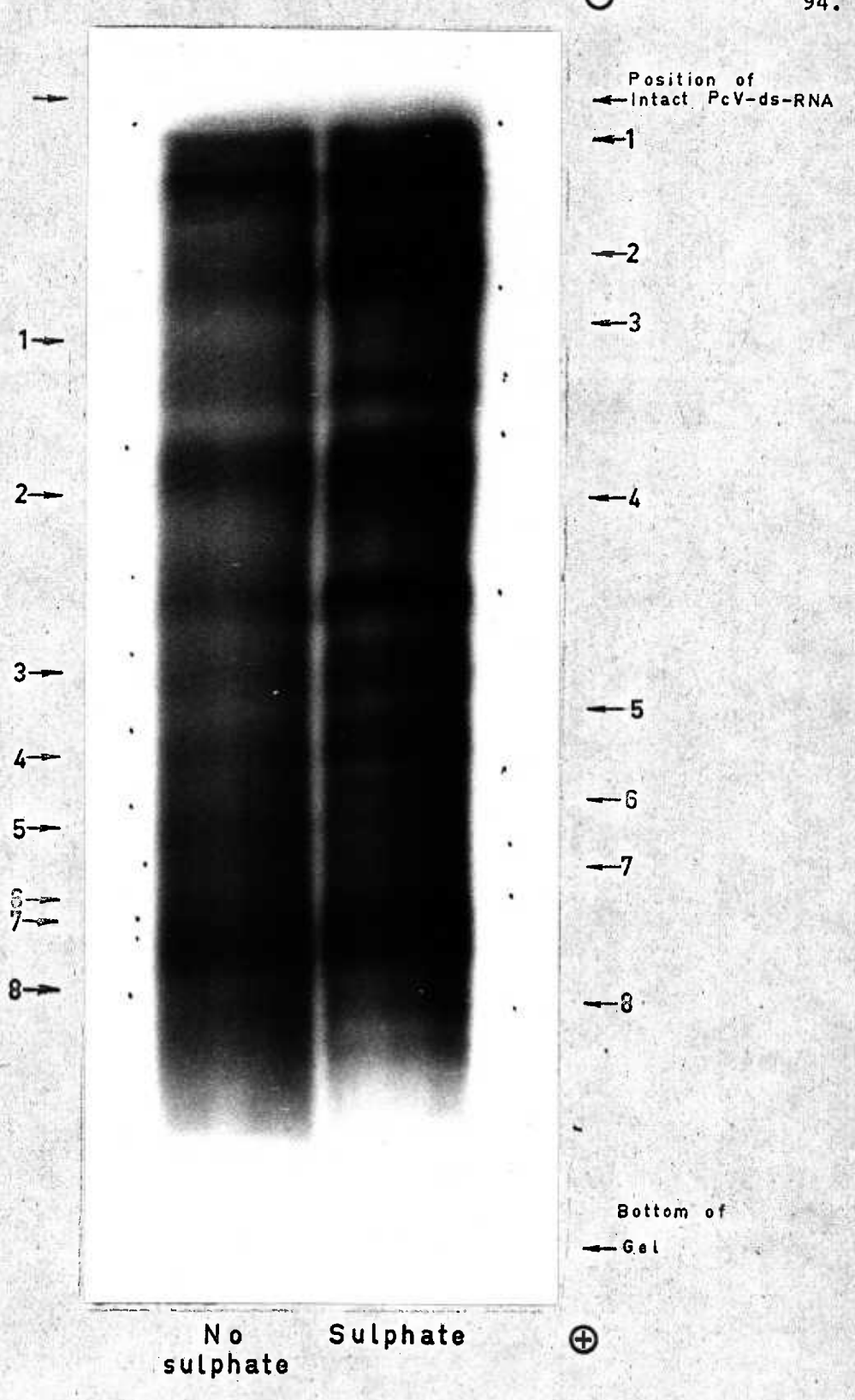


Figure 15 : Autoradiograph of Gel Electrophoresis Patterns of PcV-ds-RNA after Digestion with RNase III in the Presence and Absence of Sulphate.

The preparation of the samples is described in the Methods section. After electrophoresis, the gel was stained with toluidine blue and an autoradiograph made, 16 hr exposure. The bands marked by arrows were then carefully cut out, the RNA eluted and alkali hydrolysed, see figs. 17 & 18. Measurement of the gel and autoradiograph showed that all bands marked above coincided with bands of toluidine blue stained material.

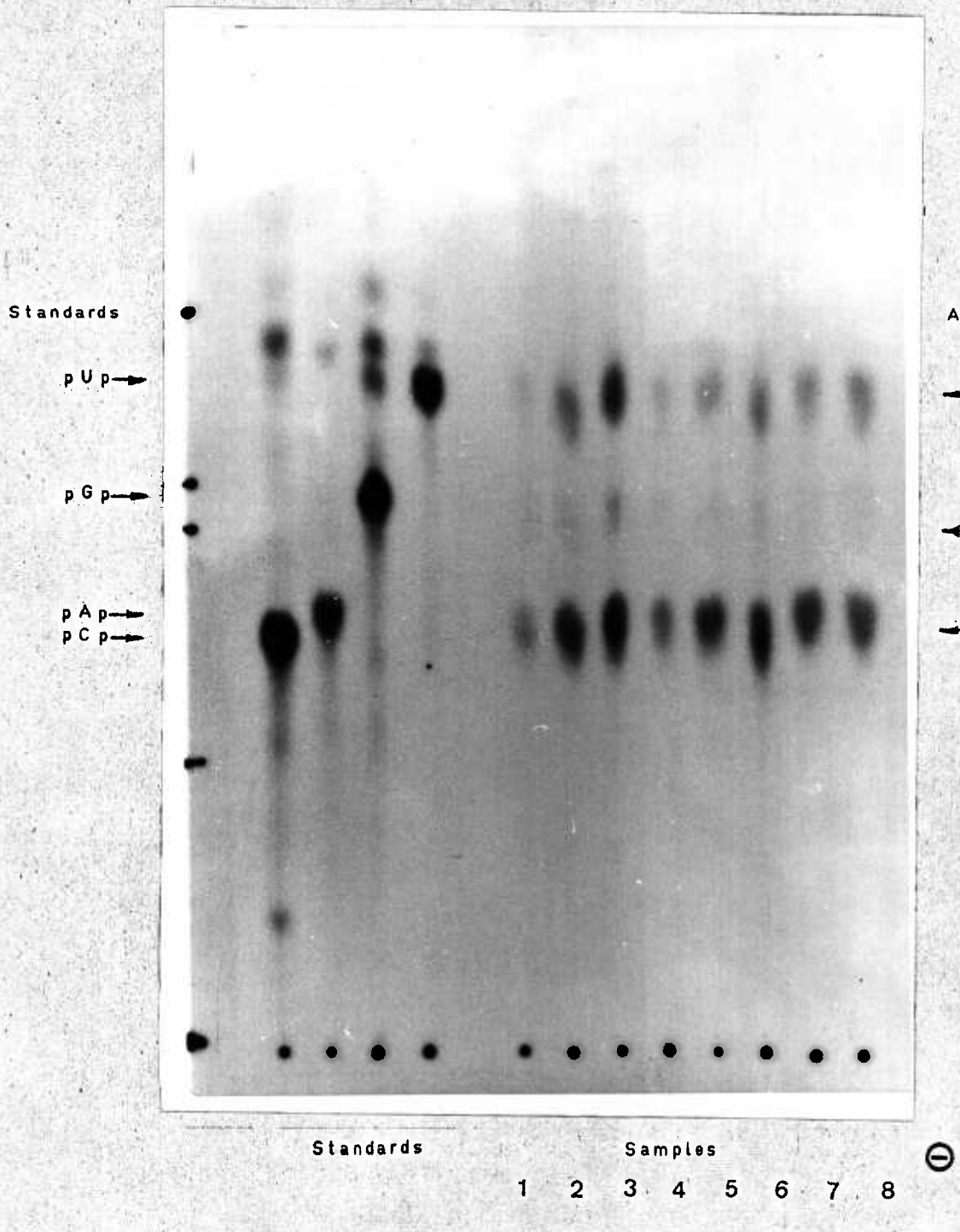


Figure 16 : Autoradiograph of Paper Electrophoretogram, Showing 5'-Ends of RNase III-digested PcV-ds-RNA.

The 8 bands (see fig. 15) cut from the RNase III digest prepared in TM buffer + 0.1M chloride, pH 7.6, were eluted from the gel as described in the Methods, section 2, hydrolysed with 0.2M sodium hydroxide, and run with suitable standards for 1 hr on 3MM paper at 2400 V, 200 mA, solvent pyridine-acetic acid, pH 3.5. After drying, the paper was exposed for 2 weeks with a Kodirex film.

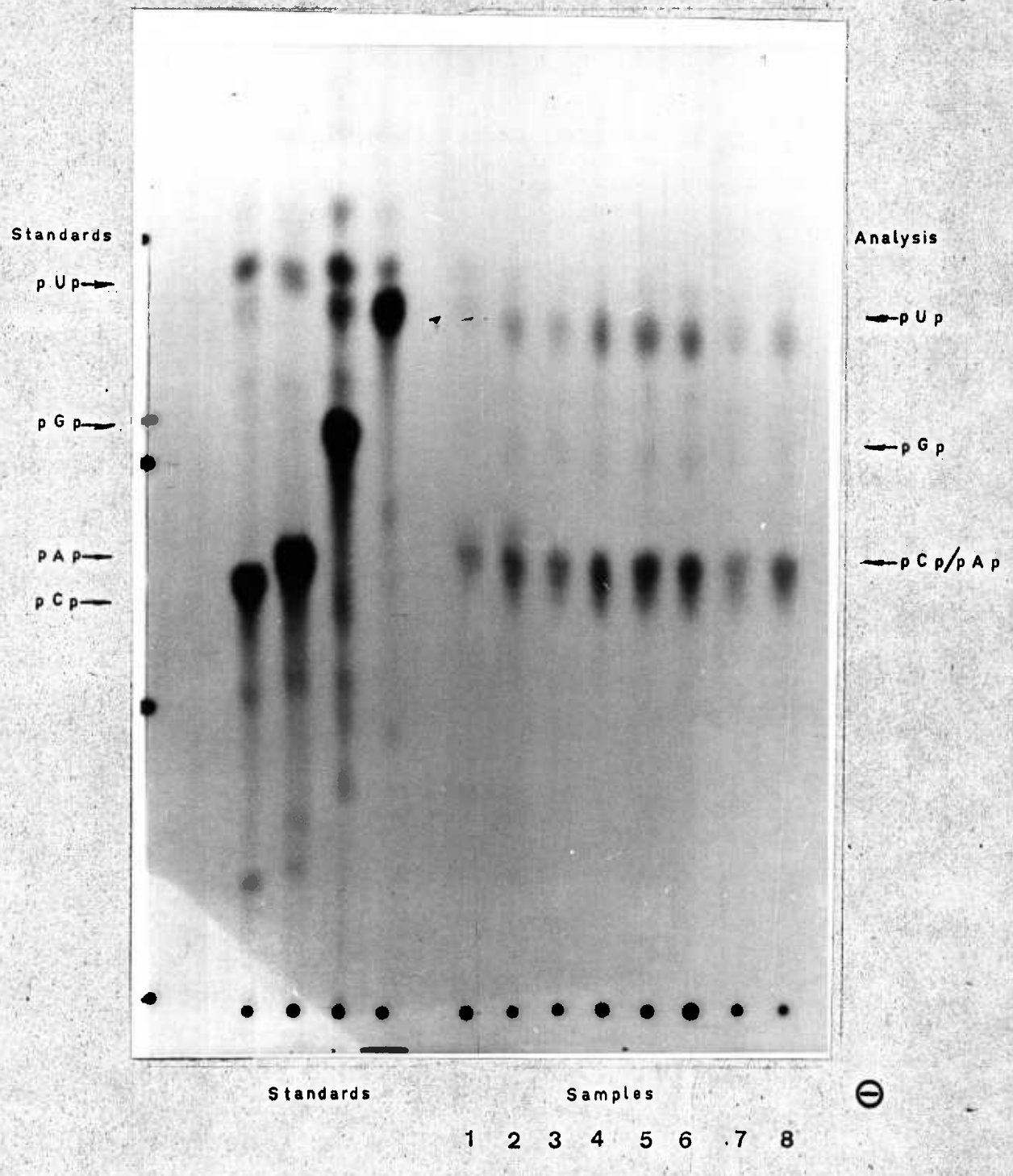


Figure 17 : Autoradiograph of Paper Electrophoretogram, Showing 5'-Ends of PcV-ds-RNA Following Digestion with RNase III in the Presence of Sulphate.

Conditions exactly as described in fig. 16, except that the original digest was prepared in TM buffer + 0.1M ammonium sulphate, not chloride.

marked on fig. 15. All 16 fragments analysed in this experiment had the same, or very similar, terminal nucleotide composition. In a second experiment, where again 8 bands were cut out from each of two samples (some bands were the same as in the first experiment, some different), similar results were obtained.

Each hydrolysate yielded a spot that could be identified by reference to the standards as uridine 3'-5'-diphosphate (pUp), and a second major spot of adenosine 3'-5'-diphosphate (pAp) showing either trailing or fusion with a small cytidine 3'-5'-diphosphate (pCp) spot. (pCp runs just behind pAp in this system). A trace of guanosine 3'-5'-diphosphate (pGp) was also visible in several hydrolysates.

To decide whether pCp was present in the hydrolysates, the pAp/pCp spots were eluted from the papers, and those from the eight digestions prepared with the addition of sulphate were pooled, as were the eight spots from the digestion without sulphate. These two pools were then re-run for a much longer time, so as to improve the separation between pAp and the possible pCp component of the original spots. This paper was then autoradiographed, and the result is shown in fig. 18, where it is clear that in fact the original spots are resolved into two components, the major being pAp, and the minor pCp. Examination of figures 16 and 17 shows that the pAp and the pUp spots were equal in intensity in each sample, at least as judged by eye, and the pCp and pGp spots were of much lower intensity.

As both pCp and pAp were detected in these experiments, the retardation phenomenon (Szekely & Loviny, pers. commun.) noted in Results section I b) is clearly irrelevant in this case.

These experiments reveal with certainty that RNase III shows base-specificity by its action in producing specific cuts in PcV-ds-RNA.

b) Pancreatic RNase digestion of ds-RNA.

Bovine pancreatic RNase is probably one of the best studied of all enzymes (see review by Barnard, 1969). The existence of an RNA-degrading activity in the pancreas was recognised over 50 years ago (Jones, 1920). The enzyme was extracted from the tissue and partially purified by Kunitz (1940), and the RNase A fraction was produced in chromatographically pure form by Hirs, Moore and Stein (1953). The specificity of panc. RNase has been studied in considerable detail, see for example Witzel and Barnard (1962a,b), Follman, Wieker and Witzel (1967), Gassen and Witzel (1967), but the outline of the specificity was

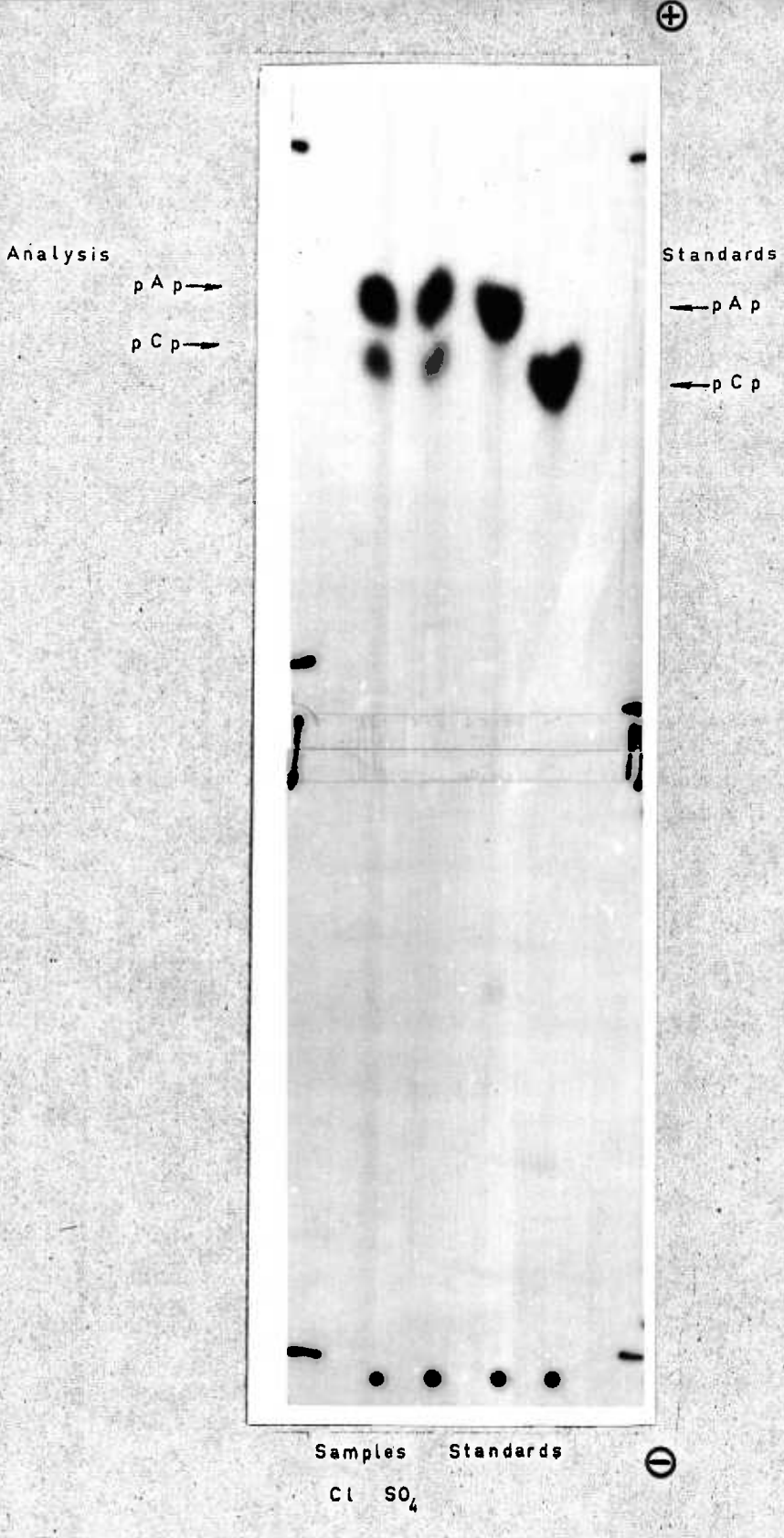


Figure 18 : Autoradiograph of Paper Electrophoretogram, Showing the 5'-Ends of RNase III digested PcV-ds-RNA.

The eight spots labelled pCp/pAp in fig. 16 were pooled, as were eight similarly labelled in fig. 17, and the pools were run together with suitable standards on 3MM paper for 2.5 hr at 2700 V, 200 mA. Exposure was for 1 week on Kodirex film.

worked out many years before. These early workers showed that panc. RNase cleaves only following pyrimidine bases, forming the pyrimidine nucleotide 2'-3'-cyclic phosphates, from which are then produced only 3'-phosphates. Linkages following purine bases, and purine nucleotide 2'-3'-cyclic phosphates are not normally substrates for panc. RNase (Markham & Smith, 1952; Brown, Dekker & Todd, 1952; Brown & Todd, 1953). However, Beers (1960) found that high concentrations of panc. RNase were capable of hydrolysing poly(A), i.e. purine-purine phosphodiester linkages are cleaved if sufficient enzyme is present.

Although ds-RNA is often characterised by its resistance to pancreatic RNase digestion at high salt concentrations (Geiduschek et al., 1962; Gomatos & Tamm, 1963a; Bellamy et al., 1967), it is important to remember that this resistance is only relative. Digestion of ds-RNA still occurs in high salt concentration solutions, although at a greatly reduced rate (Shapiro, Bellamy, August & Joklik, 1966; Bishop, Koch & Levintow, 1967). Bellamy et al. (1967) showed that resistance, as measured by degree of hyperchromic effect, varied with salt concentration and with enzyme:substrate ratio, and that seemingly complete resistance could be obtained by manipulating the conditions correctly. The earlier work was done using more sensitive techniques, measuring the sedimentation velocity (Shapiro et al., 1966) and infectivity (Bishop et al., 1967) of ds-RNA from viruses and virus infected cells. Assays depending on hyperchromicity, or on the liberation of acid- or alcohol-soluble fragments, are unlikely to show any result until the molecular weight of the fragments has been reduced below 1×10^5 daltons (Bernardi, 1968). Since the initial MW. of the ds-RNAs used in studies of RNase-resistance are generally much higher than this, then it is clearly possible for several cleavages to occur before any detectable alteration in hyperchromicity or acid solubility.

C. Dreyer and M. Szekely (pers. commun.) found that if the concentration of enzyme was raised sufficiently, to an enzyme: substrate ratio of 1:10 or even higher, then digestion occurred in SSC (ionic strength 0.195) detectable when the treated ds-RNA was examined by gel electrophoresis. During the digestion under the above conditions, no increase in A_{260} occurred. It is known (Loviny & Szekely, 1973) that if the enzyme:substrate ratio is raised to 10 µg panc. RNase:1 µg ds-RNA, then complete digestion could be achieved, even in SSC, a medium of high salt concentration. In this thesis, studies on the mode of digestion of

ds-RNA by an enzyme, panc. RNase, having a preference, although not an absolute specificity, for ss-RNA are reported.

(i) RNase sensitivity of PcV-ds-RNA batches.

Dreyer and Szekely worked with PcV-ds-RNA batch MPP 63, and it has been found in this study that the different batches of ds-RNA differed considerably in their sensitivity to panc. RNase. This was judged by viscosity measurements, which are at least as sensitive as gel electrophoresis. More recent PcV-ds-RNA batches from Beecham Research Laboratories, e.g. P2 and 5/119, were quite rapidly digested by as little as 1:100 panc. RNase in PBS (ionic strength 0.159). Fig. 19 shows the fall in η_{rel} for various ds-RNA batches, all digested under identical conditions in PBS with 1:50 panc. RNase:RNA at 30°C. After 1 hr a hyperchromic effect was also detectable, see e.g. the results given later in Table 5. The reasons for variation in sensitivity are not known. Sensitive batches of ds-RNA have a very similar Tm curve (in respect of shape, transition width, Tm and hyperchromocity) to less sensitive batches, and both resemble very closely the data for these parameters published by Buck et al. (1971). The ds-RNA of the more sensitive batches was not heavily nicked, which perhaps might alter the secondary structure in some way to increase the panc. RNase sensitivity.

Libonati and Floridi (1969) and Libonati (1971) showed that the dimerised and polymerised forms of pancreatic RNase were much more active against ds-RNA and poly A than was the normal monomeric form of the enzyme. Although the standard technique to prepare such oligomers involves lyophilisation from solution in acetic acid (Crestfield, Stein & Moore, 1962), Libonati (1969) showed that an untreated commercial preparation of panc. RNase, which had been found to have unusual activity against MS 2 replicative form ds-RNA (Billeter et al., 1966), contained oligomers, and that it was these complexes which were responsible for the higher activity. The possibility of this being the case with the panc. RNase sample used in these studies has been considered. A sample of panc. RNase was treated according to the technique of Crestfield et al. (1962) for the depolymerisation of such aggregates and then assayed against ds-RNA. No significant change was found, see Table 5. This does not disprove the possible existence of such oligomers in the panc. RNase sample used here, but it does make the possibility less likely.

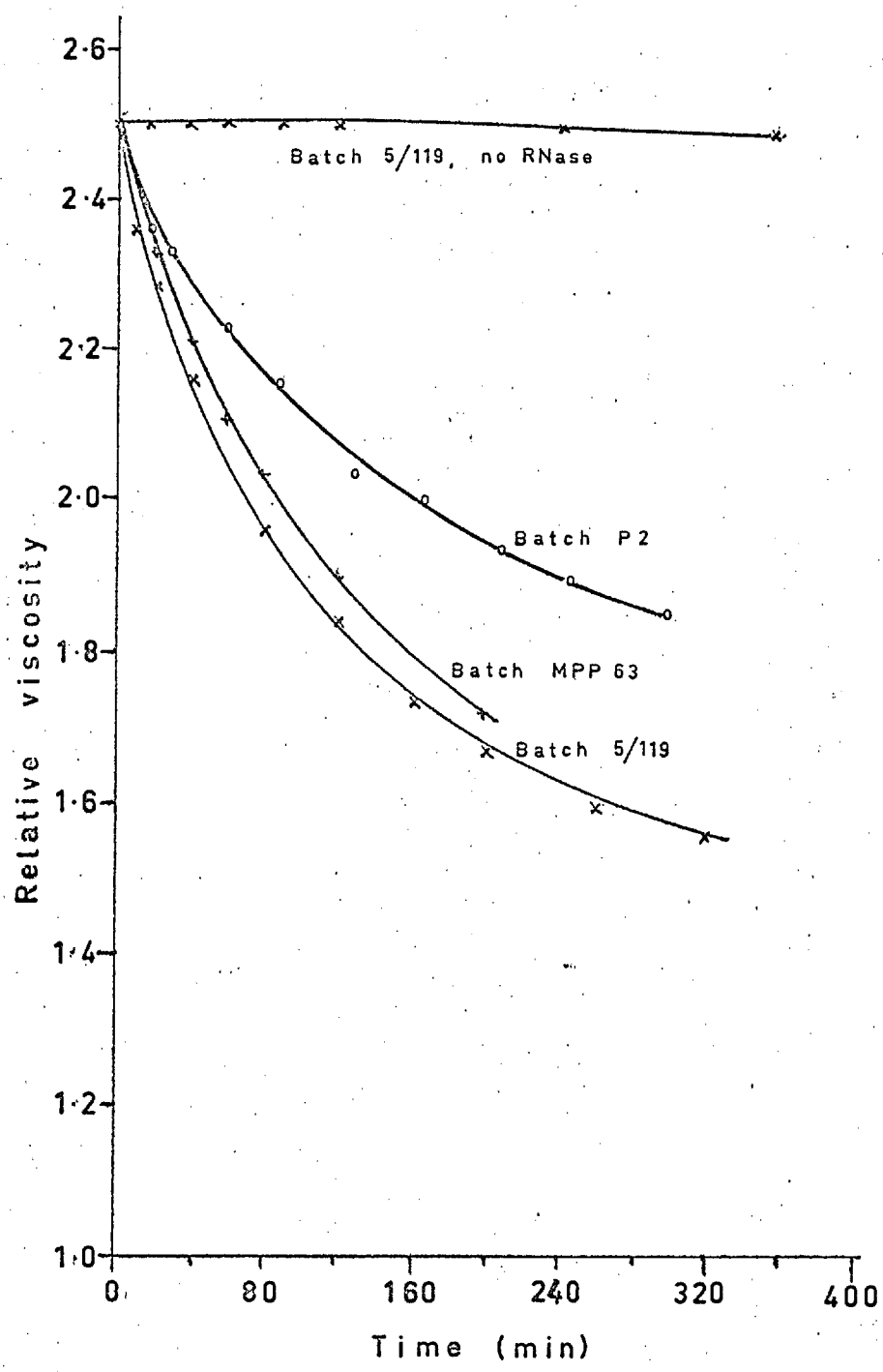


Figure 19 : Rates of Digestion of Different Batches of PcV-ds-RNA by Pancreatic RNase.

All RNA batches were dissolved in PBS, pH 7.2, at 1 mg/ml, and incubated at 37°C in an Ostwald viscosimeter with 20 µg/ml panc. RNase.

TABLE 5.

ACTIVITY OF UNTREATED AND HEAT-TREATED PANCREATIC RNase AGAINST PcV-ds-RNA

RNase	Amount	A_{260}
Untreated	50 µg	0.450
Untreated	100 µg	0.800
Heat-treated	50 µg	0.394
Heat-treated	100 µg	0.760

The indicated amounts of panc. RNase were mixed with PcV-ds-RNA, batch 5/119, so that the final RNA concentration was 500 µg/ml, volume of reaction 1 ml. After 1 hr at 37°C, the nucleic acid was precipitated by the addition of an equal volume of 12.5 % perchloric acid. The precipitate was removed by centrifugation, and the A_{260} of the supernatant determined using a blank prepared by incubation of ds-RNA without enzyme. Heat-treated panc. RNase had been treated according to Crestfield et al. (1962).

Bands of specific fragments on gel electrophoresis have only been observed in one experiment with RNA batch 5/119 when digested with 1:50 panc. RNase:RNA in PBS at 30°C. The band pattern was very clearly different from that obtained by RNase III digestion.

(ii) Mechanism of cleavage of ds-RNA by panc. RNase.

Plots of the relative viscosity (η_{rel}) of ds-RNA samples undergoing digestion by panc. RNase, fig. 19, show no lag period after the addition of the enzyme, before the viscosity starts to fall. As mentioned above, this lack of a lag period is not conclusive, because a very short lag period might not be detected. Because this question of mode of cleavage is important in understanding the activity of panc. RNase against ds-RNA, different techniques have been used to determine whether cleavage of the ds-RNA was haplotomic or diplotomic.

Denaturation experiments, to detect nicking of the individual strands in panc. RNase digestion, have yielded equivocal results. During haplotomic digestion it would be expected that the substrate molecules would be quite heavily nicked, and that on denaturation these nicks would lead to a considerable change in the sedimentation profile, and mean that the average size of the denatured fragments would be less than half the size of the native fragments. If the ds-RNA was degraded diplotomically, then no nicks could be expected, and denaturation would halve the MW. of the fragments, and cause no change in the size distribution curve (although of course the whole would be shifted to lower MWs.). To determine the mode of digestion of panc. RNase, ds-RNA was digested for varying periods with panc. RNase, and the digestion was then stopped by the addition of diethyl pyrocarbonate. Half of each digest was then denatured by reaction with formaldehyde, and the sucrose gradient profiles for native and denatured digests compared, using E.coli r-RNA as S value standards to facilitate comparison between the different runs. Fig. 20 shows the results for such a series of experiments. As digestion proceeds, it appears that the curve obtained for the denatured samples gets progressively broader than the curve for the corresponding native digest. However, if the size of the fragments is measured, a different result is obtained. If the sedimentation value of the very peak of a native digest is compared with the peak S value for the corresponding denatured digest, then a graph such as fig. 21 results. On this plot are also shown two other, calculated curves. One is the result expected if the equations relating MW. and S value for ds- and ss-DNA are used to predict

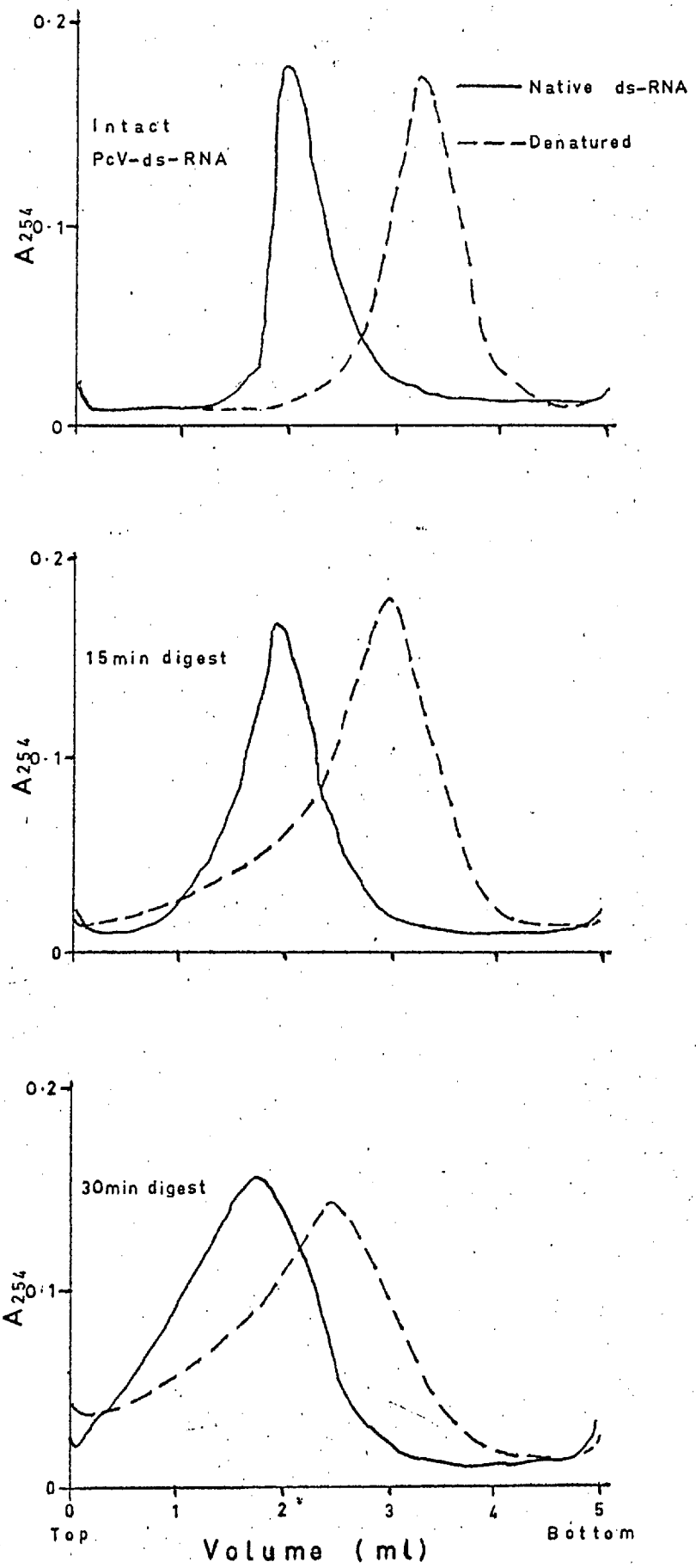


Figure 20 : continued overleaf.

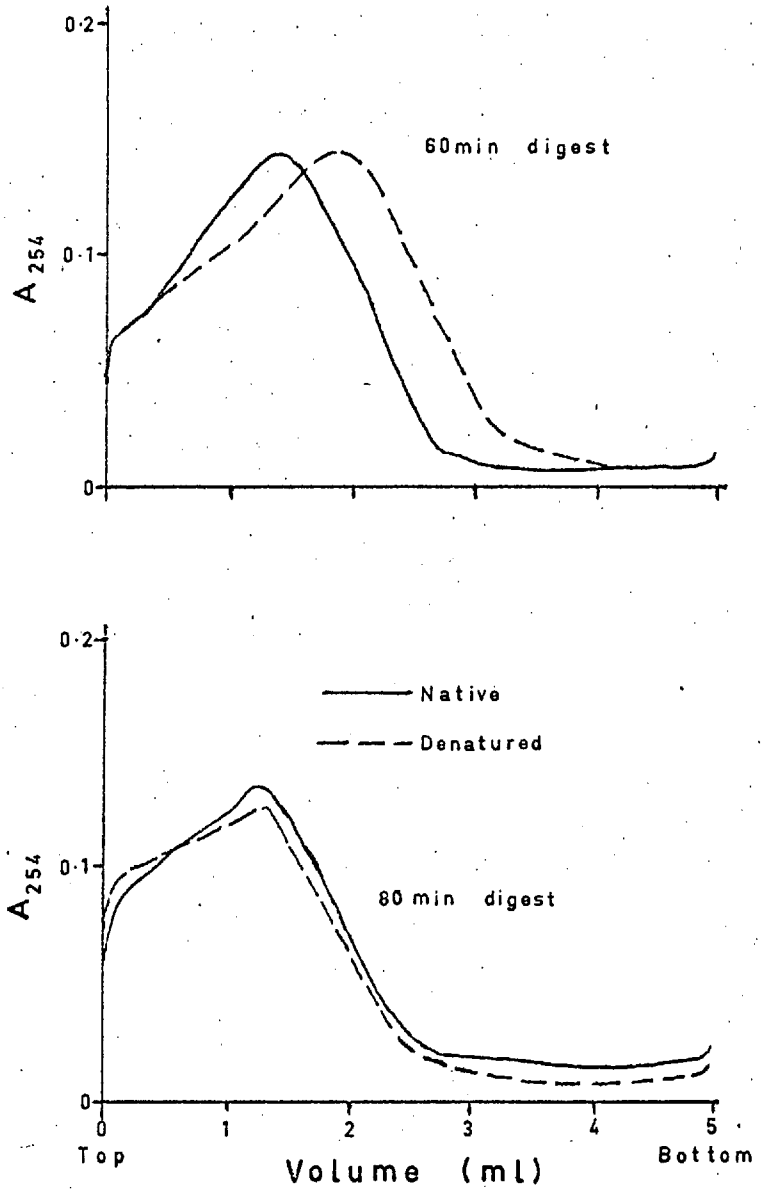


Figure 20 : Sucrose Density Gradient Analyses of Native and Denatured Pancreatic RNase-digested PcV-ds-RNA.

RNA 5/119, 1 mg/ml in PBS, was digested with panc. RNase (20 µg/ml) for the times indicated. The digestion was stopped by the addition of 10 µl/ml DEP, followed by phenol extraction and ethanol precipitation. The washed precipitates were dissolved at 1 mg/ml in 0.1M phosphate buffer, pH 7.8, and divided into 2 portions. To one was added 1.8 % neutralised formaldehyde, and both were heated to 80°C for 8 min, then cooled in ice-water. It is assumed that, like intact PcV-ds-RNA, heating alone does not alter the sedimentation profile (compare fig. 20 i) and ii)). 20 µg samples were then run on 5 ml 5 % - 20 % sucrose density gradients for 16 hr at 25,000 rpm in an SW 50.1 rotor.

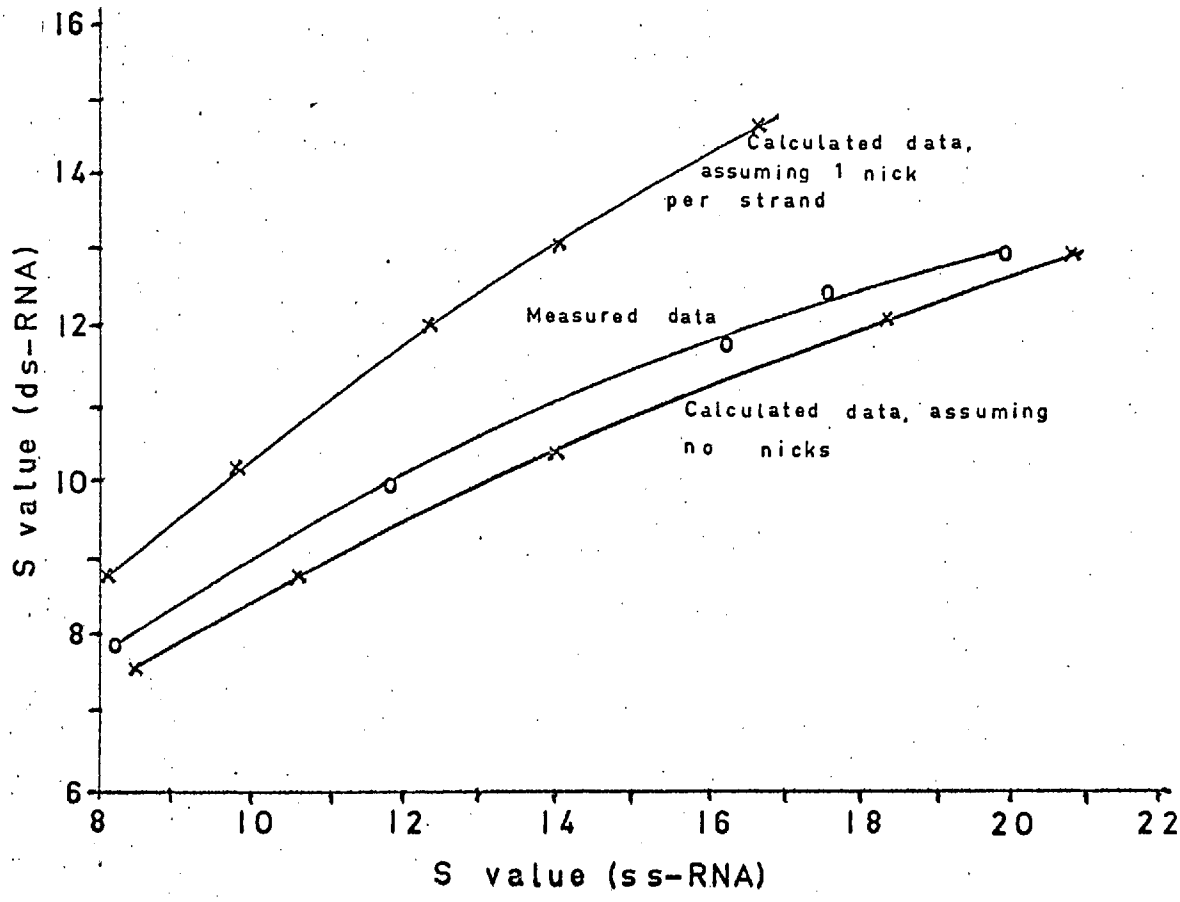


Figure 21 : Peak S Values of Native and Denatured Pancreatic RNase Digests.

S values of the peaks in fig. 20 are plotted here. Also given are calculated data for unnicked digests with one, randomly placed nick per strand. These were calculated in the following way. From the S value of the native digest was calculated an estimate of MW., using the equation of Eigner and Doty (1965). This estimate was then divided by 2 to give a MW. estimate for the constituent unnicked single strands. MW. estimates were divided by 4 to simulate one, randomly placed, nick in each strand. Approximate S value predictions were then made from these MW. estimates by use of the equation of Studier (1965).

the result obtained by the denaturation of an unnicked digest. This curve was calculated in the following way. The S value of the peak of the native digest was converted to a MW. estimate using the equation of Eigner and Doty (1965), and this result then divided by two to represent the halving of MW. that is expected on the denaturation of an unnicked ds-molecule. This result was then converted back to an estimated sedimentation coefficient for ss-RNA by use of the empirical relationships of Studier (1965), and the S value prediction plotted against the value determined for the native digest. This line does not correspond exactly with that obtained by experiment, but the calculated and experimental lines are parallel. The lack of exact correspondence is perhaps due to the use of equations derived from work on DNA. The other calculated curve shown on the figure was determined as that described above, except that one nick per strand in each fragment was assumed. To make an estimate of the effect of a single nick in each strand, MW. estimates for ds-RNA were calculated for convenient S values (using the equation of Eigner & Doty, 1965) and were divided by two, to represent the halving of MW. that is expected on the denaturation of an unnicked ds-molecule, and then by two again to simulate the effect of a single randomly placed cleavage in each single strand. (It is appreciated that this is a simplistic model, and that there is no experimental evidence to support a random cleavage.) The estimated MW. was then converted back to a prediction of S value using the relationship of Studier (1965), and plotted as described above. The purely experimental result is intermediate between these two predictions, and is as mentioned before parallel to the plot calculated for unnicked ds-fragments. As mentioned before, the peak for denatured samples is generally broader than that of the native digests, and this indicates that some nicking occurs, but from the data obtained from the size estimates it is possible to conclude that diplotomic cleavage also occurs.

So, to determine more exactly whether panc. RNase acts diplotomically or haplotomically, the intrinsic viscosities of a series of digests were measured, and from this data the number of hits necessary to cleave the ds-RNA molecule were calculated, as described above (Results section 2 a) (i)) for RNase III digestion. The result of such calculations is shown in fig. 22, which is a plot of the data of Table 6. The best straight line was calculated and found to have a slope of 1.27. This indicates that, like RNase III, the digestion of ds-RNA by panc. RNase is mainly by diplotomic cleavage.

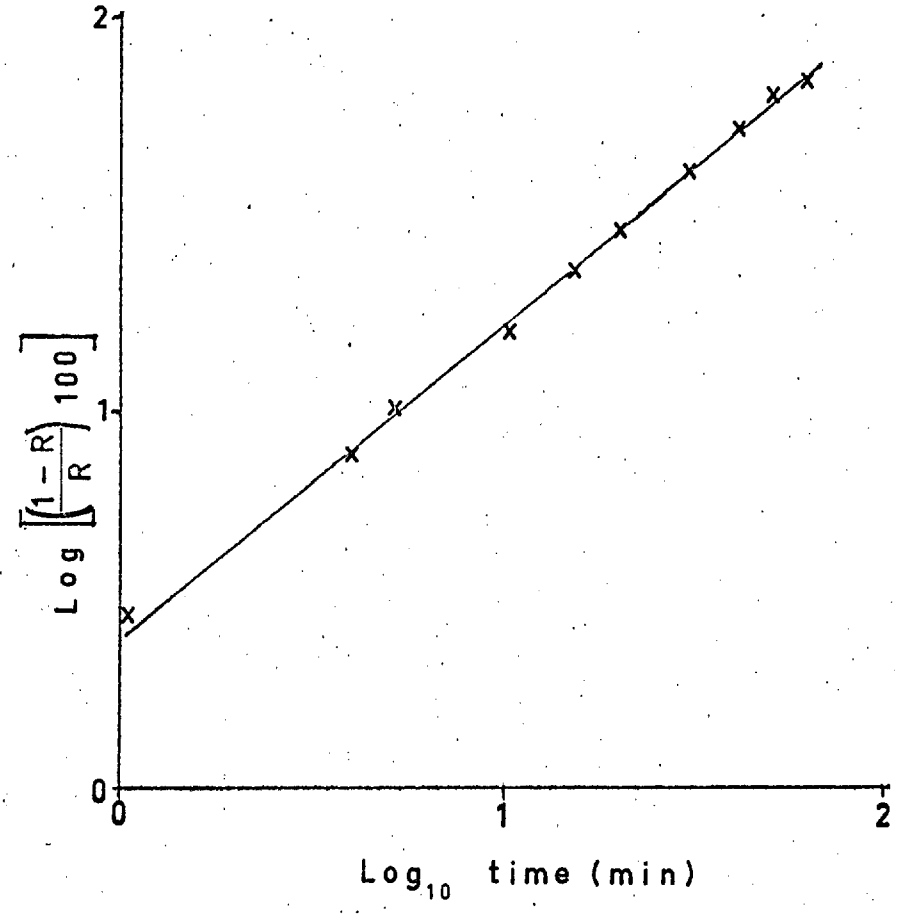


Figure 22 : Determination of the Mode of Cleavage of PcV-ds-RNA by Pancreatic RNase.

A plot according to Cavalieri and Rosenberg (1961), of the data given in Table 6. The slope of the best straight line representing the number of enzyme 'hits' necessary to cleave the double strand, calculated by the method of Least Squares, is 1.27.

TABLE 6.

VISCOSIMETRIC DATA ON THE FRAGMENTATION OF PcV-ds-RNA BY PANCREATIC RNase

Time (min)	$[\eta]$	MW. (daltons x 10^{-6})	R	$\log\left[\left(\frac{1-R}{R}\right)100\right]$	log t
0	12.78	1.43	1.000	-	-
1	12.25	1.39	0.972	0.44	0.00
4	11.55	1.33	0.930	0.88	0.60
7	10.95	1.28	0.895	1.07	0.85
10	10.50	1.24	0.867	1.18	1.00
15	9.71	1.17	0.818	1.35	1.18
20	9.25	1.12	0.783	1.44	1.30
30	8.31	1.02	0.713	1.60	1.48
40	7.47	0.95	0.664	1.70	1.60
50	6.83	0.90	0.629	1.77	1.70
60	6.33	0.85	0.594	1.83	1.78

50 ml of ds-RNA 5/119 (1 mg/ml in PBS) was warmed to 37°C and then mixed with 1 mg panc. RNase. 4 ml samples were taken at the times mentioned above, and mixed with 10 µl DEP and 0.4 ml 10 % SDS, and then extracted with 4 ml of water-saturated phenol. Following phenol extraction and ethanol precipitation the samples were dissolved in 5 ml of PBS, and the nucleic acid concentration determined by measurement of A_{260}. It was assumed that the hyperchromic effect on such limited digestion would be negligible. The viscosities were determined at 4 concentrations, as described in the Methods (section 10). MW. estimates were made using the equation of Eigner and Doty (1965).

$R = MW._t/MW._o$

c) Ultrasonic irradiation of ds-RNA.

The effect of ultrasonic irradiation (ultrasonication) on DNA was first observed by Goldstein and Stern (1950), who found a very rapid fall in viscosity during an initial period of irradiation, and then as time progressed the rate of fall decreased until the viscosity reached a final limiting value. Their results were confirmed by the work of Lee and Peacocke (1951), who concluded, on the basis of titration data, that the fall in viscosity on ultrasonication was the result of the rupture of hydrogen bonds, and that only limited cleavage of internucleotide linkages occurred. This conclusion was disputed by Laland, Overend and Stacey (1952), for their finding that U.V. absorption of DNA showed no hyperchromic effect even on prolonged irradiation, when the molecule was extensively degraded, with a considerably reduced viscosity, led them to consider that ultrasonication caused cleavage across each extended DNA molecule (or fragment) at a random point near its centre. Thus, a DNA molecule was considered to be fragmented into very approximate halves, then quarters, eighths, and so on. This was also found by Freifelder and Davison (1962).

Doty, McGill and Rice (1958) concluded from a study of the light-scattering and hydrodynamic properties of ultrasonicated DNA that even after prolonged irradiation the degree of denaturation was negligible, and that cleavage was almost entirely by the scission of both strands of the double helix at once. There was less than 1 single-strand scission per 1000 residues. Their finding that cleavage of both strands occurred simultaneously was confirmed by examination of ultrasonicated DNA in the electron miscroscope (Hall & Litt, 1958). They found that the fragments had abrupt ends, which they took to indicate that ultrasonication cut both strands at the same point, or at least very close. However, very short ss tails would probably not be seen by such an examination. Further evidence of the lack of significant denaturation was furnished by Marmur and Doty (1962), who found that ultrasonication of DNA caused only a slight fall in the Tm. Richards and Boyer (1965) found sonication cleavage of DNA to occur 90 % through C-O bond breakage and 10 % P-O bond breakage, with no detectable C-C bond cleavage. Breakdown almost certainly occurred by simple backbone scission.

The mode of action of ultrasonication on DNA was re-investigated recently by Obrenovitch and Aubel-Sadron (1971). These authors found from a study of the MW. of ultrasonicated DNA (and treating these data by the techniques of Schumaker et al., 1965, and Cavalieri & Rosenberg, 1961, as detailed above), that cleavage was purely diplotomic, at least in the first minutes of irradiation.

The action of ultrasonic waves on ds-RNA has been noted by Lampson et al. (1970) and by Shiokawa and Yaoi (1972). Both of these groups found that ultrasonication reduced the size of poly(I).poly(C), as measured by viscosity, sedimentation coefficient or gel filtration behaviour, but did not study the action in any more detail. Some of the effects of ultrasonic irradiation on ds-RNA were therefore studied. The mode of action has been shown to involve diplotomic cleavage, and the effects of sample volume and nucleic acid concentration have been determined.

Gel electrophoresis of ultrasonicated PcV-ds-RNA results in long undifferentiated smears as shown in fig. 23. As expected from data published on DNA, no specific fragments were produced. (Cleavage, although near the middle of each fragment, occurs at random sites around the centre.) However, the tendency for cleavage to occur in the central area of the molecule is reflected on these gels by the presence of a clear area beneath the residual intact ds-RNA, and above the first fragments. This clear area can be seen in four of the samples shown in fig. 23, and is marked by an arrow.

Viscosimetry of ultrasonicates showed that there was no lag period after commencement of irradiation before double-stranded cleavage occurred; see fig. 24 for a plot of the relative viscosity fall. The initial viscosity fall is very precipitous, indicating that early cleavage is very rapid, and thus it is very unlikely that any existing lag period would be detected. To test whether ultrasonication did in fact cleave both strands of the ds-RNA at once, the intrinsic viscosities of a series of ultrasonicates were determined (Table 7). These data were converted to approximate MW. estimates by use of the empirical equation of Eigner and Doty (1965). This information was then used in calculations according to Schumaker et al. (1956) and Cavalieri and Rosenberg (1961), and the results are plotted in fig. 25. The slope of the best straight line is 1.08, which, within experimental error, shows that ultrasonication fragments ds-RNA purely by diplotomic cleavage.

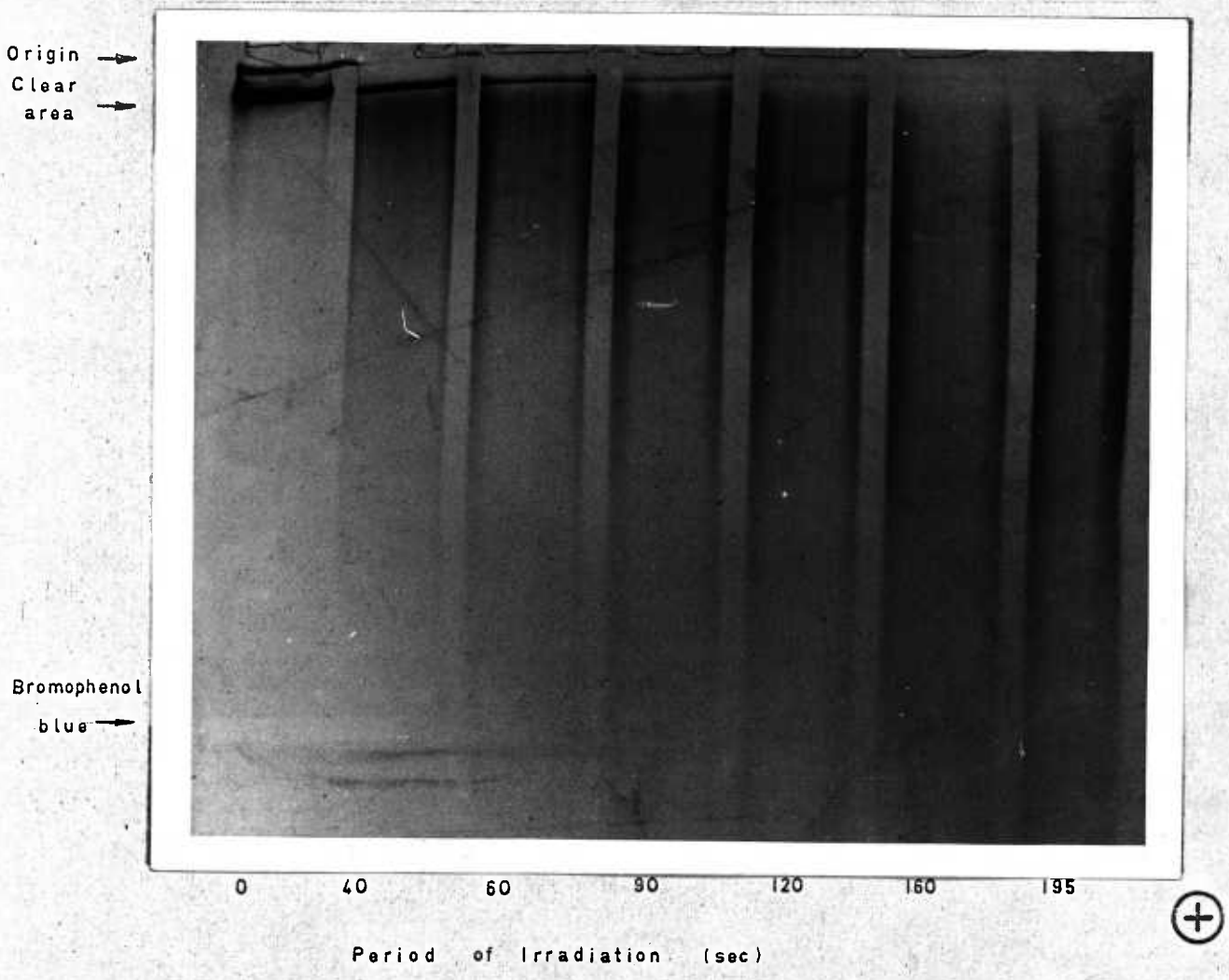


Figure 23 : Ultrasonic Fragmentation of PcV-ds-RNA.

3 ml samples of PcV-ds-RNA, at 1 mg/ml in PBS were irradiated for the times indicated above, centrifuged, and 20 µl samples were run on a polyacrylamide gel.

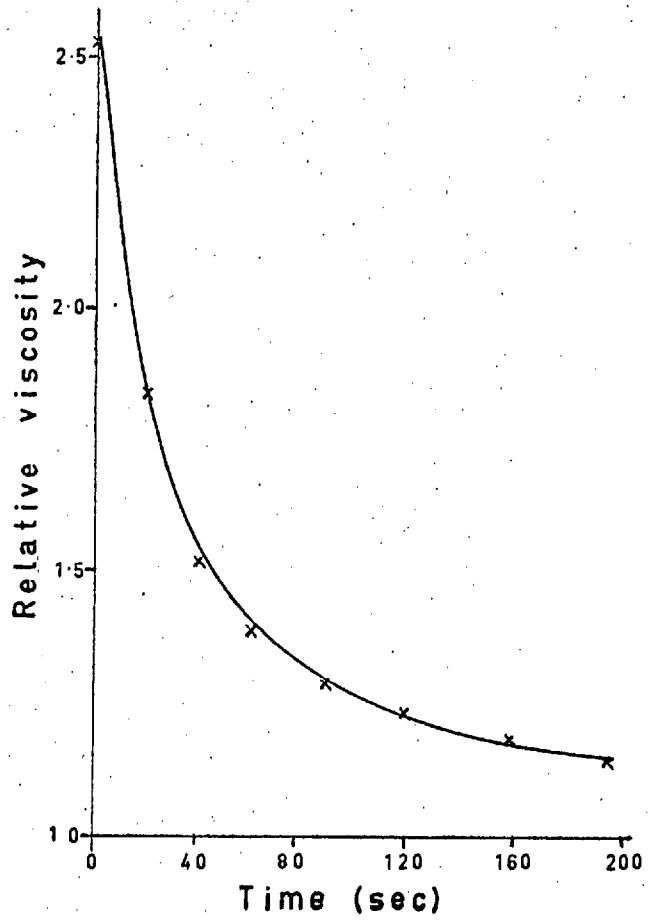


Figure 24 : Fall in Relative Viscosity of PcV-ds-RNA on Ultrasonic Irradiation.

RNA, batch MPP 63, was irradiated at a concentration of 1 mg/ml in PBS, 3 ml volume, with a 9 mm diameter probe. Transducer amplitude 7 μm peak to peak. The samples were centrifuged to remove any particles thrown off from the probe, warmed to 30°C and the viscosity determined as described in the Methods section.

TABLE 7.

VISCOSIMETRIC DATA ON THE FRAGMENTATION OF PcV-ds-RNA BY ULTRASONICATION

$[\eta]$	MW. ($\times 10^{-6}$)	R	$\log\left[\left(\frac{1-R}{R}\right)\right]$	t (sec)	log t
13.1	1.44	1	-	0	-
12.1	1.36	0.943	0.77	12	1.08
11.5	1.31	0.909	1.03	15	1.18
10.8	1.25	0.870	1.16	20	1.31
10.5	1.22	0.847	1.26	24	1.38
9.3	1.10	0.763	1.49	40	1.60
8.8	1.05	0.729	1.57	60	1.78
7.0	0.88	0.613	1.80	90	1.95
6.6	0.84	0.586	1.85	120	2.08
4.3	0.61	0.426	2.13	165	2.22
3.0	0.44	0.304	2.36	220	2.34
1.5	0.27	0.186	2.64	435	2.64

PcV-ds-RNA, batch 5/119, was irradiated in 4 ml volumes, concentration 1 mg/ml, for the times indicated. The solvent was PBS. After centrifugation, the intrinsic viscosity of each ultrasonicate was determined.

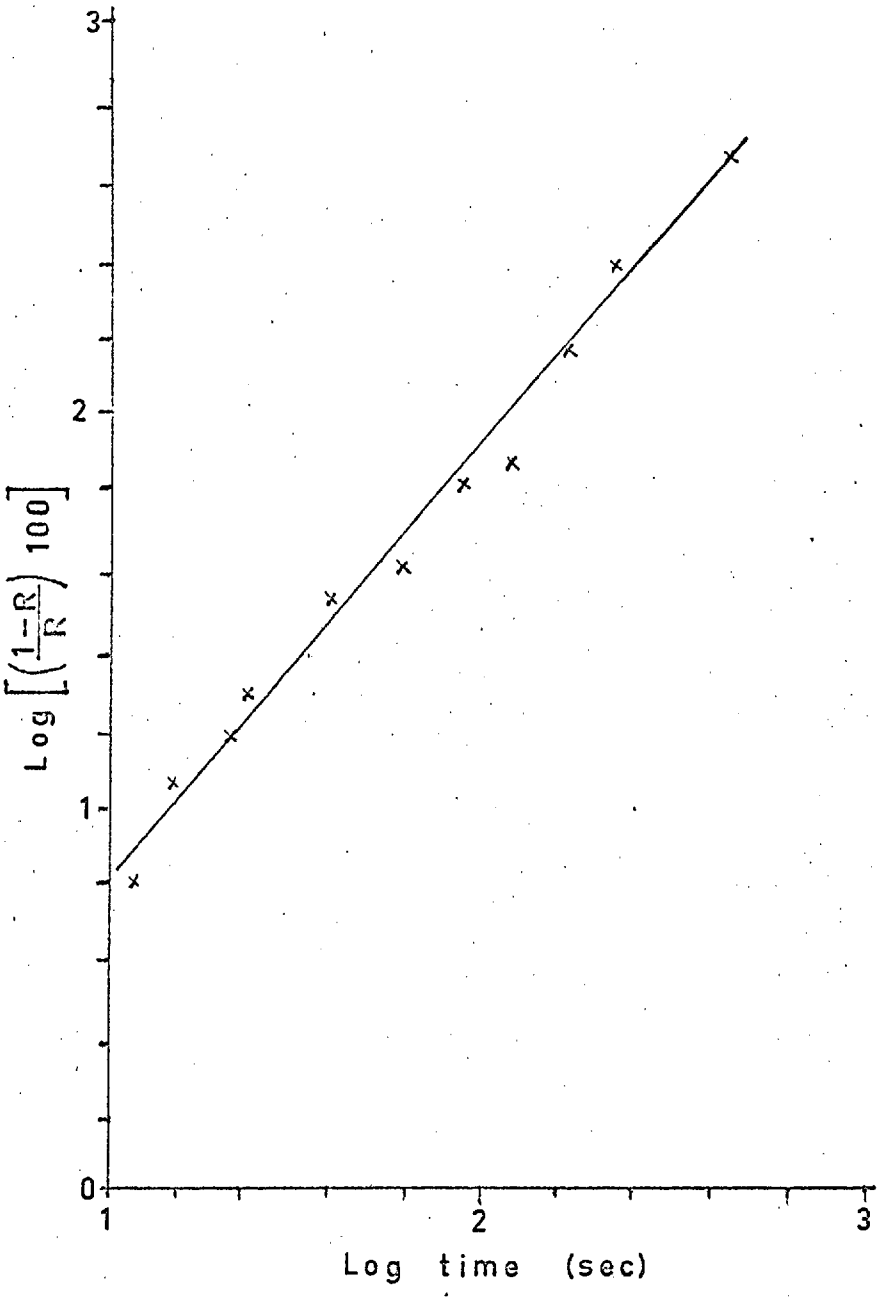


Figure 25 : Determination of the Mode of Cleavage of PcV-ds-RNA by Ultrasonic Irradiation.

A plot according to Cavalieri and Rosenberg (1961), of the data given in Table 7. The slope of the best straight line, representing the number of 'hits' necessary to cleave the double strands, is 1.08.

The effect of varying the volume of sample irradiated at a fixed concentration is shown in fig. 26, whilst fig. 27 shows the effect on the fall in viscosity of varying the sample concentration. Obviously, the data on concentration effects must be plotted as intrinsic viscosities, whilst for the effect of volume relative viscosity is adequate in giving a true estimate of the effect. Increasing either sample volume or concentration appears to decrease the efficiency of fragmentation, possibly because both alterations result in a lowering of the input energy per molecule. Also in the case of the concentration effect, increasing the amount of ds-RNA per ml increases the viscosity of the unirradiated sample. A similar self-protective effect of high DNA concentration was noted by Hershey and Burgi (1960) in work on the fragmentation of T2 phage DNA by stirring, but there is no report of systematic studies on the effects of sample volume and concentration on the fragmentation of DNA by ultrasonic irradiation. Thus it is not possible to assess the effects of the lower rigidity of ds-RNA as compared with DNA.

The conclusion which can be drawn from this work on ultrasonication of ds-RNA is that it responds in very similar ways to DNA, which is not unexpected considering the nature of the fragmentation process.

3. The Production of ds-RNA Fragments for Biological Assay.

Interest in the fragments of ds-RNA produced by the three methods of digestion used in this study arose from an interest in their biological activity, as compared with the intact ds-RNA. In this way, it was hoped that information on the effects of molecular size on the IF-inducing, antiviral, and toxic properties of ds-RNA would be obtained.

For this comparison of intact and fragmented PcV-ds-RNA, the effects of three fragmentation techniques were studied, in order to reduce the likelihood of any errors arising due to the method in which the fragments were produced.

Because intact PcV-ds-RNA contaminating a digested sample might have a disproportionate effect on the biological properties tested, it was considered important that such contamination with undigested material was kept to a minimum. On the other hand, it was desired that the fragments resemble intact PcV-ds-RNA as closely as possible, taking into account their mode of production, and so they should be as large as possible.

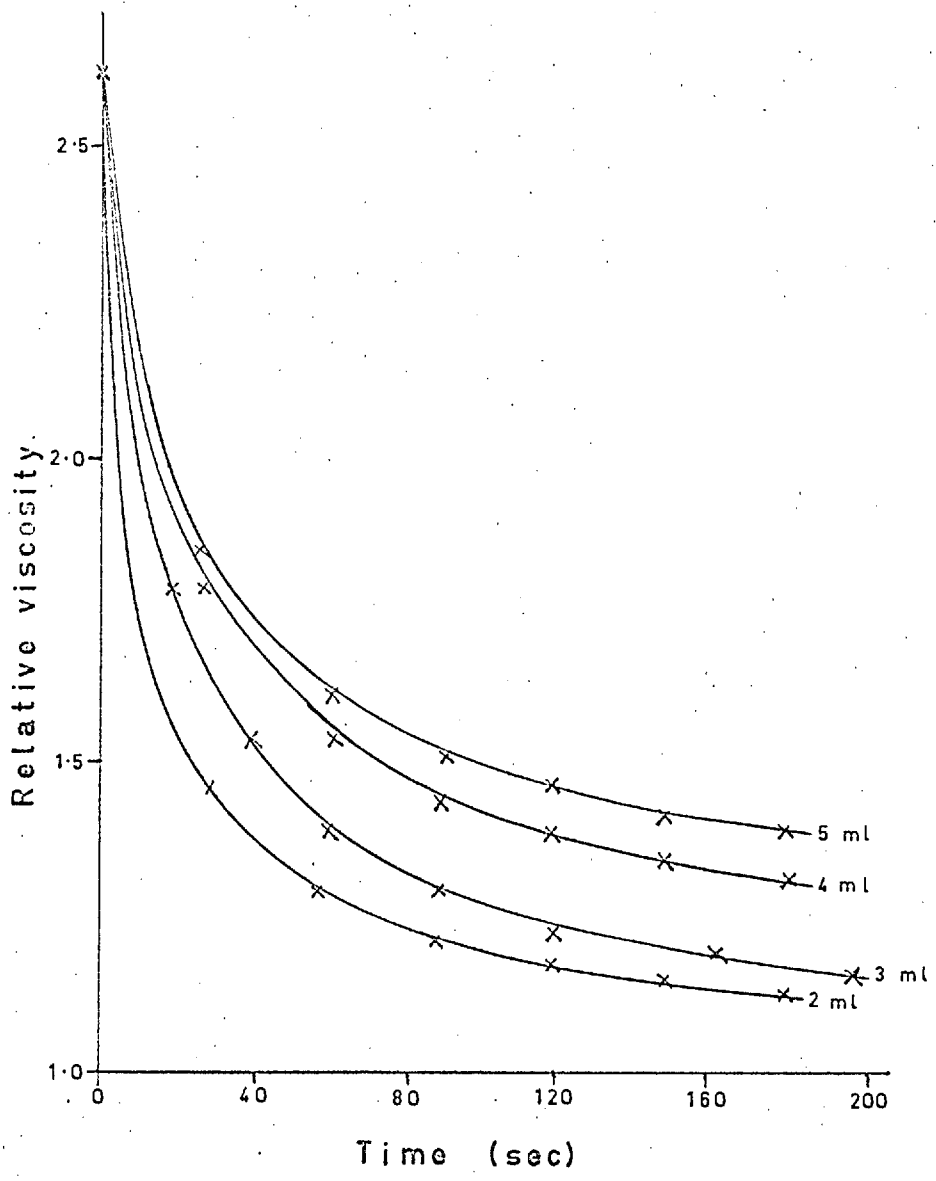


Figure 26 : Effect of Sample Volume on the Fragmentation of PcV-ds-RNA by Ultrasonic Irradiation.

Different volumes of RNA batch 5/119 solution, at a concentration of 1 mg/ml in PBS, were irradiated with a 9 mm diameter probe, transducer amplitude 7 μm. The relative viscosity was determined as described in the Methods section.

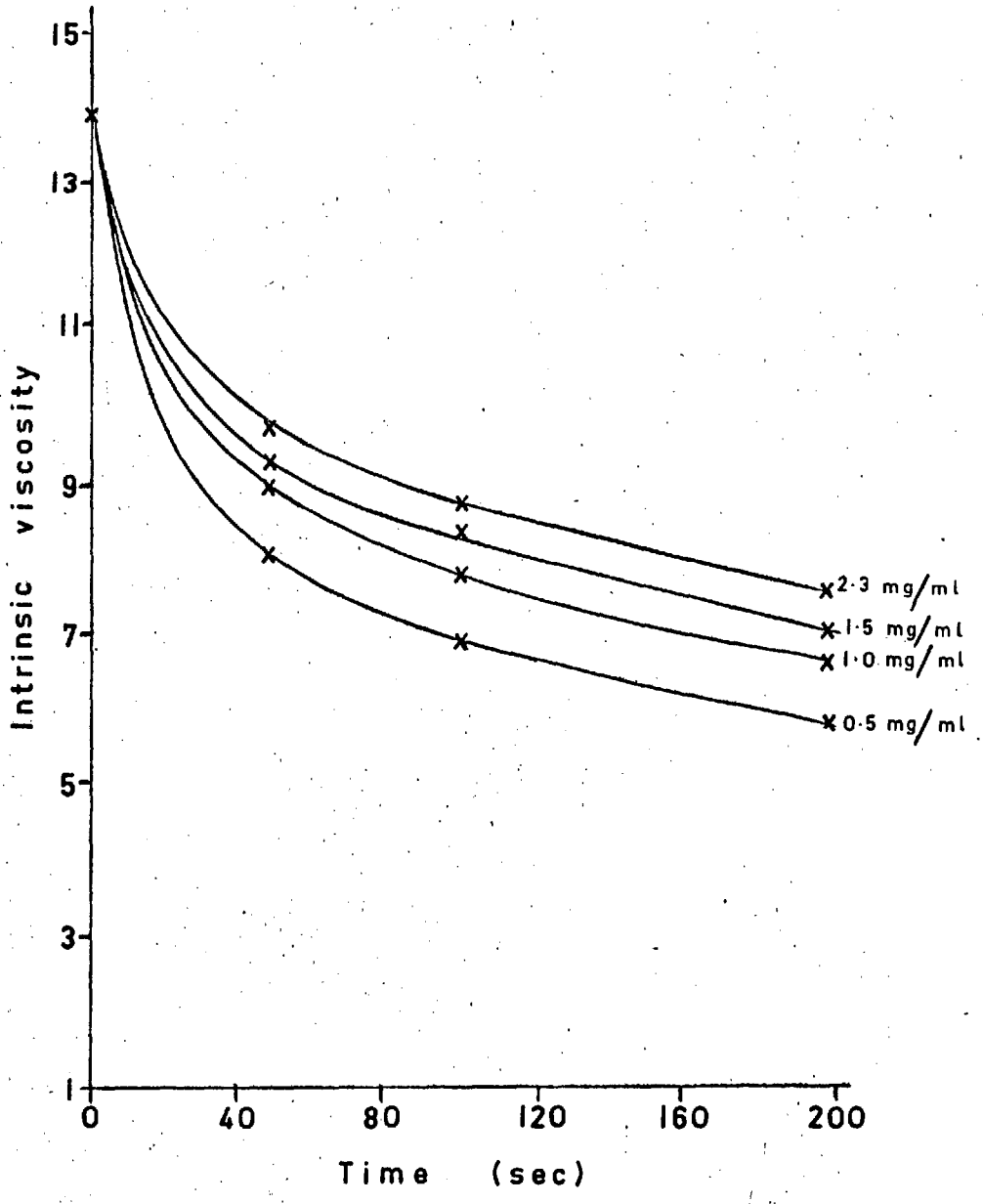


Figure 27 : Effect of Sample Concentration on the Fragmentation of PcV-ds-RNA by Ultrasonic Irradiation.

3 ml samples of PcV-ds-RNA, batch 5/119, were irradiated for 50, 100 or 200 sec, and centrifuged to remove any metal particles. The intrinsic viscosity was then determined as described in the Methods section.

Limited RNase III digests, producing specific fragments of ds-RNA were very often contaminated with intact PcV-ds-RNA, meaning that such digests containing specific fragments could not be used. Instead, the RNase III:ds-RNA ratio was raised so that the digestion proceeded somewhat further, resulting in a heterogeneous mixture of fragments yielding a gel electrophoresis pattern similar to those obtained on panc. RNase digestion or ultrasonication.

The possibility of contamination with intact ds-RNA was checked by gel electrophoresis, which after toluidine blue staining of the gel was capable of revealing the presence of as little as 0.5 µg of ds-RNA. As the standard sample size loaded onto the gels prior to electrophoresis was 20 µg, this meant that as little as 2.5 % intact ds-RNA remaining in the digest could be detected. To achieve the aim of large ds-RNA fragments with no residual intact ds-material, standard digestion conditions were developed for each of the fragmentation techniques, on the basis of the data obtained in the previous sections, and are detailed in the Methods section. The fragmented material obtained in this way was characterised as described in the following paragraphs.

a) Physico-chemical characterisation of the fragments.

(i) Double-strandedness.

As described in the Introduction, section 3 g), it is generally considered that double-strandedness is an essential requirement of an efficient RNA interferon-inducer. To test that the fragments produced by all the three digestion methods used retained the double-stranded structure of intact PcV-ds-RNA, two different techniques have been used.

Determination of the thermal denaturation curve (Tm curve) was performed on every sample prepared for biological activity assays. The complete curves for ds-RNA and three digests are shown in fig. 28. It is clear from these that all three samples of fragments, each produced by one of the different techniques, has a Tm curve which resembles very closely that obtained for the intact PcV-ds-RNA. The curves obtained for the fragments were only slightly less sharp than the control, whilst the Tm point itself is only 0.5 - 1.0°C lower for each digest than the value established for intact ds-RNA.

Thus fragmentation lowered the Tm, and increased the transition width, albeit very slightly, in each case. It has to be considered whether this was due to slight denaturation, or if such effects could result simply from the reduction in MW. That the ultrasonication of DNA,

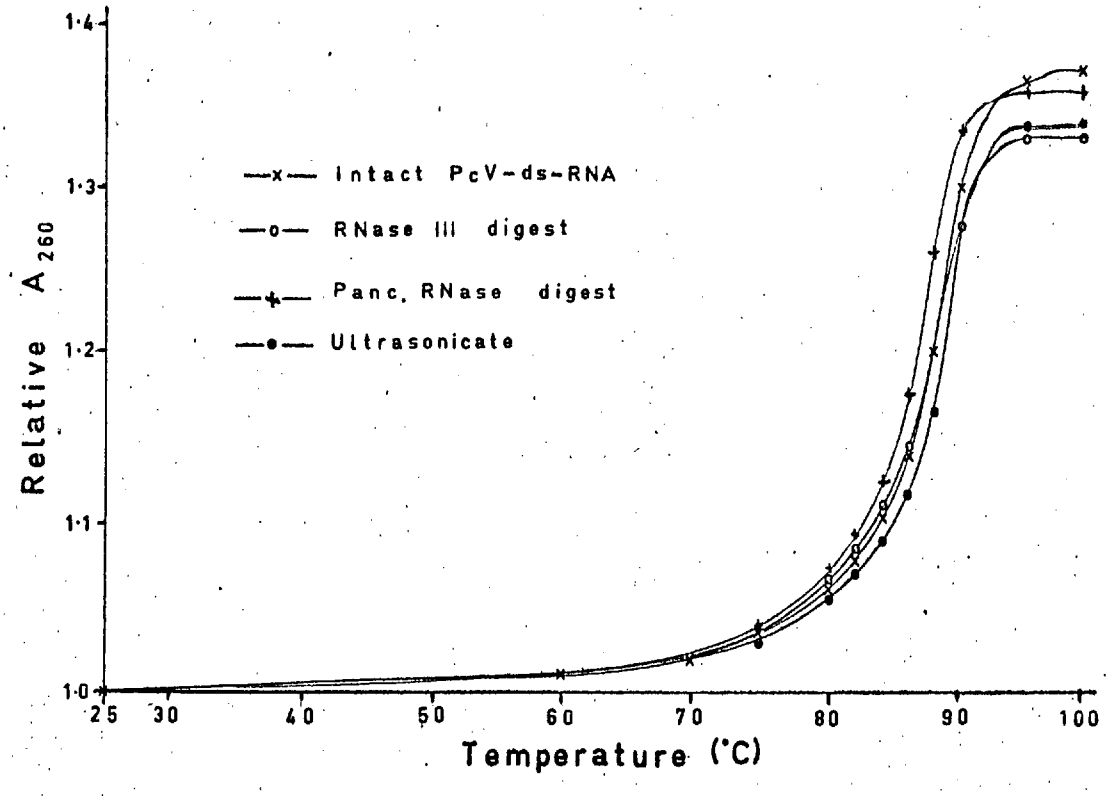


Figure 28 : Thermal Denaturation Curves of Intact and Fragmented PcV-ds-RNA.

Samples, preparation described in footnotes to fig. 30 were dissolved in 0.1 SSC, concentration 20 to 30 µg/ml. Tm curves were determined as described in the Methods section 13.

where it is known that no denaturation occurs on cleavage (see Results, section 2 c)), results in a small fall in the Tm has been shown by the work of several authors, including Marmur and Doty (1962), Mandel (1962), and Pritchard, Hughes and Peacocke (1966). Similar results were obtained on the ultrasonication of poly(I).poly(C), a synthetic ds-RNA, by Lampson et al. (1970). So, the fall in Tm is to be expected as a side-effect of the reduction in MW., and denaturation is not involved.

The second technique commonly used to check for double-strandedness of fragments was to determine their reactivity with formaldehyde at moderate temperatures. Miura et al. (1966) showed that for the ds-RNA isolated from Rice Dwarf virus, there was no appreciable reaction after incubation with 1.8 % formaldehyde at 37°C in 0.1 M sodium chloride for 22 hr, whilst the same RNA after heat denaturation (obviously thermal degradation of the sample is unimportant here) reacted very rapidly under the same conditions. The extent of the reaction was determined simply by monitoring the A_{260} of the sample from time to time. This technique has been used to examine the fragments produced in this study. The result of an experiment is shown in Table 8, the conditions (given in the footnotes to the Table) being essentially those of Miura et al. (1966). Heat denatured PcV-ds-RNA, E.coli r-RNA and heated r-RNA were included as standard ss-RNAs. Again it is clear that fragmentation by any of the three methods has not resulted in appreciable denaturation.

(ii) RNase resistance.

As resistance to digestion by nucleases has been put forward as one of the essential requirements for IF-induction (see Introduction, section 3 g) for references), the RNase resistance of the fragments was expected to be of importance in deciding the efficiency of induction. As mentioned in the Introduction, although the bulk of the work on the relationship between RNase resistance and IF-induction has been done using panc. RNase, it is probable that this is not the major nuclease active against ds-RNA either in vivo or in vitro. Therefore the sensitivity of fragments produced by ultrasonication to digestion by human serum has been assayed. The assays could not be done by viscosimetry, due to the initially low viscosity of the fragments. Therefore the production of acid-soluble nucleotides was observed. The results of such experiments are shown in fig. 29, the conditions of the assays are given in the footnotes to the figure. After an initial period when digestion of the fragments appeared to be more rapid, it appeared that fragmentation had

TABLE 8.

REACTION OF INTACT AND FRAGMENTED RNA WITH FORMALDEHYDE

Sample	Absorbance at 260 nm		
	0 hr	0.5 hr	22 hr
Control ds-RNA	0.747	0.755	0.757
Denatured control RNA	0.709	0.778	0.863
RNase III digest	0.670	0.674	0.677
Panc. RNase digest	0.713	0.715	0.717
Ultrasonicate	0.568	0.577	0.579
E.coli r-RNA	0.904	0.930	1.120
Heated r-RNA	0.899	0.945	1.120

The RNA samples, at a concentration of between 30 and 45 μg/ml, in 0.1 M sodium chloride, 0.01 M phosphate buffer, pH 7.0, were mixed with neutralised formaldehyde to 1.8 % (Miura *et al.*, 1966), placed in stoppered cuvettes, and the A_{260} determined immediately. The cuvettes were then incubated at 37°C, the A_{260} being determined after 0.5 and 22 hr incubation. The cuvettes were weighed after the first and last determination, to test for evaporation losses. In every case these were less than 2 %. Buffer plus formaldehyde, incubated at 37°C as described above, was used as a blank for the A_{260} determinations.

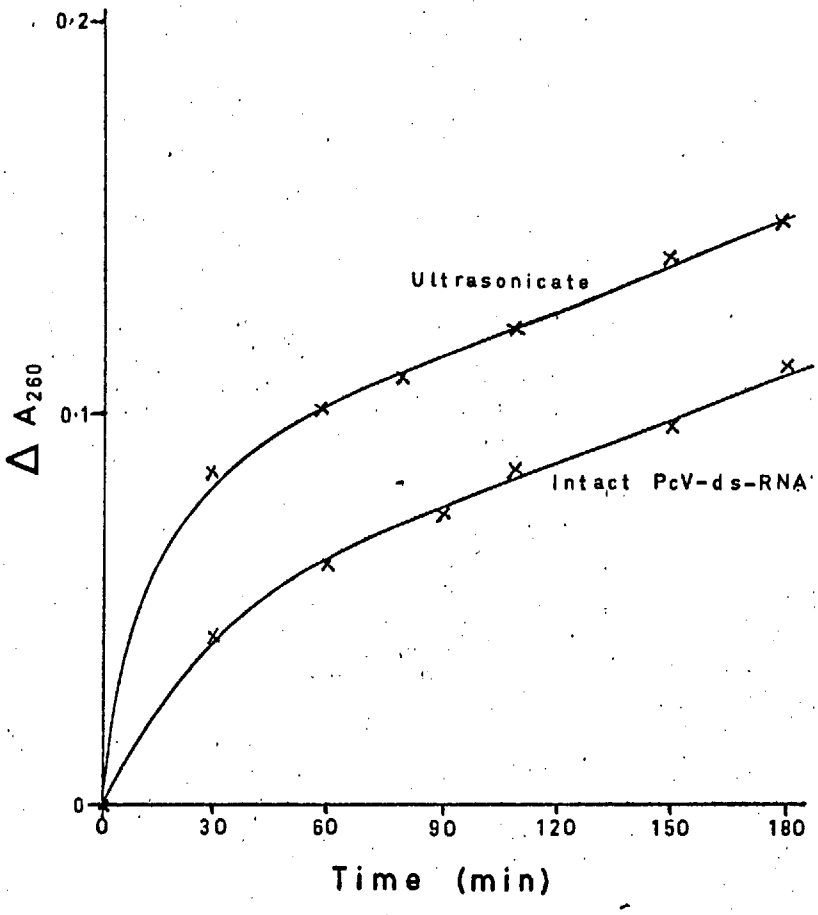


Figure 29 : Rate of Digestion of Intact and Ultrasonicated PcV-ds-RNA by Human Serum.

Intact PcV-ds-RNA, and RNA ultrasonicated at 1 mg/ml for 45 sec were mixed at 1 mg/ml with human serum and incubated at 37°C. 100 µl samples were taken at various times, precipitated by mixing with an equal volume of 12.5 % perchloric acid, stored in an ice-water bath for 5 min, and the precipitate removed by centrifugation. The A_{260} of the supernatant was determined after dilution to 500 ul with water.

little effect on the rate of digestion. This higher initial sensitivity is perhaps due to the fact that the ultrasonicates as presented for assay were very heterogeneous with regard to molecular size. This means that a fraction of the ultrasonicated sample was of such a small size as to be rendered acid-soluble on very limited digestion, during the period in which the almost monodisperse (in comparison) intact ds-RNA was being converted to a polydisperse size distribution. After this brief initial phase, however, the rate of digestion of intact ds-RNA and of the ultrasonicated sample by human serum were very similar. (Human plasma gave very similar results.) It is therefore probable that differences in the biological activities of intact and fragmented ds-RNA would not arise from the more rapid digestion of the fragments by serum nucleases.

(iii) Size range of the fragments.

To obtain rough estimates of the size range of the fragments used in the biological assays, gel electrophoresis and sucrose gradient centrifugation have been used. The first technique is very suitable for the detection of any residual intact ds-RNA, and also for showing the production of specific digestion fragments due to its high resolution. However, for examining the distribution of fragment size sucrose gradient analysis is much more suitable.

No accurate MW. determinations were made (which would be impossible with such heterogeneous digests), but instead the size range of the fragments was estimated. In gel electrophoresis experiments, the dye bromophenol blue was initially used as a marker, this being known to run slightly slower than 5S ribosomal RNA. In later experiments, the ds-RNAs of the mixed viruses of P.stoloniferum were used as MW. standards. The MWs. of these ds-RNAs as measured by gel electrophoresis are 1.01, 0.99, 0.94, 0.89 and 0.23 x 10^6 daltons (Bozarth et al., 1971). Very similar values (1.11, 1.05, 0.95, 0.88 and 0.21 x 10^6 daltons) were reported by Border et al. (1972).

A smaller component, not recorded by either of these groups of workers, has been found in this work, being especially common in samples that had been stored frozen for some time. It was thus probably a specific breakdown product of one of the larger ds-RNA components.

The MW. of the small P.stoloniferum ds-RNA is in contention. The two published estimates (Bozarth et al., 1971; and Border et al., 1972) were obtained by gel electrophoresis studies, using Wound Tumour virus ds-RNA (Bozarth et al.) (itself standardised against Reovirus ds-RNA by

Wood & Streissle, 1970)), and Reovirus ds-RNA (Border et al.) as MW. markers. In both these cases, it is a considerable extrapolation from the mobility of the fastest component of the standards to the mobility of the fast P.stoloniferum ds-RNA.

The sedimentation coefficient of the fast ds-RNA was found to be 8.9S (Kempson-Jones, 1971), which when used to obtain a MW. estimate by the relationship of Eigner and Doty (1965) results in a value of 0.63 x 10^6 daltons, almost three times the value as determined by gel electrophoresis.

The gel electrophoresis pattern of PcV-ds-RNA, the P.stoloniferum RNAs, and three digests prepared by the various techniques described earlier is shown in fig. 30. In this figure, it would appear from the published MW. estimates for the P.stoloniferum ds-RNAs, that the ultrasonicate and the RNase III digest contain material ranging from about 0.23 x 10^6 daltons downwards, whilst the panc. RNase digest is of somewhat larger MW., the maximum MW. of the material being of the order of 1 x 10^6 daltons. However, the results obtained on sucrose density gradient analysis of the same three digests conflict with the gel electrophoresis data. For the sucrose gradient analysis, P.stoloniferum ds-RNAs were not suitable standards as they were not adequately resolved. So E.coli r-RNA was used to give reference sedimentation coefficients as in earlier experiments on the gradient analysis of denatured and native intact PcV-ds-RNA. The gradient analyses of the three digests described in the footnotes to fig. 30 are shown in fig. 31. Again, conversion of the S values obtained in this way to estimates of MW. was done by the use of the relationship of Eigner and Doty (1965). The S value versus distance migrated down the gradient as shown in fig. 31, is plotted in fig. 32, and on this are shown the S values for the peaks of three digests. All fall between 7.6 and 8.6 S, which convert to MWs. of between 3.9 x 10^5 and 5.7 x 10^5 daltons respectively. In each case, there is very little material smaller than 4S (0.54 x 10^5 daltons) and very little material of 16S or larger (2 x 10^6 daltons) i.e. intact material.

The gel electrophoresis pattern, fig. 30 shows these three digests as migrating with, and faster than, the small ds-RNA of P.stoloniferum viruses. If it can be assumed that the fragments and the intact standard P.stoloniferum ds-RNA have similar structures, then the contradiction between the published MW. estimates and that established by consideration of the sedimentation coefficient becomes apparent. Taking the published

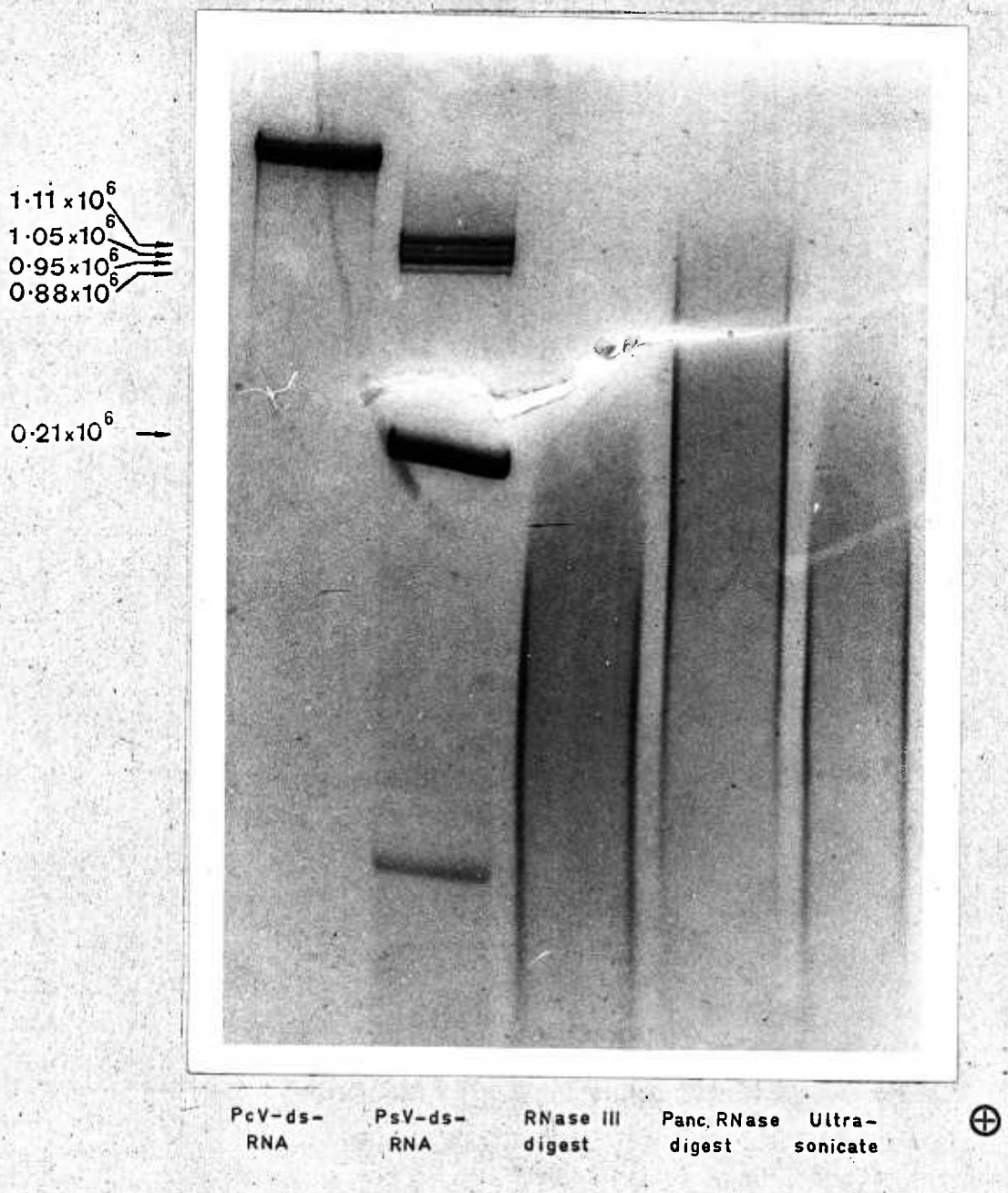


Figure 30 : Gel Electrophoresis of Digests Prepared for Toxicity and Antiviral Activity Assay.

Three digests were prepared to compare the effects of different digestion methods on the toxicity and antiviral activity of fragmented PcV-ds-RNA. Digests were prepared as described below, ds-RNA of batch 5/119 was used in each case. 20 µg samples of each digest were run on a polyacrylamide gel, intact PcV-ds-RNA and ds-RNA from P.stoloniferum viruses (PsV-ds-RNA) were run as standards.

Figure 30 : continued from overleaf.

(i) RNase III digest.

150 mg of ds-RNA, dissolved in TM buffer + 0.1M ammonium chloride, pH 7.8, at a concentration of 1 mg/ml, was digested for 1 hr at 36°C with 750 U of RNase III per mg of ds-RNA. The digestion was stopped by the addition of SDS; phenol extraction etc. were all as described in the Methods section.

(ii) Pancreatic RNase digest.

150 mg of ds-RNA was digested, with 5 mg of pancreatic RNase (i.e. enzyme:substrate ratio of 1:30). The substrate was dissolved in PBS at 1 mg/ml. After 1 hr at 36°C, digestion was stopped by the addition of DEP, and the RNase removed by phenol extraction as described in the Methods section.

(iii) Ultrasonicate.

5 x 25 ml aliquots of ds-RNA (1 mg/ml in PBS) were irradiated for 420 sec using a 19 mm diameter probe. After irradiation the samples were pooled, centrifuged to remove any particles thrown off from the probe, ethanol precipitated and dialysed into PBS.

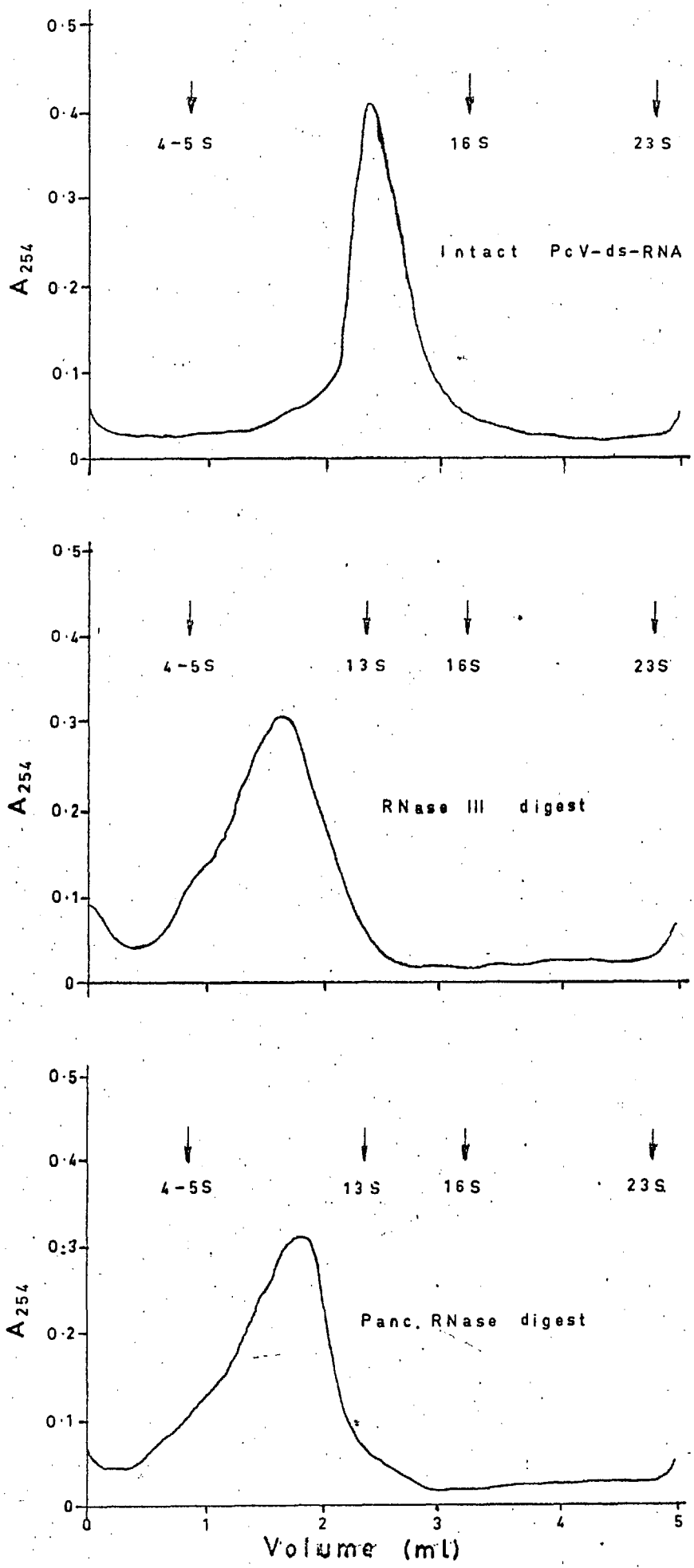


Figure 31 : continued overleaf.

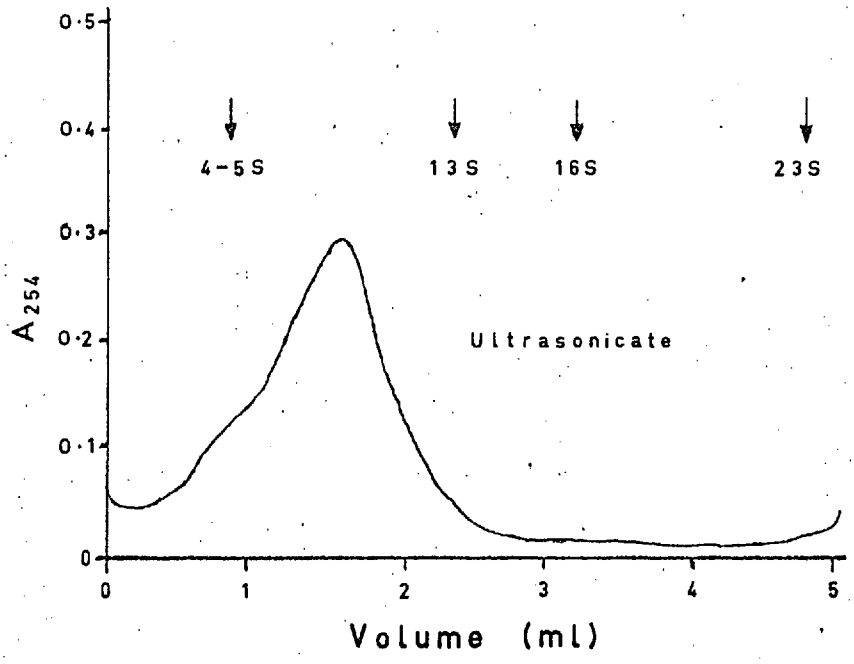


Figure 31 : Sucrose Density Gradient Analysis of Samples for Toxicity Assay.

20 µg of the samples described in fig. 30 were loaded onto 5 ml 5 % - 20 % sucrose gradients, and centrifuged at 25,000 rpm for 16 hr in the SW 50 rotor. E.coli r-RNA was run as S value markers.

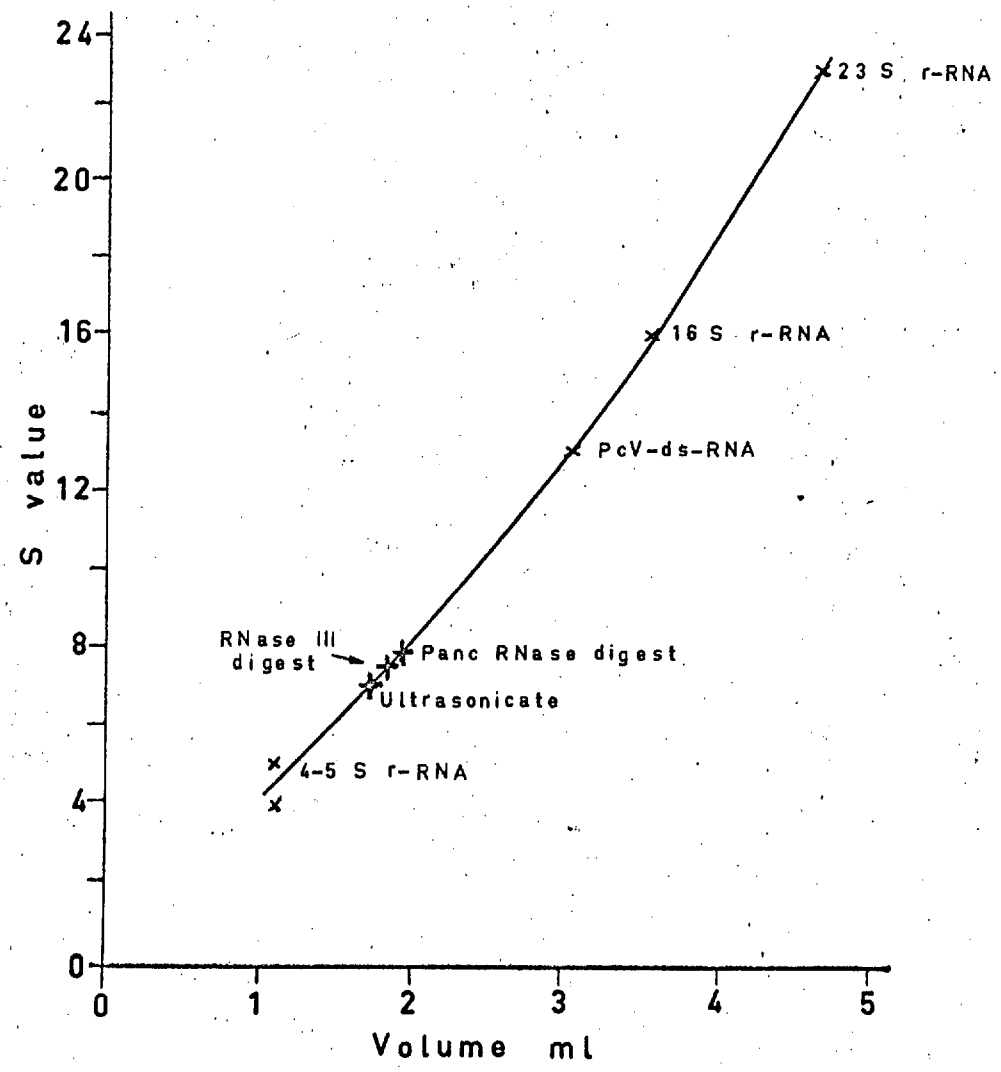


Figure 32 : A Plot of the Sedimentation Data of Fig. 31.

On this figure, the S value of the E.coli r-RNA components is plotted against the distance sedimented. The peak values for each digested sample are also marked.

values of the MW. of the P.stoloniferum ds-RNAs, it would appear that the fragments are smaller than 0.23×10^6 daltons, which is a value much smaller than the result established by sucrose gradient centrifugation analysis. However, if the MW. estimate of 0.63×10^6 daltons for the P.stoloniferum fast ds-RNA, calculated above from the data of Kempson-Jones (1971), is used when comparing the digests on gel electrophoresis, there is little conflict with the results obtained by sucrose gradient centrifugation. The reason for the discrepancy between the published MW. for the P.stoloniferum small ds-RNA, obtained by gel electrophoresis, and the result calculated here from the sedimentation coefficient, is not clear. The difference between the two types of MW. estimates is too great to be entirely due to the use of MW.-S value equations developed originally from work with ds-DNA, or the lower sensitivity and resolution of the gradient technique.

b) Toxicity and antiviral activity assays.

As mentioned in the Introduction, one of the main problems of the potential use of ds-RNAs as interferon-inducers in antiviral therapy is the toxicity (reviewed by Stinebring & Absher, 1971; and Philips et al., 1971). So, it was important to assay the toxicity of the fragments produced from PcV-ds-RNA, to assess the effect of MW. on this phenomenon. Three ultrasonicates of PcV-ds-RNA, batch P2, were prepared by ultrasonication in PBS at a concentration of 2.3 mg ds-RNA/ml, for 3, 7, and 20 min. The gel electrophoresis pattern of the three ultrasonicates is shown in fig. 33, where it can be seen that the 3 min ultrasonicate still contained some residual intact ds-RNA (approximately 5 %). To obtain a significant result the toxicity of the samples had to differ by a factor of two. Therefore, contamination with small amounts of intact RNA would not have any deleterious effect on the accuracy of the experiment. The toxicity of two of the samples, those irradiated for 3 and 7 min, was assessed by injecting the samples i.p. into groups of five mice, strain CD 1, at several dose levels, then counting the number of mice dead at each dose level at 6 days after injection. This assay was performed by Beecham Research Laboratories. The results are shown in Table 9. It is clear that there is very little effect on the toxicity, the apparent small increase found on fragmentation is below the level of sensitivity of the assay.

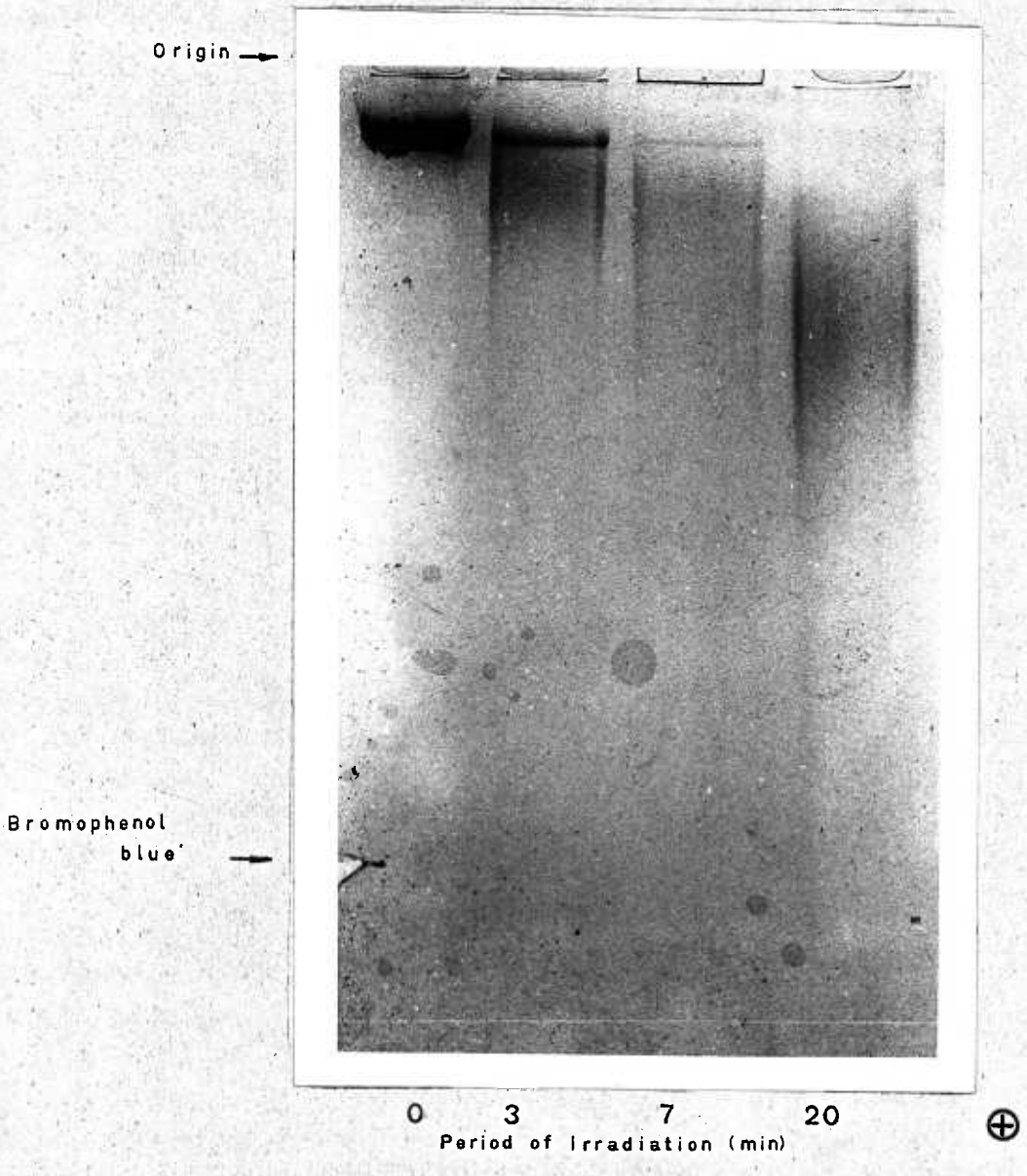


Figure 33 : Electrophoresis of ds-RNA Ultrasonicated for Toxicity Assay.

PcV-ds-RNA, batch P2, was ultrasonicated at a concentration of 2.3 mg/ml for the periods indicated above, in 5 ml volumes. 20 μg samples were run on the polyacrylamide gel.

TABLE 9.

TOXICITY OF ULTRASONICATED PcV-ds-RNA

Dose of fragments mg/kg, i.p.	Number of mice dead, out of 5		
	control	3 min ultrasonicate	7 min ultrasonicate
1	N.D.	0	0
2	N.D.	0	0
3	N.D.	0	0
5	0	0	0
10	0	0	0
20	1	3	1
30	3	4	4
40	4	5	5
80	5	5	5

Assays done in mice of strain CD 1, 5 mice at each dose level. N.D. = not done. Preparation of the samples is described in the text, see also fig. 34. The toxicity assay was performed by Beecham Research Laboratories.

The assay of antiviral activity of all three ultrasonicates was also done in CD 1 mice, and the assay results are given in Table 10. (The assay was performed by Beecham Research Laboratories.) The assay itself is of lower sensitivity than the toxicity assay, requiring a four-fold change in activity for a significant alteration to be recognisable. Clearly the data show no such change in the degree of protection afforded against the higher challenge with EMC virus. With the lower (190 LD_{50}) challenge, it would appear that an increase in protective activity occurs on limited ultrasonication. This may be due to, for example, more rapid uptake of the fragments as compared to intact PcV-ds-RNA, or to fragmentation increasing the number of effective molecules, either or both of which might more than compensate for a lower inducing capacity on a molecule-for-molecule basis. So within the limitations of the sensitivity of the assays, it would seem that fragmentation had led to no alteration in the toxicity, and a slight increase in antiviral activity.

To exclude the possibility of errors arising due to the fragmentation technique, samples digested in three different ways (but all to approximately the same extent), were assayed for toxicity and for antiviral activity. Such a comparison might also reveal the involvement of any specific portions of the ds-RNA molecule that were cleaved by one fragmentation technique and not by the others. The gel pattern of these samples is shown in fig. 30, whilst the sucrose gradient centrifugation analysis is shown in fig. 31. The size of these samples was discussed in section 3 a) (iii). In every case, only a very small fraction of the samples was smaller than 3S, which is very approximately equivalent to 2.2×10^4 daltons.

The results of the toxicity assays after both i.v. and i.p. injection of the samples (performed by Beecham Research Laboratories) are given in Tables 11 a) and b). In the case of this assay, ten mice were used at each dose level, making reasonably accurate calculation of the LD_{50} possible, but even so, the two-fold limitation on sensitivity remained. No such difference was detected on fragmentation. All methods of fragmentation gave results similar to that given by the intact unfragmented ds-RNA when given by the same route, except for the pancreatic RNase digest after i.v. injection. These fragments seem from the LD_{50} value to be more toxic than the others, even though the result is still below the level of significance. This result is probably

TABLE 10.

ANTIVIRAL PROTECTIVE EFFECT OF ULTRASONICATED PcV-ds-RNA

Sample	No. mice dead after 12 days following challenge with :	
	1900 LD_{50}	190 LD_{50}
Intact	9/10	7/10
3 min U/S	5/10	1/10
7 min U/S	6/10	1/10
20 min U/S	9/10	4/10
Untreated controls	20/20	19/20

Mice of strain CD 1 were injected with the inducer, 0.2 mg/kg i.p., 24 hr prior to injection with the stated dose of Encephalomyocarditis (EMC) virus, also i.p. The number of deaths was counted 12 days after virus challenge. Preparation of the ultrasonicates is described in the footnotes to fig. 34. The antiviral activity assay was done by Beecham Research Laboratories.
U/S = ultrasonication.

TABLE 11a.

TOXICITY OF FRAGMENTED PcV-ds-RNA ON INTRAVENOUS INJECTION

Sample	Dose (mg/kg)	No. animals dead on each day							Total dead no. in group	LD_{50}
		0	1	2	3	4	5	6		
Intact ds-RNA	2.5								0/10	
	5								0/10	
	10			1					1/10	14 mg/kg
	20		4	4	1				9/10	
	40		5	5					10/10	
	80		6	4					10/10	
RNase III digest	2.5		2						2/10	
	5		7						7/10	
	10		2	1					3/10	6 mg/kg
	20		9	1					10/10	
	40		10						10/10	
	80		10						10/10	
Panc. RNase digest	2.5								0/10	
	5		1						1/10	
	10		1						1/10	12 mg/kg
	20		6	4					10/10	
	40		8	1					9/9	
	80		9	1					10/10	
Ultra-sonicate	2.5								0/10	
	5								0/10	
	10			2					2/10	13 mg/kg
	20		7	2					9/10	
	40		8	2					10/10	
	80		10						10/10	

Samples were injected at the stated doses i.v. into CD 1 mice (18 - 22 g, male) and the number of deaths occurring on each day following injection were recorded for 6 days. The preparation of the samples is described in the footnotes to fig. 30. The toxicity assay was kindly performed by Beecham Research Laboratories.

TABLE 11b.

TOXICITY OF FRAGMENTED PcV-ds-RNA ON INTRAPERITONEAL INJECTION

Sample	Dose (mg/kg)	No. animals dead on each day								Total dead no. in group	LD_{50}
		0	1	2	3	4	5	6	7		
Intact ds-RNA	10									0/10	
	20			2						2/10	36 mg/kg
	40			7						7/10	
	80		3	5						8/10	
RNase III digest	10									0/10	
	20							1	1	2/10	34 mg/kg
	40			3	2	1				6/10	
	80			8	1	1				10/10	
Panc. RNase digest	10									0/10	
	20		2	2						4/10	41 mg/kg
	40		2	1						3/10	
	80		1	5		1				7/10	
Ultra-sonicate	10			1						1/10	
	20		2	2						4/10	25 mg/kg
	40		2	5						7/10	
	80		2	6	2					10/10	

For experimental details see Table 11a, except that injection was intraperitoneal. The assay was performed by Beecham Research Laboratories.

incorrect, however, for study of the data shows that some mice given a low dose were killed, whilst others receiving a higher dose survived. The reason for this is not known, but is unlikely to be of any importance, as the increased toxicity of the panc. RNase digest found on i.v. injection was not reflected in the results obtained by i.p. administration. However, in the i.v. assay of all the fragmented samples, it can be seen from the data given in Table 11 a) that mice given the fragmented samples died earlier than those that received the intact ds-RNA, although the total number of deaths after 7 days (the end of the experiment), and hence the LD_{50}, did not differ significantly. The antiviral protection assay results for these samples, again using EMC as the challenge virus, are given in Table 12. As before there is no significant alteration in the degree of protection conferred by any of the three batches of fragments and the intact ds-RNA.

c) Interferon-induction assays.

The interferon assay system developed by Himmelweit, basically a refinement of the dye-uptake assay method of Finter (1969), is considerably more sensitive than the antiviral activity assays described above, and the results are accurate to $\pm$ 30 % (F. Himmelweit, unpublished results). To assay for the interferon-inducing capacity, the samples were injected intravenously into mice of strain BSVS. These mice differed from the CD 1 mice used in the antiviral activity and toxicity assays in being more sensitive to bacterial and viral infection, and also the effects of injected ds-RNA.

The first digest assayed for the capacity to induce IF was an RNase III digest, prepared with an early batch of enzyme which contained quite large amounts of contaminating E.coli-derived RNA. The gel electrophoresis pattern of this digest is shown in fig. 34. There seems no reason to doubt that the contaminating RNA would also contaminate the digest, although examination of the RNase III preparation by gel electrophoresis showed that either the contaminating RNA is very heterogeneous, being too thinly spread over the gel to be detected, or is of very small size, so as to run off the gel. Such RNA, almost certainly single-stranded (if it were not it would be digested by the RNase III) would be expected to have no direct effect in itself in the IF-induction assay (Field et al., 1967a,b). It would however, dilute the ds-RNA fragments, as estimation of amounts of RNA was performed simply by determination of the A_{260} of the samples. This means that less than the

TABLE 12.

ANTIVIRAL PROTECTION BY FRAGMENTED PcV-ds-RNA

Sample	Dose (mg/kg)	No. animals dead on each day													Total dead no. in group	PD_{50}
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	
Intact ds-RNA	5															0/10	
	0.5							1								1/10	
	0.05							1		1						2/10	0.0015
	0.005							1			1					2/10	mg/kg
	0.0005						1			2		1		1		5/10	
RNase III digest	5															0/10	
	0.5															0/10	
	0.05							2								2/10	0.0015
	0.005															0/10	mg/kg
	0.0005									3		1	2			6/10	
Panc. RNase digest	5															0/10	
	0.5									1						1/10	
	0.05						1	1								2/10	0.004
	0.005					1	4	1								6/10	mg/kg
	0.0005					2	5		1	1						9/10	
Ultra-sonicate	5															0/10	
	0.5															0/10	
	0.05						2					1				3/10	0.003
	0.005							2								2/10	mg/kg
	0.0005					1	2	2		1						6/10	
Untreated controls						11	17	3	5							36/40	

All samples given i.p. into 18 - 22 g CD 1 mice, and were challenged 24 hr later with 190 LD_{50} of EMC virus. Deaths were recorded for 13 days following virus challenge. For the details of the preparation of the samples see the footnotes to fig. 31.

PD_{50} = dose protecting 50 % of group.

The assay of antiviral protection was kindly performed by Beecham Research Laboratories.

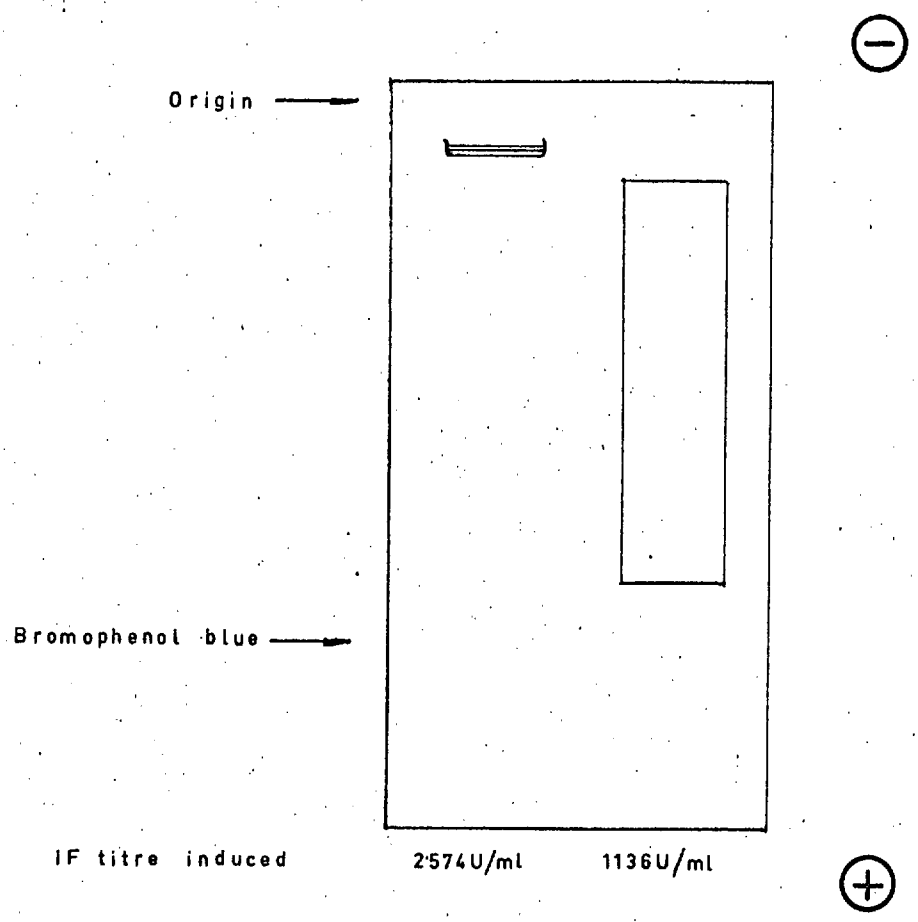


Figure 34 : Gel Electrophoresis of RNase III-digested PcV-ds-RNA for Interferon Assay.

PcV-ds-RNA, batch 27C, was digested essentially as described in the Methods section, except that semi-purified RNase III, containing relatively large amounts of RNA (about 0.5 mg/ml), was used (see text). The samples contained about 20 % of RNA derived from the enzyme preparation. The interferon titres induced by each sample are appended below the gel pattern. Interferon-inducing capacity was kindly assayed by Dr. F. Himmelweit.

standard amount of material was injected into the mice used in this assay (usually 80 µg of ds-RNA was given i.v. into each 20 g mouse, i.e. 4 mg/kg) and thus some lowering of the titre of IF induced by the fragments could be expected. This would be expected to be proportional to the amount of inactive E.coli-derived RNA included with the ds-fragments. An estimation of the level of this contamination was made on the assumption that the recovery of contaminating RNA from the purification of the fragments after digestion was the same as the recovery of the fragments. After phenol extraction, ethanol precipitation and dialysis, the yield was usually between 40 and 60 %, and in this experiment the yield was 45 %. A very approximate estimate of the amount of RNA contained in the enzyme preparation was made from the A_{260} of the preparation. The semi-purified RNase III preparation used was calculated to contain about 0.5 mg/ml of E.coli-derived RNA. From this it can be calculated that roughly 20 % of the sample presented for IF-induction assay, was in fact not fragments of PcV-ds-RNA, but instead E.coli derived. When this mixed sample was injected into the mice, it induced only 1136 Units/ml of IF, whilst the intact untreated ds-RNA induced 2754 Units/ml. These assays were kindly performed by Dr. F. Himmelweit. Fragmentation therefore reduced the IF-inducing capacity by 56 %, much more than would be expected to result simply from the dilution effect. This of course assumes that the IF titres induced are proportional to the dose of inducer, which is uncertain, and it was thus doubly important to verify this apparent reduction in IF-inducing capacity on fragmentation using PcV-ds-RNA digests containing only negligible amounts of E.coli-derived RNA. These IF titres were assayed using serum taken 12 hr after injection of the sample into the tail vein, which is about the time of the peak titre induced by PcV-ds-RNA (Buck et al., 1971). In later assays, a 10 hr induction period was used, which still corresponded fairly well with the peak titre for intact RNA.

The lowering of the IF titre was greater than could be predicted from the dilution of the fragments with intact ss-RNA. In all later experiments with RNase III, the enzyme preparations had been treated by incubation with 'Enzite' insoluble RNase, which reduced 6-7-fold the level of RNA (as measured by A_{260}) contained in the enzyme preparation (see Methods, section 3). RNase III preparations treated in this way contained between 0.03 and 0.1 mg/ml of RNA prior to ion-exchange chromatography, as estimated by measurement of A_{260}. An RNase III digest

made using this treated enzyme was prepared, and its gel electrophoresis pattern is shown in fig. 35. In this case, calculating the amount of ss-RNA contamination as described before, less than 2 % of the A_{260} of the fragmented material was accountable for by the enzyme-derived RNA. Again, when these samples were assayed for IF-inducing capacity, a considerable reduction in titre was found. Fragmented RNA induced 797 Units/ml, only 60 % of the titre induced by an equal dose of the intact ds-RNA, 1341 Units/ml.

The mice given intact RNA were, as expected, perfectly well throughout the period before sacrifice, but in the case of the mice given the fragmented material, there were several fatalities, and the survivors appeared sick, with ruffled fur and hunched backs. Of the ten mice given fragmented RNA, three died in the period before sacrifice. The deaths all occurred fairly early in the assay period, but not so soon after injection as to make a fault in injection technique a likely cause. Carry-over of phenol from the deproteinisation step after digestion was very unlikely, as the sample was ether extracted, ethanol precipitated and finally dialysed before injection. The only possible remaining cause for the toxicity is the fragmented ds-RNA itself. This indicates a greatly increased toxicity on fragmentation, a result contradicting that obtained in assays of the LD_{50} in CD 1 mice.

Two panc. RNase digests were assayed for IF-inducing ability. A drawing of the gel electrophoresis pattern of the digests is shown in fig. 36. The first digest, fragmented to roughly the same extent as the RNase III digests described above, was given to ten mice as before, i.e. 80 µg/mouse i.v. All ten died in a few hours, and so it was not possible to assay the IF-inducing capacity of the fragments. The second digest was fragmented to a much greater degree, so that part ran completely off the gel on electrophoresis under the standard conditions described in fig. 36. This only killed three out of the eleven mice to which it was given, but induced very little IF. The actual titres were 1708 Units/ml for intact control ds-RNA, and 27 Units/ml for an equal amount of the fragments. This is less than 2 % of the control titre.

The low titre induced by the latter panc. RNase digest could either be due to the more extensive fragmentation, or be a result of the method of digestion. This means that it is not possible to compare the results obtained with different sized fragments produced in different ways. To test the effect of different sizes of fragments on the IF-inducing ability, experiments were made in two different ways.

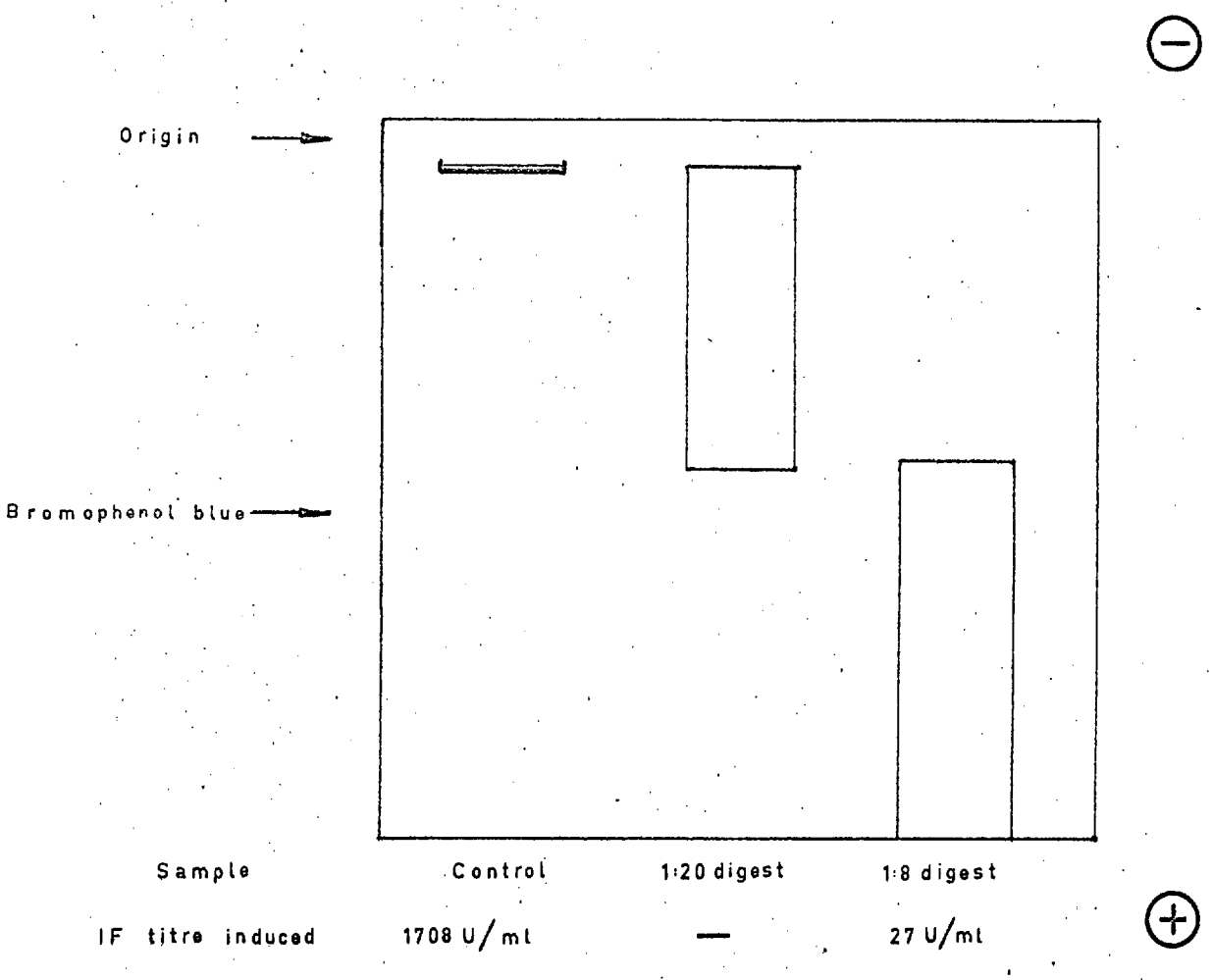


Figure 36 : Gel Electrophoresis of Pancreatic RNase Digests Prepared for Interferon Assay.

PcV-ds-RNA, batch MPP 63, was digested under the conditions described in the Methods section, with either 20 µg RNA/µg enzyme, or 8 µg RNA/µg enzyme. After purification and dialysis, the fragments were assayed for interferon-inducing capacity. The titres are given below the appropriate samples above. The capacity of samples to induce interferon was kindly assayed by Dr. F. Himmelweit.

The first technique was to prepare a large amount of RNase III-digested RNA, and then to fractionate this digest on sucrose gradients. When this was done, dividing a digest into three fractions, the IF titres induced by all three fractions were so low as to make comparison impossible. This was perhaps due to over-fragmentation of the initial digest. The sucrose gradient analysis of the digest is shown in fig. 37, and the titres induced by the three fractions are given beneath. Again, each of the three fractions, although inducing very little IF, killed some of the mice to which they were given, and it was noted that the sera of the survivors receiving fragments were very much more yellow than was the case for mice given intact ds-RNA. The sera of the latter resembled those of untreated mice. This yellow colouration might be due to the release of bile pigments into the circulation, indicating damage to the liver. Liver damage is known as a symptom of the toxicity of ds-RNA (see review by Philips et al., 1971).

The second method of examining the effect of molecular size on the IF-inducing capacity was to study a series of separate digests degraded by the same technique to differing degrees, and gave much more clear-cut results. In this experiment, small batches of ds-RNA were ultrasonicated for increasing periods of time, and the gel electrophoresis patterns are shown in fig. 38. The corresponding IF titres induced by equal amounts of the fragments are given beneath each sample pattern. It is clear that the induced IF titre falls together with the MW. of the fragments, within the ± 30 % limits of error of the IF assay. These fragments killed between 3 and 7 out of each group of 10 or 11 mice to which they were given. There did not seem to be any pattern in the number of mice which were killed by the fragments of these various sizes. Again, the sera of the mice given the fragments but surviving had a yellowish tinge, that of the mice given the intact ds-RNA did not. All the IF titres obtained in this section of the work, and given in the text and figures above, are summarised in Table 13.

The work on the biological effects of fragmentation of ds-RNA has shown that fragmentation can produce in one strain of mice an increased toxicity, and yet in another result in no significant alteration. Fragmentation by all the methods described produced a lowering of the efficiency of IF-induction, in BSVS mice. Assays in CD 1 mice of antiviral activity showed either no significant alteration or even, in one case, a slight increase in protection upon fragmentation. Increasing fragmentation caused a progressive fall in the IF-inducing capacity.

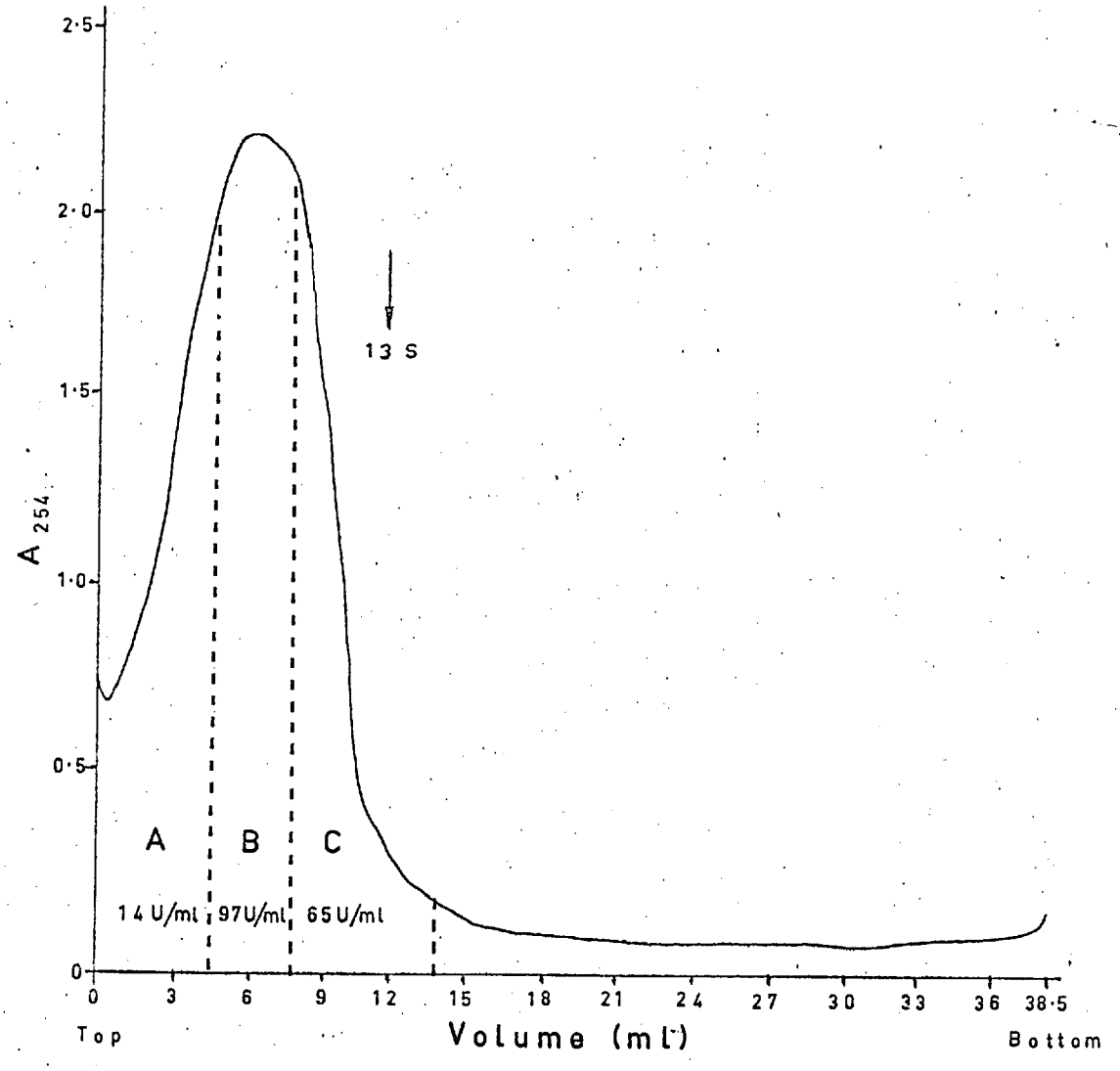


Figure 37 : Sucrose Density Gradient Fractionation of an RNase III Digest of PcV-ds-RNA.

RNA was digested with 700 U RNase III/mg nucleic acid for 1 hr at 36°C, and then phenol extracted and ethanol precipitated. The precipitate was redissolved in 0.05M acetate buffer, pH 5.2 containing 0.01M EDTA at 1 mg/ml, and 2 mg samples layered onto 37.5 ml 5 % - 20 % sucrose gradients in the same buffer. These were spun for 16 hr at 25,000 rpm in an SW 27.1 rotor, and then fractionated after passing through an Isco Density Gradient Analyser. Fractions A, B and C from 3 gradients were pooled, concentrated by ethanol precipitation, dialysed against PBS and assayed for capacity to induce interferon in mice. The IF titres induced are appended beneath each fraction, an equal dose of intact ds-RNA induced 1393 U/ml of interferon. Inducing capacity was kindly assayed by Dr. F. Himmelweit.



Figure 38 : Ultrasonication of PcV-ds-RNA and Interferon-Inducing Activity.

PcV-ds-RNA, batch MPP 63, was irradiated in 2 ml volumes at a concentration of 1 mg/ml in PBS, for 3, 5, 10 and 20 min. The samples were centrifuged, ethanol precipitated, redissolved, dialysed against PBS and the concentration adjusted to 400 µg/ml. 50 µl samples were carefully loaded onto the gel shown above, whilst the remainder was assayed for IF-inducing activity in BSVS mice. The titres induced are shown beneath the appropriate samples, and in Table 13.

TABLE 13.

INTERFERON-INDUCING ACTIVITY OF INTACT AND FRAGMENTED PcV-ds-RNA. COLLECTED DATA FROM DIFFERENT EXPERIMENTS

Sample	Described in :	IF titre induced (U/ml)
Intact	fig. 34	2574 (100)
RNase III digest		1136 (44)
Intact	fig. 35	1341 (100)
RNase III digest		797 (59)
Intact	fig. 36	1708 (100)
Panc. RNase digest		27 (1.5)
Intact	fig. 37	1393 (100)
Small fragments		14* (1)
Medium fragments		97* (7)
Large fragments		65* (5)
Intact	fig. 38	1078 (100)
Ultrasonicated for 3 min		656 (61)
Ultrasonicated for 5 min		255 (24)
Ultrasonicated for 10 min		67 (6)
Ultrasonicated for 20 min		48 (4)

All assays of inducing capacity were kindly performed by Dr. F. Himmelweit.

* Differences between these three are not significant.

Figures in parentheses are titres expressed as a percentage of the titre induced by intact PcV-ds-RNA in each experiment.

DISCUSSION

1. Studies on Intact PcV-ds-RNA.

a) Overall structure.

It has been shown in the Results, section 1 a), that PcV-ds-RNA appears to have no single-strand nicks or longer single-stranded regions, for the MW. of the denatured RNA (0.90 - 0.96 x 10^6 daltons) appears to be very close to half that of the native form. The appearance of the denatured material on sucrose density gradient centrifugation, on gel electrophoresis in a denaturing solvent (formamide), or on gel electrophoresis after reaction with formaldehyde, was homogeneous, i.e. denaturation yielded only a single MW. species, at least within the resolution of the analytical techniques used.

As gel electrophoresis of native PcV-ds-RNA will, under the conditions used in this study, reveal the presence of at least three components, it is perhaps surprising that gel electrophoresis of the denatured RNA did not also resolve separate bands. On the basis of the mobilities of formaldehyde-reacted E.coli r-RNA shown in figures 4 and 5, it would be expected that the bands produced by denaturation of PcV-ds-RNAs into ss-RNA of half the parental molecular weight would be about 1 mm apart, i.e. sufficient for detection. In one experiment, two very close bands of formaldehyde-reacted RNA were seen after electrophoresis and toluidine blue staining, but this was not repeatable, generally both electrophoresis of fd-RNA and electrophoresis in formamide revealed only one narrow band. This suggests two possibilities, neither of which fits all the facts. The first is that denaturation was not complete, and that either some denaturation-resistant area held the two strands together in a specific structure, or that specific aggregates were formed. In support of this hypothesis is the fact that formaldehyde reaction produces aggregation and cross-linking effects, the extent to which these occur being dependent on the conditions (Freifelder & Davison, 1963). As for the use of polyacrylamide gels with formamide as a denaturing solvent, Strauss, Kelly and Sinsheimer (1968) considered that under their conditions, 96 % formamide might not be sufficient to completely denature ds-RNA. However, if denaturation was not complete,

or if specific aggregates were formed, both on formaldehyde reaction and in formamide, the fact remains that these forms migrated very close to the place predicted for ss-RNA of MW. c. 1 x 10^6, both on gel electrophoresis in two different solvents and on sucrose density gradient analysis. Clearly therefore the aggregation hypothesis is untenable.

The second explanation is that PcV-ds-RNA consists of three double-stranded components, each of which is made up of one intact single strand of the same MW., base-paired to a very heavily nicked second strand of 3 differing MWs. However, no low MW. material was seen on gradient centrifugation where it would be most readily detected, and the amount of denatured RNA found in the peak on the sucrose gradient corresponded well (allowing for the hyperchromic effect upon denaturation) with the amount of RNA loaded onto the gradient.

So it seems probable that the double strands of PcV-ds-RNA consist of base-paired complexes of two complete single strands. As mentioned in the Results, an alternative hairpin-loop structure can be ruled out, for on denaturation the resultant single strand should still have a MW. of approximately 2 x 10^6 daltons, about twice the value reported here. However, it is not possible to state that PcV-ds-RNA has a completely base-paired double-helical structure, as there are at least two types of departure from base-pairing that could occur at low levels and remain undetected. Neither irregularity has any experimental evidence to support its occurrence, but equally neither can be disproved on the evidence presented here.

Firstly, it is not possible to discount the occurrence of short single-stranded tails on the ends of the PcV-ds-RNA molecules, this possibility is discussed at greater length in section b) below. The second possible type of base-pairing defect that could occur is mismatching, i.e. the occurrence of non-complementary regions which could either remain in the double-strand structure, or could be looped out. These could occur to a fairly large extent before an effect on Tm or formaldehyde reactivity would be noted, if results obtained on synthetic polyribonucleotide complexes (Uhlenbeck, Harrison & Doty, 1968) can be applied here.

b) The 5'-termini of the intact PcV-ds-RNA.

In the polynucleotide kinase labelling experiments described in the Results, section 1 b), a pyrimidine nucleotide was found as the major apparent 5'-terminal nucleotide. As mentioned before, for technical reasons it is impossible to decide finally between cytidine and adenosine, although the former is more likely. As no attempt was made to separate the 3 PcV-ds-RNA molecules in these labelling experiments, it would seem that all 3 have the same 5'-terminal. All three bands of PcV-ds-RNA were visible in autoradiographs of the labelled material, thus discounting the possibility that only 1 of the classes of ds-RNA was labelled.

A pyrimidine nucleotide has also been found as the 5'-terminal of one of the ds-RNAs isolated from the viruses of P.stoloniferum (Szekely & Loviny, unpublished results). These are the first times that pyrimidines have been found at the 5'-terminal of an RNA molecule. As described in greater detail in the Introduction, section 1 b), the 5'-ends of only 1 other ds-RNA have been determined so far. In this case, both 5'-ends of all segments were found to be guanosine diphosphate, i.e. ppGp... (Banerjee & Shatkin, 1971). The finding of a pyrimidine nucleotide as the apparent 5'-terminal of PcV-ds-RNA may mean that the polymerase activity responsible for the replication of the ds-RNA has an unusual specificity. The virions of the P.chrysogenum virus have been shown to have an associated polymerase activity (Nash, Douthart, Ellis, Van Frank, Burnett & Lemke, 1973) as have those of P.stoloniferum viruses (Lapierre, Astier-Manifacier & Cornuet, 1971). In neither case have the template specificities of the activities been investigated.

An alternative explanation for the appearance of a pyrimidine nucleotide as a 5'-terminal nucleotide is that its presence is a result of the removal of the true 5'-terminal as part of a single-strand tail by digestion with contaminating nucleases during the early part of the isolation and purification procedure, or at some later time. As mentioned above, a short tail would not be detected on the basis of the formaldehyde reaction, as the resulting A_{260} increase would be very small. The presence of small tails on the 5'-ends of Reovirus ds-RNA has been suspected (Millward & Nonoyama, 1970; see also Poonian, Schlaback & Weissbach, 1971). Banerjee and Shatkin (1971) and Shatkin (1971) were however unable to find evidence to support this, and considered 5'-end single-strand tails to be unlikely. There is no evidence for or against the existence of short ss tails on PcV-ds-RNA.

2. The Fragmentation of PcV-ds-RNA.

a) RNase III.

In this thesis, it has been shown that RNase III acts to cleave ds-RNA largely by the diplotomic mechanism (Results 2 a) (i)). As mentioned in the Results, Schweitz and Ebel (1971) reported a similar finding, based on a study of the gel electrophoresis pattern of digested poly(I).poly(C) before and after treatment with 7M urea. As mentioned before, their work was not conclusive as it depended on the assumption that 7M urea is adequate to denature ds-RNA. The technique of viscosimetry used in this thesis does not require denaturation, is able to give results on the very early stages of digestion, in which fragments of the size for biological assay were produced, and the technique is capable of distinguishing not only purely diplotomic and purely haplotomic mechanisms of cleavage, but is also able to detect a mixed mode of fragmentation when both diplotomic and haplotomic cleavages occur. This situation would be difficult to resolve by a denaturation technique as the predominant cleavage mechanism might appear to be the only one. In the case of RNase III digestion this has proved to be an important distinction.

Although diplotomic cleavage has been established as the major mode of action, evidence was found that some haplotomic cleavage also occurs. There are two possible explanations for this. The first is that the enzyme preparation did not separate some other nuclease from the RNase III, and this other nuclease acts purely by haplotomic cleavage. No activity against ss-RNA was found when RNase III preparations were assayed using r-RNA as the substrate. This RNA, although it possesses large amounts of base-paired structure, is not digested by RNase III, apparently because the ds regions are not long enough to be recognised by the enzyme (Schweitz & Ebel, 1971). This does not rule out a haplotomic nuclease, for like RNase III it could have an absolute specificity for double-stranded structures, but it does mean that it has a similar length-requirement for action. There is no evidence for or against the existence of such a second activity in the preparations of RNase III.

The alternative explanation is that the same enzyme molecule can cleave by either method. This has been shown already for acid DNase II (Bernardi & Sadron, 1964). If both digestion mechanisms were active, then some of the fragments would be expected to have short single-strand tails, but these would be relatively few, and so would not be detectable by formaldehyde reaction.

Consideration of the simultaneous appearance and later simultaneous digestion of all components of the band pattern leads to the conclusion that RNase III acted in these initial cleavages by a semi-processive mechanism, in that one intact molecule was broken down to all the specific fragments before the enzyme re-initiated on another molecule. The term 'processive digestion' was first used by Nossal and Singer (1968), in a study of the action of the exonuclease RNase II. In processive digestion, after the initial enzyme cleavage of the polymeric substrate, the enzyme molecule stays associated with the same substrate molecule, cleaving it again and again. RNase III is a semi-processive enzyme, for after cleaving a substrate molecule again and again to produce all the specific fragments, digestion terminates for a while whilst a fresh ds-RNA molecule is similarly fragmented. Only later, when all intact molecules have been fragmented specifically, does digestion of the fragments themselves occur. In a true processive digestion all susceptible sites on a substrate molecule would be cleaved before the enzyme molecule attacked another substrate molecule.

Processive fragmentation is most likely to occur with exonucleases, but a semi-processive mode of action has been described for the ATP-dependent DNase of Haemophilus influenzae strain Rd (Friedman & Smith, 1972a). This endonuclease was shown to digest a 35 S DNA to yield only 16 S fragments, the sizes being assessed by sucrose gradient centrifugation. A calculation of MWs. by the equation of Eigner and Doty (1965) for DNA of MW. greater than 2×10^6 daltons :

$$S = 0.034\ M^{0.405},$$

shows that each 35 S molecule is cleaved into approximately seven 16 S fragments. The enzyme then apparently reinitiates on these specific fragments, digesting them much further, and this second stage of digestion does not appear to be processive (Friedman & Smith, 1972b). The authors proposed a model for the initial processive activity, involving an exonucleolytic-type action with the substrate passing through a 'hole' in the enzyme molecule, and being cleaved at certain points as it passes through. Although there are certain similarities between the two-stage (processive and then random) activity of this ATP-dependent DNase and RNase III, there is insufficient evidence to draw any further parallels.

The most striking thing about RNase III digestion is the production of specific fragments in its action on ds-RNA. The basis behind this

specificity of action has been investigated. As no single-strand nicks or longer discontinuities were found in intact PcV-ds-RNA samples, the possible effects of these can be discounted. So, the basis for specificity probably resides in some more subtle structural feature of the ds-RNA which is recognised by the enzyme. Although RNase III has been shown by Robertson et al. (1968) to digest poly(A-U) and poly(G).poly(C) at very similar rates (this has also been confirmed in this study), Barnard (1969) considered that the rates of cleavage could still vary at different points on a ds-RNA molecule, even if an initial interaction between the enzyme and a specific base did not occur. In other words, although one sort of bond is cleaved more readily than another (preference or relative specificity), both are cleaved. Evidence has been obtained with panc. RNase that the relative susceptibility of the phosphodiester bonds is influenced by the base-stacking interactions of the surrounding residues (Witzel & Barnard, 1962; Follman et al., 1967).

Laskowski and his coworkers (Vanecko & Laskowski, 1962; Georgatsos & Laskowski, 1962; Laskowski, 1967) found that with DNase I, DNase II, and Snake Venom endonuclease the initial cleavages were much more specific than those occurring later on in the digestion, as assayed by a study of the terminal nucleotides of the fragments early and late in the digestion. Endonucleolytic RNases tend to be, as a group, much more base-specific in their action than DNases (Barnard, 1969). RNases often have complete (or almost complete) specificity for cleavage of the phosphodiester bond next to one particular base or base type. E.g. RNase T_1 cleaves only after G residues (Sato & Egami, 1957; Sato-Asano, 1959), bovine panc. RNase A is specific for pyrimidine residues (Markham & Smith, 1952; but see also Beers, 1960, and section 2 b) of the Results), and RNase U_2 from Ustilago sp. (Arima, Uchida & Egami, 1968a,b) cleaves only after purine residues, and preferentially after A residues. Partially as a consequence of this base specificity (but mostly because of folding of the substrate molecule), such nucleases can be extremely specific in their action, and under appropriate conditions put as little as one cleavage in an RNA molecule.

For example, Adams et al. (1969) digested R 17 bacteriophage ss-RNA with RNase T_1, and were able to produce on limited digestion fragments up to 300 bases long. This means that different susceptible linkages are cleaved at different rates, and this is presumably due to the secondary

structure of the RNA. RNase T_1 would not cleave under mild conditions any guanylate residues involved in base paired regions (review by Barnard, 1969). Also certain regions of the RNA may be protected by the conformation of the RNA, being buried inside the molecule because of the way in which it is folded, and thus are inaccessible to the action of the enzyme.

However, this does not explain how specific fragments are produced from a ds-RNA, for in such a case all bases are hydrogen bonded, and none are buried. In the case of certain DNases, the restriction enzymes, which are capable of putting one or a very few extremely specific nicks in a DNA molecule, it is known that the enzyme recognises and cuts a specific nucleotide sequence. For example, Kelly and Smith (1970) found the recognition site for a restriction enzyme purified from H.influenzae to be :

.....p G p T p Py p Pu p A p C p.....

.....p C p A p Pu p Py p T p G p.....

It is obvious that any enzyme having a recognition site on the substrate as long as this will be extremely specific in its cleavages. Restriction nucleases are, however, unusual amongst the known nucleases for requiring such a long recognition site, and RNase III does not work by such a mechanism. This is because it will produce non-specific as well as specific fragments, because it will digest a natural ds-RNA to small oligonucleotides and mononucleotides (Robertson et al., 1968), and also because it will digest poly(G).poly(C) and poly(A-U) at roughly similar rates.

The studies of the 5'-ends of the fragmented PcV-ds-RNA revealed a definite specificity of the enzyme in these early cleavages. As described in the Results, section 2 a) (iii), all four nucleosides were found to be present at the 5'-termini of specific fragments of PcV-ds-RNA, but the amounts of adenosine and uridine were greatly in excess of the quantities of cytidine and guanosine. This in itself is evidence of specificity, and it is interesting to postulate from these findings what is the nature of the site that is recognised by the enzyme. Considering only the major spots found, it seems probable that all the fragments have a similar disposition of these, i.e. that all the cleavage sites are the same. Then it is possible to draw three models for the active sites on the ds-RNA, thus :

```
--- ——(U)U—— --- ——(U)U—— --- ——(U)U—— ---
```

1a)

```
--- ——A(A)—— --- ——A(A)—— --- ——A(A)—— ---
```

or :

```
--- ——(U)U—— --- ——(A)A—— --- ——(U)U—— ---
```

1b)

```
--- ——A(A)—— --- ——U(U)—— --- ——A(A)—— ---
```

or :

```
--- ——(U)A—— --- ——(A)U—— --- ——(U)A—— ---
```

2)

```
--- ——A(U)—— --- ——U(A)—— --- ——A(U)—— ---
```

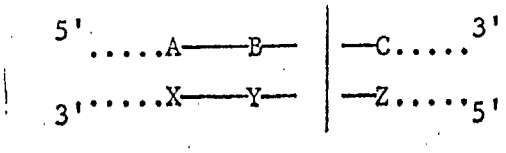
The cleavage sites on the first two models are identical.

In these diagrams, the normal letters represent the known bases, whilst the letters in parentheses represent the complementary bases predicted by base pairing. However, there must still be some further specificity in the action of RNase III on a natural ds-RNA, for it is possible to calculate approximately how frequently these patterns occur on a molecule. This is considerably more than the number of sites that are cleaved to yield the specific fragments. Assuming the MW. of each ds-RNA molecule to be 2×10^6 daltons, and assuming the proportions of all four bases to be equimolar, then 1 in every 2 base pairs will be A:U. If the arrangement of the base pairs is assumed to be random, then there is a further 1 in 2 chance that the next base pair will be A:U. This, however, can be in one of two configurations, either cis, the same way up as before, or trans, inverted. Assuming that only one of the 3 models shown above is correct, then there is a further 1 in 2 chance that this base pair under consideration will be the correct way up for the formation of a susceptible site, i.e. a total chance of $1/2 \times 1/2 \times 1/2 = 1/8$ that any given adjacent couple of base pairs will be a sensitive site. The total number of nucleotides in the RNA, assuming an average residue weight of 330, is $2 \times 10^6/330 = 6000$ bases, i.e. 3000 base pairs, and hence 3000 couples of base pairs (each base pair is a member of two couples). Of these, 1 in 8, that is approximately 370 will be of the conformation found for the active site. Consideration of the band patterns shown in figs. 9 and 10 shows that this is much greater than the actual number of sites that must be cleaved to produce an appropriate number of specific fragments. Because of the double-stranded nature of the substrate it is unlikely that some of the susceptible sites can be buried away inside the molecule. So it appears that the enzyme specificity is greater than it would seem from a simple consideration of the 5'-ends of the fragments.

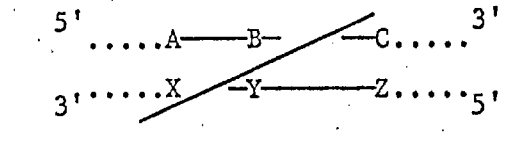
Possibly nucleotides further inside the fragments are also important in deciding which of the postulated sensitive sites are actually cleaved.

The finding of small quantities of guanosine and cytidine does not fit into the model developed above, and below. As they are found to a much lesser extent than either adenosine or uridine, it cannot be that guanosine and cytidine are frequently involved in susceptible sites. Possibly the labelling by polynucleotide kinase is not uniform, but there has been no evidence published in support of this.

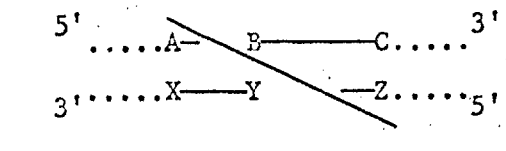
In considering the nature of the susceptible sites on the ds-RNA substrate, an assumption was made in considering solely the situation that arises if RNase III cuts straight across the ds-RNA. This was claimed to be the case by Schweitz and Ebel (1971) and is confirmed by the results given in this thesis, but it is not probable that the techniques used would be sufficiently sensitive to distinguish between the three cases below :

a) 

5'-end determination detects C and Y, which is sufficient to define the immediate nature of the susceptible site.

b) 

5'-end determination detects C and X, the base pair B-Y is not detected.

c) 

5'-end determination detects B and Y, which are necessarily complementary residues.

In case a), 5'-end determination completely defines the bases adjacent to the site of cleavage, for from the two residues actually found the complementary bases can be predicted. Situations b) and c) mean however, that determination of the 5'-ends does not yield as much information as it might at first seem about the cleavage site. In case b) the base pair B-Y is not detected, whilst in case c) it is the only one detected. It is of interest that of necessity the nucleosides detected in case c) are complementary, as were the residues found in the labelling experiments performed on RNase III digests.

Examination of fig. 12 shows that the addition of ammonium sulphate to the digestion mixture did not significantly inhibit the

digestion of PcV-ds-RNA by RNase III, at least at that stage in the fragmentation when specific fragments were produced. Instead a slight stimulation of activity was noted. This is in conflict with the findings of Robertson et al. (1968) who reported an apparent inhibition by sulphate of the digestion of Reovirus ds-RNA, at least as judged by the rate of appearance of acid-soluble fragments. This different, less sensitive, assay system may be the source of the conflict.

Addition of sulphate, or increasing the amount of chloride in the PcV-ds-RNA:RNase III digestion mixture, changed qualitatively or quantitatively the band pattern produced by limited action of RNase III on PcV-ds-RNA, but no change in the 5'-termini of the specific fragments resulted. These findings together support the hypothesis that in the digestion of PcV-ds-RNA to produce specific fragments, cleavage does not occur between G:C base pairs. The lack of change in 5'-termini when the enzyme specificity is somehow changed could also be taken to support scheme c) as the method of cleavage. However, this cannot be decided finally in the absence of 3'-end determinations on the fragmented PcV-ds-RNA.

The method, or methods, by which sulphate exerts its effect in inhibiting the digestion of poly G:C, and in changing the band pattern produced on digestion of PcV-ds-RNA (and how far these changes are related to a simple increase in ionic strength) remain unknown. The possibility exists that adding sulphate to the digestion medium, or increasing the concentration of chloride may alter the configuration of the RNase III molecule in some way so as to alter the enzyme specificity. Sulphate has been shown to have a specific effect in stabilising the structure of several proteins, including bovine panc. RNase (Von Hippel & Wong, 1964; Ginsburg & Carroll, 1965; Gerlsma, 1968), but whether a similar effect occurs with RNase III, and how this would relate to the chloride-induced change in specificity cannot be studied in the absence of really pure enzyme, which is not so far available. It is also quite possible that the salt changes may have some effect on the structure of ds-RNA and ds-polyribonucleotides, altering their susceptibility to RNase III cleavage.

b) Pancreatic RNase.

There have been very few studies on the digestion of ds-RNA by panc. RNase, and thus little is known about its mode of action on this substrate. The apparent resistance of ds-RNA to panc. RNase depends, as described in the Results, section 2 b), on the sensitivity of the assay technique. In this work three very sensitive techniques : gel electrophoresis, sucrose density gradient centrifugation and viscosimetry were used to study the fragmentation of PcV-ds-RNA by panc. RNase.

The differing sensitivity to panc. RNase of the different batches of PcV-ds-RNA could perhaps stem from the different purification procedures used in the preparation of each batch.

The sensitivity of ds-RNA to panc. RNase may well be altered by relatively simple treatment. For example, the thermal activation process (De Clercq, Wells & Merigan, 1970) which involves heating the ds-RNA polymer (the effect is most noticeable with poly(A-U)) at 37°C in the presence of Mg^{++} and Ca^{++} ions, at slightly alkaline pH (7.5), results in an up to 100-fold increase in RNase resistance. However, the RNase resistance of the activated polymer was measured by determining the antiviral activity after digestion with various amounts of panc. RNase, not a very sensitive test. Also, nothing is known about the relative activity of activated poly(A-U) fragments. Thermal activation did not apparently increase the resistance to digestion with Snake Venom phosphodiesterase, an exonuclease (De Clercq, Wells, Grant & Merigan, 1971). The antiviral activity, expressed in terms of the minimal inhibitory concentration, is also greatly increased. Because activation only occurs when the polymer is in very dilute solution, the physical basis of the phenomenon has not been properly investigated.

It is not suggested that the different batches of PcV-ds-RNA have undergone differing degrees of thermal activation during the isolation and purification procedures, but it seems reasonable to assume that if simple incubation under mild conditions can affect the panc. RNase resistance 100-fold, then other conditions encountered in the preparation of the ds-RNA might have some effects on the nuclease resistance.

For the study in this thesis of the mode of action of panc. RNase on ds-RNA, it was first necessary to determine whether the activity against ds-RNA was due to the formation of dimers and higher oligomers, which have been shown to be much more active against ds-RNA than is the monomeric form of panc. RNase (Libonati & Floridi, 1968; Libonati, 1971).

Treatment of the enzyme preparation according to Crestfield et al. (1962) to destroy dimers and higher oligomers did not result in any significant alteration in the rate of digestion of ds-RNA. This finding indicates that dimers, formed by some unknown mechanism, are probably not the source of the activity against ds-RNA, but rather that the monomers are active. However, the presence of dimers, and the destruction of deliberately preformed dimers, were not tested.

Studies of the method of cleavage of ds-RNA by panc. RNase showed that usually both strands of the nucleic acid double-helix were cleaved simultaneously. This is an unexpected finding, as it seemed more likely that a nuclease with a preference for the cleavage of single strands would, when confronted with a double-stranded substrate, act by cleaving the individual single strands, i.e. haplotomically. This however was not the case, for it was shown that panc. RNase digestion of ds-RNA does not involve a lag period before MW. reduction commences, that digests denatured by formaldehyde reaction do not contain many (although they did contain some) ss nicks which would be expected as a feature of haplotomic digestion, and finally that only 1.3 hits were necessary to cleave the ds-molecule, as assessed by the methods of Schumaker et al. (1956) and Cavalieri and Rosenberg (1961). All these results show that panc. RNase cleaves PcV-ds-RNA mainly by the diplotomic method.

This result is in conflict with the data of Erikson and Erikson (1967) who found that treatment of the replicative form ds-RNA (RF-ds-RNA) from R 17 phage-infected E.coli cells with 1:10 (RNase:RNA) panc. RNase for 10 min, in a buffer of high ionic strength, did not affect the sedimentation behaviour of the ds-RNA. However, heat denaturation of the digested RF-ds-RNA revealed the presence of several single-strand breaks. Under their conditions they found heat denaturation to cause very little apparent hydrolysis. Their finding of many single-strand nicks indicates that if digestion had progressed further, then haplotomic cleavage of the molecule would have occurred. Except for the differences between the substrate ds-RNAs (one a genome, the other an intermediate in the replication of a single-stranded RNA-containing bacteriophage) the conflict between these data and those reported in the thesis has not been resolved. The mechanism of action postulated below is based on the latter results only.

Evidence to support diplotomic cleavage of ds-RNA by panc. RNase was obtained by Bishop et al. (1967b), who found that panc. RNase destroyed the infectivity of Poliovirus RF-ds-RNA, with single-hit kinetics, i.e. cleavage of the ds-RNA was by a diplotomic mechanism.

It has been postulated that cleavage of ds-RNA by a nuclease molecule requires that the enzyme has two active sites, which act on the opposite phosphodiester bonds simultaneously (D'Alessio, Zofra & Libonati, 1972a). These authors based their hypothesis on the findings that Bovine Semen RNase (RNase BS-1), a nuclease active against both ss- and ds-RNA (Libonati & Floridi, 1969), was in fact a dimer of two panc. RNase-like subunits, and separation of these subunits destroyed the activity against ds-RNA without altering that against ss-RNA (D'Alessio, Parente, Demma, Farina & Leone, 1971; D'Alessio, Parente, Farina, La Montagna, De Prisco, Demma & Leone, 1972b). It has also been reported that artificially prepared dimers of bovine panc. RNase A have a very greatly increased activity against ds-RNA and poly A, as compared with the monomeric form (Libonati, 1971). The preparation of dimers from one active molecule of panc. RNase and one molecule inactivated by carboxymethylation of a histidine residue in the active site is quite possible; and in accord with the theory such active/inactive dimers have 50 % of the activity against ss-RNA of a normal dimer, but are virtually inactive against ds-RNA (D'Alessio et al., 1972a). Dimers with two active sites, each cleaving a single strand of the ds-RNA, would give the appearance of diplotomic action, the major mode of fragmentation found in this work. It would seem then, that there are two possibilities to explain the diplotomic cleavage of PcV-ds-RNA. Both involve what I shall call 'functional dimers', by which is meant two RNase molecules acting together, but not necessarily linked to each other in any way. The first hypothesis is that cleavage of opposite phosphodiester linkages does not occur (to any great extent) until 2 enzyme molecules are positioned so as to cleave both simultaneously. The two enzyme molecules arrive at opposite phosphodiester bonds by chance. Possibly the binding of one panc. RNase molecule makes the binding of a second to the opposite phosphodiester bond more likely.

The second, and preferred, hypothesis is that a small quantity of transient functional dimers is being formed, and that the equilibrium dimer $\rightleftharpoons$ 2 monomers is greatly in favour of the monomeric form. However, sufficient dimers (which may or may not resemble those studied by Libonati, 1971) exist to cleave the ds-RNA at an appreciable rate. Treatment of

the panc. RNase preparation according to Crestfield et al. (1962) so as to split up dimers and higher oligomers, would destroy the small equilibrium fraction of dimers postulated, but these would then re-form when the sample of enzyme was cooled down. It is postulated that these transient dimers are responsible for the diplotomic cleavage of PcV-ds-RNA by panc. RNase. Some support is given for these hypotheses by the fact that the amount of panc. RNase necessary to obtain rapid or complete digestion of ds-RNA is very great (10 µg panc. RNase/1 µg ds-RNA, Loviny & Szekely, 1973), so that the process cannot really be considered enzymic, i.e. catalytic, unless only a small fraction of the enzyme (functional dimers ?) is active at any one time.

c). Ultrasonic irradiation.

Fragmentation of ds-RNA by ultrasonic waves was found to occur by a similar mechanism to the fragmentation of DNA. This is not surprising considering the overall physical similarities between the two ds-nucleic acids.

3. The Biological Activity of Fragmented PcV-ds-RNA.

Administration of ds-RNA to animal tissue cultures or to intact animals has several effects which may or may not be related, and which were reviewed in the Introduction. The most important effects, in the current view, are the induction of IF, antiviral protection, and toxicity. The effects of fragmentation of the ds-RNA on these phenomena have been studied.

Fragmentation of PcV-ds-RNA has been found to result in a drastic fall in IF-inducing capacity, with no significant alteration, or a slight increase, in antiviral protection depending on the dose of challenge virus, and an increased toxicity in one strain of mice but no change in another.

These results, the first obtained on a natural as opposed to a synthetic ds-RNA, in part confirm and in part contradict the experiences of other authors, all working with synthetic ds-polyribonucleotide complexes. The different activities will be dealt with separately, and then together.

a) Toxicity.

Fragmentation of PcV-ds-RNA, reducing the size of the molecules from 2 x 10^6 daltons to a very approximate average of 5 x 10^5 daltons, led to no significant change in toxicity when assayed in CD 1 mice, following either i.v. or i.p. injection. However, similar and smaller fragments killed several (usually between 3 and 7, but on one occasion all and on another none) of each group of 10 or 11 BSVS mice following i.v. injection at 4 mg/kg. Intact PcV-ds-RNA at an exactly similar weight dose had no appreciable toxicity in these mice.

As described in the Introduction, section 3 g), all investigators who determined the relationship of toxicity and MW. following i.p. or i.v. injection of ds-RNA (Niblack & Walsh, pers. commun., 1970; Levy et al., 1970; Niblack & McCreary, 1971; Hamilton, 1971; Hilleman et al., 1971; Shiokawa & Yaoi, 1972) found that lower MW. ds-RNA was less toxic. There are no published reports of increased toxicity, as was found in this study using mice. Data on the toxicity in CD 1 mice injected i.p. and i.v. with intact and fragmented PcV-ds-RNA show that the route of injection, whilst very influential on the toxicity in terms of LD_{50}, did not affect the relative toxicities of intact ds-RNA and the fragments. The conflict between the results obtained in different mouse strains points out the very great importance of animal strain as well as species in this type of work. This importance of animal strain was also shown in the work of Rice, Turner, Chirigos and Spahn (1971), who found a 100-fold variation in IF-inducing ability of a poly(I).poly(C)-poly D-lysine complex, by using 8 different mouse strains.

The time of death of the BSVS and CD 1 mice is also of interest. In the toxicity assays in CD 1 mice, the deaths occurred over a period of 7 days, most dying on the first, second and third days following injection, see Tables 11a and b. When BSVS mice were given fragmented ds-RNA they died only a few hours after administration, although it is not known for how long deaths would have continued to occur had the survivors not been sacrificed for IF assay after 10 or 12 hrs. CD 1 mice given fragmented ds-RNA often showed symptoms of shock (R.C. Imrie, pers. commun.), from which they quickly recovered, only to die much later of the toxic effects as reported. A possibly similar shock phenomenon was noted by Philips et al. (1971) when injecting poly(I).poly(C) i.v. into CD 1 mice. The poly(I).poly(C) ranged in MW. from 10^5 to 10^7 daltons, with a peak value of 3 x 10^6 daltons, and mice given high doses were often shocked, and

had immediate, brief convulsions from which they soon recovered. Again, these mice died a few hours later, due to the very high doses used. It is postulated that BSVS mice, which are more sensitive, given fragmented PcV-ds-RNA were shocked, but did not recover. The intact PcV-ds-RNA did not cause such shock at the same weight dose.

b) Antiviral activity and IF-inducing capacity of the fragments.

A significant difference in the response of BSVS and CD 1 mice in terms of these effects was noted. Fragmentation sufficient to cause a great reduction in the IF-inducing capacity in BSVS mice caused apparently no decrease in antiviral activity (presumably related to interferon production, De Clercq, Nuwer & Merigan, 1970) in CD 1 mice. However, this difference and the difference in apparent toxicity on fragmentation also noted when comparing these two strains of mice may just reflect a low sensitivity of CD 1 mice to changes in the MW. of the ds-RNA.

Fragmentation of PcV-ds-RNA has been shown in this thesis to lead to a fall in the IF-inducing efficiency in BSVS mice, and the reduction in efficiency was qualitatively similar regardless of the method of fragmentation used. With ultrasonic fragmentation, there was a direct relationship between the degree of fragmentation and the efficiency of IF induction. It is therefore unlikely that specific internal regions of the ds-RNA molecule are important in the biological activity, as with each method of fragmentation acting in a different way and presumably at different sites, it would be expected that any specific regions necessary for induction of IF would be cleaved by one method of fragmentation and left intact by another. However, there is still the possibility that the specific regions are very short and frequently repeated along the strands and are therefore retained in all the digests.

These data mean that probably the PcV-ds-RNA acts to induce IF in a very similar way to the various synthetic double-stranded RNAs, where base composition has been shown not to be an absolute requirement for inducing ability (Field et al., 1967; Colby & Chamberlin, 1969; De Clercq & Merigan, 1969). In these latter studies, the efficiencies of the different inducers varied considerably, although as no mention is made of the relative sizes of the different inducers it is impossible to draw any safe conclusions from their work on the lack of any sequence or composition requirements for IF-induction.

The cause of the fall in IF-inducing ability of PcV-ds-RNA on fragmentation is not known. Although fragmented ds-RNA killed several of the mice used in IF assays, and presumably had non-lethal ill-effects on the survivors, the possibility that sick mice did not produce IF efficiently can be discounted. This was shown by the work of Buckler, DuBuy, Johnson & Baron (1971) who found that the peak titre and duration of the IF response increased with increasing dose of poly(I).poly(C) inducer, even up to toxic levels.

Digestion with serum nucleases could be a significant factor in deciding the efficiency of IF-induction by intact and fragmented ds-RNA. Although there was no significant difference in the rates of digestion of intact and ultrasonicated PcV-ds-RNA by human serum (after an initial period when digestion of the fragments appeared to be more rapid), because the size of the fragments is lower of course than that of the intact ds-RNA before digestion, it is possible that digestion at similar rates will reduce the fragmented RNA to below the threshold size for induction a long time before it produces the same effect on the intact RNA.

However, nuclease activity against ds-RNA has not been detected at significant levels in the sera of mice (Nordlund et al., 1970).

The most simple hypothesis to explain the lowered efficiency of IF production is that for some reason the fragments may simply be less efficient as IF-inducers than is the intact material. This could be either a threshold size effect, or there could be a continuous parallel decline in inducing efficiency with MW. The threshold effect would be masked in these assays by the wide size range of the fragments. In either case, however, it is not known why size is important for efficient induction. It could be that some recognition phenomenon has a size requirement; for example the triggering of the receptor site protein postulated by Colby and Chamberlin (1969) may in some way be dependent on the size of the inducer molecule. Alternatively, the MW. of ds-RNA could somehow regulate the efficiency of some uptake process, for instance from the circulation onto the surface of the IF-producing cells.

Another possible cause of the lowered efficiency of ds-RNA fragments as IF-inducers could be that alteration of inducer size may alter by some mechanism the kinetics of induction and/or IF production. The kinetics of appearance of IF in the serum of mice has been found to be dependent on the dose when poly(I).poly(C) is used as the inducer (Buckler et al., 1971). This kinetic hypothesis is supported by the results of Jameson

and Goldberg (1970). After injecting mice with complexes of poly(I) and poly(C) of different sizes, these authors found that the persistance of the induced IF in the mouse circulation was markedly dependent on the size of the inducer. By assaying for circulating IF at only one time, that of the peak titre for intact PcV-ds-RNA (Buck et al., 1971), the peak titre induced by fragments might be missed. This effect could again be an uptake or recognition phenomenon. It is not yet possible to decide between these hypotheses; the cause of the lowered efficiency of IF-induction on fragmentation is unknown. The results obtained on IF-inducing capacity and toxicity do show however, that fragmentation does not improve the prospects for the use of PcV-ds-RNA as an antiviral agent, and point out the very great importance of measuring toxicity in several animal strains and species before a safe prediction can be made as to the possible toxic effects of PcV-ds-RNA in medical or veterinary usage.

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