THE INTERACTION OF A NATURALLY-OCCURRING dsRNA WITH MAMMALIAN CELLS

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by

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ABSTRACT

dsRNA extracted from the mycophage that grows in cultures of the mould Penicillium chrysogenum is a potent interferon inducer and antiviral agent. Toxic side-effects prevent its chemotherapeutic administration to man. The effects and biochemical fate of a highly purified dsRNA from P. chrysogenum (BRL 5907) were examined in activated lymphocytes and established cell lines, using several techniques in parallel (density gradient centrifugation, hydroxylapatite chromatography and autoradiography) to allow a more thorough investigation of the cellular interaction than has previously been described. Preliminary experiments on the fate of dsRNA in the mouse were also conducted to attempt the correlation of *in vitro* with *in vivo* results.

The interaction of dsRNA with primary cultures of mouse spleen lymphocytes was profoundly influenced by the presence of foetal calf serum in the culture medium. In serum-free medium, dsRNA was toxic to stimulated lymphocytes, inhibiting DNA synthesis and cell division, when present from the initiation of transformation. Lymphocytes already undergoing blastogenesis, or those stimulated in serum-supplemented medium, were considerably more resistant to the inhibitory effects of dsRNA. Dividing lymphoid and fibroblast cell lines were likewise relatively unaffected by dsRNA, both in the presence and absence of serum.

In serum-free medium, mouse lymphocytes incorporated a low level of ^{3}H-dsRNA, and the cell-associated label retained molecular integrity for at least 24 hours. The problem of non-specific membrane binding and contamination of nuclear preparations with unlysed, non-viable cells was appreciated and investigated. In the presence of serum, the incorporation and re-utilisation of ^{3}H-dsRNA for cellular RNA and DNA synthesis was facilitated by the denaturation and degradation of dsRNA brought about by serum nucleases. Only the transformed lymphocytes incorporated significant levels of ^{3}H-dsRNA. Established cell lines incorporated far greater amounts of ^{3}H-dsRNA per cell than the lymphocytes, both in the presence and absence of serum. Under conditions where ^{3}H-dsRNA remained intact in the culture medium, it was rapidly catabolised and re-utilised by the cell lines, as a result of their greater ability, compared with mouse lymphocytes, to degrade dsRNA intracellularly.

The metabolism of dsRNA occurs at different rates in different cell types, and depends on the prevailing metabolic activity of the cells. Cytotoxicity was apparently associated with the persistence of cell-associated, undegraded dsRNA. This observation was supported by the finding that after intravenous administration of ^{3}H-dsRNA to mice, obvious early intestinal damage was accompanied by the presence of undegraded dsRNA in the ileum. The *in vivo* studies also identified the white blood cells as primary targets for dsRNA, and lend support to an indirect mechanism of toxicity.

CONTENTS

LIST OF ABBREVIATIONS

BSA	bovine serum albumen
Con A	concanavalin A
CS	caesium sulphate
DEAE-dextran	diethyl-aminoethyl dextran
dsRNA	double-stranded RNA
EAT cells	Ehrlich ascites tumour cells
FCS	foetal calf serum
HPBL	human peripheral blood lymphocytes
LPS	lipopolysaccharide
2-ME	2-mercaptoethanol
MEM	minimal essential medium
MLR	mixed lymphocyte reaction
MS cells	mouse spleen cells
PBS	phosphate-buffered saline
PHA	phytohaemagglutinin
PMNL	polymorphonuclear leucocyte
Poly A.poly U	polyadenylic acid-polyuridylic acid (double-stranded)
Poly I.poly C	polyinosinic acid-polycytidylic acid (double-stranded)
PWM	pokeweed mitogen
sd	standard deviation
srbc	sheep red blood cells
SSC	standard saline citrate solution (0.15M NaCl, 0.0015M trisodium citrate, pH7.4)
ssRNA	single-stranded RNA
Tdr	thymidine
WBC	white blood cell

INTRODUCTION

Filtrates from cultures of the Penicillium moulds have long been known to contain antiviral activity, and it was during the 1960's that the Merck, Sharpe and Dohme group discovered that the active principle was a double-stranded RNA (dsRNA), which, when purified, was a potent interferon inducer (Lampson et al, 1967). This dsRNA is contained within virus-like particles or mycophages that proliferate in the fungal mycelium (Banks et al, 1968; Kleinschmidt et al, 1968). Several such naturally-occurring dsRNAs with interferon-inducing properties have been described, one of which, obtained from mycophage-infected cultures of Penicillium chrysogenum (designated BRL 5907), has been highly purified in the laboratories of Beecham Pharmaceuticals Research Division for evaluation as a potential antiviral compound.

Synthetic double-stranded polyribonucleotides, such as the poly I.poly C duplex, were also found to be potent interferon inducers, both in vivo and in vitro (Field et al, 1967). The polymer poly I.poly C is among the most active of the synthetic inducers, and has appeal for clinical use because, unlike the naturally-occurring dsRNAs, it contains no potentially harmful genetic information. Prepared by annealing poly I with poly C, it is readily available from commercial sources, and therefore the most widely studied dsRNA inducer of interferon. However, as a consequence of the relatively simple method of preparation, considerable variety is found in the molecular weight and degree of duplex stability of different batches of poly I.poly C used by various groups. By contrast, dsRNA from P. chrysogenum is reproducibly obtained with a well-defined size and structure, and a highly base-paired secondary conformation. Furthermore, the preparation is specified free from bacterial endotoxin.

As well as being an interferon inducer and antiviral agent (Sharpe, Birch and Planterose, 1972) dsRNA from P. chrysogenum has other important biological activities which it shares with other natural dsRNAs and synthetic polyribonucleotides. These include antitumour activity in the mouse, as exemplified by its ability to suppress Lewis lung carcinoma and FS6 fibrosarcoma (Heyes and Catherall, 1974), and the inhibition of splenomegaly induced by Friend leukaemia virus (Pilch and Planterose, 1970). dsRNA enhances cellular immune responses (Naysmith and Cunnington, unpublished observations) and influences humoral immune responses; like the synthetic polynucleotides, it exerts

an adjuvant effect on antibody production when administered after an antigen, but is immunosuppressive if administered before antigen (Cunnington and Naysmith, 1975a; Schmidtke and Johnson, 1971). dsRNA also has a marked stimulatory effect on antibody titre to Newcastle disease virus (NDV) in chickens, when incorporated with the NDV antigen in an oily adjuvant to protect the dsRNA from rapid degradation (Gough et al, 1974).

The mechanism of the anti-tumour and immunoregulatory activity of dsRNAs is unresolved. An enhancement of T-cell function certainly occurs, and may be brought about either directly, perhaps through a mitogenic effect on lymphocytes (Dean, Wallen and Lucas, 1972; Ts'O et al, 1976) or indirectly, possibly through macrophage activation, as dsRNAs enhance macrophage function in vitro (Alexander and Evans, 1971) and in vivo (Cunnington and Naysmith, 1975a; Johnson and Johnson, 1971). Interferon induction by dsRNAs may also be involved, if, as has been suggested, interferon itself plays a significant role in the regulation of immune responses (Brodeur and Merigan, 1975; Johnson and Baron, 1976).

Interferon-inducing dsRNAs have toxic side-effects, particularly in mammals that are most responsive to the interferon-inducing activity, such as the rodent (Carter and De Clercq, 1974). The mouse develops weight loss, diarrhoea, thymic atrophy, splenomegaly, acute lymphopenia and other haematological changes (Philips et al, 1971; Leonard et al, 1969; Carter et al, 1976). Pyrogenicity, a local Schwartzman-like phenomenon, embryotoxicity and ocular toxicity are observed in rabbits (Absher and Stinebring, 1969; Lindsay et al, 1969). The dog is particularly sensitive to the acute toxic effects of dsRNA, doses higher than 1.0 mg/kg causing severe liver and intestinal damage, leucopenia , flaccid prostration and death (Philips et al, 1971). Poly I.poly C has been administered to man, when the most consistent side-effect has been a febrile response (Field et al, 1971, for example).

dsRNAs are directly toxic to cells, causing inhibition of protein synthesis in intact cells followed by extensive cellular destruction (Cordell-Stewart and Taylor, 1971, 1973). This cytotoxicity, which is specific for double-stranded RNAs, is further potentiated if the cells have been previously exposed to interferon (Stewart et al, 1972, 1973). The enhanced toxicity may be related to the enhanced inhibitory effect of dsRNA on protein synthesis in extracts from interferon treated L-cells as compared with untreated L-cells (Kerr, Brown and Ball, 1974). The block in protein synthesis occurs at the level of translation and involves at least two separate mechanisms.

One of these is the activation of a protein kinase that phosphorylates and inactivates the initiation factor eIF-2, and the other is the generation from ATP of the oligonucleotide pppA2'p5'A2'p5'A, which activates an endonuclease that degrades mRNA (Roberts et al, 1976; Farrell et al, 1978; Kerr and Brown, 1978). The shut-down in host protein synthesis in cells infected with lytic viruses may be related to the formation of dsRNA as a replicative intermediate, because the rate of appearance of dsRNA in infected cells parallels the decline in the rate of cellular protein synthesis (Cordell-Stewart and Taylor, 1973).

Another toxic effect of interferon inducers at the cellular level is the inhibition of DNA synthesis. dsRNAs inhibit DNA synthesis in vivo in liver cells following partial hepatectomy (Jahiel et al, 1971) and in isoproterenol-stimulated mouse salivary glands (Serota and Baserga, 1970). Poly I.poly C inhibits DNA synthesis in vitro in synchronised HeLa cells (Teng et al, 1973) and in PHA-stimulated lymphocytes (Badger, Cooperband and Green, 1972).

The biological significance of dsRNA in viral infections, and its role in host recovery, has been discussed by Carter and De Clercq (1974). dsRNA has been implicated in the replication of most, if not all, lytic RNA viruses (Ralph, 1969), and has also been demonstrated in cells infected with DNA viruses, for example vaccinia virus (Duesberg and Colby, 1969). This dsRNA may be responsible for both the pathological damage associated with a cytolytic virus infection, through a suicide-like mechanism involving destruction of the infected cells, and the stimulation of host defences against the infection, particularly interferon production, enhancement of antiviral antibody formation, and stimulation of cell-mediated immune mechanisms. Furthermore, the capacity of dsRNA to invoke a pyrogenic response may be important in limiting the spread of some respiratory tract viruses, whose rate of replication is reduced at temperatures above 37°C.

Small amounts of dsRNA have been detected in the heterogeneous nuclear RNA from normal uninfected cells (Jelinek and Darnell, 1972; Stollar et al, 1978). The function of this dsRNA is unknown, but it may play a regulatory role in normal protein synthesis in mammalian cells; very low levels of dsRNA inhibit protein translation in rabbit reticulocyte lysates (Hunter, Hunt and Jackson, 1975). This inhibition is specific for dsRNAs or double-stranded polyribonucleotides containing a helical region with a minimum of 20 base-pairs, and again involves the phosphorylation of eIF-2 (Farrell et al, 1977) and the production of the endonuclease activator pppA2'p5'A2'p5'A (Hovanessian and Kerr, 1978).

Thus dsRNAs are proving to be important biological molecules both inside and outside the field of virology. There is no doubt that their immunostimulatory and broad spectrum antiviral activities make them potentially very valuable drugs. However, their progression to the clinic for large-scale antiviral use has been prevented because of the toxic side effects associated with their administration. Naturally-occurring dsRNAs cause further concern because they contain foreign genetic information and must, if they enter cells intact, be considered as potentially harmful mutagens. Such transformation could occur conceivably through integration of sections of dsRNA directly into the host cell DNA, or by some form of reverse transcription from dsRNA to DNA, and subsequent integration of the foreign DNA.

The molecular basis for the toxic effects of dsRNA *in vivo* is not well understood. While some workers favour a simple model involving direct inhibition of protein or DNA synthesis, or both (Carter and De Clercq, 1974), others have suggested that dsRNAs, like endotoxin, pyran copolymer and other polyanions, damage the cell membranes of macrophages and granulocytes, causing the release of a number of pharmacologically active mediators including pyrogens, enzymes, oligonucleotides and lymphokines, which can modify the host in many ways (Braun, 1969).

Two acute toxic manifestations of dsRNA observed in most species are pyrogenicity and leucopenia. These effects are similar to, but have different kinetics from, those produced by endotoxin. It has been suggested that the fever is produced by the release of endogenous pyrogen from granulocytes (Stinebring and Absher, 1971), as is the case for endotoxin (Snell and Atkins, 1969). Leucopenia may arise through sequestration of white cells in the tissues, as well as a direct toxic effect on circulating WBC and haemopoietic stem cells (Degré, 1973). The WBCs most severely depleted by dsRNAs are the lymphocytes.

Thus peripheral blood leucocytes appear to be primary targets for the toxic effects of dsRNAs administered *in vivo*. These cells, together with tissue macrophages, are thought to be major producers of systemic interferon (Ho, 1977; Carter *et al*, 1977). However, surprisingly little work has been done on the interaction of dsRNA with lymphocytes, with regard to the cytotoxic effects or the incorporation and biochemical fate of the inducer. Such studies have been conducted in established or primary cell lines and Erhlich ascites tumour (EAT) cells, whose growth characteristics *in vitro* are very different to those of lymphocytes. It is important to examine the association of dsRNA

with cells, both *in vitro* and *in vivo*, in order to elucidate the mechanisms of toxicity and biological activity, so that materials with higher therapeutic ratios may be designed.

Most studies on cellular dsRNA uptake have been directed towards understanding the mechanism of interferon induction or viral RNA replication. In general, the experiments employed radio-labelled polynucleotides, such as poly I.^{3}H-poly C, which have been prepared by annealing one labelled polymer to an unlabelled complementary polymer. This may give misleading results, since some cells appear to incorporate one strand preferentially. De Clercq *et al* (1973) showed that the poly I strand was bound more tightly to rabbit kidney cells than the poly C strand. Further, while the poly I strand of a poly I.poly C duplex entered EAT cells (that is, it became resistant to external ribonuclease treatment), the poly C strand remained membrane bound; poly A.poly U, on the other hand, entered the cells as a duplex entity (Schell, 1971). Some of these findings are explained by the recent observation that the poly C strand is degraded by ribonucleases considerably faster than the poly I strand (De Clercq, 1979). ^{3}H-adenosine-labelled dsRNA from *P. chrysogenum* has a base composition A:U:G:C of approximately 1:1:1:1 (see appendix II), and as it is a naturally-occurring nucleic acid, the labelled adenosine residues should be more randomly distributed in both strands. Therefore this material has a more balanced distribution of label, as well as a more well-defined structure, than the synthetic inducers used in previous studies.

When considering the penetration of mammalian cells by dsRNA, there seems to be no doubt that high molecular weight molecules can enter cells in an intact form, since naked poliovirus dsRNA (the replicative form) is infective in cell cultures (Bishop and Koch, 1967). Whether penetration of dsRNA is actually required for interferon production remains debatable; the trigger for interferon production appears to be either a membrane event or the intracellular activity of only one or a few molecules per cell. Bausek and Merigan (1969) used radiolabelled poly I.poly C to show that interferon induction in cell cultures is triggered within one hour of exposure to inducer, and that most of the polynucleotide which binds to the cell membrane is not required for induction. A temperature-dependant stage, during which the poly I.poly C becomes resistant to ribonuclease, is also required after binding. A membrane-triggering event for induction of interferon was proposed after it was found that poly I.poly C coupled to insoluble supports, such as Sepharose, retained its inducing capacity. However,

some leakage of poly I.poly C from the matrix was always observed, therefore cellular penetration could not be ruled out (Pitha and Pitha, 1973; Bachner et al, 1975).

In many cell lines the induction of significant levels of interferon or production of infective virions occurs only if the dsRNA is present with the polycation DEAE-dextran. The uptake of labelled dsRNA has likewise been studied in the presence of DEAE-dextran (Colby and Chamberlain, 1969; Pérez-Bercoff et al, 1974; Táborský et al, 1977). In these studies, increased cellular penetration of dsRNA in the presence of DEAE-dextran, and association of intact dsRNA with nuclei, was claimed. However, for reasons that are fully described in the final discussion section of this report, these conclusions should be questioned. Indeed, there is little, if any, indisputable evidence in the literature for the intracellular persistence of high molecular weight dsRNA. Most results point to a fairly rapid catabolism of dsRNA and re-utilisation of the labelled degradation products for cellular nucleic acid biosynthesis, at least in the cell lines (Bausek and Merigan, 1969; Prose et al, 1970; Kelly and Levy, 1973). The rate of degradation may vary in different cell types; De Clercq and Stewart (1974) showed that poly I.^{3}H-poly C was degraded more rapidly in HeLa and Vero cells than in primary rabbit kidney cells. High molecular weight label was associated with the latter after a 1 hour labelling period. This differential degradative response observed between the cell types was inversely related to their ability to produce interferon, suggesting a correlation between biological activity and prolonged cellular association of undegraded inducer.

In many of these early studies it was not possible to distinguish between labelled donor RNA and cellular RNA synthesised de novo from degradation products. Therefore in autoradiographs, grains detected in the nuclei of cells following incubation with labelled poly I.poly C may not represent dsRNA in its native state. Labelling of cells in the presence of actinomycin D to inhibit de novo RNA synthesis should allow such a distinction to be made, but under these conditions incorporation of dsRNAs is severely reduced (Bausek and Merigan, 1969; Levy et al , 1970).

Alternative means of distinguishing between donor and cellular RNA have since been employed, for example, a double-labelling technique, whereby cells are exposed to ^{3}H-RNA in the presence of, say, ^{14}C-uridine, and the degree of association of ^{3}H and ^{14}C in the various RNA species is assessed. Volkin et al (1973) studied the integrity of human

lymphoblastoid ssRNA after its incorporation by human skin fibroblasts, using double-labelling and nearest neighbour analysis. In the latter method, RNA synthesised de novo was marked by partial replacement of uracil with 5-fluorouracil, and the donor RNA was labelled with ^{32}P. Uracil and 5-fluorouracil are separable as mononucleotides, therefore it was possible to determine the origin of labelled ^{32}P on the 3' or 5' position of the substituted nucleoside.

In analogous studies on the uptake and metabolism of DNA by cells in culture it is possible to distinguish donor from cellular DNA by labelling one type with the analogue 5-bromodeoxyuridine. The substituted DNA can be separated from normal DNA by its heavier buoyant density upon centrifugation to equilibrium in caesium chloride density gradients. Naturally-occurring phage and viral dsRNAs band characteristically, in caesium sulphate gradients, at a buoyant density lower than that of the RNA species found in normal mammalian cells (Szybalski and Szybalski, 1971). P. chrysogenum dsRNA typically equilibrates at a density of $1.61 g/cm^3$ in caesium sulphate, therefore this property was used as a marker to distinguish cell-associated ^{3}H-dsRNA from ^{3}H-RNA synthesised de novo from degradation products.

The primary aim of these studies was to investigate the interaction of a naturally-occurring dsRNA (BRL5907), a potent interferon inducer and toxic material in the mouse, with primary murine lymphocyte cultures in vitro, in particular its effect on DNA synthesis, the nature of its cellular incorporation and its biochemical fate inside the cells, and to compare its fate to that in an established lymphoid and a non-lymphoid cell line, so as to provide a point of reference with published work.

Freshly-prepared mouse spleen cells stimulated with concanavalin A (Con A) were used to provide a population of metabolically active lymphocytes, as this is a very popular and well-characterised system for the study of lymphocyte activation. The peak of the blastogenic response in these short-lived cultures (1 ml volume) occurs after approximately 48 hours, and the incorporation of ^{3}H-thymidine into DNA at this time has been used for many years as a measure of the response. Con A was employed as stimulant in preference to phytohaemagglutinin (PHA), as it causes less cell agglutination than PHA and induces the superior DNA synthetic response in murine spleen cells (Stobo et al, 1972). Like PHA it stimulates only the T-lymphocytes (Schumann et al, 1973) which comprise 50-55% of the splenic population (Ling and Kay, 1975a).

The immunoglobulin-secreting LDV cell line provided an example of a lymphoid cell having an infinite life-span in culture. These cells are of human origin, derived from a normal adult male, and proliferate in suspension culture. The V79 fibroblast, derived from normal Chinese hamster lung tissue, was used as a typical long-established and well-studied cell line. Growing in monolayer culture, these fibroblasts have an infinite life-span, although they are not believed to be virally transformed.

Virtually no data are available on the metabolism of dsRNAs *in vivo* with respect to the molecular integrity of the material following administration. Therefore preliminary *in vivo* experiments were also carried out in order to relate the apparent *in vitro* findings to the fate of radiolabelled dsRNA in the mouse.

It was hoped that the results would allow further understanding of the mechanism of dsRNA toxicity, as well as providing some answers to the question of whether dsRNA can penetrate cells as an intact duplex, and if so, as to how long it persists.

MATERIALS AND METHODS

1. Source of polynucleotides and radioisotopes

Double-stranded (ds) RNA and ^{3}H-adenosine-labelled dsRNA were provided by Beecham Pharmaceuticals Research Division. The material is purified from a mycophage growing in penicillin-producing strains of the fungus Penicillium chrysogenum, using essentially the same method as Banks et al (1969). Sterile dsRNA was supplied in vials in a lyophilised form, which, on reconstitution with distilled water, gave a 2 mg/ml solution in 0.15M NaCl. This sterile solution was stored at -20°C. It is chemically stable for four months at room temperature and much longer at 4°C.

^{3}H-labelled dsRNA was produced by the same methods from P. chrysogenum cultures grown in the presence of ^{3}H-adenosine. It was additionally separated from low molecular weight products by Sepharose 2B chromatography. One preparation of specific activity 30 µCi/mg, and three of 300-400 µCi/mg were available, supplied as 1-2 mg/ml solutions in 0.15M NaCl, and sterilised by chloroform treatment.

Poly I.poly C was obtained from P-L Biochemicals, Milwaukee. Poly A.poly U, yeast RNA and calf liver RNA were supplied by Sigma (London) Chemical Co. ^{3}H-poly C was obtained from Miles Biochemicals, Ltd., while the low molecular weight precursors (Me-^{3}H)-thymidine (18-25 Ci /mmol), (2-^{3}H)-adenosine (22-25 Ci/mmol), (5,6-^{3}H)-uridine (40-60 Ci/mmol) and L-(4,5-^{3}H)-leucine (40-60 Ci/mmol) were supplied by the Radiochemical Centre, Amersham.

^{3}H-DNA was prepared from ^{3}H-thymidine-labelled mouse spleen lymphocytes by Marmur extraction of the isolated nuclei and subsequent caesium chloride density gradient centrifugation. The ^{3}H-DNA had a molecular weight $>10^{6}$ and specific activity of 25-65 µCi/mg. ^{3}H-ssRNA was prepared from ^{3}H-adenosine-labelled mouse spleen lymphocytes by hot phenol extraction and purification by caesium sulphate density gradient centrifugation. The specific activity was 15 µCi/mg. Labelled nucleic acids were sterilised by chloroform treatment and stored in phosphate-buffered saline at -20°C.

2. Cell culture techniques

.1 Tissue culture media

RPMI 1640 medium was prepared from a powdered concentrate (Flow Laboratories, Irvine, Scotland), and sterilised by filtration through a Millipore 0.22 µm filter. Eagles minimum essential medium (EMEM) was prepared from sterile concentrates (Flow). Media contained 0.2% sodium bicarbonate, 1000 units/ml sodium benzyl penicillin and 100 µg/ml streptomycin sulphate (Glaxo). Foetal calf serum (FCS), obtained from Flow Laboratories, was heat-inactivated at 56°C for 30 min before use. Autoclavable solutions were sterilised for 30 min at 120°C and 15 p.s.i. Glassware was heat-sterilised for 4 hours at 160°C.

.2 Preparation of mouse spleen (MS) cell cultures

Male and female CBA mice, aged 2-3 months, were used to provide spleen cell suspensions except where stated. Mice were killed by cervical dislocation, and the spleens removed into sterile ice-cold Tyrode's solution. All subsequent procedures were carried out under sterile conditions at 4°C. The spleens were minced with fine scissors and passed twice through a 60-gauge stainless steel mesh. The cells were centrifuged at 250g, washed in Tyrode's solution , then recentrifuged and resuspended, to provide a single cell suspension of approximately 2.5×10^6 cells/ml, in RPMI 1640 medium containing antibiotics, 5% FCS, and 4 µg/ml Concanavalin A (Con A; obtained from Sigma Chemical Co.). The cells were dispensed in 1 ml volumes into flat-bottomed plastic culture vials and incubated undisturbed at 37°C in a 5% CO_2-air atmosphere for 48 or 72 hours,

DNA synthesis, used as an index of response to Con A after two days of culture, was estimated by measuring the incorporation of ^{3}H-thymidine into TCA-precipitable material. ^{3}H-thymidine (2 µCi/ml, 21 Ci/mmol) was added in a volume of 50 µl PBS per ml of culture medium for a period of 2 hours, after which incorporation was measured by the filter paper disc method (see 3.2). Results are expressed as c.p.m. incorporated into TCA-precipitable material per 2 hours per 10^6 cells. Duplicate cultures did not generally vary by more than 10%. Where 5 ml cultures were employed, the cells were incubated in glass universal bottles at an initial concentration of 1×10^6 cells/ml in medium supplemented additionally with 5×10^{-5}M 2-mercaptoethanol (2-ME).

.3 Culture of LDV cells

LDV cells, supplied initially by Dr. D. Viza, Paris, are a line of human lymphoblastoid cells of infinite life span, derived by spontaneous transformation from the peripheral blood lymphocytes of a normal healthy male. They were propagated in sealed suspension cultures under a 5% CO_2-air atmosphere, generally in 100 ml glass medical flats containing 50 ml RPMI 1640 medium supplemented with antibiotics and 10% heat-inactivated FCS. When the cells reached a density of approximately 1 x 10^6/ml they were subcultured by dilution into fresh medium at a concentration of 5 x 10^4 cells/ml. Cells in 1 ml culture were grown in flat-bottomed plastic culture vials, at an initial concentration of at least 1 x 10^5/ml.

.4 Culture of V79 fibroblasts

V79 (Chinese hamster lung) fibroblasts were routinely cultured in 100 ml glass medical flats in 10 ml EMEM containing antibiotics, 10% FCS, 2.5 µg/ml hydrocortisone and 10 µg/ml folic acid. Confluent cultures were washed with PBS and the cells removed during a 5-10 min incubation in 3 mg/ml trypsin (Difco 1:250) in 0.1M citrate, 0.15M NaCl buffer, pH 7.8. After addition of serum-containing EMEM, to inactivate trypsin, the cells were centrifuged for 5 min at 250g and resuspended in fresh medium to a concentration in the range 3 x 10^4 - 1 x 10^5 cells/ml. They were reseeded either into medical flats (10 ml) or into 35 mm diameter plastic petri dishes (1 ml), and cultured at 37°C in a 5% CO_2-air atmosphere. Freshly passaged cultures were allowed to become established for at least 16 hours before the addition of chemicals or removal of serum.

In order to test the colony-forming ability of V79 fibroblasts, trypsinised cells were seeded into 50 mm diameter plastic petri dishes at a concentration of 200 cells per dish in 5 ml EMEM with 10% FCS. After 5 days incubation, colonies were fixed in methanol, stained with carbol fuchsin and counted under a light microscope.

.5 Cell counting

Cell suspensions were diluted and fixed in 1.5% acetic acid containing methyl violet, and approximately 400 cells were counted microscopically using an improved Neubauer haemocytometer.

3. Uptake of radioactive materials

"Uptake" of labelled nucleic acids or polynucleotides refers to both their binding to the cell membrane and their entry into the cell interior. "Uptake" of low molecular weight precursors, for example ^{3}H-thymidine, refers to the label incorporated into TCA-insoluble material of the cell.

.1 Scintillation counting

The radioactivity associated with washed cell suspensions, cell fractions or extracts was measured by the filter paper disc technique, described by Bollum (1968). 50-200 µl aliquots were deposited onto 2.1 cm diameter Whatman no. 1 or 3 mm filter paper discs spiked onto stainless steel pins, the discs were allowed to dry, and were counted in 5 ml scintillation fluid consisting of 6g/l butyl PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] in toluene. Butyl PBD was supplied by Intertechnique Ltd., Uxbridge. Vials were counted in a Packard Tri-Carb liquid scintillation spectrometer, model 3385. Where a measure of TCA-insoluble radioactivity was required, the dried discs were treated in batches for 30 min with two changes of cold 10% (w/v) TCA solution, washed twice in methanol and finally in acetone. After drying at 60°C the discs were counted in scintillation fluid as above. The efficiency of ^{3}H counting on paper discs varied from 10-20%.

Low activity samples in aqueous solutions were counted in the xylene-based scintillant, Insta-Gel (Packard), at an efficiency of 25% or greater.

.2 Uptake of radioactive materials by MS cells

After an appropriate incubation period, cultures were transferred to an ice bath, and the cell aggregates dispersed in the culture medium by gentle aspiration using a Pasteur pipette. Replicate cultures were pooled and centrifuged at 250g for 10 min at 4°C. The cells were washed in two volumes of fresh RPMI 1640 with 5% FCS at 4°C, recentrifuged and resuspended in medium to

50% or less of the original culture volume. Aliquots of this cell suspension were analysed for radioactivity on 3 mm paper discs and counted in methyl violet fixative.

In the case of the low molecular weight precursor ^{3}H-thymidine, a more convenient method of measuring incorporation was employed, whereby 50 µl aliquots of the dispersed cell culture containing excess precursor were placed directly onto Whatman no. 1 discs. The discs were then treated for 30 min with 3 changes of cold 10% TCA solution, washed twice with methanol and once with acetone, then dried and counted as above. Washing with TCA satisfactorily removed unincorporated ^{3}H-thymidine, and dispensed with the centrifugation and washing steps. Duplicate cultures were processed individually and a mean result calculated.

In order to study the uptake of ^{3}H-labelled macromolecules in the absence of serum, 1 ml MS cell cultures were initiated in RPMI 1640 with 5% FCS and 4 µg/ml Con A. After 24-30 hours, the aggregated lymphocytes were redispersed by gentle aspiration, pooled into warm centrifuge tubes and pelletted at 250g for 10 min at 37°C. They were washed in warm, serum-free medium, recentrifuged and resuspended in serum-free medium with Con A at a concentration of approximately 2 x 10^{6} cells/ml. Cells lost during this procedure are preferentially dead cells (personal communication, G. Harris). The washed cells were dispensed as 1 ml cultures in plastic vials, and the incubation continued for a further 24 hours.

.3 Uptake of radioactive materials by V79 fibroblasts

At the end of the incubation period, growth medium with excess label was removed and the monolayers washed three times with PBS. The cells were removed using 3 mg/ml trypsin in citrate buffer (0.5 ml per petri dish or 5 ml per 100 ml bottle), transferred to centrifuge tubes with additional medium containing serum, centrifuged at 250g for 10 min, and resuspended in fresh medium to 50% of the original culture volume. Aliquots were analysed for radioactivity on 3 mm discs, and cell counts made in methyl violet fixative.

Cultures were placed under serum-free conditions by simply replacing the growth medium with fresh, serum-free medium, after washing monolayers twice with PBS. At the end of the labelling period, cultures were incubated for 5-10 min in medium containing

10% FCS before trypsinisation of the cells, since this procedure was found to prevent cells cultured in the absence of serum from aggregating together when trypsinised from the culture vessels.

.4 Cell fractionation and nucleic acid extraction

Washed cell suspensions were pelletted by centrifugation at 250g for 10 min, and resuspended at a concentration of $<2 \times 10^7$ cells/ml in 0.25M sucrose - 3.3 mM $CaCl_2$-0.25% (v/v) Nonidet P-40 (cell lysis buffer). After standing at 0°C for 15 min to allow plasma membrane disruption, the crude nuclear suspension was centrifuged at 700g for 10 min. The cytoplasmic fraction was drawn off, while the nuclei were washed in cell lysis buffer, recentrifuged, and resuspended in cell lysis buffer. Aliquots of cytoplasm, nuclei and nuclear washings were counted on 3 mm paper discs.

A modification of the Marmur procedure (Marmur, 1961) was used to extract nucleic acids from the nuclei and cytoplasm. Nuclei were centrifuged and resuspended in 0.15M NaCl - 0.01M EDTA - 2.5 % (w/v) sodium dodecyl sulphate (NaCl - EDTA - SDS), while cytoplasmic fractions were mixed with half a volume of 2x strength NaCl - EDTA - SDS solution. Fractions were vortexed lightly, heated at 60°C for 10 min, then cooled in an ice bath. $NaClO_4$ was added to a final concentration of 1M, followed by an equal volume of chloroform:isoamyl alochol (24:1). The mixtures were shaken at 0°C for 30 min. Low speed centrifugation separated the aqueous layer, containing nucleic acids, from the lower organic phase. The extracted nucleic acids were generally dialysed against SSC in preparation for CS density gradient analysis.

In order to avoid losses of nucleic acid during the extraction procedure, some preparations of labelled nuclei were lysed and run directly on CS gradients. The nuclei were centrifuged, resuspended in 0.2 ml SSC - 0.01M EDTA - 0.25% sarkosyl, pH 7.0, heated at 60°C for 10 min, and finally cooled. Lowering the initial density of the CS solution (to ρ = 1.58 g/cm^3) allowed separation of the DNA band from protein and sarkosyl at the top of the gradient (see 5.6).

.5 Preparation of autoradiographs

Suspensions of labelled, twice-washed cells were centrifuged at 250g for 10 min in narrow-bore conical tubes and resuspended gently to a concentration of approximately 5 x 10^6 cells/ml in 10% bovine serum albumen in saline (Sigma). The cells were dropped in thin films onto clean, dry microscope slides, rapidly air-dried, fixed in methanol for 20 min, and washed thoroughly in water for 30 min. V79 fibroblasts grown on glass coverslips were washed free of excess label with several changes of PBS before fixation in situ with methanol.

Cell pellets to be prepared for sections were washed and centrifuged as above, fixed in 2% glutaraldehyde in 0.1M phosphate buffer, pH 6.8, washed three times in the phosphate buffer, and post-fixed in 1% osmic acid in the same buffer. The pellets were dehydrated in alcohol, embedded in araldite, and 1 µM sections cut on an LKB ultratome.

Autoradiographs were made using Kodak AR10 stripping film or K5 emulsion, being exposed for 1-6 weeks at low temperature (-70°C). After development, autoradiographs were Giemsa stained.

4. In vivo experiments

Sex and weight-matched CBA mice were used, aged 5-8 weeks and weighing 20-25g. dsRNA and ^{3}H-dsRNA were administered in 0.15M NaCl, in a volume not exceeding 0.3 ml, either intraperitoneally (i.p.) or intravenously (i.v.) via the lateral tail vein. The mice were bled either from the retro-orbital sinus, or by cardiac puncture while under ether anaesthesia. Blood samples for cell counts were mixed with K^+-EDTA in plastic collection vials (Sarstedt). Plasma was collected after centrifugation at 250g for 10 min at 4°C.

Plasma and serum samples containing ^{3}H-dsRNA were counted both in Insta-Gel and on Whatman no. 1 filter paper discs in butyl PBD scintillation fluid. Samples of washed red cells were solubilised in 1.5 ml soluene 350:isopropanol (1:1), decolourised for 1 hour with 0.5 ml 30% (w/v) H_2O_2, and counted in 15 ml Insta-Gel:0.5N HCl (9:1). Quenching was estimated by internal standardisation with ^{3}H-dsRNA.

Cell suspensions in Tyrode's solution were prepared from the lymphoid organs, after preliminary mincing or teasing, using a 60-gauge stainless steel mesh. Cells were fractionated into nucleus and cytoplasm as previously described. Sections of ileum were cleaned and separated directly into nuclear and cytoplasmic fractions by homogenisation in 0.25M sucrose - 3.3 mM $CaCl_2$, using a hand homogeniser. NP40 was added to the homogenate to 0.25% final concentration. After 15 min at 0°C, the nuclei were centrifuged and washed as described previously.

5. Analytical techniques

.1 Dialysis of dsRNA

Dialysis tubing (Scientific Supplies) was boiled twice in $NaHCO_3$ solution to remove impurities, rinsed and boiled twice in distilled water. The tubing was stored under absolute ethanol and softened in sterile distilled water before use. dsRNA solutions were dialysed against 20 volumes of distilled water, or phosphate-buffered saline (PBS) under aseptic conditions, then readjusted to 0.15M NaCl by further dialysis against 0.15M NaCl.

The concentration of dsRNA solutions was not found to change as a result of dialysis. The concentration was checked by measurement of the absorbance at 260 nm (A_{260}) in a Pye Unicam SP500 spectrophotometer, using a conversion factor of 50 (i.e. a 50 µg/ml solution gives an A_{260} of 1.0 O.D. unit).

.2 Polyacrylamide gel electrophoresis

Cylindrical gels (6 mm diameter and 80 mm height) were prepared using a gel solution of 2.61% acrylamide, 0.14% NN'-methylene-bisacrylamide, 0.625% agarose, 0.4% tetramethyl-ethylene-diamine (TEMED), and 0.04% ammonium persulphate in gel buffer (0.04M tris, 0.02M sodium acetate trihydrate, 0.002M EDTA, pH 7.8). Electrophoresis was carried out in gel buffer for 3 hours at 4 mA per tube. dsRNA samples, in 100 µl maximum volume, were layered onto the gels in 0.05M NaCl, 0.001M EDTA, pH 7.0, containing 0.2% sodium lauryl sulphate, 5% sucrose and bromophenol blue as marker. Gels were fixed in 7% acetic acid, or stained for 1 hour with 0.1% methylene blue in 7% acetic acid, and then destained in 7% acetic acid. Absorbance at 260 nm was measured in a Gilford linear transport scanner. All chemicals were supplied from B.D.H. Chemicals, Poole, Dorset.

.3 Sucrose density gradient centrifugation

5-20% linear sucrose gradients in 5 ml polypropylene tubes were formed by freezing 20% (w/v) solutions of sucrose in 0.1M tris-HCl buffer, pH 7.6, containing 1M NaCl, and allowing them to thaw for at least 6 hours at 0°C. Samples to be analysed were carefully layered on to the gradients, with or without 100 µg unlabelled dsRNA as A_{260} marker, and overlaid with light paraffin oil. Centrifugation was always carried out at 100,000g at 5°C for 16 hours, in an MSE Superspeed 65 centrifuge.

Tubes were pierced at the bottom and 4-6 drop fractions collected. The fractions were diluted with water and the absorbance read at 260 nm. Radioactivity was estimated either by counting 0.2 to 0.5 ml aliquots directly in 5 ml Insta-Gel for low activity samples, or, for higher activity samples, by drying 0.05 to 0.2 ml aliquots onto 3 mm paper discs. The discs were counted in butyl PBD scintillation fluid.

Sedimentation coefficients (s) were calculated from the position of the centre of the bands relative to the meniscus, and the molecular weights determined using the equations of Studier (1965), assuming that the conformations of ssRNA and dsRNA are the same as those of denatured and native DNA, respectively. Since this assumption is never completely justified, molecular weights for RNA are approximations.

.4 dsRNA melting profile

dsRNA was diluted to <40 µg/ml in 0.1x strength SSC (standard saline citrate solution). The dsRNA solution and 0.1x SSC reference solution were placed in stoppered cells of 1 cm light path in a Unicam SP1800 spectrophotometer equipped with a thermostatically-controlled heating block and temperature programmer. The block was heated at a linear rate of 0.5°C per minute from 30-100°C, and the difference in absorbance at 260 nm (A_{260}) between sample and reference was plotted as a function of temperature.

The A_{260} is constant with temperature until the point of denaturation of dsRNA is reached, when a fairly sharp increase to a higher constant A_{260} value occurs. The melting temperature (Tm) is the temperature at which 50% of this increase in A_{260} has taken place. The hyperchromicity (HC) is calculated from the difference in absorption at 260 nm and 320 nm, at the new and old

absorbance values:

$$\text{i.e. } HC = \frac{(A_{260}-A_{320})_{100°C}}{(A_{260}-A_{320})_{30°C}} \cdot 100\%$$

.5 Hydroxylapatite chromatography of RNA

Hydroxylapatite crystals were prepared according to the method described by Miyazawa and Thomas (1965). Briefly, 2L each of 0.5M $CaCl_2$ and 0.5M Na_2HPO_4 were dripped continuously, with stirring, into 100 ml water at room temperature, at the rate of 4.4 ml/min. The precipitate was then washed four times in water, boiled for 1 hour in 40% NaOH, rewashed, then brought to boil three times in 0.01M sodium phosphate buffer, pH 6.8. The precipitate was filtered and dried at 37°C. Columns were prepared by packing 150 mg dry hydroxylapatite into 2 ml syringe barrels (Gillette), fitted at the bottom with porous polythene discs. They were maintained at 60°C in a thermostatically-controlled aluminium heating block, which was bored to house twenty syringe barrels.

dsRNA and ssRNA were separated by stepwise elution with phosphate buffers; RNA samples to be tested (2,000-20,000 c.p.m.) were loaded onto the columns in 0.012M potassium phosphate buffer, pH 6.8 in a volume of 1 ml, and a further 2 ml of buffer was added to wash through low molecular weight material. ssRNA was then eluted with 3 ml 0.125M potassium phosphate buffer, pH 6.8, followed by dsRNA elution in 3 ml 0.25M potassium phosphate buffer, pH 6.8. Finally 3 ml 0.5M potassium phosphate buffer, pH 6.8 was applied to clear the columns, which were re-equilibrated with 0.012M buffer ready for re-use. Each column was used not more than four times. The eluted fractions were collected in scintillation vials, and the radioactivity counted after the addition of 7 ml Insta-Gel. When mixtures of RNA and DNA were analysed by this technique, dsDNA eluted in the same fraction as dsRNA.

.6 Caesium sulphate (CS) density gradient centrifugation

3.0g solid Cs_2SO_4 (Koch-Light) was added to 3.3 ml of nucleic acid sample in SSC. Where labelled nucleic acid was analysed, 50-100 µg each of unlabelled dsRNA and calf thymus DNA (Sigma, Type 1) were generally added as A_{260} markers. After complete dissolution of Cs_2SO_4, the refractive index (η_D) of the solution was measured on an Abbe 60 refractometer (Bellingham and Stanley)

and adjusted to 1.380 (for RNA and DNA) or 1.382 (for RNA only) with SSC, to give an initial density of 1.58-1.61 g/cm^3. The solutions were overlaid with light paraffin oil and centrifuged to equilibrium for 64-72 hours at 100,000g and 18°C in an MSE Superspeed 65 centrifuge.

The tubes were pierced at the bottom and 4-drop fractions collected. Some fractions (1 in 10) were used to measure refractive index (η), while the remaining fractions were diluted with water and used for measurement of A_{260} and radioactivity, as described for sucrose density gradient centrifugation analysis. The density of the fractions (ρ) was calculated according to the equation for Cs_2SO_4 solutions, $\rho = 13.6986.\eta - 17.3233$, corrected for the density of SSC, ($\rho = 0.03$).

RNA and DNA were recovered from the gradients by dialysis of the required fractions against SSC to remove Cs_2SO_4, concentration by evaporation in an air stream from the dialysis sac, and redialysis against a buffer appropriate to the subsequent experimentation.

.7 Enzymic digestion

Except where otherwise stated, all enzymic digestions were carried out at 37°C in 0.01M tris-HCl buffer, pH 7.5, containing 10 mM $MgCl_2$, using a high enzyme:substrate ratio. Pancreatic DNase I (Sigma) was used, at a concentration of 50 µg/ml for 2 hours, to digest DNA to short oligonucleotides. Snake venom phosphodiesterase (Sigma) at a concentration of 50 µg/ml digested oligodeoxynucleotides and ssRNA completely to 5'mononucleotides in 1-2 hours. Bovine pancreatic RNase I (Sigma), at 50 µg/ml for 2 hours, was used for the rapid degradation of ssRNA and the slower, less complete digestion of dsRNA (Edy et al, 1976).

The extent of digestion of ^{3}H-labelled RNA or DNA was estimated by measuring the fall in TCA-precipitable radioactivity using the filter paper disc method (see 3.1) and by analysing the digestion products by sucrose density gradient centrifugation or by hydroxylapatite chromatography.

The activity of pancreatic RNase or of endogenous ribonucleases in cell extracts was prevented by the addition of sarkosyl (Ciba-Geigy) to 0.25% (w/v) or sodium dodecyl sulphate to 2.5% (w/v).

.8 Paper chromatography

Paper chromatography of 5'mononucleotides, formed from ^{3}H-labelled DNA as a result of sequential digestion by DNase I and snake venom phosphodiesterase, was performed by descending chromatography on Whatman no. 1 paper. The enzyme digest was applied with 5'AMP and 5'dAMP markers (10^{-7}M), and run for 24 hours at room temperature in ethanol-ammonium acetate-borate buffer; a 70:30 mixture of 90% ethanol with a solution containing 1M ammonium acetate, 0.01M EDTA-disodium salt, adjusted to pH 9.0 with 0.880 ammonia solution and finally saturated with sodium tetraborate.

After drying the paper and marking the U.V.-absorbing areas, the chromatograms were cut into strips and then into 0.5 or 1 inch sections which were counted in butyl PBD scintillation fluid, using solvent-treated paper squares for background c.p.m.

.9 Alkaline hydrolysis

The percentage of NaOH-insoluble label in cell fractions or extracts was used as a measure of the per cent DNA, since NaOH hydrolyses RNA but not DNA, to a mixture of 2' and 3'mononucleotides. A 0.2 ml aliquot of the sample was mixed with 0.1 ml 3N NaOH (final concentration 1N NaOH) and incubated at 60°C for 1 hour. TCA-precipitable label was measured before and after hydrolysis, by the filter paper disc technique (Whatman no. 1 discs). ^{3}H-dsRNA and cytoplasmic label from ^{3}H-RNA- or ^{3}H-adenosine-labelled cells were always >98% NaOH-soluble, while ^{3}H-DNA was always 100% NaOH-insoluble.

SECTION I - THE NATURE OF dsRNA FROM P. CHRYSOGENUM.

1. Physical properties

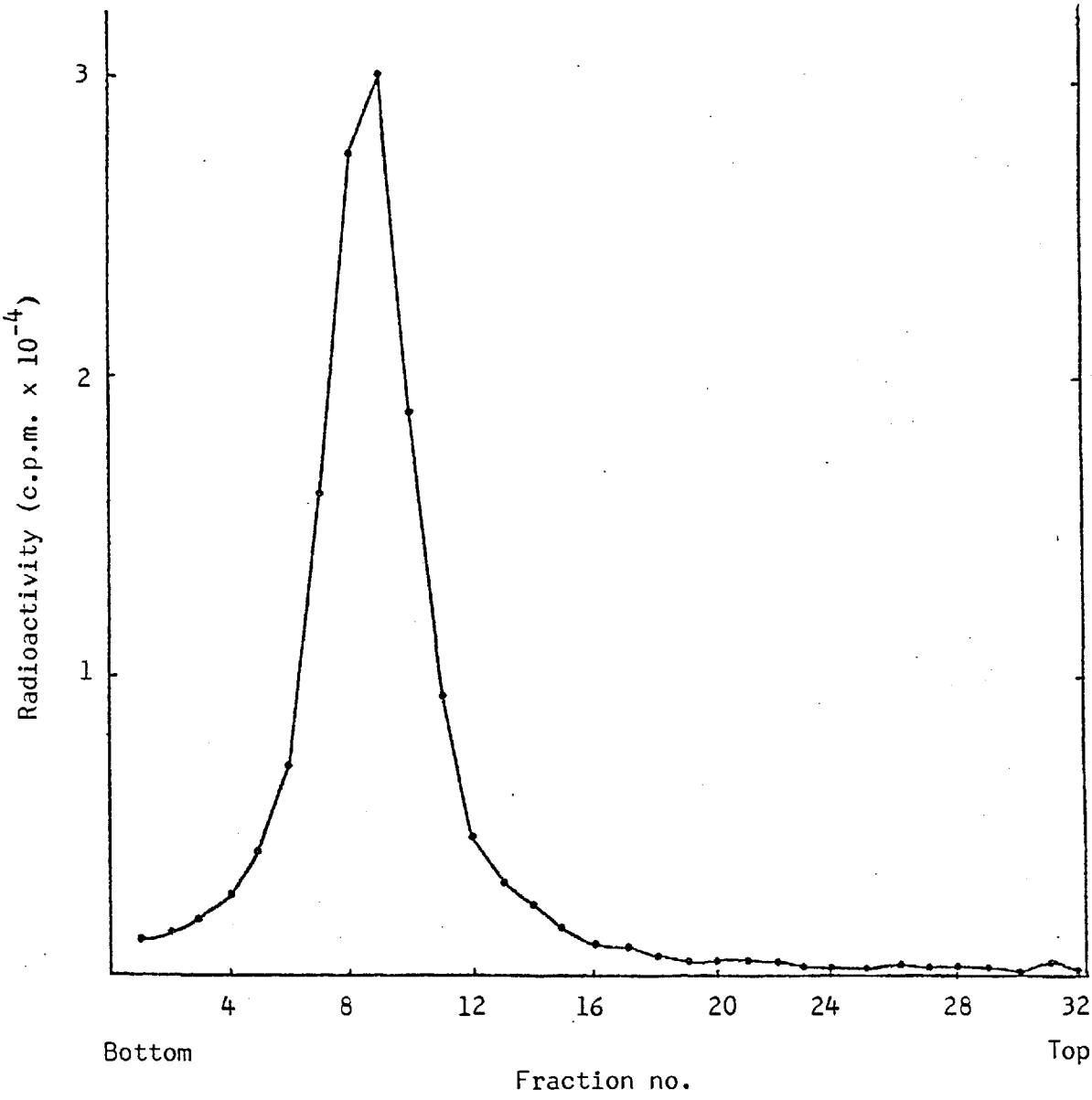
The dsRNA extracted from P. chrysogenum - associated mycophage has a well defined structure, consisting of three genome segments with molecular weights of 1.89, 1.99 and 2.18 x 10^6 (Wood and Bozarth, 1972). These segments are stable, near-perfect double-stranded molecules, except for a short single-stranded region at the 5' terminus of each (Yazaki and Miura, 1977), and are just resolvable as three separate bands by polyacrylamide gel electrophoresis. The material elutes as a single peak (A_{260}) during sepharose 2B chromatography. Its purity may be assessed from the analytical data detailed in Appendix II. The studies reported in this section were conducted to confirm the properties of the starting material to be used for the work, that is, to show that the unlabelled and ^{3}H-adenosine-labelled RNAs were double-stranded in nature and of high molecular weight.

The behaviour of dsRNA on sucrose density gradients, as run routinely during these studies, is shown in Figure 1. A single peak was obtained, giving a value for the molecular weight in the range 1.9 to 2.4 x 10^6 daltons, generally 2.1 x 10^6, but nearer 2.4 x 10^6 in this case. Since the actual value obtained varied slightly on different gradients, an unlabelled dsRNA marker was usually included in any test sample as a reference.

The banding pattern of dsRNA on polyacrylamide gels was confirmed (see Figure 38, Appendix I for a typical result), demonstrating the presence of the three mycophage genome segments in the preparation.

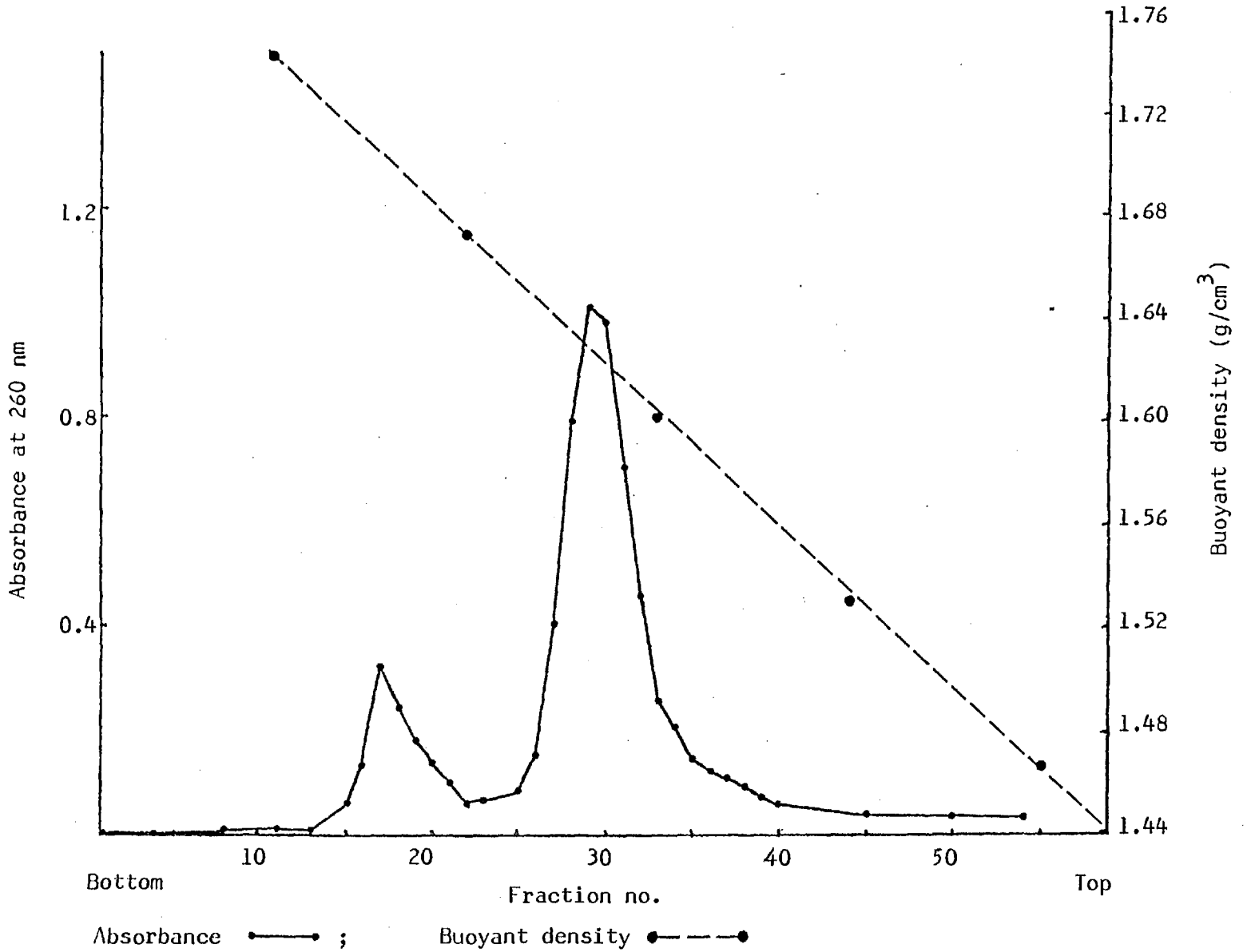
The equilibrium position of dsRNA on caesium sulphate (CS) density gradients, is shown in Figure 2. The material was always found to band as a sharp peak at a density of 1.61 (range 1.60 to 1.62). A smaller peak, representing approximately 15% of the total absorbance at 260nm was present with a density of 1.70. This small band was also presumed to be RNA in nature, since it had an A_{280}/A_{260} ratio of 2. ^{3}H-dsRNA preparations showed an identical banding pattern in caesium sulphate gradients. Reproducible, linear CS density gradients were consistently obtained throughout the course of the work. Calf thymus DNA routinely banded at a density of approximately 1.43.

Figure 1 - Sucrose density gradient centrifugation of ^{3}H-dsRNA



1 µCi ^{3}H-dsRNA (310 µCi/mg) in 50 µl PBS was centrifuged at 100,000 g and 5°C for 16 hours in a 5-20% neutral sucrose gradient containing 1M NaCl, as described in Materials and Methods. 50 µl samples of each fraction were counted after drying on to filter paper discs.

Figure 2 - Caesium sulphate density gradient centrifugation of dsRNA



The melting profile, that is, the increase in absorbance at 260 nm with increasing temperature, was typical of a double-stranded RNA molecule. (The curve is plotted in Figure 39(a), Appendix I). The material showed a fairly sharp increase in A_{260}, with a Tm in 0.1 x SSC at about 85.5°C, and a hyperchromicity of 141%. The double-stranded nature of the RNA was verified by subjecting the labelled preparation to hydroxylapatite chromatography. The results of three separate runs are given in Table 1, showing the almost complete association of label with double-stranded RNA.

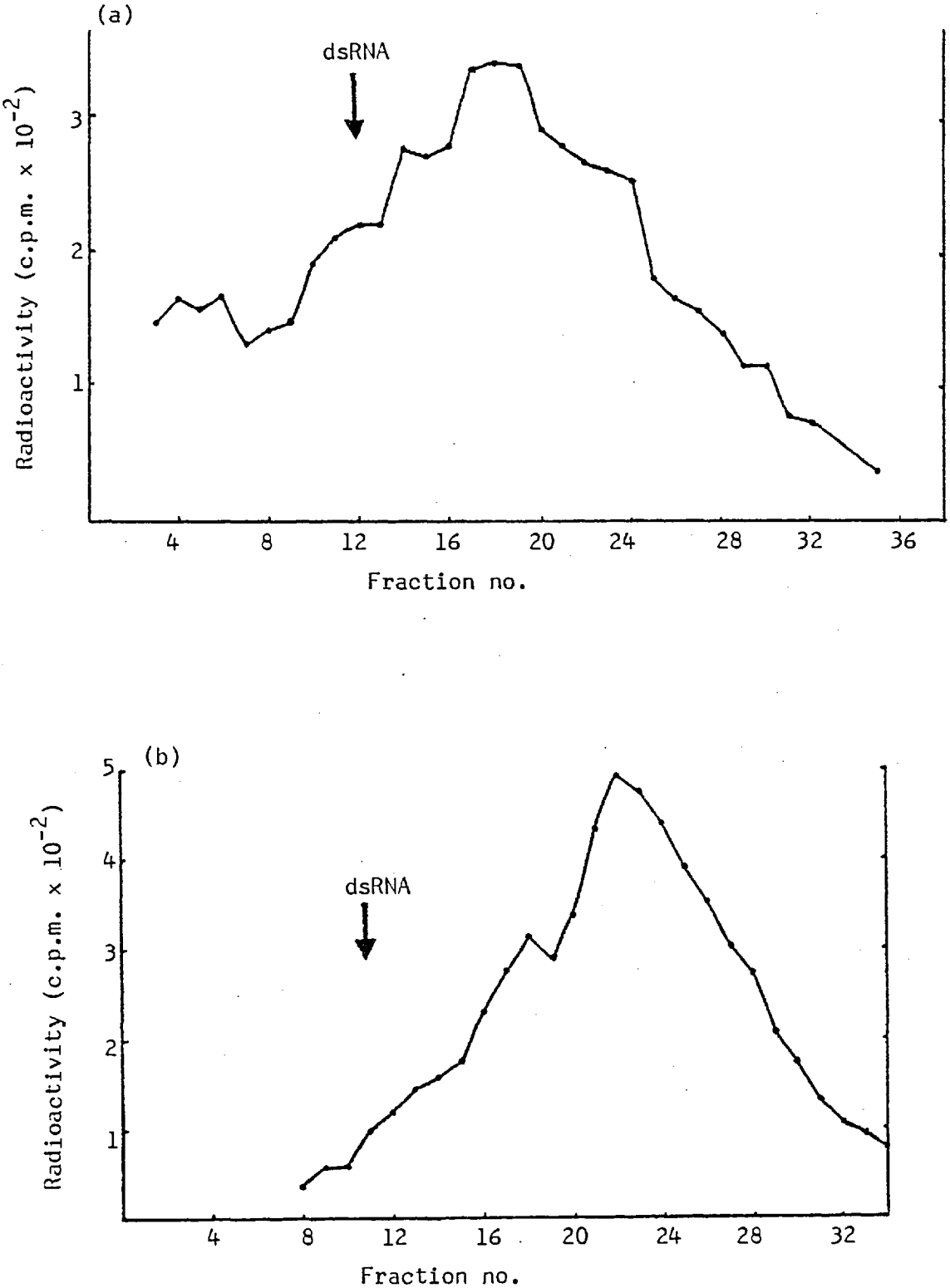
	% low mol. wt. ^{3}H-c.p.m.	Macromolecular ^{3}H-c.p.m.	
		% ssRNA	% dsRNA
Expt. 1	0.75	2.0	98.0
" 2	0.20	0.9	99.1
" 3	0.07	0.9	99.1

Table 1 - Hydroxylapatite chromatography of ^{3}H-dsRNA

Low molecular weight substances were eluted in 0.012M potassium phosphate buffer, pH 6.8. ssRNA was eluted in 0.125M and dsRNA in 0.25M potassium phosphate buffer, pH 6.8.

dsRNA was both denatured and degraded as a result of heat treatment, to an extent depending on the dsRNA concentration, the salt concentration of solvent, and the length of time at high temperature. For example, the change in molecular weight of ^{3}H-dsRNA produced by two different heating schedules is shown in Figures 3(a) and (b). The dsRNA band on sucrose density gradients was both spread and shifted to a position of lower density. After heat-treatment for 20 min in 0.03M NaCl (Figure 3 b), a considerable drop in size had occurred, the molecular weight values calculated from the centre of the peak being 1.2×10^5 for ssRNA or 2.4×10^5 for dsRNA. Hydroxylapatite chromatography revealed that the majority of label was in fact associated with ssRNA after 20 min heat treatment (Table 2), therefore the lower value is probably more applicable. The lack of complete denaturation was due to the high concentration of the dsRNA solution used (300 µg/ml); re-annealing of ssRNA upon cooling is more rapid in concentrated solutions, particularly with such a homogenous material.

Figure 3 - Sucrose density gradient centrifugation of heat-treated ^{3}H-dsRNA



^{3}H-dsRNA was analysed on 5-20% neutral sucrose gradients after the following treatments:

a) 7 min. at 100°C in 0.15 M NaCl, 50 µg/ml, rapid freezing.

b) 20 min. at 100°C in 0.03 M NaCl, 300 µg/ml, rapid freezing.

The arrows mark the normal position of untreated dsRNA.

Sample	% low mol. wt. ^{3}H-c.p.m.	Macromolecular ^{3}H-c.p.m.	
		% ssRNA	% dsRNA
^{3}H-dsRNA	0.07	0.9	99.1
^{3}H-dsRNA in 0.15M NaCl, heated 7 min	4.2	47.6	52.4
^{3}H-dsRNA in 0.03M NaCl, heated 20 min	13.0	71.2	28.8

Table 2 - Hydroxylapatite chromatography of heat-treated ^{3}H-dsRNA

^{3}H-dsRNA at 50 µg/ml in 0.15M NaCl, and 300 µg/ml in 0.03M NaCl was heated for 7 and 20 min, respectively, at 100°C, then immediately frozen at -15°C. Samples were analysed as described for Table 1.

2. The effect of RNase, DNase and pronase on dsRNA

The effect of various enzymes on the molecular integrity of ^{3}H-dsRNA is shown in Table 3. As expected, DNase and pronase did not hydrolyse the material. Pancreatic RNase I, in 0.01M tris-HCl buffer, had no appreciable effect until 2 hours. Heat-denatured dsRNA was more susceptible than untreated material to RNAse attack; hydrolysis to TCA-soluble products began instantaneously, confirming that the substrate was largely single-stranded. With dsRNA as substrate, the total radioactivity in the reaction mixture aliquots was found to decrease as the sampling time increased, although TCA-insoluble radioactivity did not decrease relative to the total radioactivity. Since this phenomenon did not occur with DNase or pronase, it was thought to be due to internal quenching of label, as a result of the RNase binding to the substrate without hydrolysing it to any large extent. After a 2 hour incubation with RNase, dsRNA remained approximately 60% macromolecular according to the TCA-precipitability measurements.

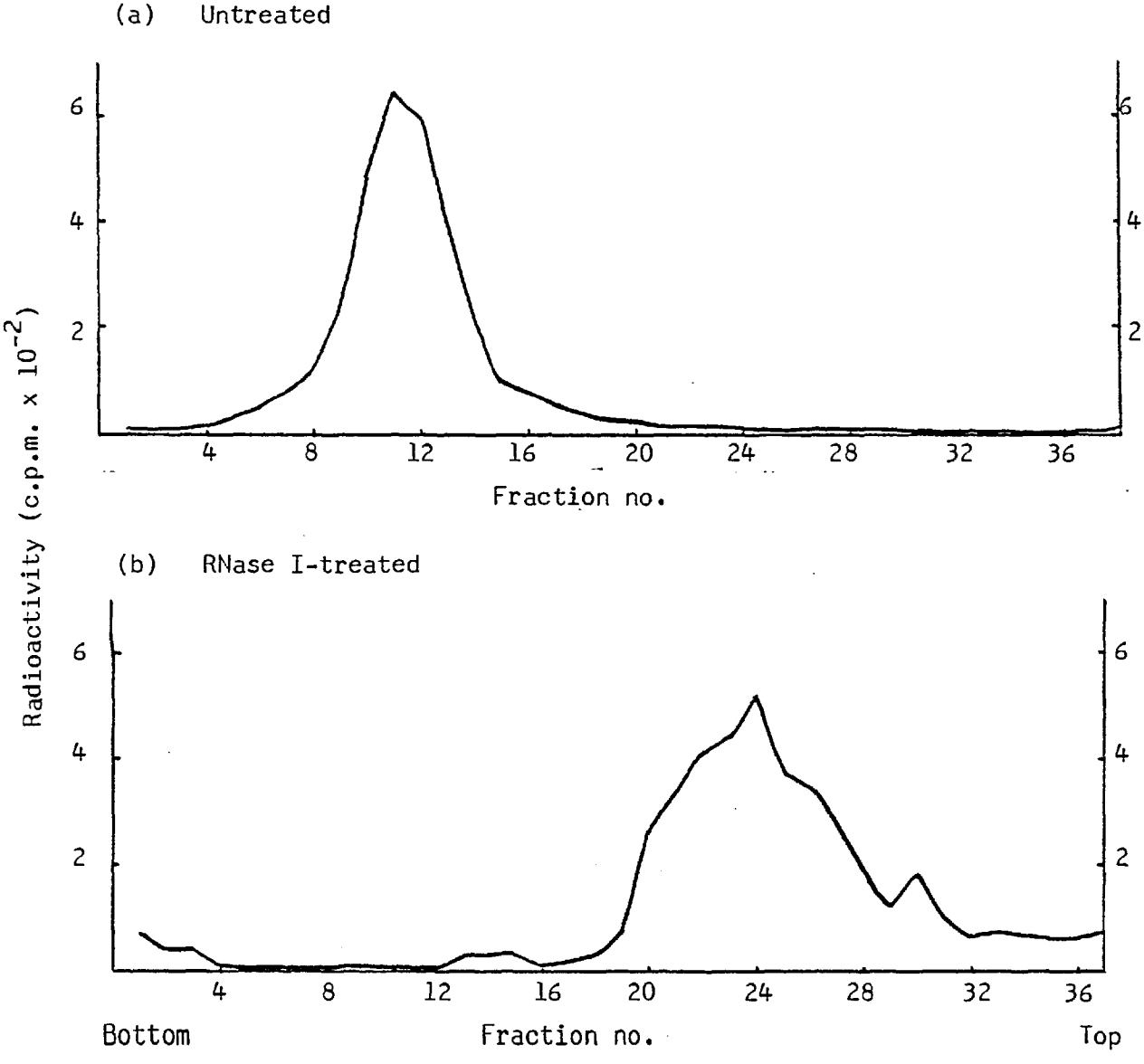
Following RNase treatment of native dsRNA for 2 hours, the products were analysed by sucrose density gradient centrifugation and hydroxylapatite chromatography. Figure 4 shows the fall in molecular weight to a smaller species (4×10^5 for dsRNA or 2×10^5 for ssRNA), with no obvious appearance of low molecular weight fragments at the top of the sucrose gradient. However, when the RNase digestion products were analysed by hydroxylapatite chromatography, 55% of the radioactivity eluted in the unadsorbed, low molecular weight fraction, and of the remainder 82% eluted as

Table 3 - The effect of DNase, pronase and RNase on ^{3}H-dsRNA

Substrate	Enzyme	Incubation time	^{3}H-c.p.m./ 50 µl reaction mixture: Total	^{3}H-c.p.m./ 50 µl reaction mixture: TCA-precipitable
^{3}H-dsRNA	DNase I	-E	11,480	11,580
		+E, 0 min.	11,150	11,240
		+E, 30 "	11,740	11,470
		+E, 120 "	10,950	11,740
^{3}H-dsRNA	Pronase	-E	11,220	11,420
		+E, 0 min.	10,800	9,850
		+E, 30 "	10,970	11,390
		+E, 120 "	10,840	10,790
^{3}H-dsRNA	RNase I	-E	10,480	11,560
		+E, 0 min.	10,280	10,050
		+E, 30 "	8,430	9,310
		+E, 120 "	6,830	6,940
^{3}H-dsRNA, heated 20 mins. at 100°C, in 0.03M NaCl	RNase I	-E	6,920	6,500
		+E, 0 min.	3,040	1,440
		+E, 30 "	2,610	1,000
		+E, 120 "	2,510	940

The substrate was diluted to 0.5 ml in 0.01M tris-HCl buffer, pH 7.5 with 10 mM $MgCl_2$, at 37°C. 50 µl aliquots were counted after being dried onto paper discs (-E). Then 25 µl of enzyme solution at 1 mg/ml was added at a final concentration of 62.5 µg/ml (+E) and 50 µl aliquots were counted on discs, with and without subsequent TCA treatment, at 0, 30 and 120 min of incubation. Loss of TCA-precipitable c.p.m. relative to total c.p.m. is indicative of enzymic hydrolysis.

Figure 4 - The effect of RNase I on the molecular weight of dsRNA.



^{3}H-dsRNA was incubated with buffer alone (a), or pancreatic RNase I at 625µg/ml (b), for 2h as described in Table 3. Aliquots of the reaction mixture were analysed directly by sucrose density gradient centrifugation. 50 µl aliquots of each fraction were counted after drying onto filter paper discs.

ssRNA, 18% as dsRNA. Hydroxylapatite chromatography was therefore the more sensitive method for measuring dsRNA degradation, as some of the non-adsorbed material in the first elution must have remained TCA-insoluble.

Sucrose density gradient centrifugation confirmed that the molecular weight of dsRNA was unaffected by incubation with DNase and pronase.

3. The effect of foetal calf serum on dsRNA

During investigations on the incorporation of ^{3}H-dsRNA by murine lymphocytes, it was found that degradation of the dsRNA took place during long incubations in serum-containing medium. Bovine serum is known to contain a ribonuclease capable of degrading dsRNAs (Stern, 1970; Norlund et al, 1970), therefore resistance of P. chrysogenum dsRNA to heat-inactivated FCS, as used throughout these experiments, was examined using several methods.

Initially, ^{3}H-dsRNA was incubated in RPMI 1640 medium, with or without 5% FCS, and the loss in TCA-precipitability with time was measured by the filter paper disc technique. This was found to be unsatisfactory for quantitative measurements because radioactivity on discs that were not treated with TCA was quenched by culture medium components. Qualitatively, however, the ratio of TCA-precipitable to total radioactivity decreased as the incubation time in the presence of serum increased. (See Table 4, I).

Hydroxylapatite chromatography of FCS-treated ^{3}H-dsRNA (Table 4, II) revealed that after a 24 hour incubation in medium containing 5% FCS, 23% of the ^{3}H-dsRNA was hydrolysed to low molecular weight products, and only 27% of the macromolecular nucleic acid remained as dsRNA. The degradation was time-dependent, very little hydrolysis occurring during the first three hours.

After incubating ^{3}H-dsRNA in medium with 5% FCS for 24 hours, the digestion mixture was dialysed and analysed by CS density gradient centrifugation. The radioactivity profile is shown in Figure 5 . The buoyant density of ^{3}H-dsRNA became spread from the initial value of 1.61, an observation consistent with degradation.

Table 4 - Degradation of ^{3}H-dsRNA in culture medium containing 5% FCS.

I. TCA-precipitability

Incubation time (hrs.)	TCA	^{3}H-c.p.m./50 µl medium containing No FCS	5% FCS
0	-TCA	20,830	20,520
0	+TCA	21,550	21,940
6	-TCA	22,460	12,940
6	+TCA	22,200	12,580
24	-TCA	21,520	7,280
24	+TCA	22,980	4,610

^{3}H-dsRNA (310 µCi/mg) was incubated at 2 µCi/ml in complete RPMI 1640 containing 0 or 5% FCS. At the times shown 50 µl aliquots were withdrawn and counted on paper discs, with ("+TCA") and without ("-TCA") cold 10% TCA treatment. Results are the means of triplicate cultures.

II. Hydroxylapatite chromatography

Incubation time in 5% FCS (hrs.)	% low mol. wt. ^{3}H-c.p.m.	Macromolecular ^{3}H-c.p.m.	
		% ssRNA	% dsRNA
0	0.3	2.4	97.6
3	1.7	14.1	85.9
6	19.0	36.3	63.7
24	23.0	73.0	27.0
^{3}H-dsRNA control	0.07	0.9	99.1

^{3}H-dsRNA (310 µCi/mg) was incubated at 1 µCi/ml in complete RPMI 1640 medium with 5% FCS. At the times shown, 100 µl samples were added to 0.9 ml 0.012M potassium phosphate buffer pH 6.8, containing 0.25% sarkosyl, and frozen before hydroxylapatite chromatography.

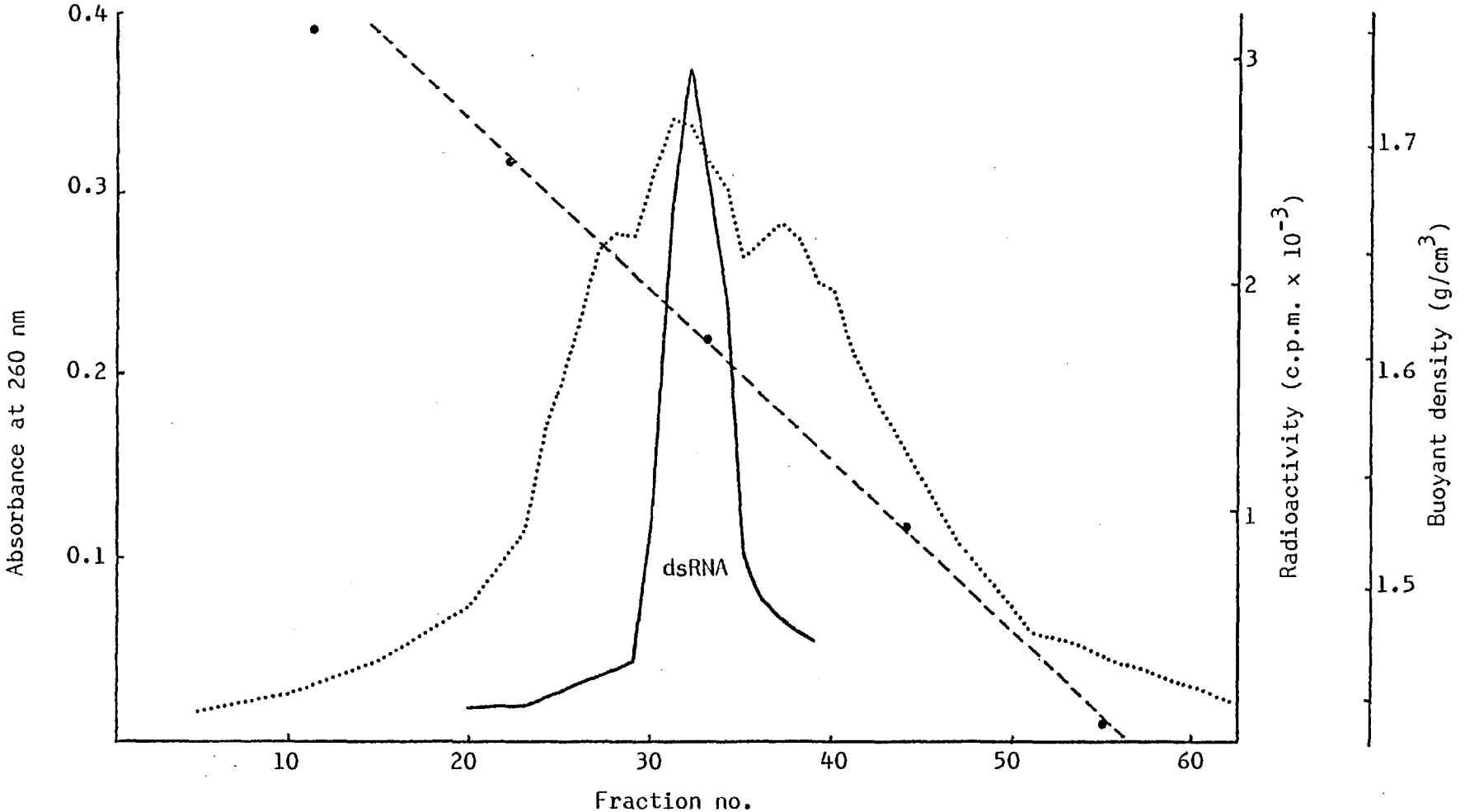


Figure 5 - Effect of serum on the CS density gradient profile of ^{3}H-dsRNA.

^{3}H-dsRNA (310 µCi/mg) was incubated at 1 µCi/ml for 24 hours in complete RPMI 1640 medium containing 5% FCS. Digestion was stopped by the addition of sarkosyl to 0.25%. The incubation mixture was dialysed for 2 hours against SSC to remove low molecular weight products, then analysed by CS density gradient centrifugation in the presence of unlabelled dsRNA as A_{260} marker.

——— absorbance at 260 nm; ····· radioactivity; •— — —• buoyant density

In order to study the fate of intact ^{3}H-dsRNA in the absence of extracellular degradation, while at the same time attempting to preserve good lymphocyte viability, the possibility of replacing 5% FCS with 0.5% FCS or 1% bovine serum albumen (BSA; Sigma, fraction V) was investigated. Table 5 (I) shows the fall in total and TCA-precipitable c.p.m. after incubation of ^{3}H-dsRNA in medium containing either 0.5% FCS or 1% BSA. Although there was clearly no evidence of gross degradation, the fall in "-TCA" values with time probably indicates binding of protein to dsRNA.

The molecular weight of ^{3}H-dsRNA after 24 or 48 hours incubation was measured by sucrose gradient centrifugation. Table 5 (II) shows the values obtained for the sedimentation coefficient(s), and molecular weight. The position of the unlabelled dsRNA marker varied slightly in different gradients, but a progressive drop in molecular weight could nevertheless be detected, decreasing to approximately half in the presence of both 0.5% FCS and 1% BSA. The position of ^{3}H-dsRNA on sucrose gradients after a 48 hour incubation is shown in Figure 6. (The sucrose gradient profile of ^{3}H-dsRNA incubated in culture medium alone was unchanged; see, for example, Figure 19 d). The molecular weights were calculated for double-stranded, and not single-stranded, RNA. This assumption was justified from the results of hydroxylapatite chromatographic analysis of the 48 hour incubation products (see Table 5 [III]), when it was shown that the majority of the initial ^{3}H-dsRNA remained macromolecular and double-stranded.

Concentrations of FCS as low as 0.5% were therefore able to cause subtle changes in the structure of ^{3}H-dsRNA during prolonged incubation, resulting in a 50% fall in molecular weight without overt hydrolysis to nucleotide products. Furthermore, limited degradation in medium containing 1% BSA was likewise observed. Although the BSA preparation used (Sigma, fraction V) was supplied 96-98% pure, Stern (1970) found enzyme activity against poly I.poly C in a similar preparation of BSA (Armour, fraction V). It was not therefore used as a serum substitute, and experiments were conducted either in normal growth medium or in medium containing no serum at all.

Table 5 - Degradation of ^{3}H-dsRNA in culture medium containing 0.5% FCS or 1.0% BSA.

I. TCA-precipitability

Incubation time (hrs.)	TCA	^{3}H-c.p.m./50 µl medium containing: 0.5% FCS	1.0% BSA	No protein
0	-TCA	22,300	21,100	20,740
0	+TCA	25,900	24,150	24,630
6	-TCA	22,240	20,560	N.T.
6	+TCA	25,930	25,260	N.T.
24	-TCA	20,620	18,320	20,090
24	+TCA	25,120	23,070	24,030
48	-TCA	20,200	18,790	N.T.
48	+TCA	22,900	20,040	N.T.

II. Sucrose density gradient centrifugation

Culture medium	Incubation time (hrs.)	^{3}H-dsRNA		dsRNA marker	
		S value*	Mol. wt.	S value	Mol. wt.
0.5% FCS	24	11.85	1.4×10^6	14.2	2.4×10^6
0.5% FCS	48	10.5	1.0×10^6	13.5	2.1×10^6
1.0% BSA	24	11.3	1.2×10^6	14.8	2.6×10^6
1.0% BSA	48	10.1	0.9×10^6	13.9	2.2×10^6

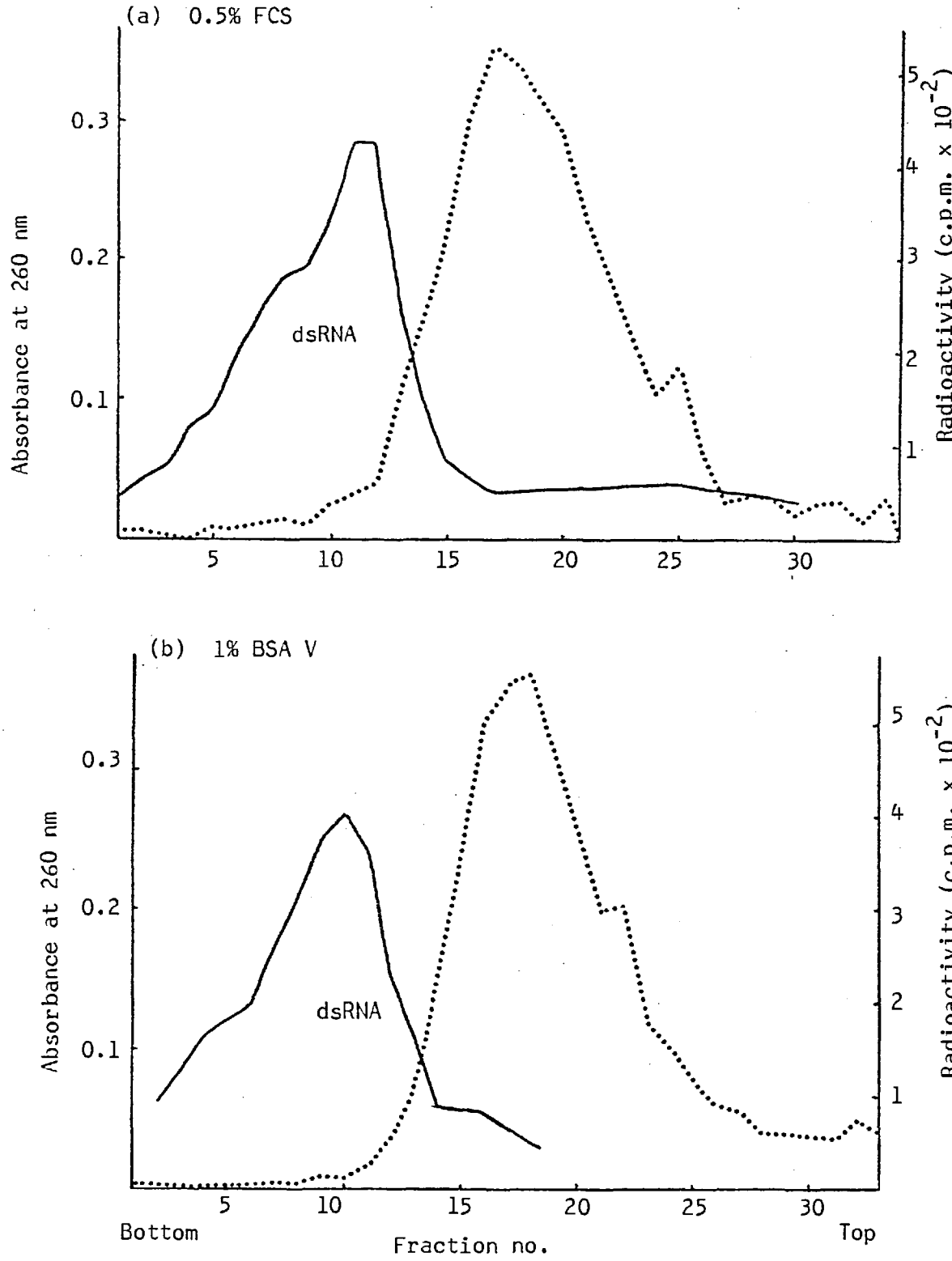
III. Hydroxylapatite chromatography

Culture medium	% low mol.wt. ^{3}H-c.p.m.	Macromolecular ^{3}H-c.p.m. % ssRNA	% dsRNA
0.5% FCS, 48 hrs.	2.3	7.4	92.6
1.0% BSA, 48 hrs.	5.1	16.2	83.8
^{3}H-dsRNA control	0.07	0.9	99.1

^{3}H-dsRNA (310 µCi/mg) was incubated at 2 µCi/ml in complete RPMI 1640 medium containing 0.5% FCS or 1.0% BSA. At timed intervals, 50 µl aliquots were counted on paper discs, with ("+TCA") or without ("-TCA") cold 10% TCA treatment. At 24 and 48 hours, aliquots were treated with sarkosyl to a final concentration of 0.25%, then analysed on neutral sucrose gradients in the presence of 100 µg unlabelled dsRNA as A_{260} marker. (Fractions were counted in Insta-Gel). Samples of the 48 hour incubation mixtures were added to 0.012M potassium phosphate buffer pH 6.8, containing 0.25% sarkosyl, and frozen before hydroxylapatite chromatography.

N.T. Not tested. * S value = sedimentation coefficient.

Figure 6 - Effect of 0.5% FCS and 1% BSA on the molecular weight of ^{3}H-dsRNA



^{3}H-dsRNA was incubated in RMPI 1640 medium containing 0.5% FCS (a), or 1% BSA, V (b) for 48 hours as described in Table 5 . The digestions were stopped by the addition of sarkosyl to 0.25%, and aliquots of the reaction mixture analysed by sucrose density gradient centrifugation in the presence of unlabelled dsRNA as A_{260} marker.

—— absorbance; ·····radioactivity

4. Discussion

The results reported in this section provided reassurance that the P. chrysogenum dsRNA as supplied was RNA in nature, 98-100% double-stranded, with a molecular weight of about 2×10^6 daltons. It was heat-denaturable, particularly at low salt concentrations, when it became susceptible to rapid hydrolysis by ribonuclease. Considerable degradation occurred during prolonged incubation in 5% foetal calf serum. The characteristic band at a density of 1.61 in CS density gradients provided an ideal marker by which to follow the biochemical fate of dsRNA in cells.

SECTION II - THE EFFECTS AND FATE OF dsRNA IN CON A-STIMULATED MOUSE SPLEEN CELLS.

1. THE EFFECTS OF dsRNA ON LYMPHOCYTE TRANSFORMATION IN VITRO

CBA mouse spleen (MS) cell suspensions in 1 ml culture were stimulated to transform in RPMI 1640 medium containing 5% FCS and Con A at a concentration of 4 µg/ml. The peak period of DNA synthesis, as measured by ^{3}H-thymidine incorporation into TCA-precipitable material, occurred between 40 and 60 hours. At this time, more than 70% of the cells had become large "blast" cells, and typically were clumped together in large cell aggregates. The number of cells per culture fell during the first 24 hours, then returned to the initial levels by 40-60 hours. No net increase in cell number is generally seen in these 1 ml cultures, since mitotic arrest occurs (Harris and Olsen, 1976).

Final cell numbers, as estimated by haemocytometer counting, were somewhat variable from one culture to another. However, this was generally reflected in differences in ^{3}H-thymidine incorporation per culture, so that the specific activity, or ^{3}H-c.p.m./10^{6} cells, was more reproducible. ^{3}H-thymidine incorporation per 10^{6} cells at 48 hours also varied between individual experiments, probably as a result of differences in the serum and the mice used.

.1 The effect of dsRNAs and ssRNAs on MS cell transformation

The effect of P. chrysogenum dsRNA on MS cell transformation was compared to that of other double- and single-stranded RNAs (Table 6). At a concentration of 100 µg/ml, ssRNA from various sources had no effect on final cell numbers, and DNA synthesis was inhibited by 27% or less. At the same concentration, the synthetic double-stranded polymers also had no effect on cell division, but inhibited ^{3}H-thymidine incorporation more severely than the ssRNAs. P. chrysogenum dsRNA was considerably more inhibitory to transformation than the other dsRNAs; at 100 µg/ml, blast cell formation was completely prevented and DNA synthesis was reduced virtually to background levels. This inhibitory property of dsRNA could be abrogated by heat-treatment at 100°C for 20 min in 0.03M NaCl, a procedure which resulted in approximately 70% denaturation and 13% solubilisation of the dsRNA (as was shown in Table 2).

Table 6 - The effect of various ds and ssRNAs on the Con A-induced transformation of MS cells

RNA	RNA conc$^{n.}$ (µg/ml)	Cell number x 10^6/ml	% inhibition ^{3}H-Tdr incorporation
-	0	2.26	-
dsRNA	10 100	1.82 0.72	0 95
Poly I:poly C (ds)	10 100	2.1 2.2	32.5 51
Poly A:poly U (ds)	10 100	2.0 2.0	8 46
Calf liver RNA (ss)	10 100	2.0 2.2	0 27
Yeast RNA (ss)	10 100	2.25 2.2	6 16
Heat-denatured dsRNA (ss) *	10 100	2.2 2.3	7 17

MS cells were prepared in 1 ml cultures at 2.5 x 10^6 cells/ml in medium containing 5% FCS and 4 µg/ml Con A. The various RNAs were added at 0 h. Cultures were pulsed with 2 µCi/ml ^{3}H-Tdr from 48-50 h, when incorporation into TCA-precipitable material was measured. Control cultures receiving no RNA incorporated 26,200 c.p.m. per 10^6 nucleated cells.

* Heat treatment: 20 min at 100°C in 0.03M NaCl.

Further experiments revealed that this toxicity of P. chrysogenum dsRNA for lymphocytes in serum-supplemented medium was due not to the dsRNA itself, but to a dialysable impurity present in the preparation. Because the results of this work are not directly relevant to the problems under study they are presented separately in Appendix I. In summary, dsRNA was found to inhibit the initial stages of mouse or human lymphocyte transformation, through a direct effect on the cells rather than on the lectin stimulant. After dialysis, dsRNA lost most, if not all, of this inhibitory activity. No structural change appeared to take place in the dsRNA molecule as a result of dialysis, suggesting that a low molecular weight impurity was responsible for the inhibition. This impurity was not a general cytotoxic agent, since cells already transformed by Con A (at 40 hours of culture) were considerably more resistant to the effect of undialysed dsRNA than freshly-prepared MS cells.

It was clear that dsRNA was not as toxic to stimulated lymphocytes as was initially supposed, therefore the effects of dialysed dsRNA were studied.

.2 The effect of dialysed dsRNA on unstimulated and Con A-stimulated MS cells

The dose response effect of dialysed dsRNA is shown in Table 7. The lower half of the Table refers to Con A-stimulated MS cells. Inhibition of DNA synthesis was not significant in this particular experiment, although concentrations of 100 µg/ml or more, in other experiments, resulted in up to 30% inhibition of ^{3}H-thymidine incorporation. The upper half of Table 7 shows the effect of dialysed dsRNA on unstimulated MS cells. It can be seen that in the absence of Con A, MS cells were stimulated to some degree, possibly by the transferrin component of foetal calf serum (Vogt et al, 1969). ^{3}H-thymidine incorporation, as well as cell numbers, were lower than in Con A-stimulated cultures. However, in cultures without Con A, dialysed dsRNA at 100 µg/ml appeared to have an enhancing effect on both cell replication and ^{3}H-thymidine incorporation. This "mitogenic" effect of dsRNA was not as great as that of Con A, and no synergistic stimulation between the two was observed.

Table 7 - The effect of dialysed dsRNA on Con A-stimulated and unstimulated MS cells

	Conc$^{n.}$ of dsRNA (µg/ml)	Cell number x 10^6/ml	^{3}H-Tdr uptake c.p.m./10^6 cells
Unstimulated MS cells	0	1.36	16,519
	1	1.32	15,359
	10	1.28	19,667
	100	1.62	30,712
Con A-stimulated MS cells	0	1.80	73,377
	1	1.56	79,532
	10	1.60	76,087
	100	1.50	71,580

MS cells in 1 ml culture (2.4 x 10^6 cells/ml) were incubated with or without 4 µg/ml Con A, in the presence of dialysed dsRNA. ^{3}H-Tdr incorporation was measured over a two-hour pulse at 48 h with 2 µCi/ml ^{3}H-Tdr.

Mitogenic effects of double-stranded polynucleotides on lymphocytes have been reported; poly I.poly C was shown to be mitogenic for mouse spleen cells, although to a lesser degree than PHA, at a concentration of 10-50 µg/ml (Dean, Wallen and Lucas, 1972; Ts'o et al, 1976).

.3 The effect of serum on the dsRNA-induced inhibition of MS cell transformation

As a consequence of the finding that FCS contains nuclease activity capable of degrading dsRNA (see section I), the effect of dsRNA on Con A-stimulated MS cells in the absence of serum was investigated. Con A does transform MS cells in the complete absence of serum, although DNA synthesis and cell division are lower than in FCS-containing cultures. Loss of lymphocyte viability in serum-free medium has been described (Ling and Kay, 1975d).

Table 8 shows the effect of both dialysed and undialysed dsRNA on ^{3}H-thymidine incorporation and cell numbers, in the presence and absence of serum. The dose-dependent inhibition of transformation by undialysed dsRNA preparations was more pronounced in the absence of serum. Serum-free cultures exposed to 100 µg/ml undialysed dsRNA from 0 hours contained no viable cells at 48 hours; only cell debris was observed, having no incorporation of ^{3}H-thymidine above background levels.

The inhibitory effect of dialysed dsRNA on lymphocyte transformation was also enhanced in serum-free medium. The dramatic fall in cell numbers at 100 µg/ml was indicative of the toxicity of dsRNA itself in the absence of serum. The nature of this effect was different to that of undialysed dsRNA, since the suppression of cell division was not accompanied by a corresponding fall in specific thymidine incorporation, that is, cells which survived dsRNA treatment went on to transform and synthesise DNA normally. Thus the cytotoxic effects of dsRNA on MS cells were manifested only in serum-free culture medium, and only at concentrations of 30 µg/ml or greater. If addition of dialysed dsRNA was delayed, the inhibitory effect was largely abrogated. When cultures were stimulated with Con A in the presence of serum for 30 hours, and then exposed to dialysed dsRNA in the absence of serum, the inhibitory effect was not as great as in cultures both depleted of serum and treated with dsRNA from the initiation of

Table 8 - The effect of dsRNA on MS cell transformation in the presence and absence of foetal calf serum.

dsRNA	dsRNA concn. (µg/ml)	0% serum		5% serum	
		Cell number x 10^6/ml	^{3}H-Tdr uptake/10^6 cells	Cell number x 10^6/ml	^{3}H-Tdr uptake/10^6 cells
None	-	1.30	50,440	2.00	98,400
Undialysed	10	1.22	43,200	1.75	91,180
	30	0.58	860	1.40	83,260
	100	0	110	1.10	130
Dialysed	10	1.23	44,290	1.74	97,070
	30	0.76	57,310	1.84	89,040
	100	0.21	54,830	1.72	84,560

1 ml MS cell cultures were prepared at 2.5 x 10^6 cells/ml in RPMI 1640 medium, with or without 5% FCS, in the presence of 4 µg/ml Con A and various concentrations of dialysed or undialysed dsRNA as shown. ^{3}H-Tdr incorporation into DNA was measured during a 2 hour pulse at 46-48 hours, and cell counts estimated microscopically at 48h. Results are the average of duplicate cultures.

transformation. Following delayed addition of 100 µg/ml dsRNA, cell replication was unaffected, although DNA synthesis was inhibited by 26% (Table 9).

.4 Discussion

Many of the early results obtained were difficult to interpret following the subsequent unexpected discovery, in dsRNA preparations, of a dialysable impurity which inhibited lymphocyte transformation. Only after removal of this substance by dialysis could the effect of dsRNA on lymphocyte transformation be investigated.

Dialysed dsRNA inhibited DNA synthesis in the presence of serum, only at a concentration of 100 µg/ml. The degree of inhibition varied from 0-30% in different experiments, probably reflecting both the inherent variability of the system, and the efficiency of dialysis of different batches of dsRNA. This was generally accompanied by slight suppression of cell numbers at 48 hours. Cells stimulated in the absence of serum were, on the other hand, far more susceptible to the inhibitory effects of dsRNA. ^{3}H-thymidine uptake measurements showed that although the cell numbers were significantly reduced, those which survived dsRNA treatment behaved normally with respect to this parameter. The intact material therefore seems to have a cytotoxic effect on primary cultures of MS cells, to which a sub-population of lymphocytes is resistant, so that blast cell formation is not completely suppressed.

Thus P. chrysogenum dsRNA was toxic to primary lymphocyte cultures in two ways. The first effect, expressed in the presence of FCS, prevented the initiation of transformation, and was removed from the preparation by simple dialysis. The second, expressed in the absence of serum only, did not prevent initiation of transformation completely, but nevertheless resulted in toxicity at concentrations of 30 µg/ml and above, when the dsRNA was present from 0 hours. These findings were borne in mind when studying the fate of ^{3}H-adenosine-labelled dsRNA in MS cells. Since ^{3}H-dsRNA was additionally purified by Sepharose 2B chromatography, it was unlikely to contain the dialysable impurity found in the unlabelled material. In any event, the amount of ^{3}H-dsRNA added per culture was always less than 10 µg/ml and, in serum-free medium, was always added after 30 hours of transformation when the cells were more resistant to dsRNA cytotoxicity.

Table 9 - The effect on MS cell transformation of delayed addition of dialysed dsRNA in the absence of serum

Conc^{n}. of dsRNA (µg/ml)	Cell number x 10^6/ml	^{3}H-Tdr uptake c.p.m./culture	^{3}H-Tdr uptake c.p.m./10^6 cells
0	1.84	70,932	38,550
10	1.96	69,139	35,275
50	2.11	65,302	30,948
100	2.19	62,330	28,461

1 ml MS cell cultures were prepared in medium containing 5% FCS and 4 µg/ml Con A. After 30 h cells were pooled, washed in serum-free medium, and resuspended at 1.8 x 10^6 cells/ml in serum-free medium. dsRNA was added at the concentrations shown, and ^{3}H-Tdr incorporation measured from 52-54 h. Results are the average of duplicate cultures.

2. THE UPTAKE AND FATE OF ^{3}H-dsRNA IN CON A-STIMULATED MOUSE SPLEEN CELLS

The uptake and biochemical fate of ^{3}H-adenosine-labelled dsRNA was studied in the presence and absence of foetal calf serum, using the methods described in Section I. Significant catabolism and re-utilisation of label was observed only in serum-supplemented medium, therefore it was under these conditions that the effects of unlabelled precursors and hydroxyurea on the fate of ^{3}H-dsRNA were examined.

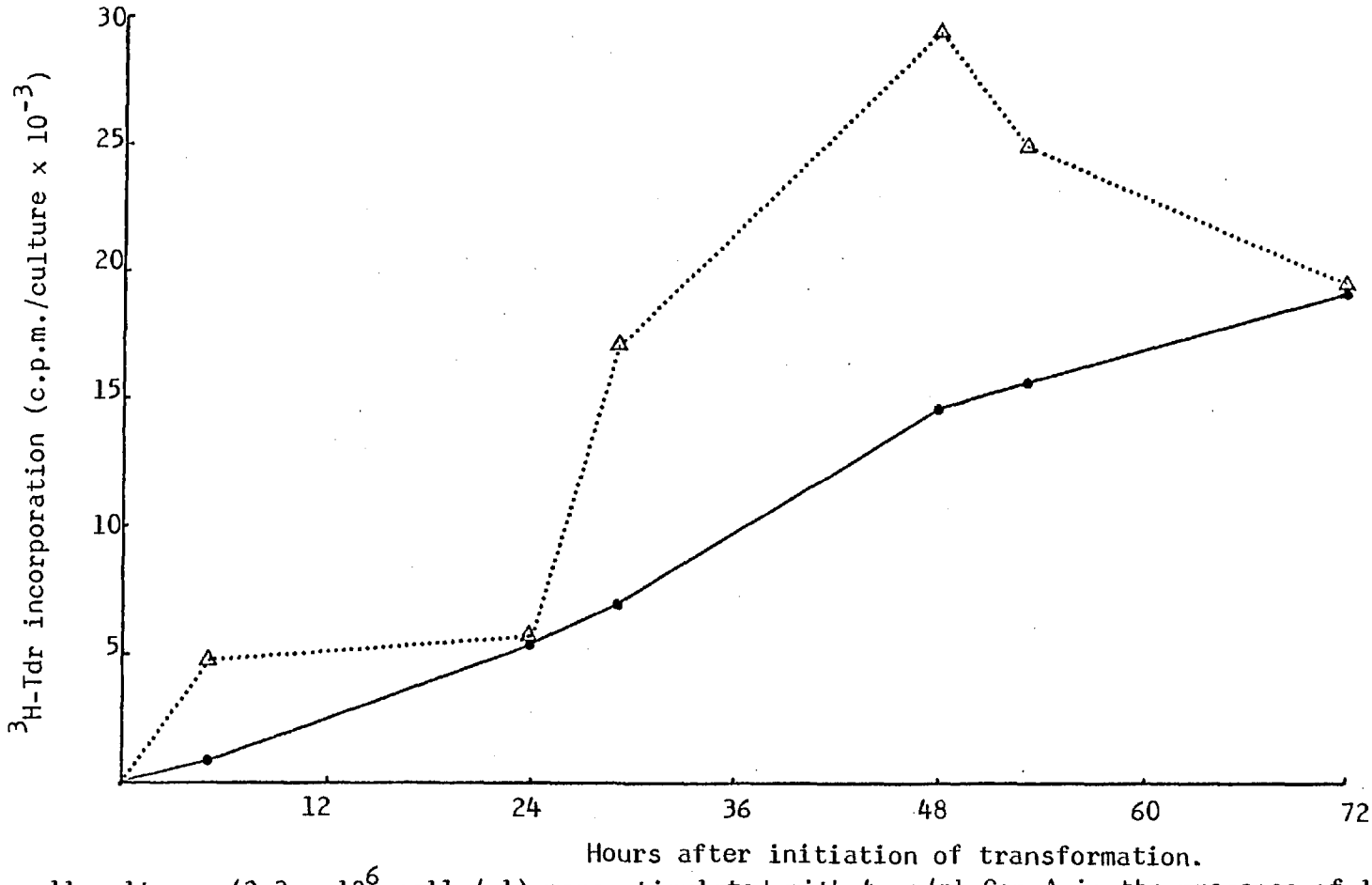
.1 Incorporation of ^{3}H-dsRNA in the presence of serum

The uptake of 10 µg/ml ^{3}H-dsRNA (30 µCi/mg) into TCA-insoluble material over the course of transformation in 1 ml Con A-stimulated MS cells is shown in Figure 7. Incorporation of ^{3}H-thymidine during a 1 hour pulse, measured in parallel cultures treated with 10 µg/ml unlabelled dsRNA, verified that transformation was unaffected by dsRNA at this concentration. The rate of uptake of ^{3}H-dsRNA was slightly greater during the period of active DNA synthesis (24-48 hours). By 72 hours, 14% of the original ^{3}H-dsRNA had been incorporated by the cells. Cells prevented from transforming by the addition of 50 µg/ml unlabelled, undialysed dsRNA at 0 hours did not incorporate ^{3}H-thymidine or ^{3}H-dsRNA.

The intracellular localisation of ^{3}H-dsRNA was studied by measuring the label in nuclear and cytoplasmic cell fractions in pooled, replicate cultures of MS cells, pulsed with ^{3}H-dsRNA for 3 (49-52), 6 (46-52) or 22 (30-52) hours. The distribution of ^{3}H-adenosine and ^{3}H-uridine, both of which are utilised for RNA and DNA synthesis, was examined in parallel cultures for comparison. Table 10 shows the pattern of incorporation.

Where no solubilisation of label occurred, the c.p.m. figures for TCA-precipitable radioactivity were often greater than those for total radioactivity probably due to quenching of the latter by phenol red-containing tissue culture medium. The quenching agents were removed from the discs during TCA treatment. Therefore the degree of TCA-precipitability in these experiments could not be regarded as completely quantitative until corrected for the quenching of untreated samples. Likewise, the total c.p.m. for (nucleus + cytoplasm + nuclear washings) was not equal to the c.p.m. of the whole cell fraction, because sucrose-$CaCl_2$-NP40 lysis buffer quenched the radioactivity by 20-25%. The percentage of

Figure 7 - Uptake of ^{3}H-dsRNA and ^{3}H-thymidine by Con A stimulated MS cells in 1 ml culture



1 ml MS cell cultures (2.3_{3} x 10^{6} cells/ml) were stimulated with 4 µg/ml Con A in the presence of 10 µg unlabelled dsRNA or 10 µg ^{3}H-dsRNA (30 µCi/mg). At the times shown, duplicate cultures labelled with ^{3}H-dsRNA were pooled, and the incorporation into TCA-insoluble material in the cells was measured. Cultures treated with unlabelled dsRNA were pulsed for 1 hour prior to harvest with 2 µCi/ml ^{3}H-thymidine, and incorporation into DNA measured.

●——● ^{3}H-dsRNA incorporation (c.p.m./culture)

△·····△ ^{3}H-Tdr incorporation (c.p.m./culture/hr)

Table 10 - The intracellular distribution of ^{3}H-dsRNA, ^{3}H-adenosine, and ^{3}H-uridine in Con A-stimulated MS cells.

^{3}H-label	Inc. time (h)	^{3}H-cpm incorporated/10^{6} cells				% of label in nucleus[2]	% of nuclear label as DNA[3]
		Cells	Nucleus	Cytoplasm	Nuclear wash		
^{3}H-dsRNA (1.9 µCi/ml)	3	1,960 (1,907)[1]	230 (338)	168 (186)	2 (32)	57.5	39
	6	3,681 (3,765)	475 (530)	382 (177)	7 (58)	55	53
	22	9,446 (8,372)	4,205 (3,996)	1,985 (1,134)	67 (93)	67	67
^{3}H-adenosine (2 µCi/ml)	3	11,361 (5,437)	2,791 (3,193)	4,855 (694)	134 (84)	36	57
	6	42,264 (27,861)	16,998 (19,879)	15,535 (2,437)	624 (240)	51	71
	22	160,845 (149,058)	77,706 (98,206)	34,396 (19,112)	1,215 (940)	68.5	76
^{3}H-uridine (2 µCi/ml)	3	2,928 -	1,073 (1,358)	1,045 (289)	28 (52)	50	54
	6	7,928 (6,230)	3,427 (3,669)	1,960 (927)	88 (86)	63	46
	22	30,732 (33,954)	18,597 (18,786)	6,672 (5,970)	196 (244)	73	56

1 ml MS cell cultures in 5% FCS-containing medium, stimulated with 4 µg/ml Con A, were labelled with ^{3}H-dsRNA (5 µg and 1.0 µCi/ml), ^{3}H-adenosine (2 µCi/ml, 5 µM) or ^{3}H-uridine (2 µCi/ml, 5 µM) either from 30-52 hours (5 cultures; 22 hr pulse), from 46-52 hours (8 cultures; 6 hr pulse) or from 49-52 hours (10 cultures; 3 hr pulse). At 52 hours, the cultures were pooled, washed, and the incorporation of label measured as described in Materials and Methods. The mean cell number per culture at 52 hours was 2.76 (± 0.2 S.D.) x 10^{6}. Marmur extracts from the nuclei were run on CS density gradients (see Figure 8).

1. TCA-precipitable c.p.m. are shown in parentheses under total c.p.m.
2. % of label in nucleus is calculated as % of the total label in (nucleus + cytoplasm + nuclear washings).
3. % DNA was calculated from the % of label in the nuclear Marmur extract resistant to NaOH hydrolysis. All cytoplasmic label was 95-100% NaOH solubilised. Background c.p.m. after NaOH hydrolysis of ^{3}H-dsRNA was 50 c.p.m. All samples contained >250 c.p.m. before NaOH hydrolysis.

total cellular radioactivity in the nucleus was calculated directly from the total c.p.m. in (nucleus + cytoplasm + nuclear washings) since these fractions were counted in the same lysis buffer.

The results show that all three substances were taken up and distributed such that by 22 hours, approximately 70% of the total radioactivity was found in the nucleus. Label accumulated in the nucleus with time, and the nuclear label became gradually converted to DNA with time. There was evidence of degradation of ^{3}H-dsRNA to TCA-soluble material in the cytoplasm at 6 and 22 hours, while label in the nucleus remained largely TCA-insoluble. ^{3}H-dsRNA in the cell supernatant began to show an increase in TCA-solubility only after 22 hours. A clear, progressive, nuclear accumulation of label was observed with the precursors ^{3}H-adenosine and ^{3}H-uridine. TCA-precipitability of radioactivity in the cytoplasm, as well as in the nucleus, increased as the labelling time increased.

After 22 hours the nuclear label in ^{3}H-adenosine-labelled cells was significantly more resistant to NaOH hydrolysis than that in ^{3}H-uridine-labelled cells. This difference was borne out in the CS gradient profiles shown in Figures 8a and b. While ^{3}H-adenosine was utilised predominantly for DNA synthesis, ^{3}H-uridine was utilised for RNA and DNA synthesis to approximately the same extent. The CS profile of the nuclear extract from ^{3}H-dsRNA-labelled cells at 22 hours suggested that radioactivity was being lost from dsRNA of density 1.61 and appearing in heterogeneous RNA and DNA (Figure 8c). DNA formed a sharp band at a density of approximately 1.43 in CS gradients.

Nuclei were prepared from pooled cell cultures that had been labelled with ^{3}H-dsRNA (10 µg/ml) from 0 to 48 hours of transformation. Total nucleic acids were extracted using the Marmur procedure, and analysed by CS density gradient centrifugation (Figure 9). After 48 hours, more than 90% of the radioactivity was associated with the DNA band (assuming similar recovery of RNA and DNA during Marmur extraction). No ^{3}H-dsRNA at ρ = 1.61 was present. The DNA-containing fractions were recovered from the appropriate gradient fractions, and 93% of this radioactivity was found to be resistant to NaOH solubilisation, suggesting that ^{3}H-dsRNA was not simply co-centrifuging with DNA on the CS density gradient, but had been converted to ^{3}H-DNA.

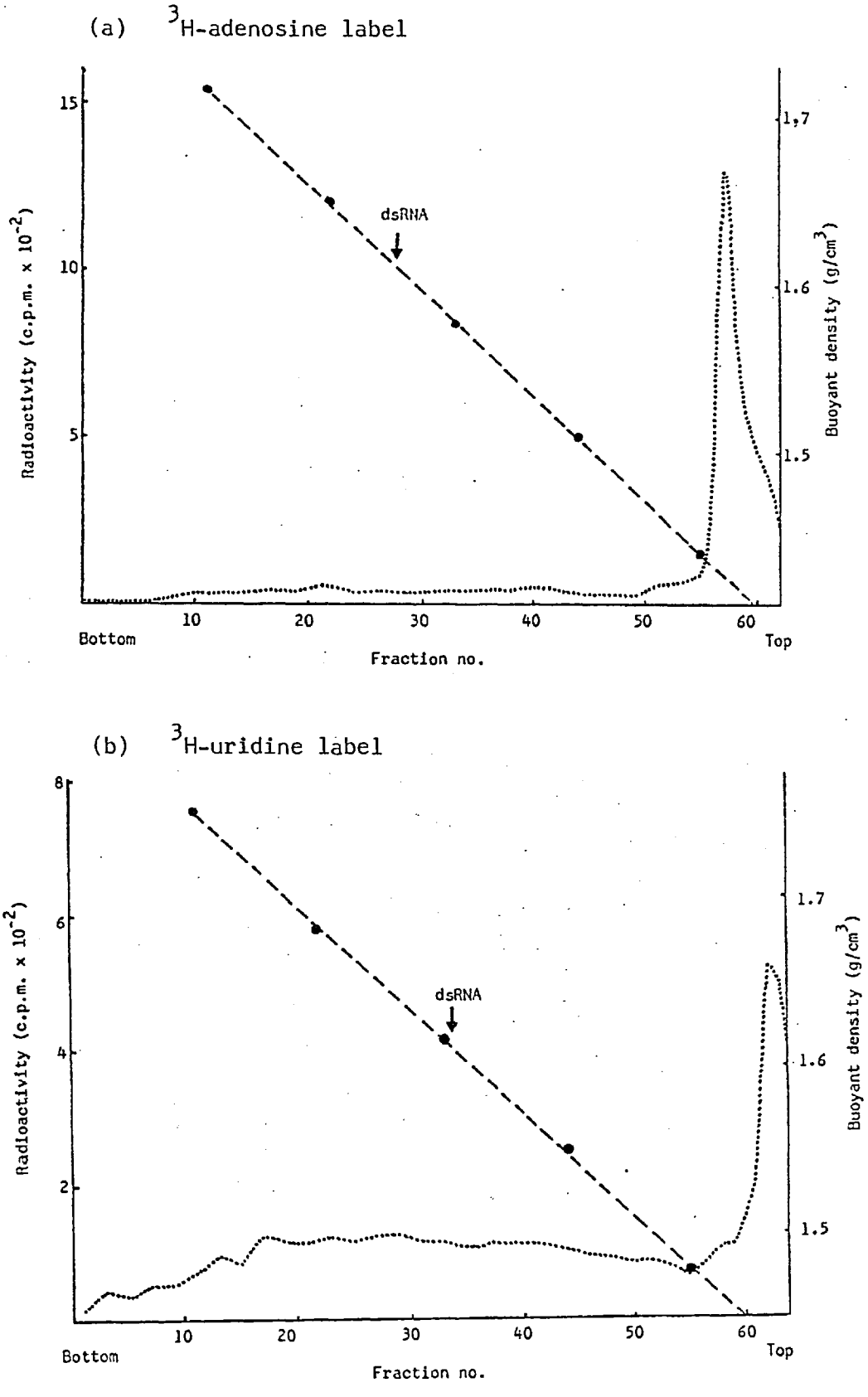
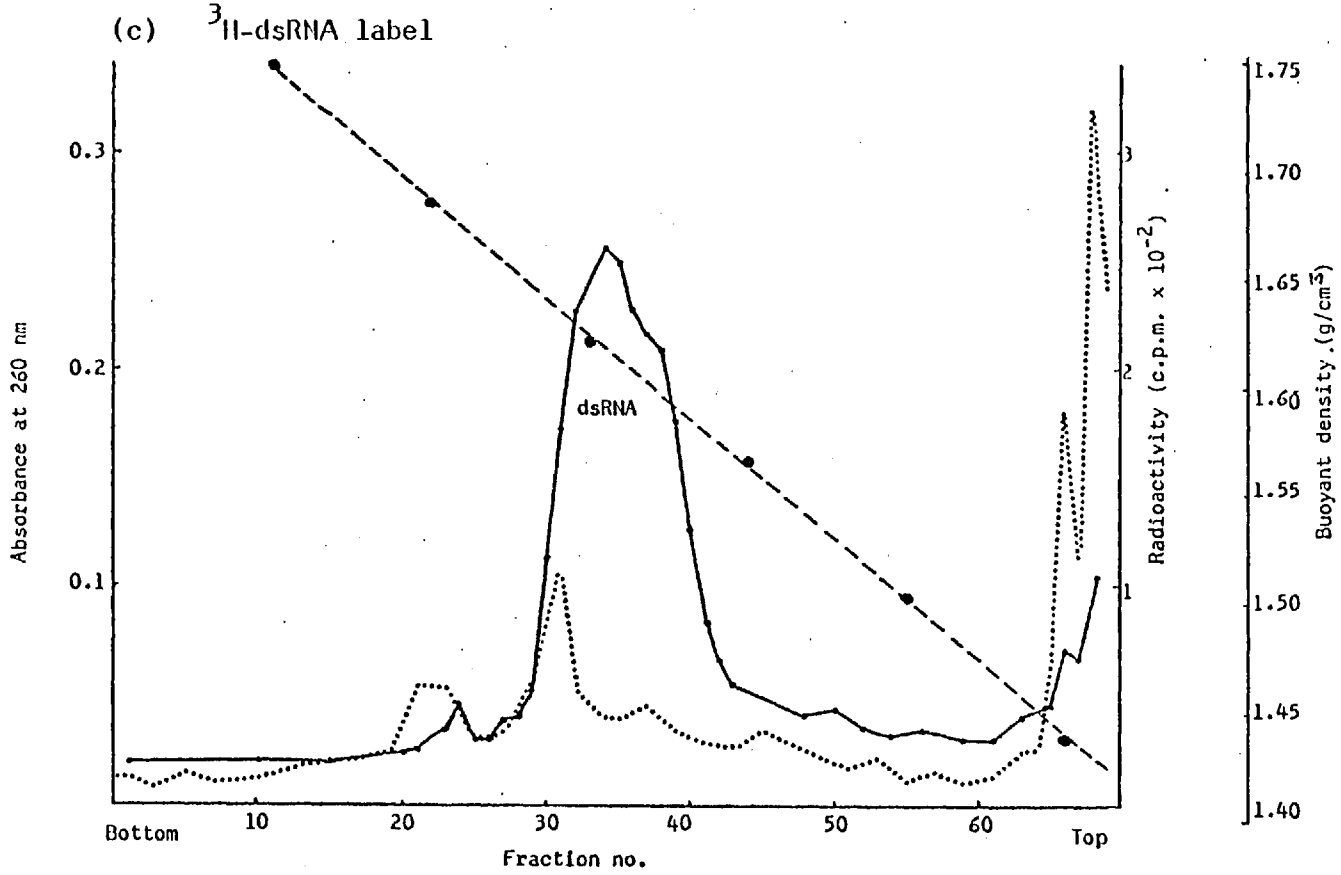


Figure 8 - CS density gradient centrifugation of nuclear extract from MS cells labelled for 22 hours with ^{3}H-adenosine, ^{3}H-uridine and ^{3}H-dsRNA in the presence of serum.

Cont/.....

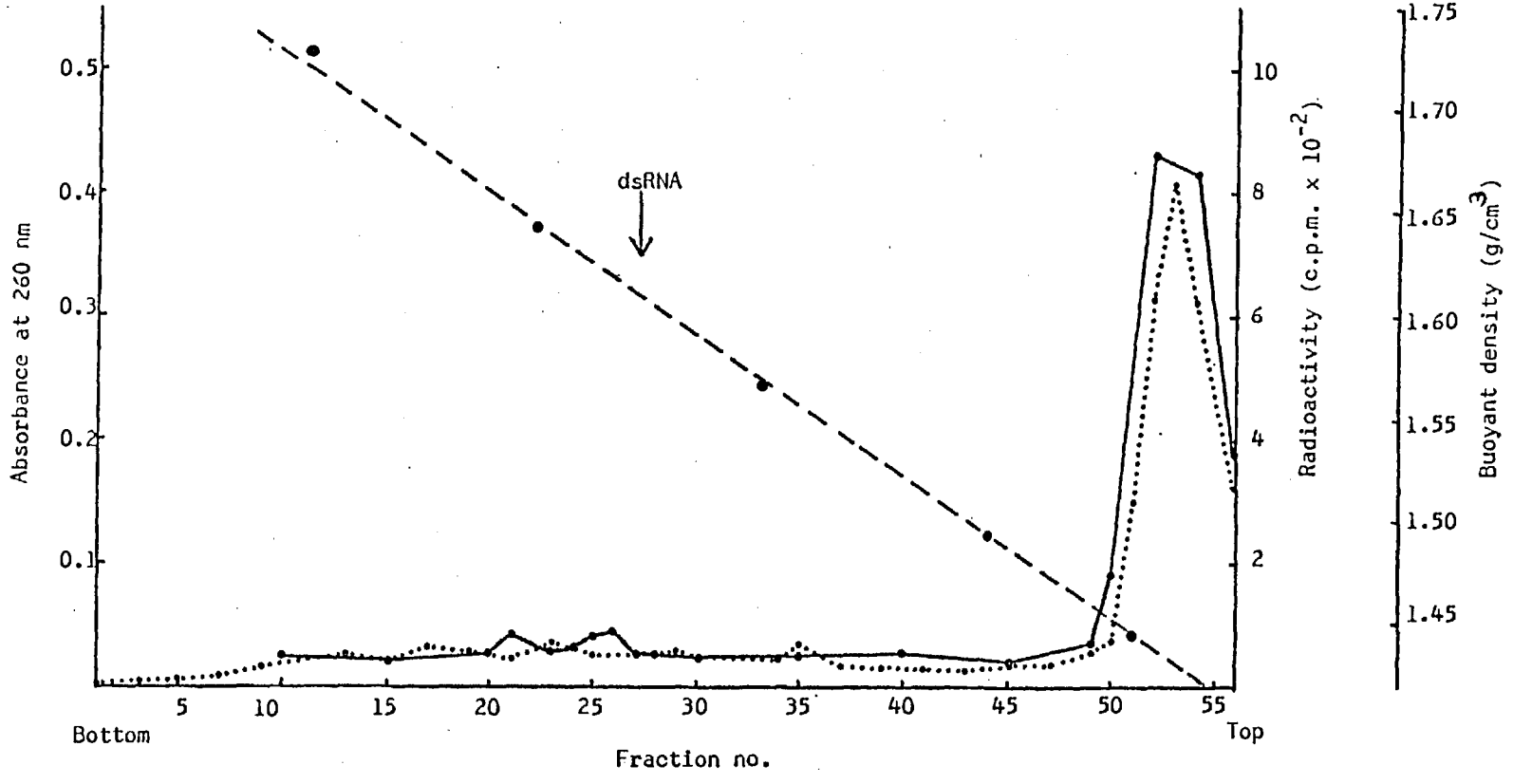
Figure 8 (cont.)



1 ml MS cell cultures were labelled for 22 hours (30-52h) with ^{3}H-adenosine (a), ^{3}H-uridine (b), or ^{3}H-dsRNA (c) as described in Table 10. Nucleic acids were extracted from the isolated nuclei and subjected to CS density gradient centrifugation. Unlabelled dsRNA was included as A_{260} marker in gradient 8 (c). DNA bands at a density of approximately 1.43 g/cm^{3}.

——• absorbance at 260nm; ····· radioactivity; •— — —• buoyant density

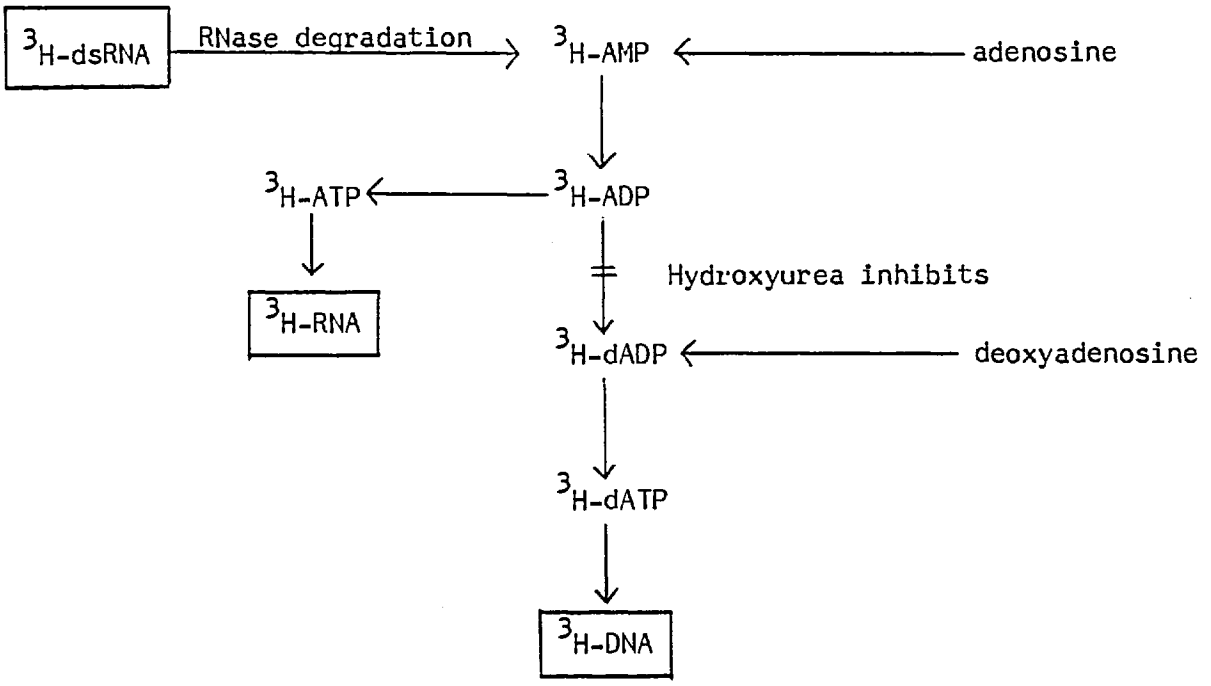
Figure 9 - Caesium sulphate density gradient centrifugation of the nucleic acid extracted from ^{3}H-dsRNA-labelled MS cells.



1 ml MS cell cultures (2.66 x 10^6 cells/ml) in medium containing 5% FCS and 4 µg/ml Con A, were incubated with 0.3 µCi/ml ^{3}H-dsRNA (30 µCi/mg) from 0-48h. Labelled nucleic acid was extracted from the nuclei of pooled, washed cells, and analysed by CS density gradient centrifugation.

•——• Absorbance at 260nm; ····· radioactivity; •— — —• buoyant density

Figure 10 - Possible pathways for the breakdown and re-utilisation of ^{3}H-adenosine-labelled dsRNA.



The salvage pathways which may be used in the breakdown and re-utilisation of ^{3}H-dsRNA by MS cells are shown in Figure 10. Reduction of the ribonucleotide diphosphates to deoxyribonucleotide diphosphates is catalysed by ribonucleoside diphosphate reductase, an enzyme which is characteristically inhibited by hydroxyurea. If ^{3}H-dsRNA were metabolised by the pathways in Figure 10, hydroxyurea should inhibit the conversion of ^{3}H-dsRNA to ^{3}H-DNA but not to ^{3}H-RNA. Another prediction would be that unlabelled adenosine, in large excess, should inhibit ^{3}H-dsRNA incorporation by becoming phosphorylated in the cell to AMP and so diluting the pool of ^{3}H-AMP formed during ^{3}H-dsRNA degradation. Nucleosides, and not nucleotides, were used for these competition experiments since the cell membrane is relatively impermeable to the phosphorylated precursors.

.2 The effect of unlabelled nucleosides and hydroxyurea on ^{3}H-dsRNA and ^{3}H-adenosine incorporation

In MS cells treated with 5 mM hydroxyurea, DNA synthesis, as measured by ^{3}H-thymidine incorporation, was virtually abolished (Table 11) and an 18% decrease in cell number per culture at 48 hours occurred. Hydroxyurea inhibited ^{3}H-dsRNA incorporation into total nucleic acid by 58%.

Table 11 - The effects of cold adenosine, uridine and hydroxyurea on ^{3}H-dsRNA and ^{3}H-Tdr incorporation in Con A-stimulated MS cells.

Additions	^{3}H-dsRNA uptake		^{3}H-Tdr uptake	
	cpm/10^{6} cells	% inhibition	cpm/10^{6} cells	% inhibition
None (control)	1,980	0	44,870	0
Adenosine, 20 µm	1,320	33	69,210	0
Adenosine, 100 µm	750	62	58,840	0
Uridine, 20 µm	1,820	8*	43,430	0
Uridine 100 µm	1,780	10*	43,600	0
Hydroxyurea, 1 mM	830	58	1,350	97
Hydroxyurea, 5 mM	830	58	510	99

1 ml MS cell cultures, stimulated with Con A, were labelled in the presence of FCS from 30 to 48 hours with ^{3}H-dsRNA (0.3 µCi/ml; 30 µCi/mg) or ^{3}H-Tdr (2 µCi/ml; 5 µM). Cold adenosine, uridine or hydroxyurea, also present from 30-48 hours, were added immediately before the labelled materials. Incorporation of label (TCA-precipitable) was measured at 48 hours. Results are the average of duplicate cultures, and are expressed as % inhibition of the untreated control.

* not significantly different from controls.

A large molar excess of unlabelled uridine had no significant effect on either ^{3}H-dsRNA or ^{3}H-thymidine incorporation. Thymidine monophosphate (TMP) is synthesised from UMP by reduction and methylation, in the absence of exogenously-supplied thymidine. Excess uridine failed to suppress thymidine incorporation, suggesting that the thymidine kinase pathway of TMP synthesis prevails when the cells are supplied with thymidine.

Excess unlabelled adenosine did not inhibit ^{3}H-thymidine incorporation by Con A-stimulated MS cells; in fact this parameter was enhanced. Adenosine has been shown to stimulate lymphocyte proliferation at concentrations below 100 µM (Carson and Seegmiller, 1976). Unlabelled adenosine inhibited ^{3}H-dsRNA incorporation in a concentration-dependent fashion, as was predicted from Figure 10. However, only 5% of the initial amount of ^{3}H-dsRNA was actually taken up by the cells, therefore the "^{3}H-adenosine equivalents" generated by breakdown of ^{3}H-dsRNA should have theoretically been diluted out by the large excess of cold adenosine (100 µM), so that a greater degree of inhibition might have been anticipated. Several possibilities could explain this observation: (1) that the AMP generated from dsRNA degradation and from exogenous adenosine were not freely accessible to the same pool, (2) that a considerable fraction of the cell-associated ^{3}H-dsRNA remained undegraded, or (3) that ^{3}H-dsRNA catabolism and re-utilisation involved pathways other than those shown in Figure 10. Further experiments suggested that the second and third possibilities were unlikely explanations.

The results summarised in Table 12 show that 50 µM unlabelled adenosine inhibited the incorporation of ^{3}H-dsRNA and ^{3}H-adenosine to approximately the same degree (60%). Although the lack of complete inhibition of ^{3}H-dsRNA incorporation may be explained by precursor compartmentation effects, the lack of total inhibition of ^{3}H-adenosine uptake may not, and is more likely to be due to the fact that ^{3}H-adenosine uptake was not linear, at this concentration, over the 24 hour period.

Incorporation of ^{3}H-dsRNA and ^{3}H-adenosine by MS cells was also inhibited by 50 µM unlabelled deoxyadenosine. The percentage of total nuclear label that was DNA (stable to NaOH hydrolysis) was lower in cells treated with deoxyadenosine than in those treated with adenosine, an observation consistent with the fact that deoxyadenosine competes at a later stage than adenosine on the pathway to ^{3}H-DNA synthesis.

Table 12 - Modulation of ^{3}H-dsRNA and ^{3}H-adenosine uptake and distribution in stimulated MS cells.

(a) ^{3}H-dsRNA incorporation

Addition	c.p.m./ 10^6 cells	% inhibition	% of label in nucleus	% of nuclear label as DNA*
None (control)	4,930	0	64	59
50µM Adenosine	2,160	56	62	70
50µM Deoxyadenosine	2,100	57	63	55
3mM Hydroxyurea	1,940	61	53	4

(b) ^{3}H-adenosine incorporation

Addition	c.p.m./ 10^6 cells	% inhibition	% of label in nucleus	% of nuclear label as DNA*
None (control)	69,060	0	79	89
50µM Adenosine	28,020	60	68	81
50µM Deoxyadenosine	36,650	47	63	74
3mM Hydroxyurea	30,640	56	50	2

1 ml MS cell cultures, stimulated with Con A in serum-containing medium, were treated with unlabelled nucleosides, dsRNA (dialysed) or hydroxyurea at 30 hours of transformation. Cultures were then labelled with ^{3}H-dsRNA (0.78 µCi/ml, 383 µCi/mg) or ^{3}H-adenosine (1 µCi/ml, 24 µCi/mmol) from 30 to 48 hours. At 48 hours, cells from triplicate cultures were pooled, washed, and the intracellular distribution of incorporated radioactivity determined (as Table 10).

* determined by NaOH hydrolysis of whole nuclei. All samples contained >380 c.p.m. before hydrolysis (50 c.p.m. background after RNA hydrolysis)

3mM hydroxyurea inhibited incorporation of both ^{3}H-adenosine and ^{3}H-dsRNA by MS cells during an 18 hour labelling period, and in both cases preferentially suppressed incorporation of label into the nuclear fraction. When the nuclear label was subjected to alkaline hydrolysis, less than 5% was DNA in nature, while in control cultures without hydroxyurea, the amount of DNA was 59% in ^{3}H-dsRNA-labelled cells, or 89% in ^{3}H-adenosine-labelled cells.

In order to investigate the effects of hydroxyurea more fully, Con A-stimulated MS cells were incubated for 30 min with 5mM hydroxyurea, a concentration previously shown to abolish DNA synthesis, before addition of ^{3}H-dsRNA or ^{3}H-adenosine. The distribution of label in these cells after 18 hours is shown in Table 13. The proportions of ^{3}H-dsRNA in the nucleus, in the absence and presence of hydroxyurea, were 58% and 46% respectively. Cells again took up high levels of ^{3}H-adenosine, which was distributed in a similar way to ^{3}H-dsRNA. The proportions of NaOH-resistant label in the nuclei were lower than in previous experiments, although the % DNA figures calculated from the CS density profiles (64 and 70% for ^{3}H-dsRNA and ^{3}H-adenosine respectively) were somewhat closer.

The absence of NaOH-resistant label in hydroxyurea-treated cells was confirmed by CS density gradient centrifugation of the nuclear extracts, when no radioactivity was found in the DNA bands (see Figure 11). These results demonstrated that the appearance of label in the DNA of MS cells labelled with ^{3}H-dsRNA was a metabolic process requiring a functional DNA-synthesising system, and was not simply an association, covalent or otherwise, of ^{3}H-dsRNA with MS cell DNA. This conclusion was confirmed by verification of the deoxyribose nature of the labelled adenosine moiety in the ^{3}H-DNA, as described below.

.3 Nature of DNA in ^{3}H-dsRNA-labelled MS cells

DNA-containing fractions (50-58) from the CS density gradients shown in Figures 11(a) and (c) were pooled, dialysed, concentrated and digested sequentially with pancreatic DNase I and snake venom phosphodiesterase. Digestion to TCA-soluble products (5' mononucleotides) was complete, as shown in Table 14.

Table 13 - The intracellular distribution of ^{3}H-dsRNA and ^{3}H-adenosine in Con A-stimulated MS cells in the presence and absence of 5mM hydroxyurea (HU).

^{3}H-label (30-48 hrs)	5mM Hydroxyurea	^{3}H-c.p.m. incorporated/10^6 cells				% of label[2] in nucleus	% of nuclear[3] label as DNA
		Cells	Nucleus	Cytoplasm	Nuclear Wash		
^{3}H-dsRNA (5 µCi/ml)	-	31,260 (30,790)[1]	16,060 (17,700)	10,860 (6,940)	920 (810)	58	35
	+	16,570 (14,870)	7,730 (7,920)	7,810 (4,620)	1,110 (1,180)	46	0
^{3}H-adenosine (2 µCi/ml)	-	186,700 (174,320)	90,230 (98,580)	54,120 (38,200)	7,360 (7,640)	59	57
	+	116,460 (103,380)	54,370 (51,160)	54,200 (28,530)	11,090 (10,970)	56	0.7

1 ml MS cell cultures were set up at 2.4 x 10^6 cells/ml in serum-containing medium with 4 µg/ml Con A. After 29 hours, the cultures were pooled, washed to remove dead cells, and resuspended at 2.1 x 10^6 cells/ml in fresh medium with serum and Con A, with or without 5mM hydroxyurea (HU). After 30 minutes, replicate cultures (10 without HU, 20 with HU) were labelled with ^{3}H-dsRNA (5 µCi/ml, 310 µCi/mg) or ^{3}H-adenosine (2 µCi/ml, 5 µM) from 30-48 hours. At 48 hours, replicate cultures were pooled, and the intracellular distribution of radioactivity determined as described in Materials and Methods.

1. TCA-precipitable c.p.m. are shown in parentheses under total c.p.m.
2. % of label in nucleus is calculated as a % of total label in (nucleus + cytoplasm + nuclear washings).
3. % DNA was calculated from the % of label in the nuclei resistant to NaOH hydrolysis. Cytoplasmic label was 97-100% NaOH solubilised. Background c.p.m. after NaOH hydrolysis of ^{3}H-dsRNA was 95 c.p.m.; all samples analysed contained >2,700 c.p.m. before hydrolysis.

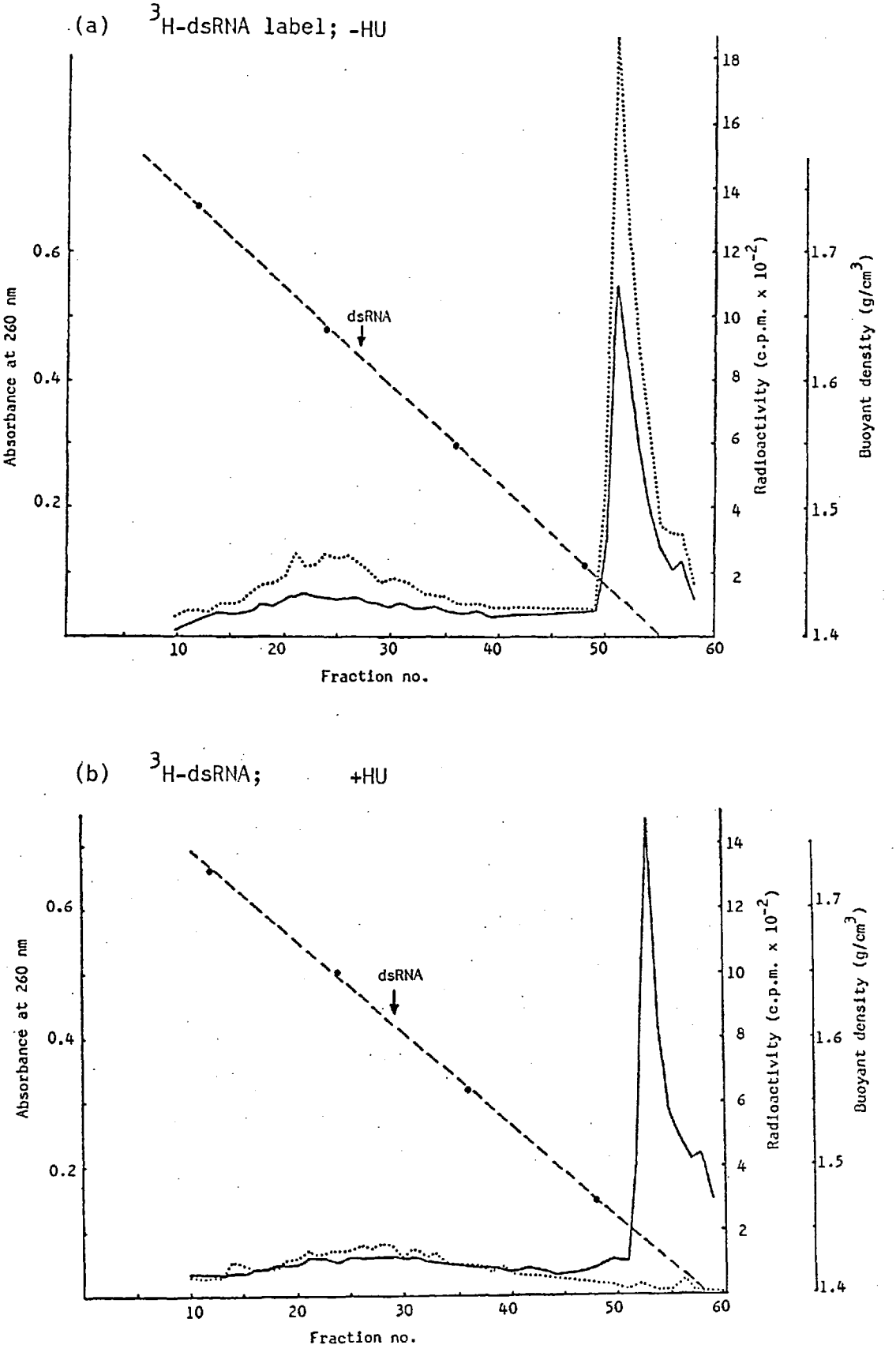
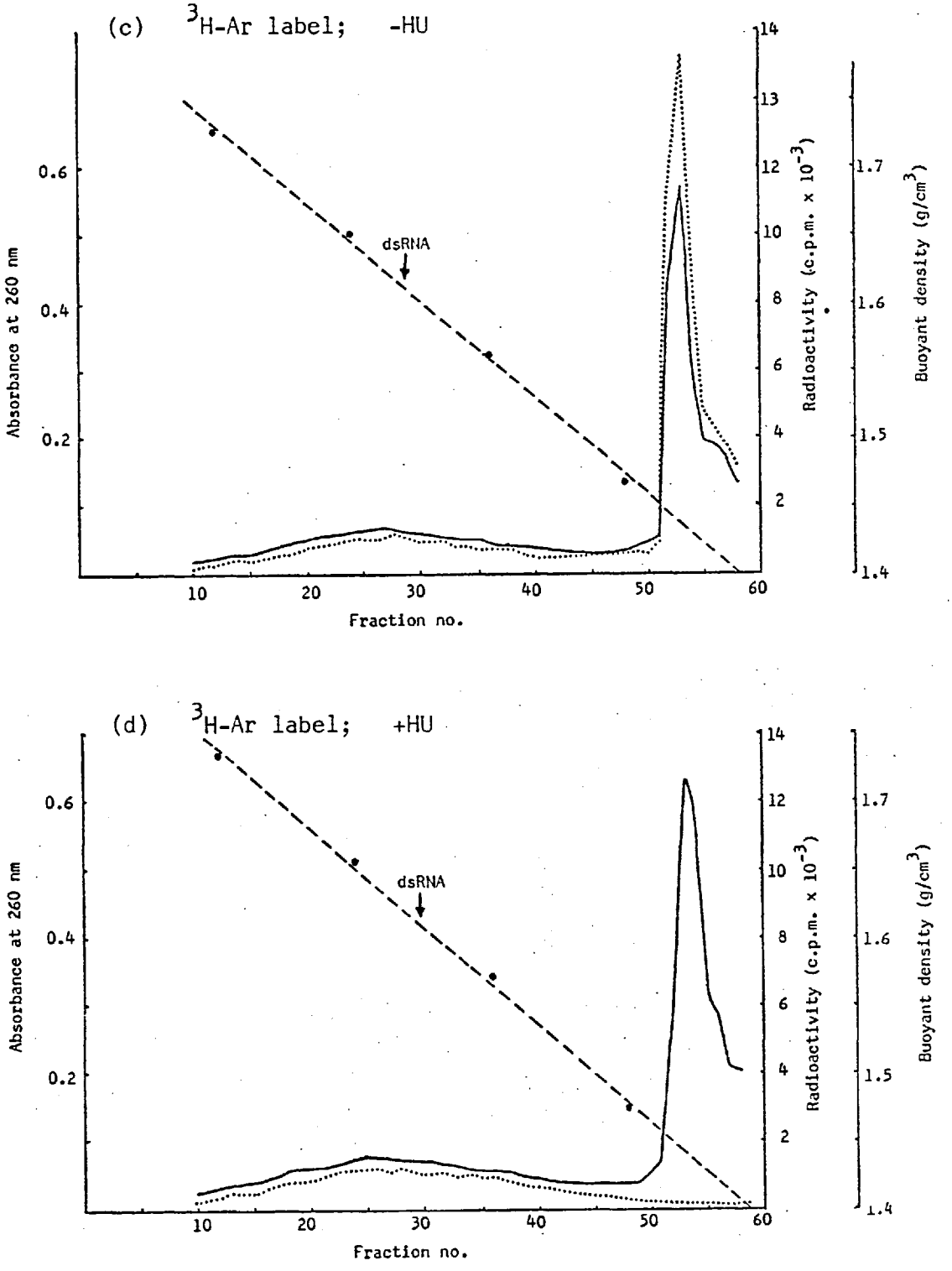


Figure 11 - CS density gradient centrifugation of the nuclear extracts from MS cells labelled for 18 hours with ^{3}H-dsRNA and ^{3}H-adenosine in the presence and absence of hydroxyurea (HU).

Cont/.....

Figure 11 (cont.)



1 ml MS cell cultures were labelled with 5 µCi/ml ^{3}H-dsRNA (a and b) or 2 µCi/ml ^{3}H-adenosine (c and d) for 18 hours in the presence (b and d) or absence (a and c) of 5mM hydroxyurea as described for Table 13 . Nucleic acids were extracted from the nuclei isolated from pooled cell cultures and subjected to CS density gradient centrifugation.

—— absorbance at 260 nm; ·····radioactivity; •– – –• buoyant density.

Table 14 - Enzymic digestion of ^{3}H-DNA from ^{3}H-dsRNA- and ^{3}H-adenosine-labelled MS cells.

Label	^{3}H-DNA - c.p.m./50 µl enzyme digest			
		-Enz	+Enz; 0 min	+Enz; 210 min
^{3}H-dsRNA	-TCA	3,408	1,203	729
	+TCA	3,330	378	36
^{3}H-Ar	-TCA	17,801	6,656	2,607
	+TCA	15,756	4,138	52

DNA recovered from the CS gradients shown in Figures 11(a) and (c) was digested in 0.5 ml 0.01M tris-HCl buffer, pH7.5 containing 10mM $MgCl_2$, with 25 µg pancreatic DNase for 2 hours, then 25 µg snake venom phosphodiesterase for 1.5 hours. 50 µl aliquots of reaction mixture were counted on paper discs, before and after TCA treatment, at the beginning and end of digestion.

The products were analysed by descending paper chromatography in a borate-containing solvent. The presence of borate in the solvent allows separation of 5' ribonucleotides and 5' deoxyribonucleotides, by forming a complex with the former through the 2' and 3' OH groups on the ribose ring, thus retarding their elution. The chromatography results are shown diagramatically in Figure 12. Hydrolysed DNA from ^{3}H-dsRNA-labelled cells contained labelled adenosine as 5'dAMP — no 5'AMP was present. Some label ran as 5'dGMP, just behind 5'dAMP.

DNA extracted from ^{3}H-adenosine-labelled cells served as a control for these techniques. This was also digested to produce mainly 5'dAMP. Less than 1% of the radioactivity ran as 5'AMP, which probably represented a trace RNA contaminant in the DNA isolated from the CS density gradient. Such an amount was not detectable by NaOH hydrolysis of the DNA, due to the lack of precise quantitation of TCA-precipitability on paper discs. It may alternatively be derived from RNA "primers" in DNA, that is, short stretches of RNA covalently linked to very much longer sequences of DNA, which have been reported to act as initiation sites for DNA replication during the S phase of the cell cycle, and which do not alter the buoyant density of the DNA during equilibrium density gradient centrifugation in caesium sulphate (Neubort and Bases, 1974).

Figure 12 - Paper chromatographic analysis of the DNA digests from ^{3}H-dsRNA and ^{3}H-adenosine labelled MS cells

Position of Markers	^{3}H-c.p.m.	
	^{3}H-dsRNA-labelled cells	^{3}H-adenosine-labelled cells
Origin	4	6
	0	4
	4	15
	0	25
5'AMP	3	8
	7	7
	5	3
	5	9
5'dGMP	20	17
	106	67
	153	129
	65	798
	225	2,573
	288	1,353
5'dAMP	68	51
	0	6
	1	4
	5	5
	42	7
	1	4
	2	2
	2	1
	3	11
	6	13
	2	19
Solvent Front	0	2

DNA-containing bands from the CS gradients shown in Figures 11(a) and (c) were digested with pancreatic DNase I and snake venom phosphodiesterase (see Table 14 for details). The digestion products were analysed by descending paper chromatography in ethanol-ammonium acetate-borate solvent, in the presence of 5'AMP and 5'dAMP markers.

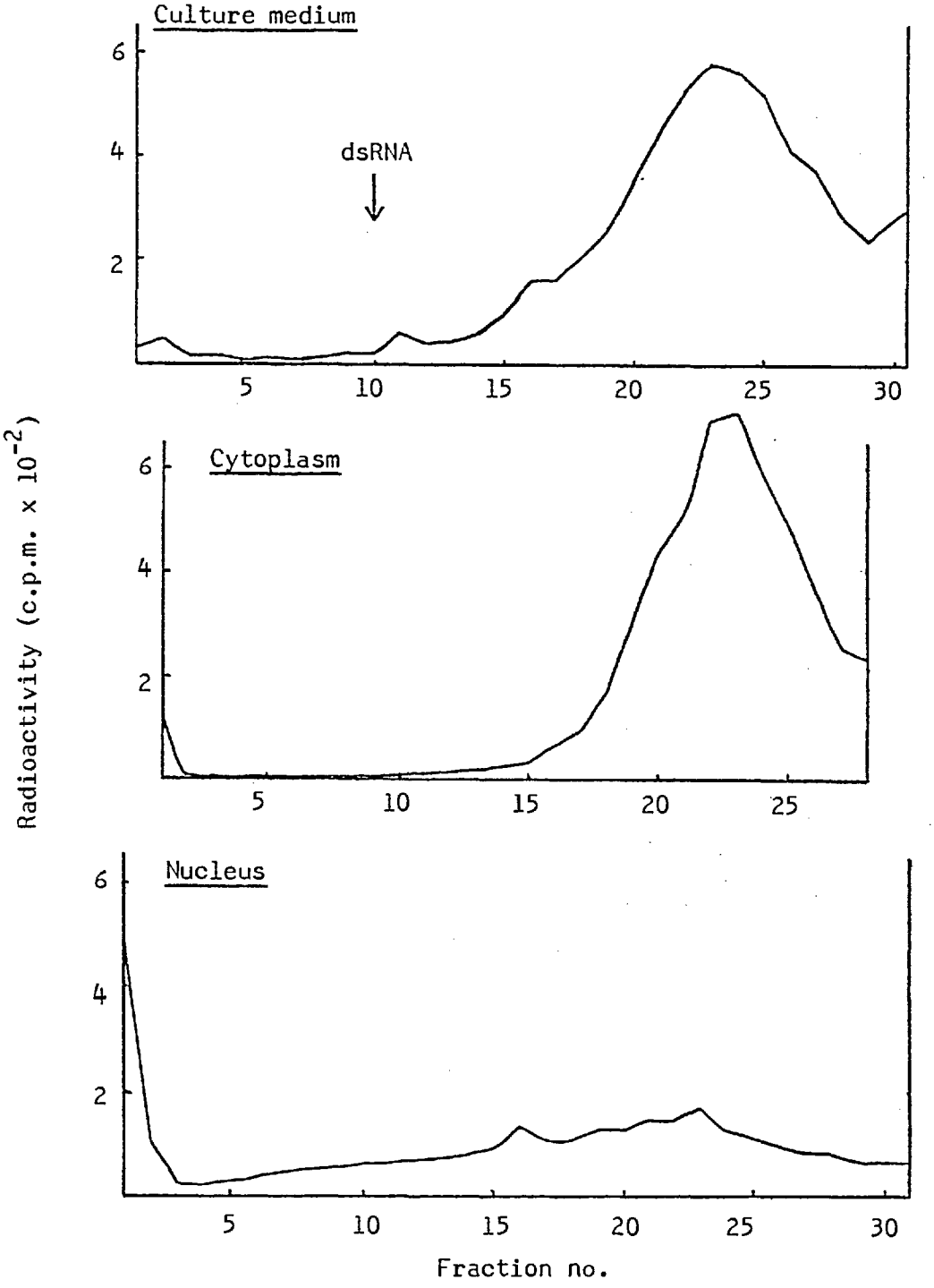
.4 Nature of RNA in ^{3}H-dsRNA-labelled MS cells

After labelling stimulated MS cells with ^{3}H-dsRNA for 18 hours, the molecular weights of the labelled RNA species in the cell culture supernatant, nuclear and cytoplasmic fractions were determined by sucrose density gradient centrifugation. The results, shown in Figure 13, revealed that very little, if any, of the original ^{3}H-dsRNA remained in the culture medium; most of it had been degraded to material of molecular weight 1×10^{5} (value for ssRNA). Labelled RNA in the cell cytoplasm had also been degraded to a size of approximately 6×10^{4}. The nuclear RNA gave a more heterogeneous profile, with no obvious predominant size species. Radioactivity at the bottom of this gradient (nuclear fraction) was probably DNA of much higher molecular weight.

RNA remaining in the culture medium and RNA extracted from the cytoplasm by the Marmur procedure were analysed by hydroxylapatite chromatography. Nuclear RNA was separated from DNA on a CS density gradient but was unfortunately lost by non-specific degradation during recovery, and therefore was not available for analysis. As seen from the results in Table 15, RNA in both the culture medium and cell cytoplasm was approximately 20% converted to low molecular weight material, and of the remaining macromolecular material, 85-90% was single-stranded. The RNA in the culture medium was also analysed by CS density gradient centrifugation (see Figure 14). The buoyant density was found to have spread throughout the gradient from the initial value of 1.61, a further indication of non-specific degradation.

These changes in the RNA in the culture medium were very similar to those produced by FCS alone (see Section 1.3). Therefore, it was likely that during prolonged incubations, MS cells took up increasing amounts of labelled degradation products of mean molecular weight 1×10^{5}, rather than intact dsRNA of 2×10^{6} molecular weight. Since the biological activity of dsRNAs is decreased as the molecular weight is reduced (Edy *et al*, 1974; Morahan *et al*, 1972) it is important to study the fate of biologically active ^{3}H-dsRNA under conditions where it remains intact in the culture medium. For this reason the uptake of ^{3}H-dsRNA was investigated in Con A-stimulated MS cells cultured in serum-free medium.

Figure 13 - Sucrose density gradient centrifugation of labelled RNA in the culture medium, cell cytoplasm and nucleus of MS cells labelled for 18 hrs with ^{3}H-dsRNA in serum-containing medium



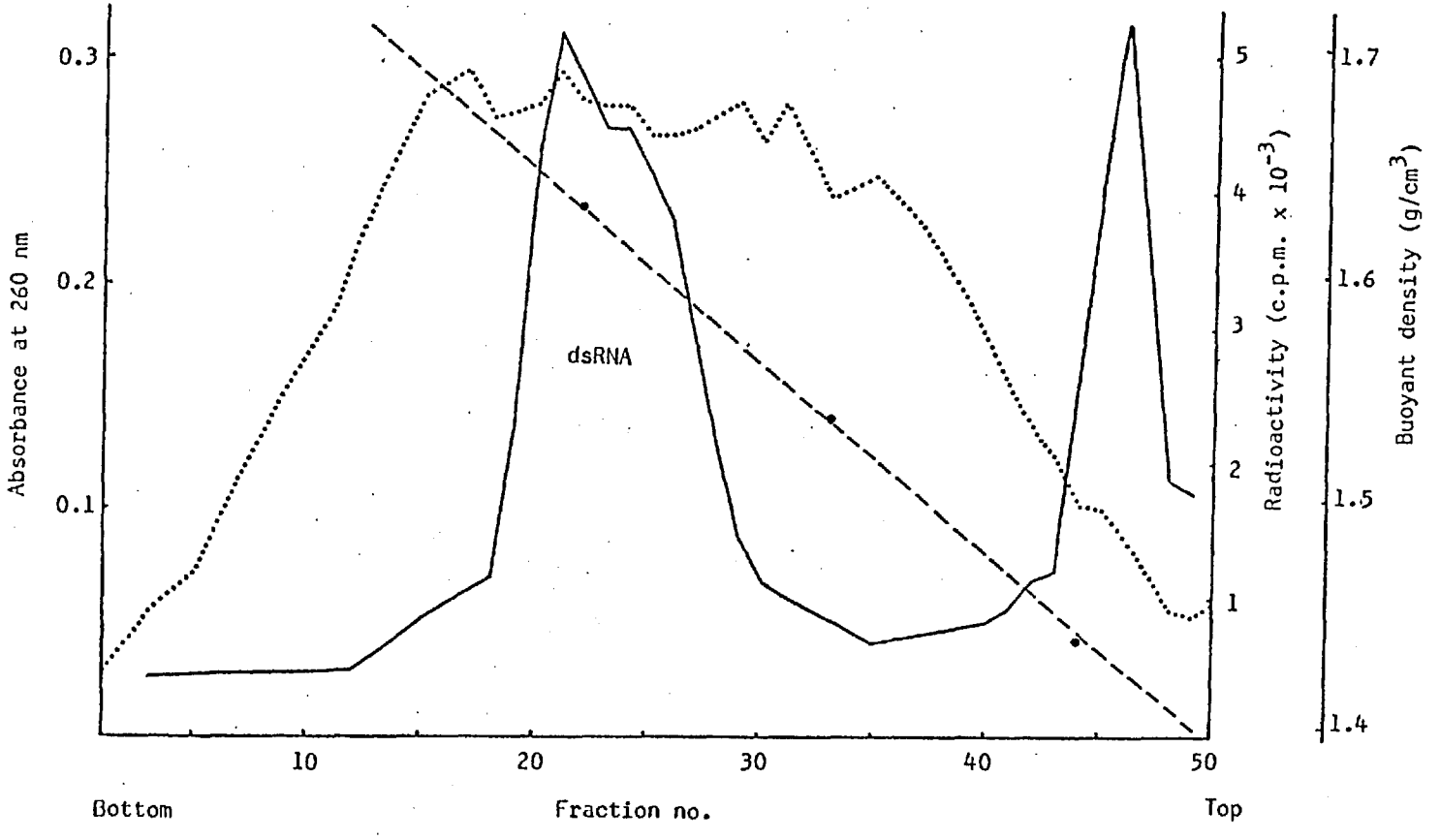
Con A-stimulated MS cells, in serum-containing medium, were labelled with ^{3}H-dsRNA for 18 hours, as described in Table 13 (except that hydroxyurea was not present). 50-150µl samples of (a) culture medium, (b) cytoplasm or (c) nuclei were analysed by neutral sucrose density gradient centrifugation. Fractions were counted on filter paper discs.

Table 15 - Hydroxylapatite chromatography of ^{3}H-RNA in culture medium and cytoplasm of MS cells labelled with ^{3}H-dsRNA in serum-containing medium.

^{3}H-RNA	% low mol. wt. ^{3}H-c.p.m.	Macromolecular ^{3}H-c.p.m.	
		% ssRNA	% dsRNA
Culture medium	23	89	11
Cytoplasm	19	87	13
^{3}H-dsRNA control	0	2	98

MS cells in serum-containing medium were labelled with ^{3}H-dsRNA for 18 hours, as described in Table 13. Samples of the culture medium and Marmur-extracted RNA from the cell cytoplasm were applied to hydroxylapatite columns in 0.012M potassium phosphate buffer pH 6.8, and analysed as described in Materials and Methods. ^{3}H-dsRNA was chromatographed concurrently as a control.

Figure 14 - CS density gradient centrifugation of the ^{3}H-labelled material remaining in the culture medium after labelling MS cells for 18 hours with ^{3}H-dsRNA.



MS cells in 1 ml cultures were labelled for 18 hours with ^{3}H-dsRNA (2µCi/ml; 310 µCi/mg) in serum-containing medium. At the end of the incubation period the cells were centrifuged and a sample of the supernatant was analysed, with unlabelled dsRNA and DNA markers, by CS density gradient centrifugation.

——— absorbance at 260 nm; ····· radioactivity; •— — —• buoyant density

.5 Incorporation of ^{3}H-dsRNA in the absence of serum

MS cells were washed free of serum after 30 or 40 hours of transformation, then reincubated in serum-free medium for the labelling period. 2-mercaptoethanol (2-ME) was added to serum-free medium at 5×10^{-5}M, to improve the lymphocyte response (Broome and Jeng, 1973). Preliminary experiments showed that 2-ME marginally improved MS cell viability between 30 and 60 hours of culture, and that the level of ^{3}H-dsRNA incorporation was not affected by 2-ME per se. However, the level of incorporation was severely reduced in the absence of serum.

Table 16 shows the uptake of ^{3}H-dsRNA and ^{3}H-DNA, for comparison, by Con A-stimulated MS cells in the presence and absence of FCS. Because the increase in cell number per culture from 30 to 60 hours was small in serum-free medium, it was important to verify that the cells at this time remained active with regard to other parameters, such as DNA synthesis. Therefore a third set of cultures were pulsed with ^{3}H-thymidine for the last two hours of the labelling period. ^{3}H-thymidine uptake per culture at 62-64 hours was lower in serum-free than in serum-containing cultures, but cell numbers were also lower, so that ^{3}H-thymidine incorporation/10^{6} cells (specific activity) was similar in both situations. Incorporation of ^{3}H-DNA was likewise quantitatively similar in the presence and absence of serum. However, ^{3}H-dsRNA incorporation was much lower in serum-free than in serum-supplemented cultures.

The distribution of ^{3}H-dsRNA in the cells after a 24 hour pulse was also affected by the presence of serum in the medium. While 68% of the material was present in the nuclear fraction of MS cells labelled in medium with 5% serum, 85% was found in this fraction of cells labelled under serum-free conditions. From analysis of the nuclear fractions by NaOH hydrolysis, it was seen that in the former case, 43% of the label was DNA, while in the latter, all the label was RNA.

^{3}H-RNA remaining in the medium after 6 and 24 hour labelling periods was analysed on hydroxylapatite columns, together with samples of the total nucleic acid extracted from the isolated cell nuclei. The results, shown in Table 17, indicate that while ^{3}H-dsRNA was progressively degraded in the medium of serum-containing cultures, ^{3}H-dsRNA in the medium of serum-free cultures remained

Table 16 - The effect of serum on the incorporation of ^{3}H-dsRNA and ^{3}H-DNA by MS cells.

		-SERUM			+SERUM		
		^{3}H-dsRNA 6 hrs.	^{3}H-dsRNA 24 hrs.	^{3}H-DNA 24 hrs.	^{3}H-dsRNA 6 hrs.	^{3}H-dsRNA 24 hrs.	^{3}H-DNA 24 hrs.
^{3}H-c.p.m./ 10^{6} cells	Cells	1,520 (1,770)	4,030 (4,230)	27,590 (26,490)	2,590 (3,190)	26,440 (22,950)	27,120 (28,800)
	Nucleus	470 (740)	3,010 (2,950)	20,700 (21,600)	1,030 (1,360)	14,250 (14,950)	20,630 (22,250)
	Cytoplasm	400 (790)	370 (650)	780 (1,610)	950 (740)	6,390 (3,550)	660 (790)
	Nuclear wash	60 (630)	130 (280)	810 (1,140)	290 (430)	500 (880)	460 (700)
% of label in nucleus		51	85	93	45	68	95
% of label NaOH-insoluble	Medium	0	0	83	0	0	100
	Nucleus	0	0	88	0	43	81
	Cytoplasm	0	1	0	0	0	0
^{3}H-Tdr incorporation/10^{6} cells		26,380			29,730		

1 ml MS cell cultures were set up at 2.9 x 10^{6}/ml in medium containing 5% FCS, 4 µg/ml Con A, and 5 x 10^{-5}M 2-ME. After 40 hours, the cells were pooled, washed in serum-free medium and resuspended at 2.1 x 10^{6} cells/ml in medium with 2-ME, with or without 5% FCS. Cells were then labelled for 6 or 24 hours with 2 µCi/ml ^{3}H-dsRNA (310 µCi/mg) or 2 µCi/ml ^{3}H-DNA (50 µCi/mg). Control cultures receiving no nucleic acid were pulsed with 2 µCi/ml ^{3}H-Tdr for 2 hours (62-64 hours of cultures). Incorporation of label was measured as described in Materials and Methods. Numbers in parentheses are TCA-insoluble c.p.m./10^{6} cells.

Table 17 - Hydroxylapatite chromatography of ^{3}H-dsRNA in the culture medium and nuclei of ^{3}H-dsRNA-labelled MS cells.

Test material		% soluble ^{3}H-c.p.m.	Macromolecular ^{3}H-c.p.m.	
			% ssRNA	% dsRNA
Culture medium -serum	6 h	0.4	0.6	99.4
	24 h	0.5	0.6	99.4
Culture medium +serum	6 h	10.1	83.3	16.7
	24 h	42.4	97.0	3.0
Nuclei -serum	24 h	2.3	13.0	87.0
Nuclei +serum	24 h	8.7	67.1	32.9
^{3}H-dsRNA control		0.75	2.0	98.0

MS cells were labelled with ^{3}H-dsRNA for 6 or 24 hours, in the presence or absence of serum, as described in Table 16. Nucleic acids were extracted from the cell nuclei (Marmur) and dialysed against 0.012 M potassium phosphate buffer for hydroxylapatite analysis.

completely double-stranded, even after a 24 hour incubation period. Label associated with the nuclei of cells cultured in the absence of serum was 99% macromolecular, 87% of which was double-stranded, while in cells cultured in the presence of serum the nuclear label was more degraded and denatured, and the amount eluting in the double-stranded fraction could be attributed entirely to DNA.

This data suggested that ^{3}H-dsRNA was incorporated by mouse lymphocytes in an intact, double-stranded form in serum-free culture medium. Uptake and incorporation into cellular nucleic acid was facilitated by FCS, which denatured and degraded dsRNA to a more assimilable form.

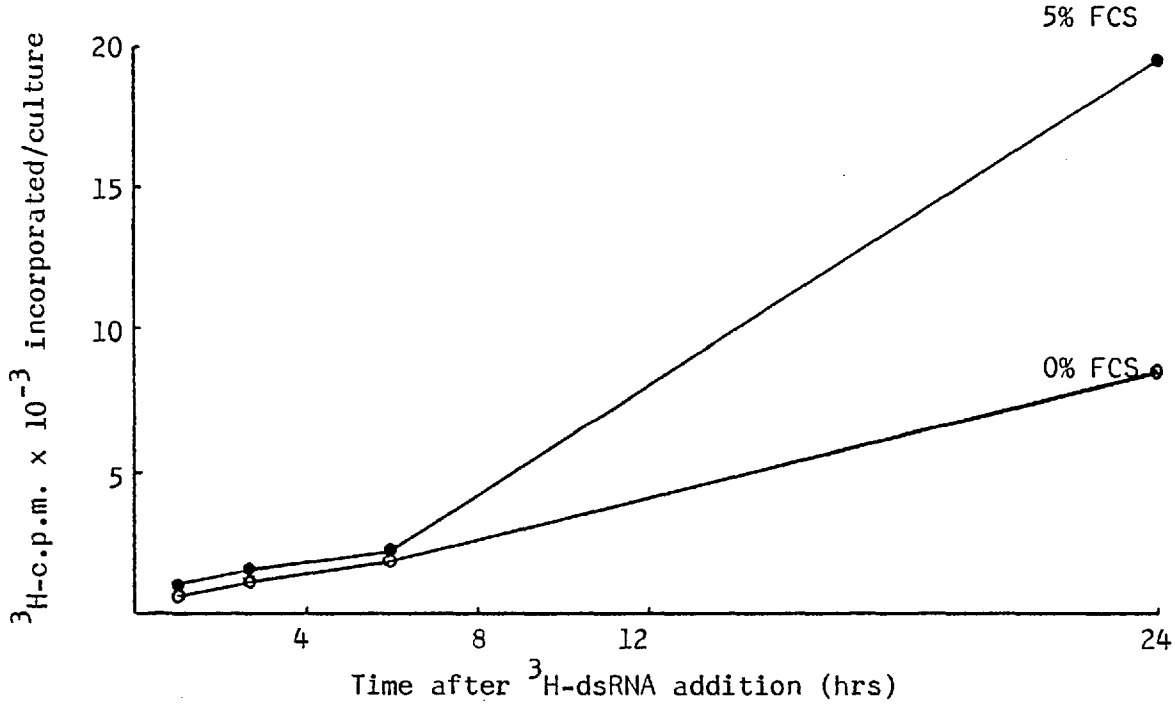
Uptake of ^{3}H-DNA was not influenced by the presence of serum in the medium and was considerably greater than uptake of ^{3}H-dsRNA in terms of number of molecules incorporated per cell (specific activity of the ^{3}H-dsRNA used was five times higher than that of ^{3}H-DNA). The effect of FCS on the molecular integrity of ^{3}H-DNA was not investigated, but no degradation to TCA-soluble material occurred in the medium of serum-containing cultures after 24 hours, suggesting that the ^{3}H-DNA remained intact. Stimulated MS cells can therefore differentiate between dsRNA and native DNA, as well as between dsRNA and ssRNA.

The kinetics of ^{3}H-dsRNA incorporation in the presence and absence of serum is shown in Figure 15. The higher level of incorporation observed in the presence of serum became significant only after 6 hours. After this time the ^{3}H-dsRNA would have been progressively degraded by serum nucleases (as was shown in Table 4). Although on this occasion the serum-containing cultures took up approximately 3-fold more ^{3}H-dsRNA than serum-free cultures, this value was generally nearer 10-fold [mean of 9.2 $\pm$ 4.8 (sd) in nine experiments].

.6 Incorporation of ^{3}H-ssRNAs in the absence of serum

Table 18 shows the results of two experiments in which the uptake and fate of ^{3}H-ssRNAs were compared to that of ^{3}H-dsRNA in serum-free culture conditions. The fate of ^{3}H-poly C, and two homologous ^{3}H-ssRNA species (prepared from ^{3}H-adenosine labelled MS cells) was seen to be the same as that of ^{3}H-dsRNA in serum-containing cultures, that is, they became partly degraded to

Figure 15 - Kinetics of ^{3}H-dsRNA incorporation into TCA-insoluble material by MS cells in the presence and absence of serum



1 ml MS cell cultures (2.2 x 10^{6} cells/ml) were stimulated with Con A in medium containing 5% FCS and 5 x 10^{-5} 2-ME. After 40 hours, the cells were pooled, washed, and resuspended at 2 x 10^{6} cells/ml in medium containing 2-ME and 0% or 5% FCS. 2 µCi/ml ^{3}H-dsRNA (310 µCi/mg) was added at 41 hours, and at intervals thereafter incorporation into the cellular TCA-insoluble material was measured.

Table 18 - The incorporation of various ^{3}H-RNAs by stimulated MS cells in serum-free culture medium

		Experiment I		Experiment II		
		^{3}H-poly C[1]	^{3}H-dsRNA[2]	^{3}H-ssRNA[3]	^{3}H-ssRNA[4]	^{3}H-dsRNA[2]
^{3}H-c.p.m. per 10^{6} cells	Medium	8,070 (910)	171,400 (191,100)	70,250 (31,040)	22,970 (8,730)	345,100 (349,600)
	Cells	2,570 (2,740)	5,470 (6,460)	1,730 (1,581)	1,750 (1,470)	1,240 (1,690)
	Nucleus	750 (820)	1,690 (1,970)	870 (1,090)	980 (1,020)	1,210 (1,470)
	Cytoplasm	360 (300)	390 (410)	430 (100)	410 (180)	60 (160)
	Nuclear wash	70 (70)	70 (90)	0 (27)	0 (17)	0 (38)
% of label in nucleus		63	79	67	70	90
% of nuclear label as DNA		42	0	55	48	0

For Legend see next page

Legend to Table 18

1 ml MS cell cultures were set up at 1.6 x 10^6/ml (Experiment I) or 1.9 x 10^6/ml (Experiment II) in medium containing 5% FCS, 4 µg/ml Con A and 5 x 10^{-5}M 2-ME. After 30 (I) or 40 (II) hours, cells were pooled, washed and resuspended in serum-free medium with 2-ME at 1.8 (I) or 2.1 (II) x 10^6 cells/ml. ^{3}H-RNAs (see below) were then added for 24 hours, after which incorporation and distribution was measured as previously described. TCA-insoluble c.p.m. are shown in parentheses under total c.p.m. For NaOH hydrolysis, all nuclei samples contained 300-850 c.p.m., while background c.p.m. after control ^{3}H-RNA hydrolysis was 45 c.p.m. All ^{3}H-RNAs incorporated were initially 100% TCA-precipitable. Cultures were treated for 24 hours with:

1. ^{3}H-poly C; 0.2 µCi/ml, 25 µCi/mg.
2. ^{3}H-dsRNA; 1.0 µCi/ml, 310 µCi/mg.
3. ^{3}H-ssRNA; 0.5 µCi/ml, 7 µCi/mg*.
4. ^{3}H-ssRNA; 0.15 µCi/ml, 5.5 µCi/mg*.

* 3 and 4 were prepared from the cytoplasm and nuclei, respectively, of ^{3}H-adenosine-labelled MS cells.

Notes. In experiment I, cells maintained good viability from 30-54 hours, and an increase in cell number occurred. ^{3}H-Tdr was efficiently incorporated from 52-54 hours in parallel cultures treated with unlabelled dsRNA or poly C.

I

cold RNA (30-54 hrs)	Cell no. at 54 hrs.	^{3}H-Tdr uptake/10^6 cells
-	1.84 x 10^6/ml	38,550
poly C, 8 µg/ml	1.92 x 10^6/ml	33,890
dsRNA, 10 µg/ml	1.96 x 10^6/ml	35,280

However, in experiment II, a net decrease in cell number occurred from 40-64 hours. No estimates of ^{3}H-Tdr incorporation at 64 hours were made.

II

^{3}H-RNA (40-64 hrs)	Cell no. at 64 hrs
dsRNA	1.51 x 10^6/ml
ssRNA^3	1.52 x 10^6/ml
ssRNA^4	1.65 x 10^6/ml

TCA-soluble material in the medium and cell cytoplasm, 60-70% of the incorporated label became nucleus-associated, and the nuclear label became converted to DNA. In contrast, ^{3}H-dsRNA was again associated preferentially with the nucleus, and no conversion of the incorporated label to DNA occurred.

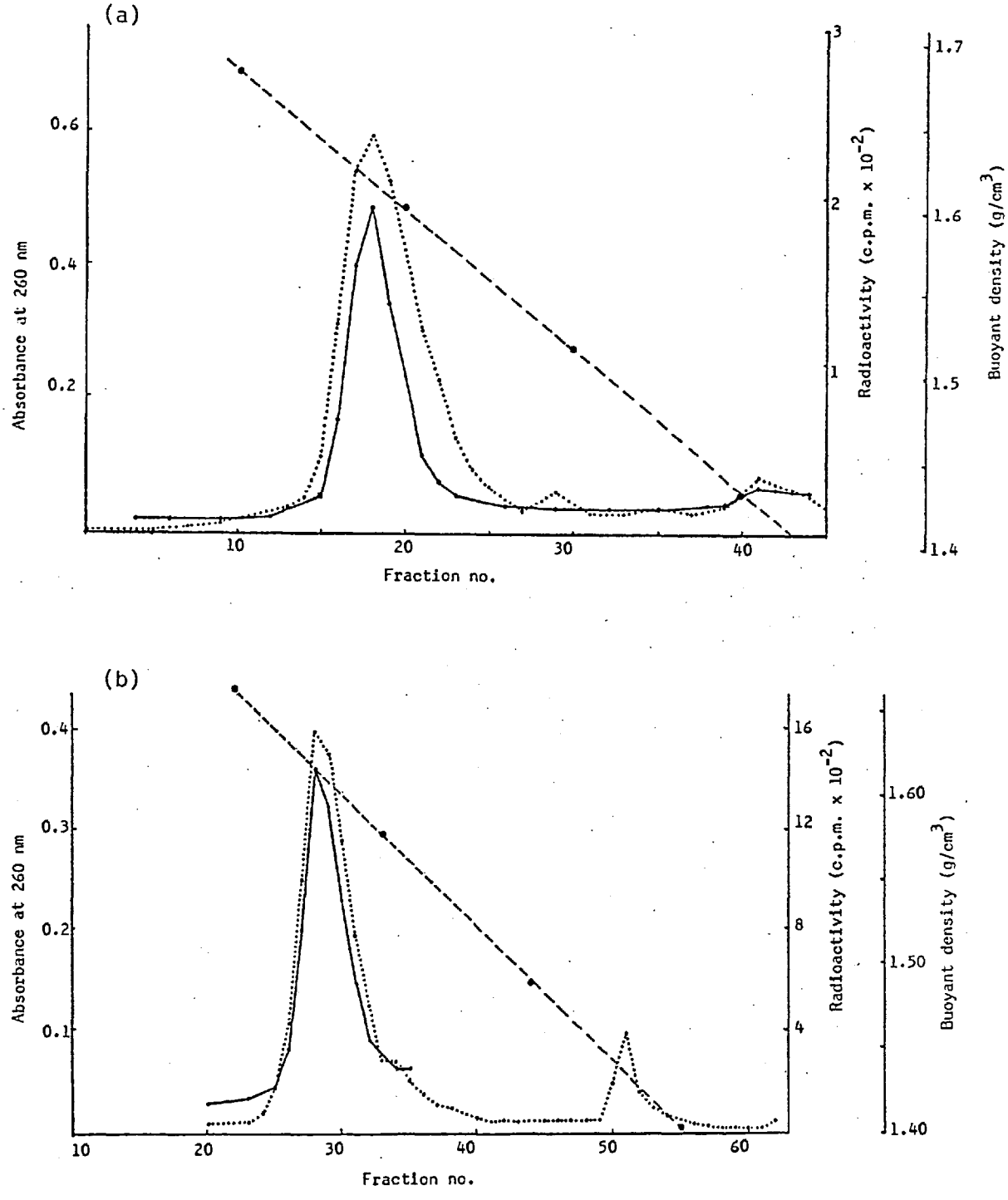
Parallel measurements of ^{3}H-thymidine uptake in cultures incubated with unlabelled dsRNA or poly C (see legend to Table 18) indicated that cells treated with both RNAs were synthesising DNA normally. Therefore the reason for the lack of conversion of ^{3}H-dsRNA, and not ^{3}H-ssRNA, to DNA must have been the inability of the MS cells to metabolise ^{3}H-dsRNA. Conversion of label from exogenously-supplied ^{3}H-RNA to DNA always correlated with TCA-solubilisation of the ^{3}H-RNA remaining in the medium and in the cytoplasm.

.7 Nature of RNA in MS cells labelled with ^{3}H-dsRNA in serum-free medium

After labelling MS cells for 24 hours with ^{3}H-dsRNA in serum-free medium, total nucleic acids were extracted from the nuclear fraction of the cells by the modified Marmur procedure and analysed by CS density gradient centrifugation. The distribution of label in these gradients is shown in Figure 16(a) and (b), representing two typical experiments. In these two experiments, incorporation of ^{3}H-dsRNA per 10^{6} cells was quantitatively the same (3,900 c.p.m./10^{6} cells), and no loss in cell number occurred during the labelling period. The intracellular distribution of label is summarised in Table 19.

In the first experiment [Figure 16(a), and Table 19(a)] almost all of the nuclear label was intact ^{3}H-dsRNA with a buoyant density of 1.61. Only a trace of label was found at the DNA position on the gradient; this was not detectable by NaOH hydrolysis of the nuclei. In the second experiment [Figure 16(b) and Table 19(b)], a slightly larger peak of labelled DNA was present, although again most of the label was intact ^{3}H-dsRNA. Some solubilisation of ^{3}H-dsRNA took place in the cell cytoplasm in the second, but not the first, experiment. It is reasonable to speculate that this solubilisation resulted in the availability of a small pool of low molecular weight labelled degradation products for MS cell DNA synthesis. The apparent low level of cytoplasmic

Figure 16 - CS density gradient centrifugation of the nuclear extract from MS cells labelled with ^{3}H-dsRNA in the absence of serum.



1 ml MS cell cultures, resuspended to (a) 2 x 10^6 cells/ml and (b) 3.3 x 10^6 cells/ml, were labelled with ^{3}H-dsRNA for 24 hours in serum-free medium as described for Table 19 . Marmur-extracted nucleic acid from the isolated nuclei were analysed by CS density gradient centrifugation, with unlabelled dsRNA as A_{260} marker.

—— absorbance at 260 nm; ····· radioactivity;

•– – –• buoyant density

Table 19 - The incorporation of ^{3}H-dsRNA by MS cells in the absence of serum.

		Expt. (a)	Expt. (b)
^{3}H-c.p.m. per 10^{6} cells	Cells	3,940 (3,920)	3,920 (4,360)
	Nucleus	2,640 (2,900)	2,440 (2,670)
	Cytoplasm	510 (670)	490 (370)
	Nuclear wash	190 (230)	50 (50)
% of label in nucleus		79	82

1 ml MS cell cultures were initiated in medium containing 5% FCS, 4 µg/ml Con A and 5×10^{-5}M 2-ME. After 40 hours, the cells were washed and resuspended in serum-free medium with 2-ME at 2×10^{6} cells/ml [Expt. (a)] or at 3.3×10^{6} cells/ml [Expt. (b)]. The cells were then labelled for 24 hours with 2 µCi/ml ^{3}H-dsRNA (310 µCi/mg), and the intracellular distribution of label measured. TCA-insoluble radioactivity is shown in parentheses. The nucleic acids extracted from the nuclear fractions were subjected to CS density gradient centrifugation (see Figures 16 (a) and (b)).

^{3}H-dsRNA degradation observed in the second experiment, but not in earlier ones, may have been due to the fact that on this occasion the cell suspensions contained a particularly high number of cells during the labelling period, providing a higher concentration of cellular endonuclease per culture.

The molecular weight of ^{3}H-dsRNA in the nuclei of MS cells labelled in the absence of serum was measured by sucrose density gradient centrifugation. As shown in Figure 17, the molecular weight fell from 2.2×10^{6} (original) to a mean size of 1.4×10^{6}. ^{3}H-dsRNA therefore seems to survive in the nuclei of transforming MS cells at a high molecular weight and in double-stranded form (hydroxylapatite chromatographic analysis of the nucleus-associated label showed it to be 87-92% double-stranded in separate experiments).

The ^{3}H-dsRNA remaining in the serum-free culture medium at the end of the labelling period was virtually intact (99.4% dsRNA, see Table 17). Further analysis of this material by sucrose density gradient centrifugation gave the profile shown in Figure 18. A slight drop in molecular weight occurred, to a size larger than that determined for the nucleus-associated label. The value of 1.7×10^{6} calculated from this gradient for the major labelled species may have been a slight underestimate since a value of only 1.90×10^{6} was calculated for the unlabelled dsRNA marker.

The sharpness of this peak on the sucrose gradient implied that a rather specific cleavage of the input ^{3}H-dsRNA occurred, during which 8% of the molecule was removed, either in a single break or by controlled exonuclease activity. There was no evidence from the gradient of a shorter fragment, or of other labelled low molecular weight products. It may be that such products were taken up preferentially by the cells. The following observations suggested that MS cells in serum-free medium did produce an enzyme capable of bringing about this limited cleavage of ^{3}H-dsRNA. When the cells were cultured without dsRNA for 24 hours in serum-free medium, this "conditioned" medium was collected and found to be capable of effecting the same small change in size of ^{3}H-dsRNA, while control medium that had not been in contact with cells was inactive. This change was temperature-dependent; no decrease in molecular weight occurred at 0°C. The cells themselves, when lysed by sonication, were also able to bring about the same reduction in size of ^{3}H-dsRNA. These results are presented in Figure 19(a) to (d).

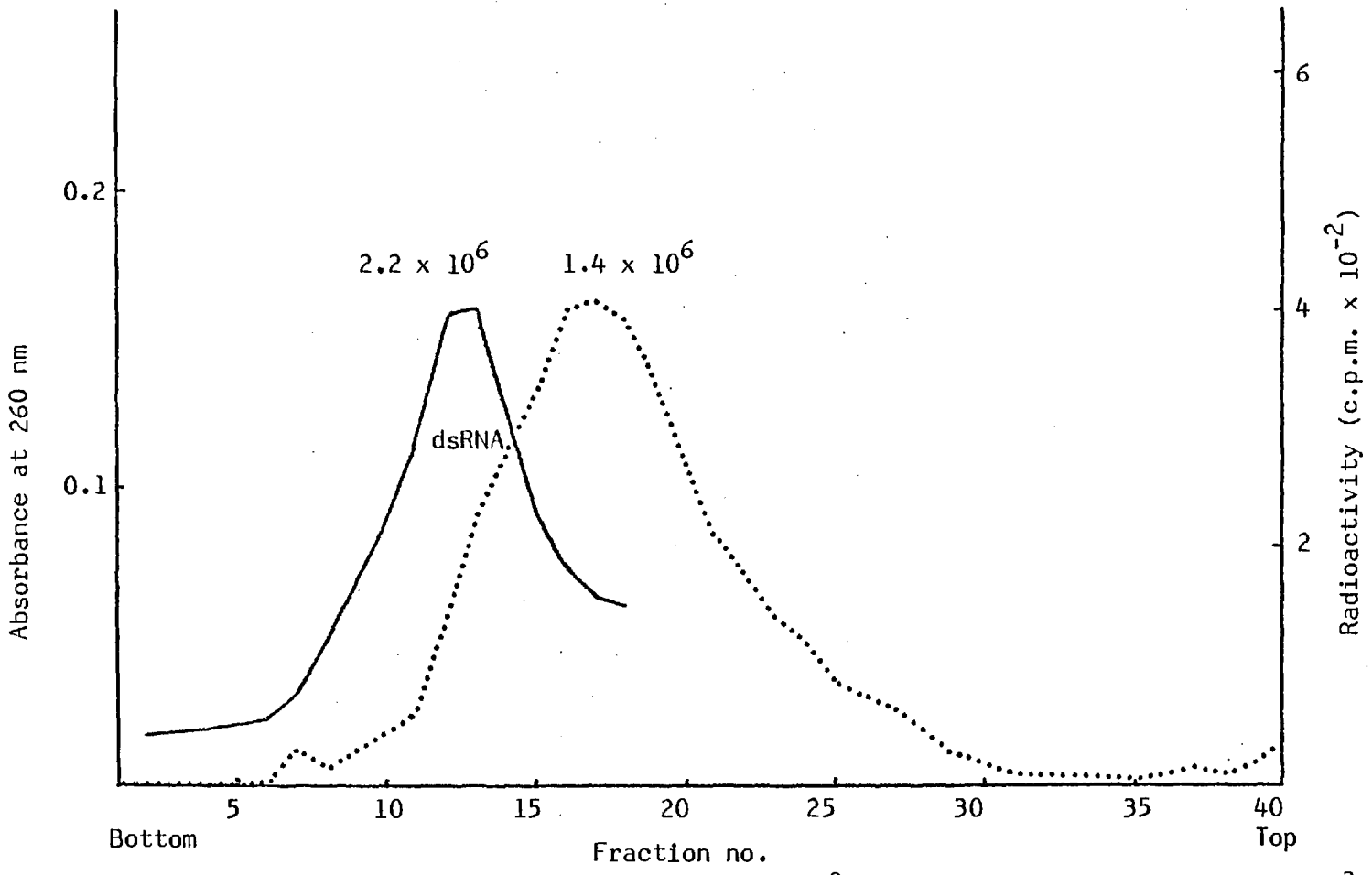


Figure 17 - Sucrose density gradient centrifugation of the nuclear ^{3}H-dsRNA in MS cells labelled with ^{3}H-dsRNA in the absence of serum

The nuclei from MS cells labelled with ^{3}H-dsRNA for 24 hours in serum-free medium were lysed in SSC-EDTA-sarkosyl, heated at 60°C for 10 mins, then cooled before direct analysis by sucrose density gradient centrifugation in the presence of unlabelled dsRNA as A_{260} marker.

——— absorbance at 260 nm; ····· radioactivity

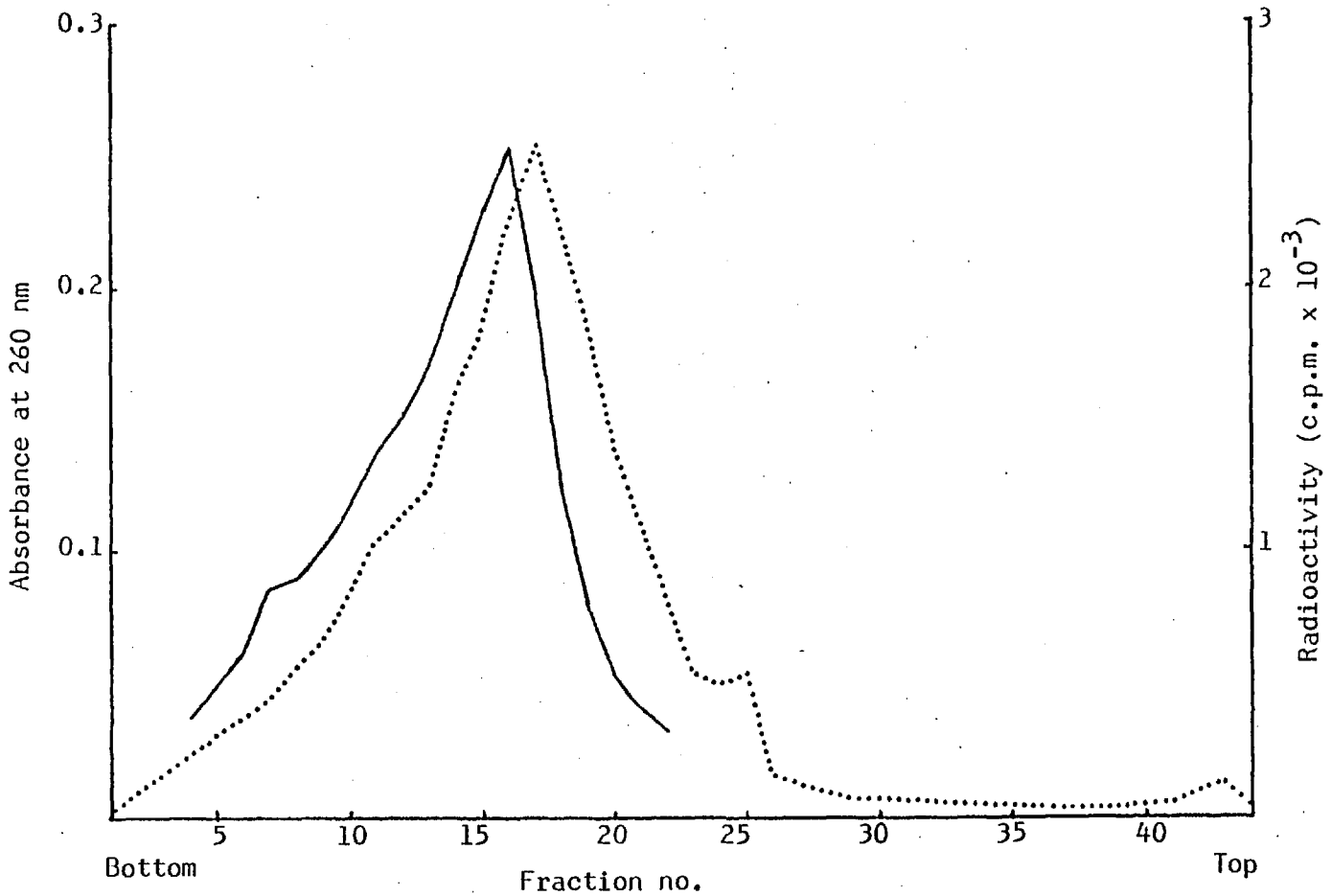
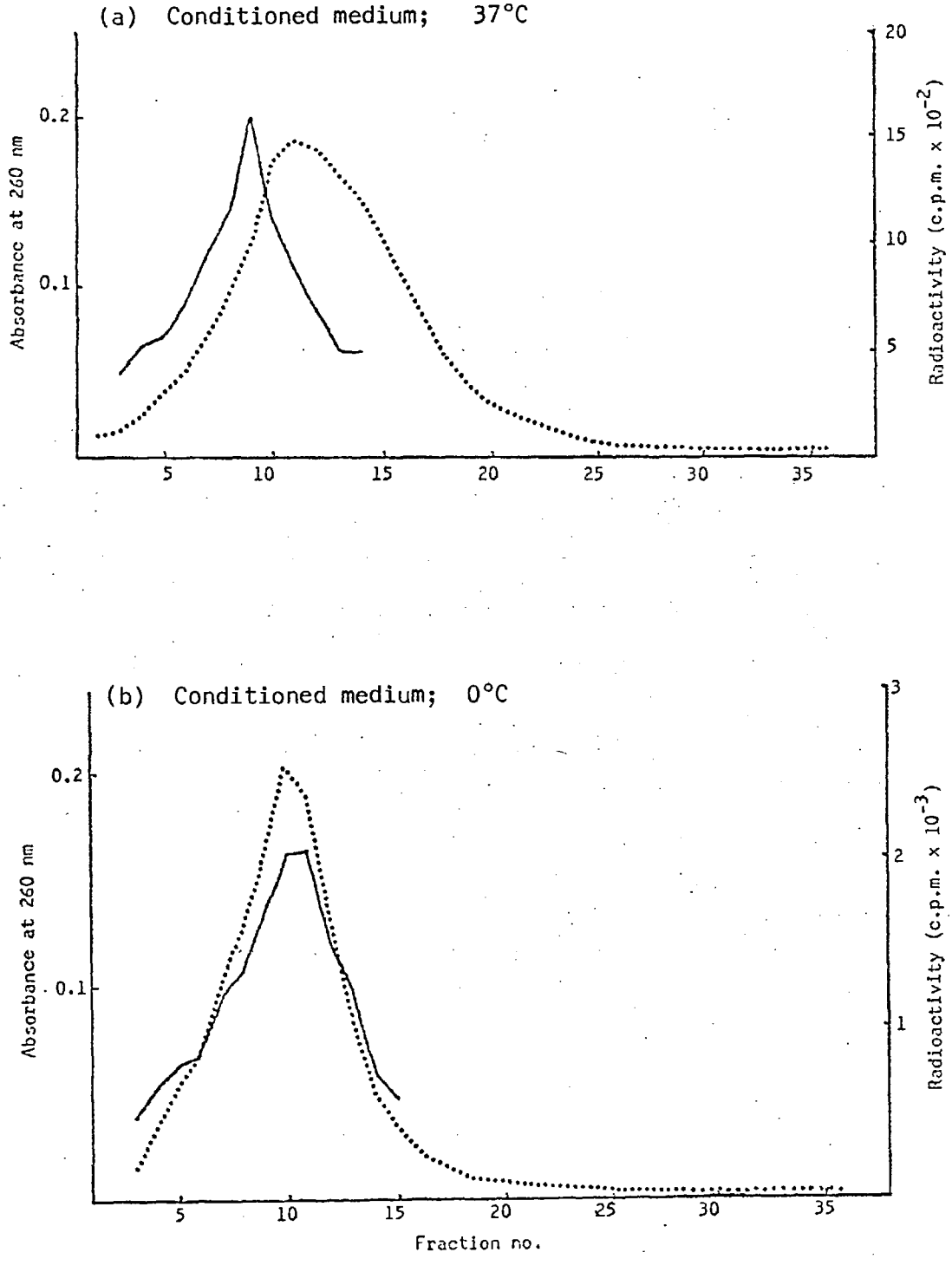


Figure 18 - Sucrose density gradient centrifugation of the ^{3}H-dsRNA remaining in the serum-free culture medium

MS cells were labelled with 2 µCi/ml ^{3}H-dsRNA (310 µCi/mg) for 24 hours in serum-free medium. At the end of the labelling period the ^{3}H-RNA remaining in the culture medium was analysed by sucrose gradient centrifugation in the presence of unlabelled dsRNA as A_{260} marker.

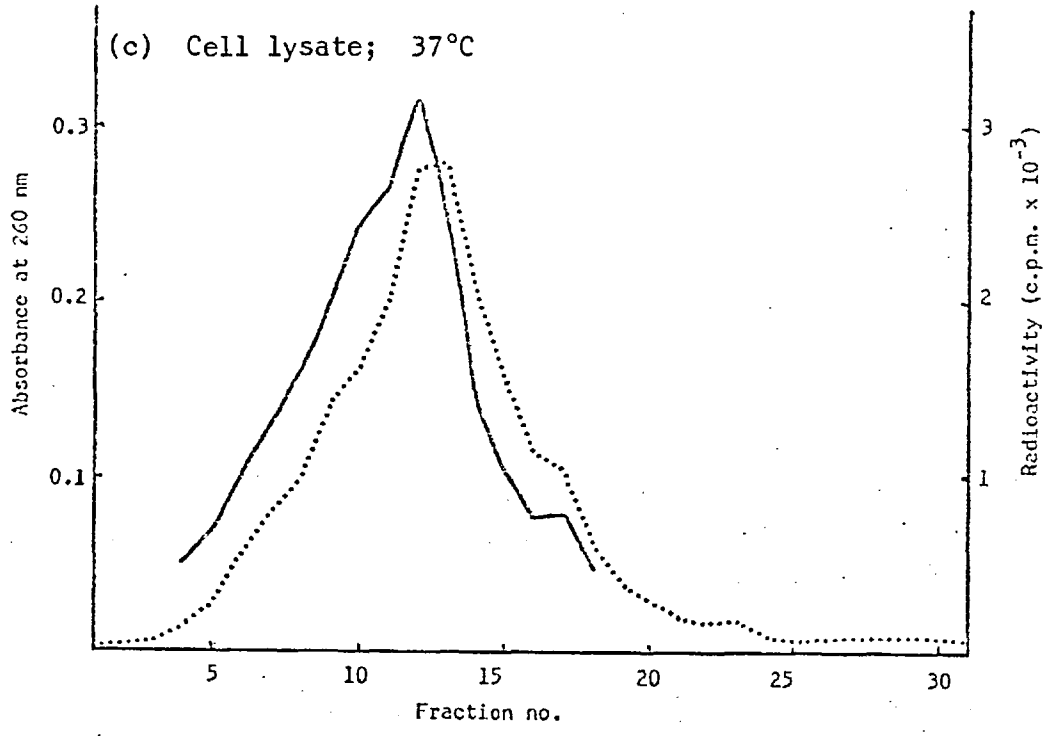
——— absorbance at 260 nm; ····· radioactivity

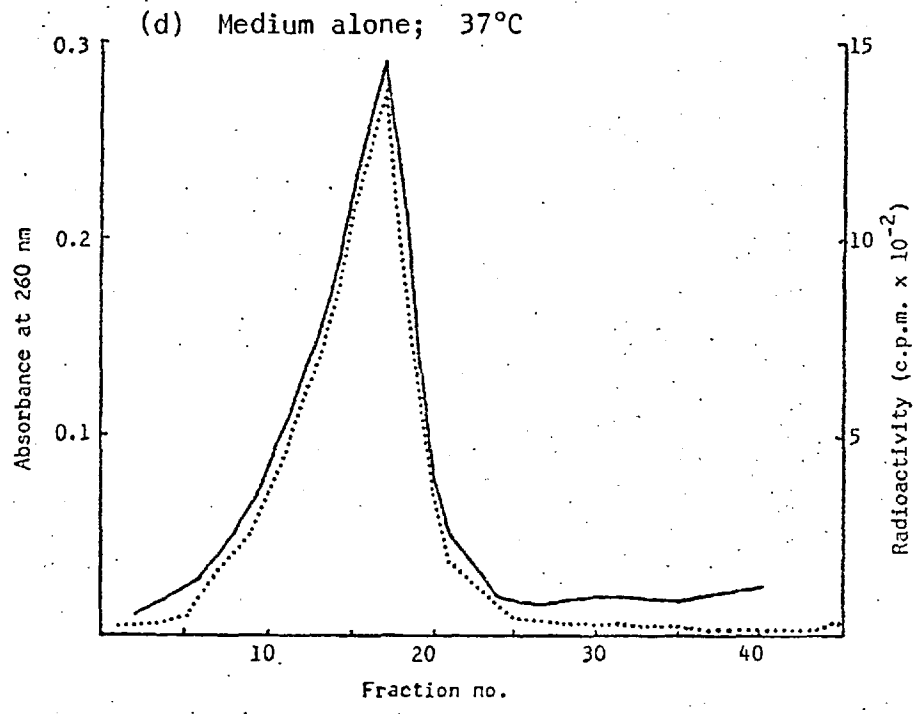
Figure 19 - The effect of MS cell lysate and conditioned medium on the molecular weight of dsRNA.



Cont/.....

Figure 19 (cont.)





30 hours after the initiation of transformation, MS cells were washed, resuspended at a concentration of 2 x 10^6 cells/ml in serum-free medium, and incubated for a further 24 hours. The conditioned medium from these cultures was collected after centrifugation of the cells, while the cells were resuspended in fresh medium and lysed by sonication for 10 secs. ^{3}H-dsRNA (310 µCi/mg) was incubated at 1 µCi/ml for 24h in (a) conditioned medium at 37°C, (b) conditioned medium at 0°C, (c) cell lysate at 37°C, (d) fresh serum-free medium at 37°C. The products were analysed by sucrose density gradient centrifugation.

—— A_{260} of dsRNA marker; ····· radioactivity.

The shift to a slightly lower molecular weight position on sucrose gradients was not therefore due simply to a temperature-induced conformational change in the dsRNA, but depended on the presence of a cell-derived product. This may be a ribonuclease which has limited activity against dsRNA, perhaps only at one or two susceptible points in the helix. Such an enzyme would account for the greater degree of solubilisation of ^{3}H-poly C and ^{3}H-ssRNA compared with ^{3}H-dsRNA, in the culture medium of MS cells labelled in the absence of serum.

.8 Distinction between membrane binding and cellular incorporation

It was important to know whether the apparent low level association of high molecular weight, double-stranded ^{3}H-dsRNA with the nuclei was real, or resulted from binding of ^{3}H-dsRNA to membrane components which centrifuged with the nuclei after cell lysis. This problem was approached first by investigating the uptake of ^{3}H-dsRNA at 0°C (binding only) or 37°C (binding and incorporation), secondly by autoradiographic analysis of labelled cultures, and thirdly by measuring ^{3}H-dsRNA uptake by non-viable cells.

(i) Uptake of ^{3}H-dsRNA at 0°C and 37°C

As a preliminary control, the non-specific association of ^{3}H-dsRNA with cells or cell fractions was measured. MS cells labelled for 24 hours with 1 µCi/ml ^{3}H-dsRNA were fractionated and analysed for radioactivity as normal, while unlabelled cultures were fractionated immediately after the addition of 1 µCi/ml ^{3}H-dsRNA at 0°C. As concluded from the data in Table 20, only 0.2% of the input ^{3}H-dsRNA centrifuged with the cells at 0°C, 46% of which subsequently centrifuged with the nuclei. In contrast, 3.2% of the input ^{3}H-dsRNA was incorporated by the same number of cells at 37°C, and 79% was nucleus-associated. Therefore, approximately 6% of the 3.2% incorporated by the cells at 37°C could be accounted for by loosely associated label inadequately removed by washing.

The amount of ^{3}H-dsRNA becoming cell-bound during a 24 hour incubation at 0°C was considerably larger (see Table 21). After measuring the radioactivity in the cells at the end of the labelling period, they were then washed again in a large molar excess of unlabelled dsRNA, in order to displace ^{3}H-dsRNA loosely or reversibly bound to the cell membranes. The results showed that binding at 0°C

Table 20 - Association of ^{3}H-dsRNA with MS cells during cell fractionation.

		^{3}H-dsRNA (1 µCi/ml) addition:	
		30-54 hrs; 37°C	54 hrs; 0°C
TCA-insoluble ^{3}H-c.p.m. per 10^{6} cells	Medium	191,100	221,900
	Cells	6,460	490
	Nucleus	1,970	110
	Cytoplasm	390	70

MS cell cultures were labelled with ^{3}H-dsRNA for 24 hours as described for Experiment 1, Table 18 . Immediately before fractionation, parallel unlabelled cultures were treated with ^{3}H-dsRNA at 0°C. Cells were fractionated as previously described, and the intracellular distribution of label measured.

Table 21 - Uptake of ^{3}H-dsRNA by viable and heat-killed MS cells at 0°C and 37°C in the presence and absence of serum.

Cells	5% FCS	Temp. °C	^{3}H-c.p.m./10^{6} cells	
			Unwashed cells	dsRNA-washed cells
Control	-	0	1,670	920
"	-	37	3,160	2,370
"	+	0	1,130	720
"	+	37	26,400	24,490
Heat-killed	-	0	2,030	1,140
"	-	37	30,650	18,020
"	+	0	11,540	7,580
"	+	37	50,700	36,550

Con A-stimulated MS cells in 1 ml cultures were washed and resuspended in medium with 0% or 5% FCS after 40 hours of cultures. Cells were heat-killed by treatment at 60°C for 40 min, while control cultures were incubated at 37°C for 40 min. The cultures were then adjusted to 37°C or 0°C before addition of 2 µCi/ml ^{3}H-dsRNA (310 µCi/mg). After a 24 h labelling period, duplicate cultures were pooled, washed and resuspended for radioactivity and cell number measurements. The cells were washed again in 1 ml medium containing 100 µg/ml unlabelled dsRNA before re-measurement of cell-associated radioactivity.

was quantitatively similar in the presence and absence of serum, and that 35-45% of this was displaced by cold dsRNA, assuming no further cell loss during washing. In serum-free medium, cells took up nearly twice as much ^{3}H-dsRNA at 37°C than at 0°C, but only 25% of this was displaceable.

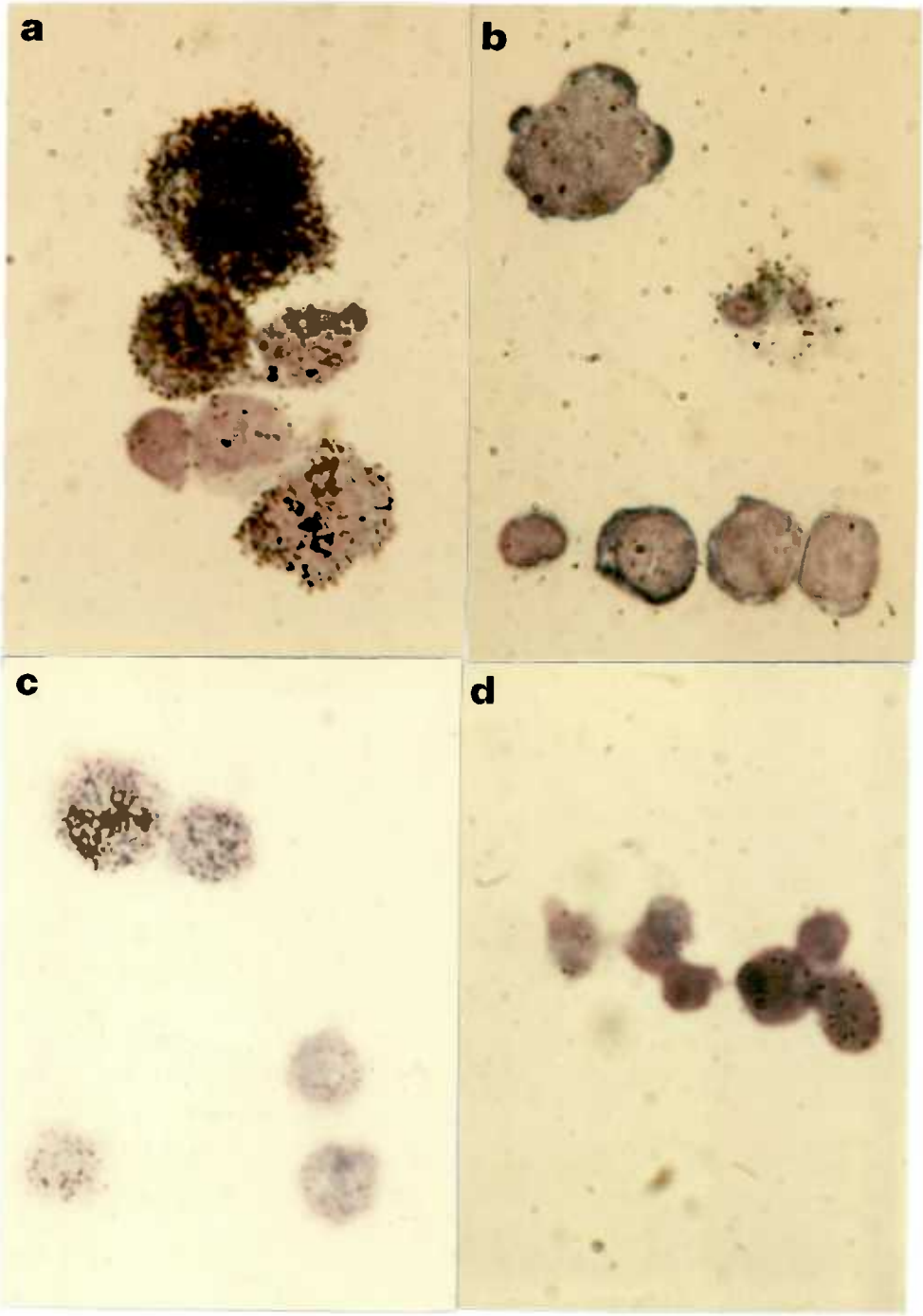
Pancreatic ribonuclease has been used by others (Bausek and Merigan, 1969) to remove membrane-bound ^{3}H-RNA from labelled cells. However, its poor activity against dsRNAs in physiological ionic strength media would tend to preclude the use of RNase for this purpose. Indeed, MS cells labelled with ^{3}H-dsRNA in serum-free medium lost no label after a further incubation with 100 µg/ml RNase I for 30 min at 37°C (data not shown).

The results shown in Table 21 indicate that 75% of the ^{3}H-dsRNA taken up by MS cells in serum-free medium is irreversibly cell-associated (not removable by washing with unlabelled dsRNA), and that approximately 60% of this results from metabolic uptake (i.e. incorporation at 37°C). These observations were highly relevant when considering the autoradiographic analysis.

(ii) Autoradiography

Con A-stimulated MS cells labelled from 40-64 hours of transformation with ^{3}H-dsRNA in the presence or absence of serum were washed twice in medium and prepared for autoradiography as described in Materials and Methods. Cells cultured in the presence of serum were generally well labelled with a high labelling index. Medium and large transformed lymphocytes contained the most grains, but labelling throughout the culture as a whole was rather heterogeneous. Small non-transformed lymphocytes were mainly unlabelled, or very lightly so. A typical field is shown in Figure 20(a). The cells appear blurred because of the different planes of focus of the cells on the glass and the grains in the photographic emulsion. Both nucleus and cytoplasm were labelled, and there was a tendency for grains to accumulate over the nucleus in well-labelled blast cells, as in ^{3}H-adenosine-labelled cells [Figure 20(c)]. This pattern of labelling confirms the biochemical data showing breakdown and re-utilisation of ^{3}H-dsRNA in MS cells. Autoradiography detects only labelled macromolecular material in cells, since low molecular weight species, such as ^{3}H-adenosine itself, are lost during the fixation procedure.

Figure 20 - Autoradiographs of labelled MS cells



(a) Con A-stimulated MS cells labelled for 24h with 2 µCi/ml ^{3}H-dsRNA (310 µCi/mg) in medium containing 5% FCS. (21 day exposure, 1300-fold magnification).

(b) As (a), except the cells were labelled in serum-free medium.

(c) Con A-stimulated MS cells labelled for 6h with 2 µCi/ml ^{3}H-adenosine (400 mCi/mmol) in medium containing 5% FCS. (21 day exposure, 520-fold magnification).

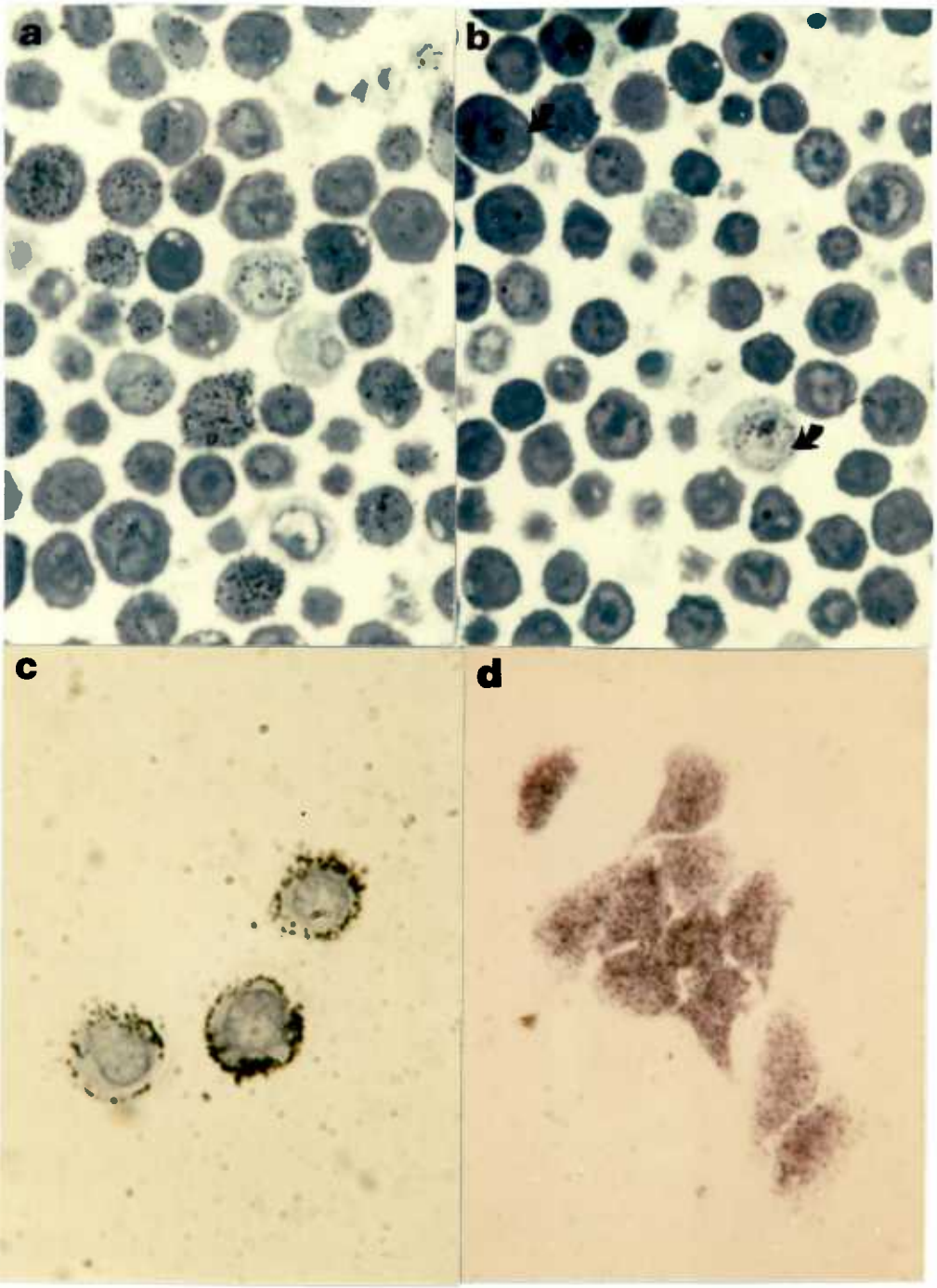
(d) Isolated nuclei from Con A-stimulated MS cells labelled as in (b).

Cells incubated with ^{3}H-dsRNA in the absence of serum were very lightly labelled in autoradiographs, with a low labelling index. Large blast cells and medium-sized lymphocytes were labelled, but with fewer grains per cell than those cultured in serum-supplemented medium [see Figure 20(b)]. Labelling was again heterogeneous; small, non-transformed lymphocytes were unlabelled. However, more careful analysis showed that approximately 60-70% of the grains were associated with medium and large cells that were apparently dead, that is, they stained very poorly with Giemsa and contained pyknotic nuclei or no nuclei at all. There was not, as the biochemical data would have predicted, a major localisation of the grains in the nuclei of labelled cells. The distribution of grains on individual cells was random and occasionally patchy. These findings raised the possibility that the nuclear fraction prepared in NP40-lysis buffer and subjected to CS density gradient centrifugation contained dead cells or cell debris associated with intact ^{3}H-dsRNA.

Autoradiographs of isolated nuclei showed that a large proportion of the grains were indeed associated with unlysed, apparently non-viable cells and cell debris. However, genuine nuclear labelling was also present, as exemplified in Figure 20(d). Because of the heterogeneous nature of these cultures, it was difficult to establish quantitatively how much of the nucleus-associated ^{3}H-dsRNA was actually incorporated into the nuclei, and how much was ^{3}H-dsRNA associated with dead cells. Although approximately 50% of the cellular uptake in serum-free medium was due to membrane binding (Table 21), all of the ^{3}H-dsRNA extracted from the nuclear fraction of these cells was shown to be intact ^{3}H-dsRNA by density gradient analysis. Therefore, it is reasonable to conclude that 50%, at least, of the observed ^{3}H-dsRNA uptake reflected genuine metabolic incorporation by transformed cells, and that ^{3}H-dsRNA can persist in a high molecular weight form in these cells for up to 24 hours.

Autoradiographs of cell pellet sections were prepared from MS cells labelled for 24 hours in the presence and absence of serum, with the aim of distinguishing intracellular grains from those localised on the membrane, that is, to address the question of whether the grains seen in drop preparation autoradiographs were "in" or "on" the lymphocytes. Figure 21(a) shows part of a section from cells labelled with ^{3}H-dsRNA in 5% FCS; the cells were

Figure 21 - Autoradiographs of ^{3}H-dsRNA-labelled MS cells and V79 fibroblasts



(a) Cell pellet section of Con A-stimulated MS cells labelled for 24h with 2 µCi/ml ^{3}H-dsRNA (310 µCi/mg) in medium containing 5% FCS. (21 day exposure, 520-fold magnification).

(b) As (a), except the cells were labelled in serum-free medium.

(c) Heat-killed MS cells, labelled with ^{3}H-dsRNA as in (a). (42 day exposure, 520-fold magnification).

(d) V79 fibroblasts labelled for 24h with ^{3}H-dsRNA (310 µCi/mg) in serum-free medium. (21 day exposure, 520-fold magnification).

heterogeneously but highly labelled, showing clear intracellular grains. Sections from cells labelled in serum-free medium [see Figure 21(b)] were only lightly labelled and photographed poorly. Large and medium-sized cells were lightly labelled (arrowed), and again the grains were mainly intracellular, as in the lightly-staining blast cell to the right-of-centre in photograph 21(b). These sections support the conclusion that ^{3}H-dsRNA is incorporated by MS cells in serum-free medium, that is, under conditions where the material retains macromolecular integrity and therefore biological activity.

(iii) Uptake of ^{3}H-dsRNA by non-viable MS cells

Following the observation that many of the more heavily-labelled cells in serum-free culture appeared to be non-viable, the uptake of ^{3}H-dsRNA by heat-killed cells was measured, and autoradiographs of the labelled cells prepared. Cells were killed by heat-treatment at 60°C for 40 min, after which they were non-viable as judged by the eosin exclusion test. As shown in Table 21, cellular uptake of ^{3}H-dsRNA was dramatically increased compared with that in control cultures under all conditions, particularly in the absence of serum at 37°C. Up to 44% of the cell-associated label was displaced by unlabelled dsRNA.

In autoradiographs, the distribution of label in heat-killed cells was very different from controls. Most of the cells were labelled, with all of the grains localised peripherally on the cell membranes. Transformed cells were very heavily labelled indeed [Figure 21 (c)]. These labelled, heat-killed cells did not lyse satisfactorily in the usual cell lysis buffer, and nuclear preparations contained mainly unlysed cells, again with solely peripheral labelling, while isolated nuclei were unlabelled.

Therefore, although dead cells took up considerably more ^{3}H-dsRNA than viable cells, the cellular association was entirely different. It is likely that heat-treatment induced alterations in the membrane proteins which increased their affinity for dsRNA. Cells killed by heat-treatment thus behaved differently to those which lost viability naturally in culture, providing no useful information on the problem of whether MS cells in serum-free medium died and subsequently bound ^{3}H-dsRNA, or died primarily as a result of ^{3}H-dsRNA accumulation.

.9 Discussion

The incorporation and metabolism of ^{3}H-dsRNA by MS cells was found to be considerably facilitated by the presence of foetal calf serum in the culture medium. Under these conditions a low level of uptake of intact ^{3}H-dsRNA at early times would become progressively masked by the incorporation of degradation products. Results of the competition experiments described in the early part of this section should be regarded with caution since during these long incubations, some degradation of ^{3}H-dsRNA would have occurred. In addition, nucleoside precursors such as adenosine and uridine may themselves be degraded in cell cultures at rapid and different rates (I. Olsen, personal communication, 1979) so that competition experiments would be influenced by varying rates of availability as well as utilisation.

The lack of total inhibition of ^{3}H-dsRNA uptake by 100 µM unlabelled adenosine may therefore be the result of more rapid breakdown of the nucleoside compared to ^{3}H-dsRNA in the culture medium, or alternatively it may be due to the fact that AMP produced from exogenous adenosine does not mix freely with AMP derived from the catabolism of dsRNA. In support of the latter suggestion, Kay and Handmaker (1970) found that unlabelled uridine did not chase out ^{3}H-uridine from RNA in stimulated lymphocytes, that is, exogenous uridine did not have as ready access to the pool of nucleotides used for RNA synthesis as did those from degraded RNA. The fact that a fraction of the adenosine label from ^{3}H-dsRNA was re-utilised as guanosine during DNA synthesis, while ^{3}H-adenosine was not, likewise suggests that separate nucleotide pools exist.

The isolation of nuclei from cells labelled with exogenous nucleic acid has been widely used as an indication of intracellular penetration. However, it has been shown that this criterion can be misleading in view of the secondary, non-specific association of exogenous polynucleotide with nuclei, which may occur during cell fractionation procedures (Janik and Greko, 1976). In this particular study, the uptake of a labelled polydeoxyribonucleotide by Vero cells was examined, and nuclei were isolated by homogenisation. 50-75% of the observed nuclear association following a 2 hour labelling period could be obtained simply by fractionation of cells in the presence of label. Therefore polynucleotide released from the cell membrane during fractionation could be misinterpreted as coming from the cell interior. Non-specific association of ^{3}H-dsRNA with

MS cell nuclei during detergent fractionation was shown to account for only 6% of the observed nuclear incorporation during a 24 hour labelling period (Table 20). Therefore any loosely membrane-bound ^{3}H-dsRNA, released during cell fractionation, would have contributed only a small percentage to the nucleus-associated radioactivity. Perhaps dsRNA is adsorbed less readily than ssDNA to isolated nuclei, or less readily to NP40-prepared nuclei than to those prepared mechanically. Isolated MS cell nuclear preparations did, however, contain a significant amount of unlysed dead cells and debris, and much of the label was associated with these in autoradiographs of isolated nuclei.

SECTION III - THE EFFECTS AND FATE OF dsRNA IN ESTABLISHED CELL LINES.

1. THE EFFECT OF dsRNA ON THE REPLICATION OF ESTABLISHED CELL LINES

.1 The effect of dsRNA on the replication of V79 cells

V79 fibroblasts are maintained as monolayer cultures with a doubling time of 12-14 hours in serum-containing medium. Table 22 shows the effect of dialysed dsRNA on cell division and DNA synthesis, in the presence and absence of serum, in 1 ml petri dish cultures. ^{3}H-thymidine incorporation was measured during a 1 hour pulse at the end of the period of exposure to dsRNA, and cell numbers were estimated by haemocytometer counting.

In the presence of serum, dsRNA had no toxic effect on the replication of V79 cells; they continued to divide and synthesise DNA at the normal rate, with a doubling time of approximately 14 hours. In the absence of serum, cell division in control, untreated cultures was significantly reduced; very little further cell division occurred after the removal of serum, and DNA synthesis was inhibited. dsRNA did not cause a further reduction in cell replication in this situation. ^{3}H-thymidine incorporation was lower in dsRNA-treated than untreated cultures, but since the effect did not increase with increasing concentrations of dsRNA, it was not thought to be a true toxic effect of dsRNA. The reason for the lack of a dose-dependent effect is unknown.

The effects of dsRNA on the division and colony-forming ability of V79 cells in 10 ml cultures is summarised in Table 23. FCS at a level of 0.5% was added to one set of cultures in order to minimise dsRNA degradation in the medium, while at the same time allowing some cell division to take place. After treatment with dsRNA (dialysed) for 24 hours, there was a low level of inhibition of cell division, both in the presence and absence of serum, but only at a concentration of 100 µg/ml dsRNA. In the cultures containing 10% FCS, 100 µg/ml dsRNA apparently inhibited cell division by nearly 50%. However, in two other experiments (e.g. Tables 22, 24), there was no difference in cell yields between cultures treated with 100 µg/ml dsRNA and untreated cultures.

Table 22 - The effect of dsRNA on the replication of V79 fibroblasts in the presence and absence of serum

dsRNA conc$^{n.}$ (μg/ml)	No FCS		10% FCS	
	Cell number x 10^5 /culture	^{3}H-Tdr uptake c.p.m./10^5 cells	Cell number x 10^5 /culture	^{3}H-Tdr uptake c.p.m./10^5 cells
0	1.86	19,715	5.28	31,311
10	1.80	11,901	5.38	37,934
30	2.10	11,000	5.05	41,636
100	2.00	11,137	5.32	39,862

V79 fibroblasts were seeded at 6 x 10^4 cells/ml onto plastic petri dishes in MEM containing 10% FCS. After 24 hours, monolayers were washed with PBS and incubated for a further 20 hours with dialysed dsRNA in MEM with or without 10% FCS. Monolayers were then washed and labelled for 1 hour with 2 μCi/ml ^{3}H-Tdr in MEM + 10% FCS. The cells were washed well with PBS, removed from the dishes by trypsinisation, and duplicate cultures were pooled for measurements of ^{3}H-Tdr incorporation into DNA, and cell number estimations.

Table 23 - The effect of dsRNA on the colony-forming ability of V79 fibroblasts

0-24 hr. pretreatment		Cell number/culture ($\times 10^6$) at 24 hrs.	Number of colonies per 200 cells
dsRNA conc$^{n.}$ (μg/ml)	% FCS		
0	10	4.55	146
1		4.95	164
10		4.65	186
100		2.25	203
0	0.5	2.65	108
1		3.27	96
10		2.49	115
100		1.95	126
0	0	*	
1		1.32	92
10		1.07	98
100		0.90	110

V79 fibroblasts were grown in 10 ml cultures in glass medical flats from an initial concentration of 5×10^5 cells/culture, in MEM + 10% FCS. 18 hours after seeding, the cells were washed and incubated for a further 24 hours with dialysed dsRNA in the presence of various concentrations of FCS. The cells were then washed, removed by trypsin treatment, counted on a haemocytometer, and plated onto plastic petri dishes at 200 cells/dish in 5 ml MEM + 10% FCS. Cell colonies were fixed and counted after 5 days.

* Culture contaminated.

The colony-forming ability of V79 cells treated with dsRNA was measured after removal of the dsRNA by washing and re-culturing in medium containing 10% FCS (Table 24). The number of colony-forming cells decreased slightly as the level of serum in the pre-treatment phase was reduced, but was unaffected by previous exposure to a high concentration of dsRNA.

These results showed that dsRNA was not overtly cytotoxic to V79 fibroblasts. In serum-containing medium, cell replication, DNA synthesis and colony-forming ability were not significantly inhibited. In serum-free medium all three parameters were lower than in serum-containing medium, but no real dose-dependent, reproducible inhibitory effects of dsRNA itself were observed. These results were in agreement with those obtained in the LDV lymphoblastoid cell line, discussed below.

.2 The effect of dsRNA on the replication of LDV cells

LDV cells in 1 ml culture were unaffected by a 24 hour exposure to dialysed dsRNA, as may be seen from the results in Table 25. At concentrations up to 100 µg/ml, dsRNA had no significant effect either on the increase in cell number per culture or on the rate of ^{3}H-thymidine incorporation. The rate of ^{3}H-thymidine incorporation in these cultures was rather variable, falling within the range 55-95 x 10^{3} c.p.m./10^{6} cells, but was not altered by dsRNA in a dose-dependent manner.

Cell division was inhibited by the omission of serum from the growth medium. The long-term effect of dsRNA on LDV cell replication was examined by exposing the cells to dsRNA in serum-free medium for 24 hours, then washing out the dsRNA and re-culturing the cells in fresh growth medium containing serum. The results, shown in Table 26, revealed no cytotoxic effects during the first 24 hour exposure period. However, cells treated with 100 µg/ml dsRNA grew more slowly than control cells during the subsequent 48 hours, although they returned to a normal rate of division by day 6.

.3 Discussion

After the discovery of the dialysable impurity in dsRNA preparations, only dialysed material was examined for its effect on the growth of established cell lines. The results showed that in 1 ml cultures, dsRNA had no effect on cell replication. The removal of serum created a more serious arrest of cell division

Table 24 - The effect of 100 µg/ml dsRNA on the colony-forming ability of V79 cells

24 hour pre-treatment	Cell no./culture at 24 hours	Day 2		Day 3	
		No. of colonies per 200 cells	Average cell number per colony	No. of colonies per 200 cells	Average cell number per colony
dsRNA; 0% FCS	6.1×10^6	78	3.6	80	28
dsRNA; 0.5% FCS	5.9×10^6	120	4.1	85	35
dsRNA; 10% FCS	1.5×10^7	110	4.9	90	55
– 10% FCS	1.3×10^7	109	4.6	81	53

Cultures were initiated in 100 ml glass flats at 10^5 cells per bottle in 10 ml medium containing 10% FCS. After 64 hours, the medium was replaced with fresh medium containing 0, 0.5 or 10% FCS and 100 µg/ml dsRNA. One control culture received 10% FCS but no dsRNA. After 24 hours the cells were removed from the glass by trypsinisation, counted and plated on petri dishes, into fresh medium with 10% FCS, at 200 cells per plate. Colonies were fixed and counted after a further 2 or 3 days.

Table 25 - The effect of dsRNA on cell division and DNA synthesis in LDV cells

dsRNA conc$^{n.}$ (µg/ml)	% FCS in culture medium					
	0		1.0		10.0	
	Cell number x 10^5/ml	^{3}H-Tdr uptake c.p.m./ 10^6 cells	Cell number x 10^5/ml	^{3}H-Tdr uptake c.p.m./ 10^6 cells	Cell number x 10^5/ml	^{3}H-Tdr uptake c.p.m./ 10^6 cells
0	2.13	80,600	3.82	63,600	3.67	54,900
1	2.53	66,500	3.70	64,400	3.35	67,800
10	2.80	78,700	3.03	95,500	3.80	56,100
100	2.55	66,200	2.73	85,600	3.73	89,900

LDV cells were washed twice in serum-free medium and dispensed into 1 ml cultures at 1.53 x 10^5 cells/ml in medium containing 0,1 or 10% FCS (0 hours). The cells were incubated with dialysed dsRNA for 24 hours, after which they were washed twice and resuspended in medium containing 10% FCS. ^{3}H-Tdr was added at a concentration of 1 µCi/ml (23 Ci/mmol) from 26-28 hours, and incorporation into TCA-insoluble material was measured. The number of cells per culture was estimated microscopically at 28 hours.

Table 26 - The effect of exposure to dsRNA on the replication of LDV cells

dsRNA (µg/ml)	Cell number x 10^5/ml		
	Day 1	Day 3	Day 6
0	2.21	4.20	9.80
1	1.91	4.88	9.20
10	2.00	4.23	8.60
100	1.91	1.40	12.60
No dsRNA 10% FCS	3.40	4.38	10.50

LDV cells were washed free of serum and dispensed into 5 ml cultures at 2 x 10^5/ml in serum-free medium. The cells were incubated with dialysed dsRNA for 24 hours, after which cell numbers were determined ("Day 1"). Cultures were then washed free of dsRNA and diluted into fresh medium with 10% FCS at 6 x 10^4 cells /ml. Further cell counts were made after 48 hours ("Day 3"), at which time the cultures were diluted 2-fold into fresh medium with 10% FCS, and final cell counts were made 72 hours later ("Day 6"). Results are the average of duplicate cultures.

than treatment with a high concentration (100 µg/ml) of dsRNA. The uptake of ^{3}H-thymidine by V79 cells was apparently depressed 40% by dsRNA, but not in a dose-dependent manner, and only in serum-free medium. This was not accompanied by a fall in cell number, and is therefore unlikely to represent a true cytotoxic effect. Other workers have reported both stimulatory and inhibitory effects of poly I.poly C on ^{3}H-thymidine incorporation by cell lines, in the absence of an effect on total cell population (Levy et al, 1970; Badger, Cooperband and Green, 1972).

In the larger volume cultures, there was some evidence of a growth inhibitory effect of dsRNA at 100 µg/ml in serum-free medium. This was reversed upon further culture in serum-supplemented medium, in which any residual dsRNA would be degraded. On analysis of the results from several experiments, growth-inhibitory effects were often observed in those experiments where the cell doubling rate in control cultures was lower than normal, and where the cells were therefore in sub-optimal conditions. For example, in Table 26 there was no increase at all in cell number during the 24 hour period in serum-free medium, and growth inhibitory effects were not seen until cell division commenced. Where cell growth was optimal, there were no significant inhibitory effects of the dsRNA on this parameter in either cell line.

2. THE UPTAKE AND FATE OF ^{3}H-dsRNA IN ESTABLISHED CELL LINES

The incorporation of ^{3}H-dsRNA was found to be very similar, both quantitatively and qualitatively, in V79 and LDV cells. V79 fibroblasts were used for most of the experiments reported, because this cell line provided actively dividing cultures more reliably than the LDV line.

.1 Incorporation of ^{3}H-dsRNA in the presence and absence of serum

The cellular incorporation of ^{3}H-dsRNA by dividing V79 and LDV cells is shown in Table 27, As in MS cell cultures, incorporation was considerably greater in serum-containing medium. The lymphoid cells took up more ^{3}H-dsRNA per cell than the fibroblasts in the absence of serum, and similar levels in the presence of serum. The intracellular distribution of label incorporated by LDV cells in serum-free medium is shown in Table 28. Approximately 50% of the label was associated with the nucleus after a 24 hour labelling period. Incorporation of ^{3}H-dsRNA was

Table 27 - The incorporation of ^{3}H-dsRNA by LDV and V79 cells in the presence and absence of serum

Cell type	Serum	Incubation time (hours)	Cell no. x 10^5 per culture	^{3}H-dsRNA uptake c.p.m./ 10^5 cells
LDV	-serum	4	4.1	2,240
		24	4.0	12,080
	+serum	4	6.4	4,920
		24	3.4	43,100
V79	-serum	4	6.3	1,330
		24	5.0	4,920
	+serum	4	8.4	4,700
		24	8.4	56,740

LDV cells were dispensed into 1 ml cultures at 2.5 x 10^5 cells/ml in medium containing 0 or 10% FCS. 1 ml V79 cell cultures, in the log phase of growth, received fresh medium containing 0 or 10% FCS. All cultures were labelled with 2 µCi/ml ^{3}H-dsRNA (350 µCi/mg) for 4 or 24 hours, after which the incorporation into TCA-insoluble material was measured and cell numbers per culture were estimated.

Table 28 - The incorporation of ^{3}H-dsRNA by LDV cells in the absence of serum

Conc$^{n.}$ of ^{3}H-dsRNA (µCi/ml)	Cell no. x 10^{5} per culture	^{3}H-c.p.m. incorporated/10^{6} cells				% of label in nucleus
		Cells	Nucleus	Cytoplasm	Medium	
0.2	2.3	7,740 (7,650)	3,460 (4,600)	3,310 (4,560)	32,980 (27,780)	51
1.0	2.5	17,590 (16,550)	7,420 (8,700)	8,290 (6,640)	224,500 (206,100)	48
2.0	2.0	29,330 (27,850)	14,570 (15,600)	14,060 (8,060)	538,300 (576,200)	50

LDV cells were washed free of FCS, dispensed into 1 ml cultures at 3 x 10^{5} cells/ml in serum-free medium, and incubated with ^{3}H-dsRNA (350 µCi/mg) for 24 hours. Cells from triplicate cultures were pooled and washed, and the intracellular distribution of ^{3}H-dsRNA was analysed as described in Materials and Methods.

linear with concentration between 0.2 and 2.0 µCi/culture. At the highest level, label in the cell cytoplasm was partly degraded to TCA-soluble fragments, while the label remaining in the medium was entirely TCA-precipitable, an observation suggesting that intracellular degradation of dsRNA was taking place.

The incorporation and fate of ^{3}H-dsRNA and the precursor ^{3}H-adenosine by V79 fibroblasts in the presence and absence of serum was measured both in 1 ml petri dish cultures (Table 29) and in 10 ml glass bottle cultures (Table 30). In petri dishes the cells divided slowly, with an average doubling time of 19.5 hours, while in the larger cultures a normal doubling rate of 14 hours was observed. On removal of serum, the cells failed to divide further to any significant degree.

As the labelling time increased, ^{3}H-dsRNA accumulated in the nucleus, and the nuclear label became increasingly NaOH-resistant. Cells labelled in the absence of serum took up far more ^{3}H-dsRNA than an equivalent number of MS cells. This label became solubilised in the cytoplasm and converted to DNA in the nucleus, while the material remaining in the medium was undegraded. The data suggested that V79 cells were able to degrade and re-utilise ^{3}H-dsRNA even in the absence of serum nucleases, and this was subsequently confirmed by CS density gradient analysis.

The incorporation of ^{3}H-adenosine by V79 cells also increased as the labelling time increased, and the label became progressively associated with the nucleus. A larger fraction of the nuclear label became resistant to NaOH than in ^{3}H-dsRNA-labelled cells, as was also true for mouse lymphocytes. V79 cells apparently incorporated more ^{3}H-adenosine per 10^{6} cells in the absence than in the presence of serum. This observation was reproducible, being a result of the fact that although serum-free cultures as a whole took up less ^{3}H-adenosine than serum-containing cultures, the former contained lower cell numbers, particularly after a 24 hour period, so that the incorporation per 10^{6} cells was actually higher. This suggests that the cells labelled for 24 hours in serum-containing medium had not incorporated ^{3}H-adenosine to maximum capacity, that is, the amount of ^{3}H-adenosine was rate-limiting. Indeed, less than 10% of the input label remained in the culture medium after 24 hours. The uptake of ^{3}H-adenosine in serum-containing cultures may also have been affected by dilution of the label with cold adenosine present in the serum, by binding to

Table 29 - The incorporation of ^{3}H-dsRNA and ^{3}H-adenosine by V79 cells in 1 ml petri dish cultures

^{3}H-label	Serum	Incubation time (hours)	Cell no. x 10^{5} per culture	^{3}H-c.p.m. incorporated/10^{5} cells			% of label in nucleus	% of nuclear label as DNA
				Cells	Nucleus	Cytoplasm		
^{3}H-dsRNA 0.2 µCi/ml (350 µCi/mg)	-Serum	4	1.2	550 (470)	130 (140)	150 (110)	38	NT*
		24	1.9	2,250 (2,280)	830 (970)	500 (240)	59	NT
	+Serum	4	1.3	2,690 (2,700)	740 (800)	930 (320)	40	NT
		24	4.8	6,830 (8,000)	4,730 (4,860)	700 (500)	85	16
^{3}H-adenosine 1 µCi/ml, 5 µM	-Serum	4	1.4	48,860 (34,000)	13,800 (13,800)	20,960 (2,770)	38	42
		24	1.5	83,680 (76,600)	32,900 (34,970)	12,960 (3,760)	89	57
	+Serum	4	1.6	31,690 (27,670)	11,430 (13,570)	13,280 (1,620)	45	42
		24	3.7	38,070 (39,850)	25,549 (26,560)	3,330 (2,230)	87	44

V79 fibroblasts were seeded into 35 mm petri dishes at 1 x 10^{5} cells/ml in medium containing 10% FCS. After 20 hours, the medium was removed, and the cells washed with PBS and reincubated for 4 or 24 hours with ^{3}H-dsRNA (0.2 µCi/ml, 350 µCi/mg) or ^{3}H-adenosine (1 µCi/ml, 5 µM) in medium containing 0 or 10% FCS. At the end of the labelling period cells were harvested from the dishes, counted and analysed for incorporated radioactivity as described in Materials and Methods. Figures in parentheses are the TCA-insoluble c.p.m./10^{5} cells. % label in nucleus is calculated as a % of the total c.p.m. in (nucleus + cytoplasm + nuclear wash). % DNA was calculated from the % of nuclear label resistant to NaOH hydrolysis. Results are from pooled triplicate cultures. (All samples contained >1,700 c.p.m. before NaOH treatment and background c.p.m. after control RNA hydrolysis was 30 c.p.m. Cytoplasmic fractions contained <1% NaOH-resistant label. *N.T. - not tested; c.p.m. too low.)

Table 30 - The incorporation of ^{3}H-dsRNA and ^{3}H-adenosine by V79 cells in 10 ml glass bottle cultures

^{3}H-label	Serum	Incubation time (hours)	Cell no. x 10^{6} per culture	^{3}H-c.p.m. incorporated/10^{6} cells				% of label in nucleus	% of nuclear label as DNA
				Cells	Nucleus	Cytoplasm	Nuclear wash		
^{3}H-dsRNA 1 µCi/ml (350 µCi/mg)	-Serum	4	7.1	4,940 (4,630)	1,380 (1,600)	1,280 (730)	70 (180)	50	9
		24	1.7	32,890 (28,430)	14,610 (15,400)	9,380 (5,270)	460 (930)	59	22
	+Serum	4	7.4	23,040 (20,520)	10,020 (10,960)	7,100 (3,540)	420 (930)	57	16
		24	7.5	295,820 (285,970)	166,300 (192,680)	58,270 (46,330)	2,990 (3,170)	73	28
^{3}H-adenosine 1 µCi/ml, 5µM	-Serum	4	6.5	200,650 (148,950)	82,970 (88,080)	83,270 (18,170)	3,600 (1,490)	48	36
		24	1.0	905,270 (741,830)	385,720 (405,340)	202,900 (89,530)	9,880 (5,330)	56	61
	+Serum	4	7.5	165,720 (131,350)	81,670 (87,870)	60,820 (15,470)	2,660 (1,100)	64	42
		24	5.2	320,590 (341,600)	197,250 (232,080)	68,390 (65,780)	3,280 (3,350)	73	51

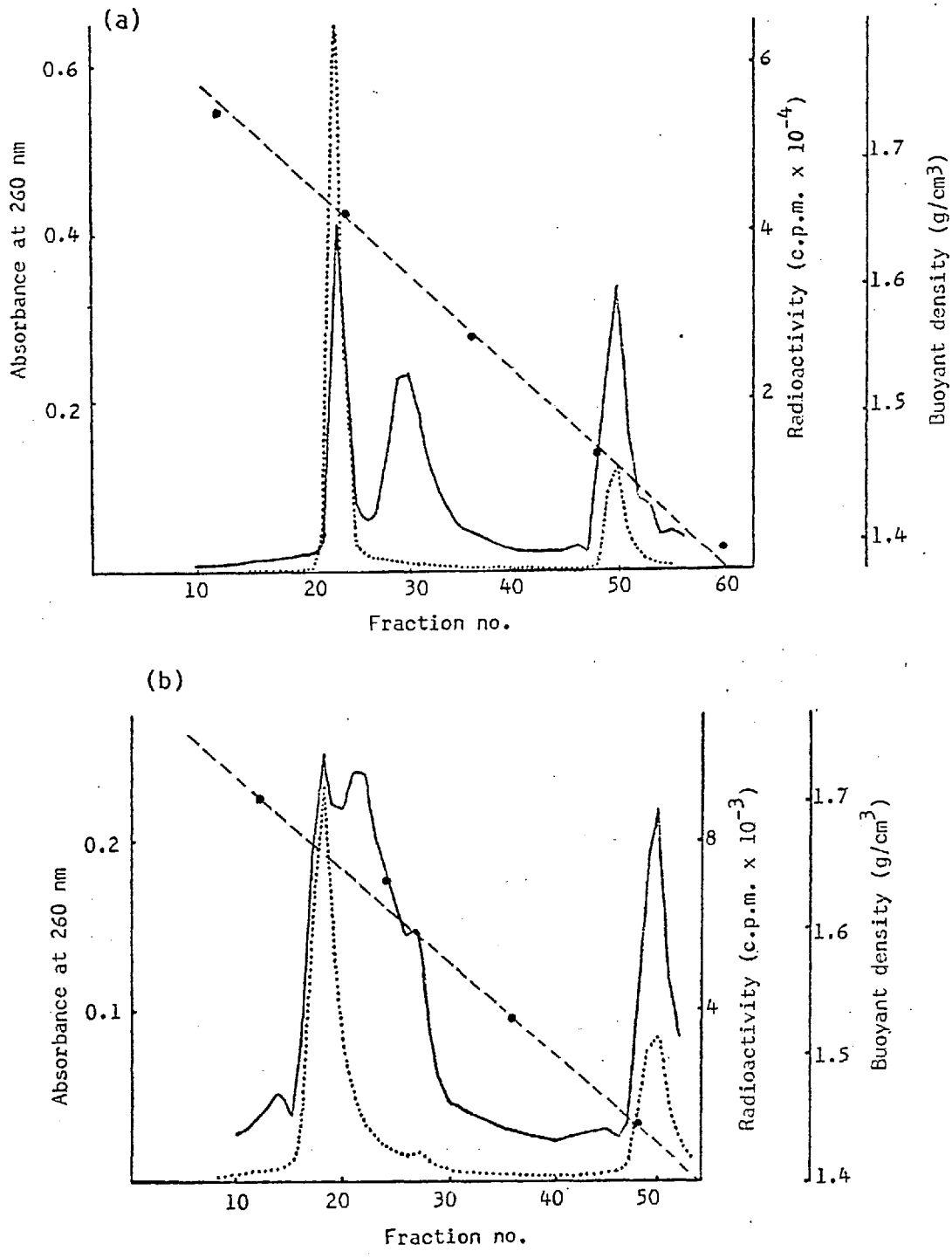
V79 fibroblasts were seeded into 100 ml medical flats in 10 mls medium containing 10% FCS at 6 x 10^{4} cells/ml. After 28 or 48 hours, the medium was removed, and the cells washed with PBS and reincubated in medium containing 0 or 10% FCS with ^{3}H-dsRNA or ^{3}H-adenosine as above. Cells were harvested at 52 hours, counted and analysed for incorporation and distribution of radioactivity, as described in Materials and Methods. Remaining details as for Table 29.

serum proteins,or by the presence of adenosine-degrading enzymes in the medium. Forsdyke (1968, 1971) showed that ^{3}H-uridine uptake by PHA-stimulated lymphocytes was inhibited in the presence of serum, due to the presence of a competing nucleoside component in the serum.

Figure 22(a) shows the distribution on a CS density gradient of the nucleic acids in the nuclei of V79 cells labelled with ^{3}H-adenosine for 4 hours. Identical profiles were obtained whether the cells were labelled in the presence or absence of serum. Unlike MS cells, most of the label was found in the sharp band of V79 RNA (at a density of 1.66-1.67) rather than in the DNA. The nuclei from these cells were known to contain approximately 40% NaOH-resistant radioactivity (Tables 29 and 30), however, the size of the labelled DNA band in the CS gradient profile did not account for 40% of the total label, for reasons that were not understood. The nuclei were lysed in sarkosyl-containing buffer and analysed directly, therefore a sample of these nuclei was also subjected to Marmur extraction and the protein-free nucleic acids analysed on a CS density gradient [Figure 22(b)]. The RNA/DNA ratio was unaltered by extraction, although the RNA band became slightly more spread in buoyant density. Total recovery of nucleic acids during extraction was 75%.

The nature of the label in V79 cell nuclei from ^{3}H-dsRNA-labelled cells is shown in Figures 23-26. Figures 23 and 24 show the nuclear label from the 1 ml cultures in the presence and absence of serum respectively. Label associated with the nuclei was gradually converted from dsRNA to cellular ssRNA and DNA. Appearance of label in DNA was stimulated by the presence of serum. Figures 25 and 26 show the distribution of label in the glass-attached cells. In these, more rapid utilisation of label occurred and there was no evidence of the presence of ^{3}H-dsRNA in the cells, even at the earlier time point. The slower conversion of ^{3}H-dsRNA to cellular nucleic acids in the 1 ml petri dish cultures as compared to the 10 ml cultures was probably related to the physiological state of the cells. In the small cultures cell growth was slower; an extended lag phase was observed after seeding the cells into fresh cultures, and even in the presence of 10% serum cell numbers had doubled only twice in 44 hours. In the larger cultures, cell division was far more rapid (doubling time 14 hours). In addition,the 4 hour labelling period was timed

Figure 22 - CS Density gradient centrifugation of nuclear label in V79 cells labelled with ^{3}H-adenosine.

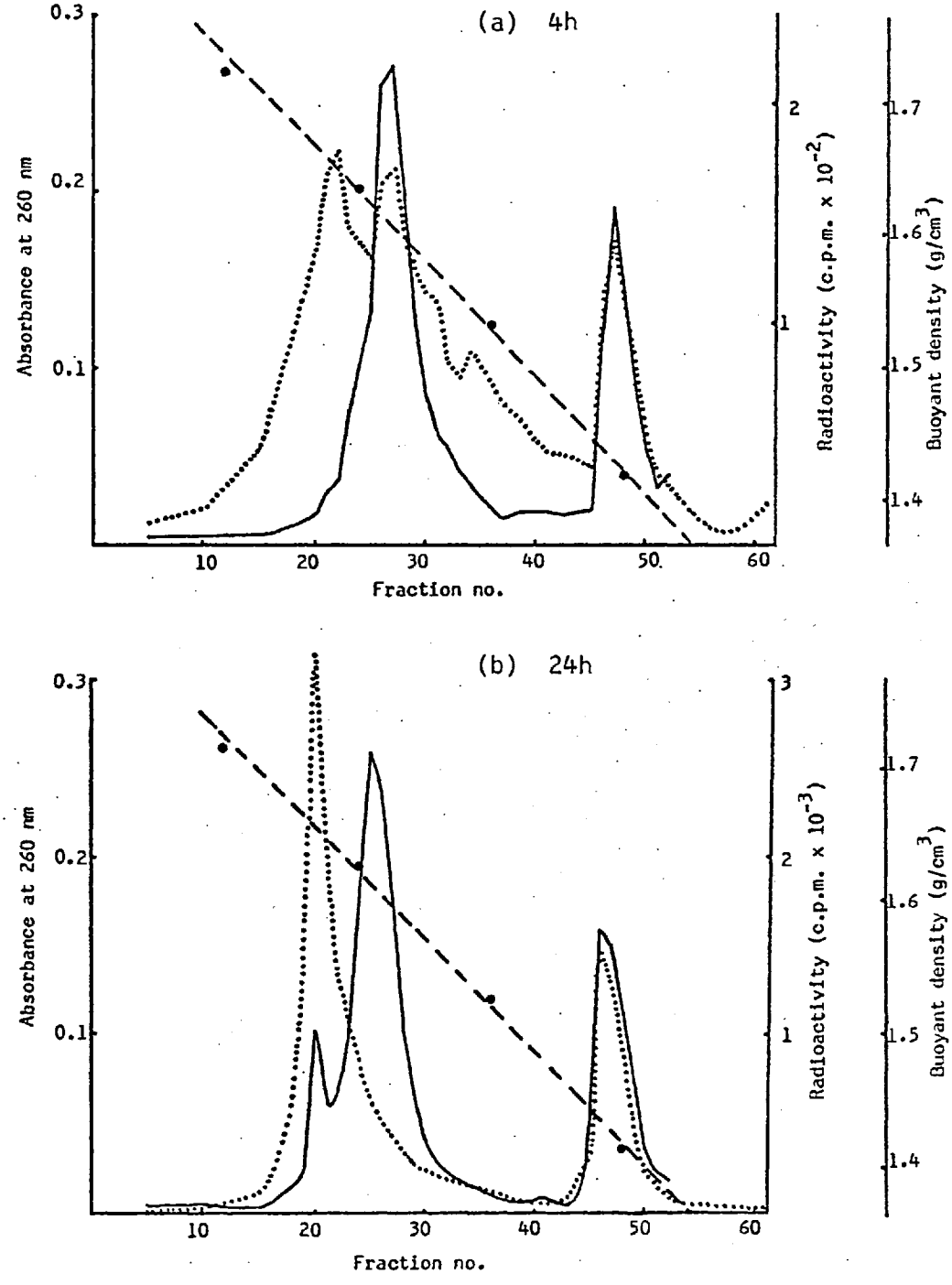


V79 cells in 10 ml culture were labelled with ^{3}H-adenosine (1 µCi/ml, 5 µM) for 4 hours in serum-free medium. The cells were washed thoroughly, removed from the culture surface by trypsinisation, and the nuclei isolated. Nucleic acids were either analysed directly after lysing nuclei in SSC-EDTA-sarkosyl (a), or following Marmur extraction (b). dsRNA and calf thymus DNA markers were present.

——— absorbance at 260nm; ····· radioactivity;

•— — —• buoyant density.

Figure 23 - CS density gradient analysis of the nuclear label in V79 cells after ^{3}H-dsRNA uptake by 1 ml cultures in the presence of serum.

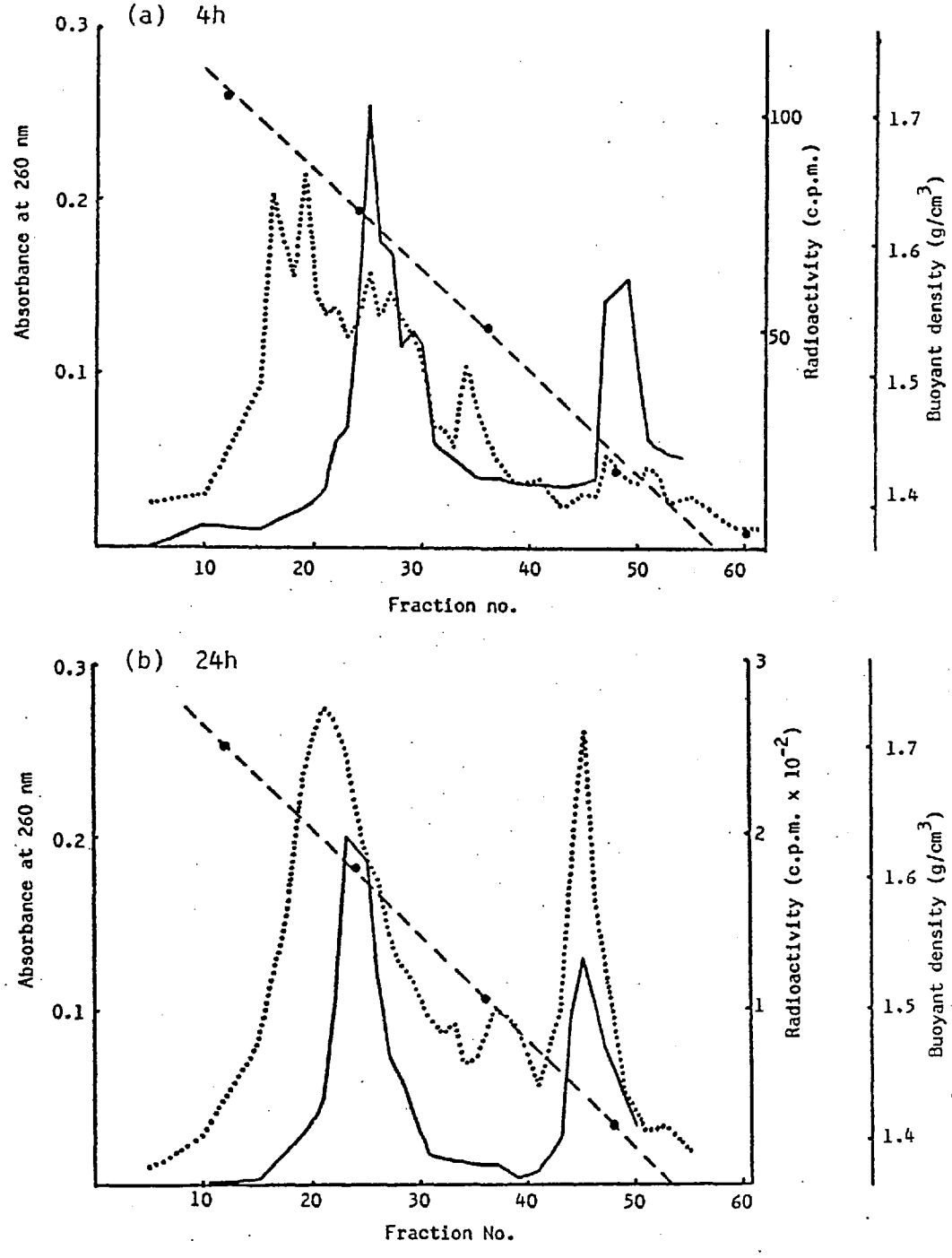


V79 cells in 1 ml petri dish cultures were labelled for (a) 4 hours, or (b) 24 hours with ^{3}H-dsRNA in medium with 10% FCS. (Experimental details as for Table 29). Nuclei were isolated and lysed in SSC-EDTA-sarkosyl before CS density gradient centrifugation in the presence of dsRNA and DNA markers.

——— absorbance at 260 nm; ····· radioactivity;

•— — —• buoyant density.

Figure 24 - CS density gradient analysis of the nuclear label in V79 cells after ^{3}H-dsRNA uptake by 1 ml cultures in the absence of serum.

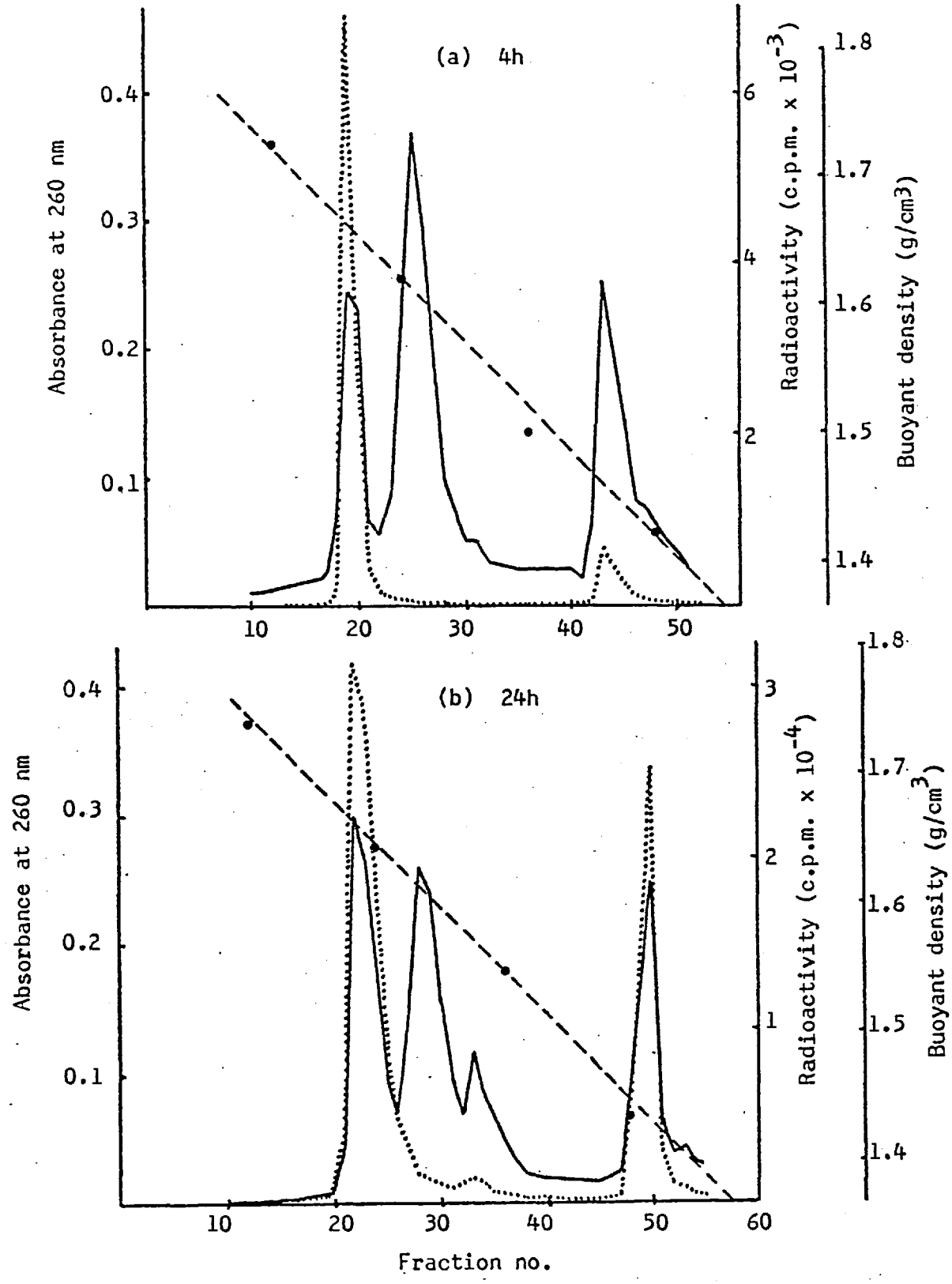


V79 cells in 1 ml petri dish cultures were labelled for (a) 4 hours, or (b) 24 hours with ^{3}H-dsRNA in medium without serum. (Experimental details as for Table 29). Nuclei were isolated and lysed in SSC-EDTA-sarkosyl before CS density gradient centrifugation in the presence of dsRNA and DNA markers.

——— absorbance at 260 nm; ····· radioactivity;

•— — —• buoyant density.

Figure 25 - CS density gradient analysis of the nuclear label in V79 cells after ^{3}H-dsRNA uptake by 10 ml cultures in the presence of serum.

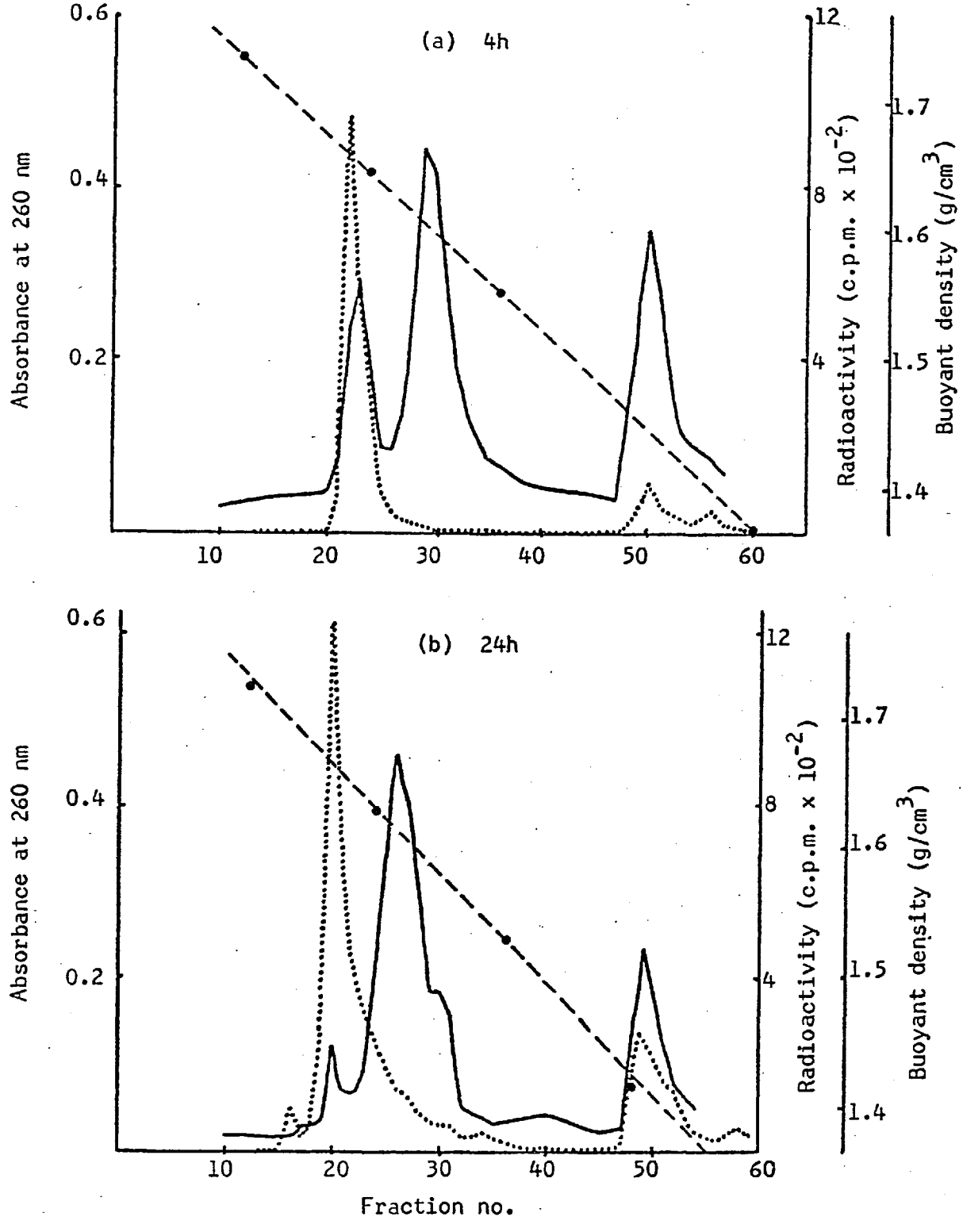


V79 cells in 10 ml glass bottle cultures were labelled for (a) 4 hours, or (b) 24 hours with ^{3}H-dsRNA in medium containing 10% FCS. (Experimental details as for Table 30). Nuclei were isolated and lysed in SSC-EDTA-sarkosyl before CS density gradient centrifugation in the presence of dsRNA and DNA markers.

——— absorbance at 260 nm; ····· radioactivity;

•— — —• buoyant density.

Figure 26 - CS density gradient analysis of the nuclear label in V79 cells after ^{3}H-dsRNA uptake by 10 ml cultures in the absence of serum.



V79 cells in 10 ml glass bottle cultures were labelled for (a) 4 hours, or (b) 24 hours with ^{3}H-dsRNA in serum-free medium. (Experimental details as for Table 30). Nuclei were isolated and lysed in SSC-EDTA-sarkosyl before CS density gradient centrifugation in the presence of dsRNA and DNA markers.

——— absorbance at 260 nm; ····· radioactivity;

●— — —● buoyant density.

to coincide with the last 4 hours of the 24 hour period, so that the cultures used were of a higher cell density.

The fate of ^{3}H-dsRNA in LDV cells was identical to that in the V79 cells. ^{3}H-dsRNA was gradually degraded and re-utilised for the synthesis of LDV cell nucleic acids. Like the V79 cells, more label was re-utilised for RNA than for DNA synthesis. LDV RNA formed a sharp band on CS density gradients at a value of 1.64-1.66g/cm^{3}.

.2 Uptake and fate of ^{3}H-dsRNA in V79 cells at stationary phase

The fate of ^{3}H-dsRNA was briefly examined in confluent cultures of V79 fibroblasts, where the proportion of cells engaged in cell division is reduced and the level of DNA synthesis is much lower than in logarithmic cultures. 10 ml cultures were set up at a high seeding density in glass bottles, and grown for 3 days to confluence (low frequency of mitosis) before labelling with ^{3}H-dsRNA for 4 or 24 hours in fresh medium with or without serum.

The distribution of label in the cells is shown in Table 31. The design of the experiment was the same as that reported in Table 30 except that in the latter, cultures were in the log phase of growth with a high frequency of mitosis. After a 4 hour labelling period,incorporation per 10^{6} cells was lower in the high density cultures than in dividing cultures. After 24 hours, the cells in serum-containing, high density cultures had incorporated more than 98% of the input ^{3}H-dsRNA; therefore, because of the higher cell numbers in these cultures, the incorporation per 10^{6} cells was lower than in dividing cultures.

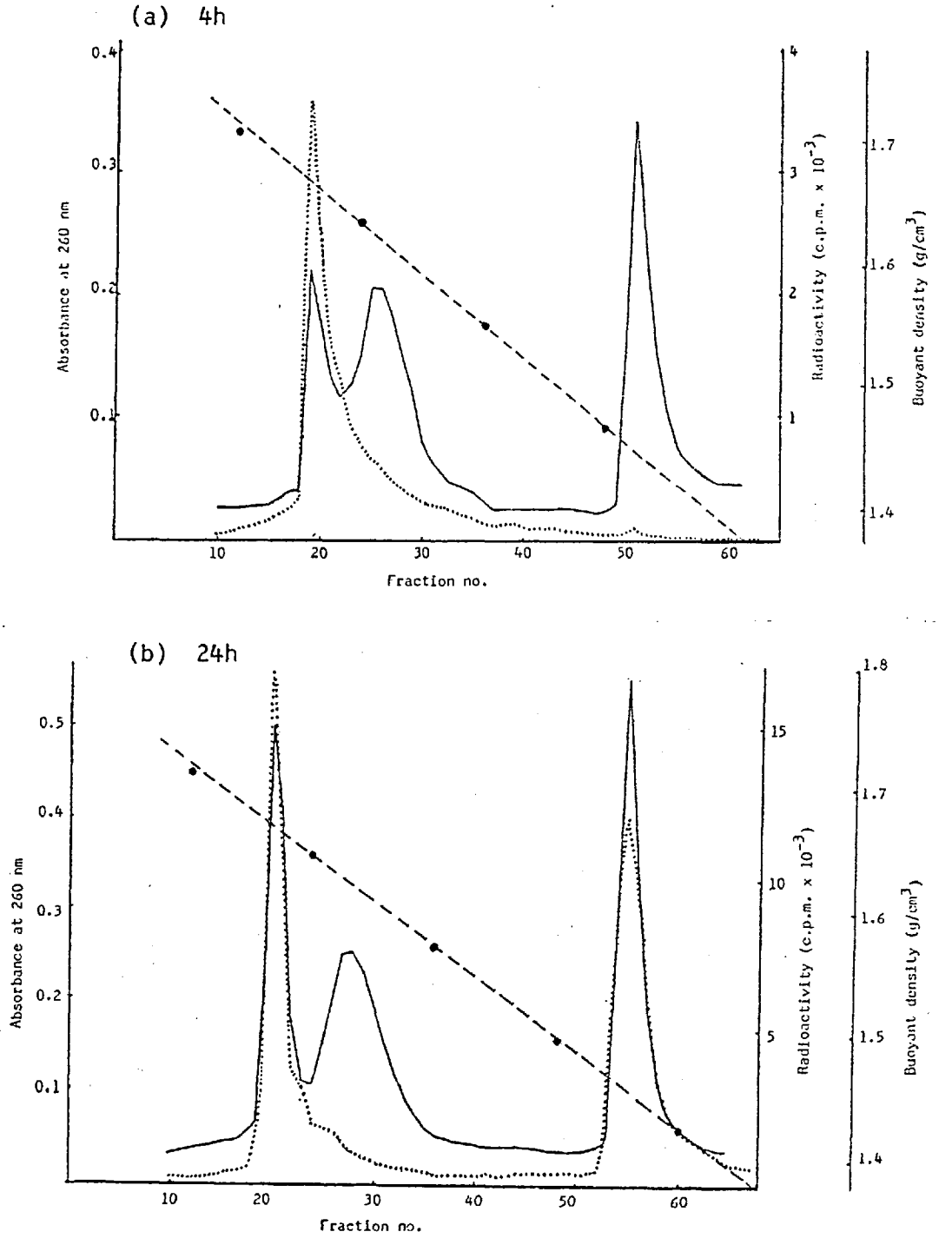
Alkaline hydrolysis of the nuclei revealed that no label was present in the DNA after a 4 hour pulse with ^{3}H-dsRNA, while a normal level of conversion occurred after a 24 hour pulse. Figures 27 and 28 show the nature of the nuclear label (unextracted) on CS density gradients. After the 4 hour labelling period, ^{3}H-dsRNA was undergoing degradation in the cells and conversion to ssRNA, even though no labelling of DNA occurred in these cells. After the 24 hour labelling period, conversion to DNA was taking place, in agreement with the results of alkaline hydrolysis. This conversion was accelerated in the presence of serum, where the rate of cell division was higher.

Table 31- The incorporation of ^{3}H-dsRNA by V79 cells at stationary phase

Serum	Incubation time (h)	Cell no. x 10^{7} per culture	^{3}H-c.p.m. incorporated/10^{6} cells				% of label in nucleus	% of nuclear label as DNA
			Cells	Nucleus	Cytoplasm	Nuclear wash		
-Serum	4	2.28	1,960 (1,750)	1,040 (1,180)	600 (190)	70 (30)	60	0
	24	2.24	29,690 (26,360)	15,970 (20,070)	7,460 (3,200)	580 (480)	66	27
+Serum	4	2.27	15,420 (13,210)	8,020 (9,540)	4,630 (1,490)	260 (220)	62	1
	24	3.43	63,730 (68,850)	47,610 (49,100)	8,180 (5,400)	1,240 (1,070)	83	34

V79 fibroblasts were seeded into 100 ml medical flats in 10 ml medium containing 10% FCS at 1 x 10^{5} cells/ml. After 72 or 92 hours, cells were washed with PBS and reincubated in fresh medium containing 0 or 10% FCS, with 1.0 µCi/ml ^{3}H-dsRNA (350 µCi/mg). Cells were harvested at 96 hours, counted and analysed for incorporation and distribution of radioactivity as described in Materials and Methods.
Remaining details as for Table 30.

Figure 27 - CS density gradient analysis of the nuclear label in V79 cells at stationary phase after ^{3}H-dsRNA uptake in the presence of serum.

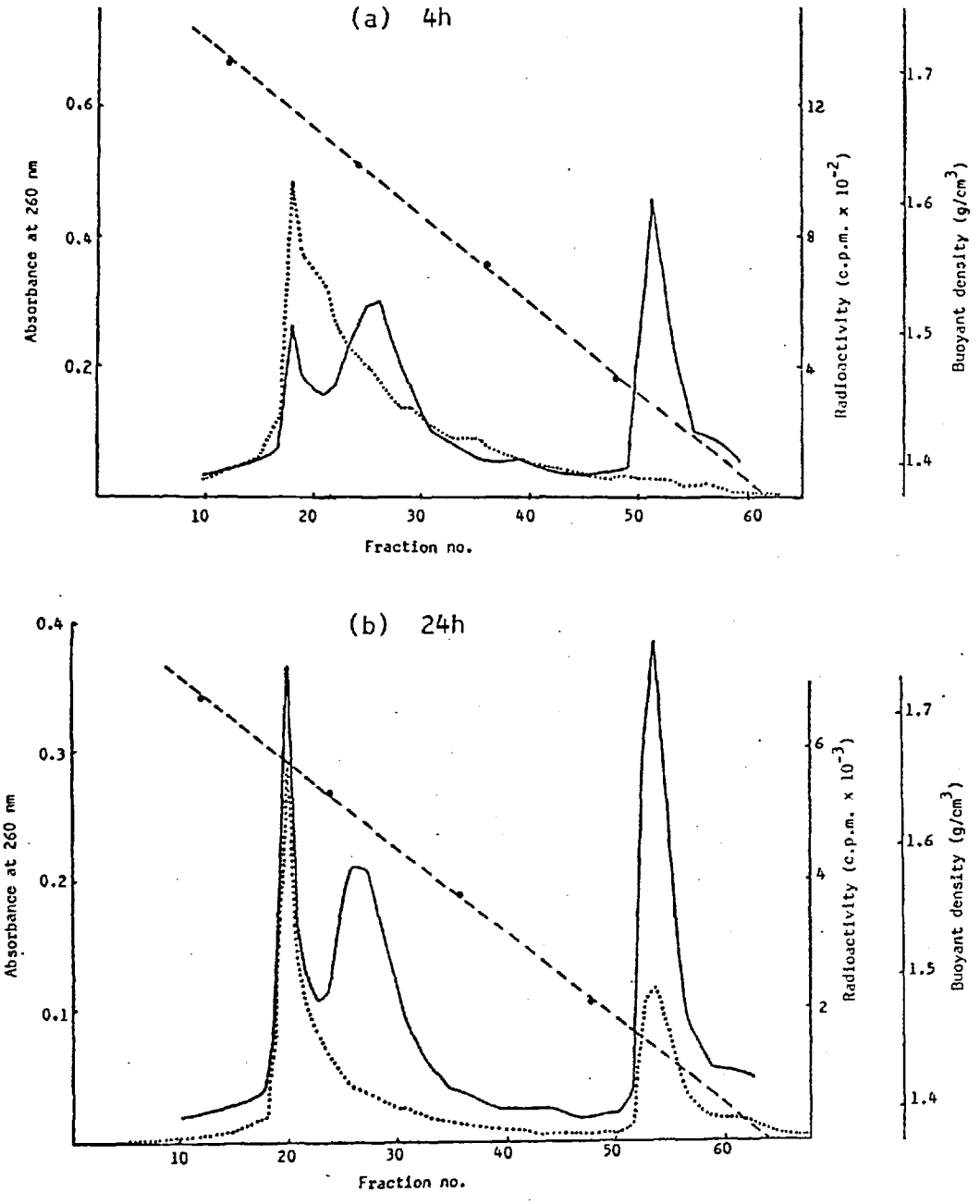


V79 cells in 10 ml glass bottle cultures were grown to stationary phase (>4 x 10^5 cells/cm^2) then labelled with 1 µCi/ml ^{3}H-dsRNA, in medium containing 10% FCS, for (a) 4 hours, or (b) 24 hours, as described for Table 31. Isolated nuclei were lysed in SSC-EDTA-sarkosyl before CS density gradient centrifugation in the presence of dsRNA and RNA markers.

——— absorbance at 260 nm; ..... radioactivity;

•— — —• buoyant density.

Figure 28 - CS density gradient analysis of the nuclear label in V79 cells at stationary phase after ^{3}H-dsRNA uptake in the absence of serum.



V79 cells in 10 ml glass bottle cultures were grown to stationary phase (>4 x 10^5 cells/cm^2) then labelled with 1 µCi/ml ^{3}H-dsRNA in serum-free medium for (a) 4 hours or (b) 24 hours, as described for Table 31. . Isolated nuclei were lysed in SSC-EDTA-sarkosyl before CS density gradient centrifugation in the presence of dsRNA and DNA markers.

——— absorbance at 260 nm; ····· radioactivity;

•— — —• buoyant density.

.3 Nature of the labelled RNA in V79 cells

RNA from the nuclei of cells in 1 ml cultures labelled for 24 hours with ^{3}H-dsRNA or ^{3}H-adenosine was collected by pooling the appropriate fractions from CS density gradients of the nuclear lysates. The RNA was concentrated, dialysed, and analysed by hydroxylapatite chromatography, the results of which are shown in Table 32.

Table 32 - Hydroxylapatite chromatographic analysis of the labelled RNA in V79 cells incubated with ^{3}H-dsRNA and ^{3}H-adenosine.

Labelling conditions	% low Mol. wt. ^{3}H-c.p.m.	Macromolecular ^{3}H-c.p.m.	
		% ssRNA	% dsRNA
^{3}H-dsRNA -FCS	7.8	81.2	18.8
" +FCS	6.9	85.0	15.0
^{3}H-adenosine -FCS	11.8	88.3	11.7
" +FCS	14.5	83.9	16.1
^{3}H-dsRNA control	0.2	1.5	98.5

1 ml cultures of dividing V79 cells were labelled with 2 µCi ^{3}H-dsRNA (350 µCi/mg) or 1 µCi ^{3}H-adenosine (200 mCi/mmol) for 24 hours in the presence and absence of serum. The nuclei were lysed and run on CS density gradients. The RNA-containing fractions were pooled, concentrated and dialysed against 0.012M potassium phosphate buffer, pH 6.8, for hydroxylapatite chromatography. A sample of ^{3}H-dsRNA itself was also analysed as control.

The nuclei of cells labelled with ^{3}H-dsRNA were found to contain a significant proportion of dsRNA (15-19%). However, nuclei of ^{3}H-adenosine-labelled cells contained a similar proportion of dsRNA (12-16%). This is unlikely to be a true value for the amount of RNA occurring naturally in a double-stranded configuration in the cell, and is probably an artefact arising from the manipulations. Hydroxylapatite chromatography thus confirmed the density gradient analysis that no detectable levels of undegraded P. chrysogenum ^{3}H-dsRNA persisted in the nuclei of V79 cells after a 24 hour labelling period, either in the presence of absence of serum.

The ^{3}H-dsRNA remaining in the serum-free V79 culture medium after 24 hours was precipitable by TCA, and when analysed by CS density gradient centrifugation, retained its original sharply-defined density value of 1.61 g/cm^{3}. It seemed likely that V79 cells contained ribonuclease activity capable of degrading ^{3}H-dsRNA in serum-free medium. "Conditioned" medium was prepared by culturing V79 cells in serum-free medium for 24 hours. The cell-free medium, when incubated with ^{3}H-dsRNA for a further 24 hours, caused a reduction in its molecular weight, as shown by sucrose density gradient analysis of the product (Figure 29). The molecular weight fell from 1.9 to 1.24 x 10^{6}, a drop of 35%. This decrease was greater than that occurring after incubation of ^{3}H-dsRNA with MS cell-conditioned medium. The incubation products were also analysed by hydroxylapatite chromatography when approximately 83% of the total label eluted in the dsRNA fraction (Table 33).

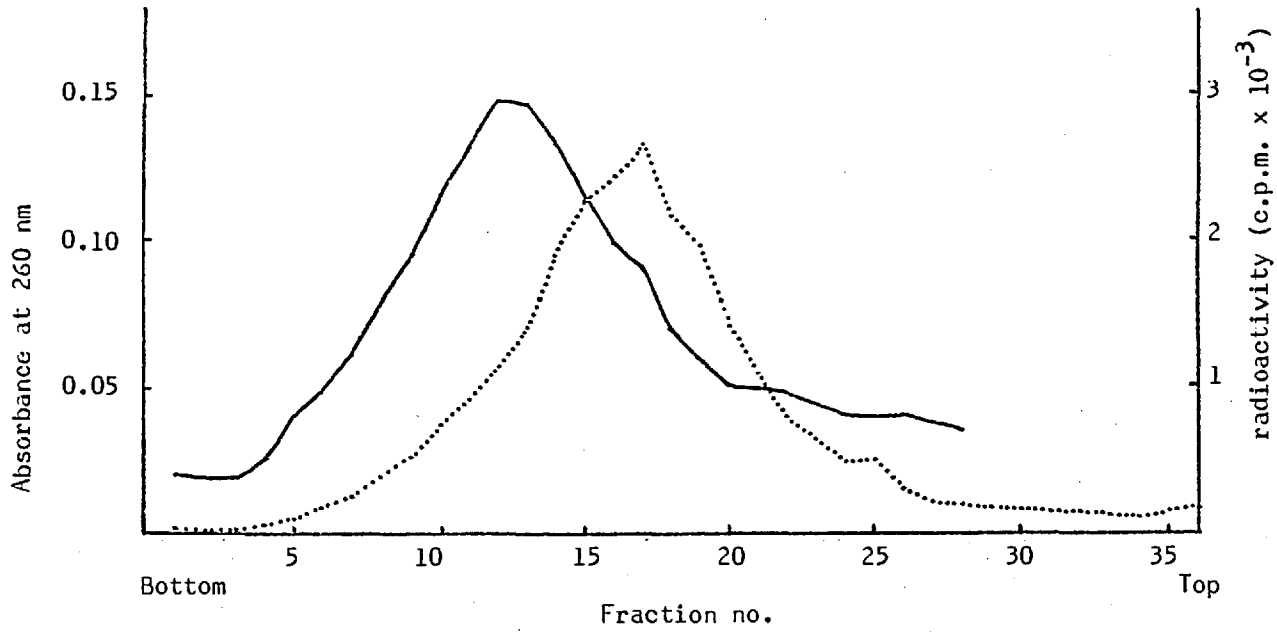
Table 33 - Hydroxylapatite chromatographic analysis of ^{3}H-dsRNA after incubation with V79 cell-conditioned medium.

Incubation of ^{3}H-dsRNA	% low Mol. wt. ^{3}H-c.p.m.	Macromolecular ^{3}H-c.p.m.	
		% ssRNA	% dsRNA
Conditioned medium	8.3	9.3	90.7
Medium alone	1.0	3.6	96.4

(see Figure 29 for details)

The results in Table 34 show that V79 cells contain ribonuclease activity capable of degrading ^{3}H-dsRNA. During incubation with sonicates of whole cells or nuclei, no rapid degradation to TCA-soluble products took place, but the radio-activity of samples not treated with TCA became quenched. This quenching was found previously to correlate with a fall in molecular weight of the ^{3}H-dsRNA, and was thus indicative of ribonuclease activity. ^{3}H-dsRNA incubated with the isolated cell cytoplasmic fraction was, on the other hand, rapidly degraded to TCA-soluble products, suggesting that the nuclei contained an RNase inhibitor which prevented rapid hydrolysis of dsRNA in whole cell sonicates.

Figure 29 Sucrose density gradient centrifugation of ^{3}H-dsRNA after incubation with V79 cell-conditioned medium



Dividing V79 cell cultures were incubated in serum-free medium for 24 hours. The medium was clarified by centrifugation and incubated for a further 24 hours with 2 µCi/ml ^{3}H-dsRNA (350 µCi/mg). The reaction products were run on a 5-20% neutral sucrose gradient in the presence of unlabelled dsRNA as A_{260} marker.

——— absorbance at 260 nm; ····· radioactivity.

Table 34 - The effect of V79 cells and conditioned medium on the TCA-precipitability of ^{3}H-dsRNA

^{3}H-dsRNA incubation	TCA	^{3}H-c.p.m./50 µl reaction mixture at:		
		0h	4h	24h
Medium alone	-TCA	25,520	27,750	28,880
	+TCA	28,160	33,060	34,620
"Conditioned" medium	-TCA	24,450	26,900	23,320
	+TCA	25,870	29,150	27,450
Cell sonicate	-TCA	26,460	17,940	15,140
	+TCA	28,490	23,000	16,280
Nuclear fraction	-TCA	30,660	20,900	16,080
	+TCA	32,020	24,990	16,210
Cytoplasmic fraction	-TCA	27,260	8,200	7,040
	+TCA	31,000	4,050	1,000

V79 cells were grown in 100 ml medical flats and harvested before confluence was reached after a 24h incubation in serum-free medium. The conditioned medium from these cells was clarified by centrifugation and incubated with ^{3}H-dsRNA. The cells were resuspended to 2 x 10^{6}/ml in fresh medium and sonicated for 10 seconds, or to 2 x 10^{6}/ml in cell lysis buffer. The cytoplasm was reserved while the nuclei were resuspended to the same volume and sonicated for 10 seconds. Reaction mixtures of 1 ml volume contained 1 µCi ^{3}H-dsRNA. At 0, 4 and 24 hours, 50 µl aliquots were counted by the paper disc method, before and after TCA treatment.

As discussed in Section II, sonicates of Con A-stimulated MS cells caused neither TCA-solubilisation nor quenching of ^{3}H-dsRNA after a 24 hour incubation, and lowered the molecular weight by only a small fraction. V79 fibroblasts apparently contain higher levels of nucleolytic activity than MS cells, which is consistent with the greater ability of the former to incorporate and catabolise ^{3}H-dsRNA under conditions where the ^{3}H-dsRNA remains intact in the medium.

.4 Autoradiography

Dividing fibroblasts cultured on glass coverslips were labelled with ^{3}H-dsRNA in the presence and absence of serum. After three washes the cells were fixed *in situ* on the coverslips and developed with K 5 emulsion. The cultures were found to be well labelled, with the grain density increasing considerably between 4 and 24 hours. Every cell took up ^{3}H-dsRNA to approximately the same level. No unlabelled cells were present at either time point. Grains were located both in the cytoplasm and nucleus, with a tendency towards grain accumulation in the nucleolar regions where active turnover of RNA occurs, confirming the pattern of breakdown and re-utilisation of ^{3}H-dsRNA in these cells. Fibroblasts labelled in the absence of serum had fewer grains per cell than those labelled in the presence of serum, but the distribution was the same. Figure 21(d) shows the grain distribution in V79 cells labelled for 24 hours in the absence of serum.

When high density confluent cultures of V79 fibroblasts were labelled with ^{3}H-dsRNA for 24 hours in serum-free medium, some cells were found free in the culture medium at the end of the labelling period. These cells accounted for less than 10% of the total cell yield, while in serum-containing cultures, free cells accounted for <1% of the total cell yield. These non-adherent cells were collected by centrifugation of the medium, and found to consist entirely of small, lightly-staining cells with small or pyknotic nuclei and poorly-defined, ruffled membranes. They had incorporated a lower level of radioactivity (15,000 c.p.m./10^{6} cells) than that associated with adherent viable cells from the same culture (30,000 c.p.m./10^{6} cells). Smears of the non-adherent and viable cells removed from the glass with trypsin were prepared for autoradiography. The dead cells contained fewer grains per cell

than the viable cells, with a more heterogenous distribution; some cells contained mainly nuclear label while others contained mainly cytoplasmic label. There was no correlation between cell death and the accumulation of label in V79 cells.

Cultures of LDV cells were also homogeneously labelled in autoradiographs. Like the V79 fibroblasts, every cell took up ^{3}H-dsRNA, the grain density being higher in cells cultured in the presence than in the absence of serum.

.5 Discussion

Cultures of established cell lines uniformly incorporated ^{3}H-dsRNA, uptake being greater in serum-supplemented than in serum-free cultures. Cell-associated label was shown to be partially degraded and re-utilised within 4 hours, as evidenced by the presence of TCA-soluble products in the cytoplasm and the appearance of label in cellular RNA and DNA. The kinetics of this rapid dsRNA catabolism could now be studied more suitably by labelling the cells with high specific activity ^{3}H-dsRNA for a short period (0.5-1.0 hour), and examining the incorporated label during subsequent "chase" periods in the absence of labelled dsRNA.

The newly-synthesised RNA in V79 and LDV cells formed a very sharply-defined band on CS density gradients. The formation of such bands is due to aggregation of ssRNA, and probably results from the "salting out" effect of SO_4^{2-} which, at high concentrations, decreases the solubility of the bases so that they associate intermolecularly in solutions of ssRNA (Robinson and Grant, 1966). The aggregation, which may be accompanied by precipitation in the form of a visible white band in the gradient, is indicated by a skewed peak which is steeper on the high density side (Williams and Vinograd, 1971). Double-stranded RNA does not seem to aggregate in caesium sulphate.

MS cell RNA, labelled from ^{3}H-adenosine or from ^{3}H-dsRNA degradation products, showed no such aggregation in CS gradients, but exhibited a more heterogeneous buoyant density profile. Although the MS RNA and DNA was generally extracted from the nuclei prior to CS gradient centrifugation, the extraction procedure itself was unlikely to have produced this large spread in density of the RNA, since the sharpness of V79 ssRNA bands was only slightly spread as a result of Marmur extraction [Figure 22(b)]. Banding of dsRNA was unaffected by Marmur extraction (Figure 16).

The persistence of some intact ^{3}H-dsRNA (ρ = 1.61 g/cm^{3}) in the established cell lines was observed after a 4 hour labelling period only when cell growth was suboptimal (see Figures 28, 29, for example). Even in these cells the label was undergoing conversion to cellular nucleic acid. Although in the MS cell system much of the intact ^{3}H-dsRNA found in the nuclear fraction was associated with non-viable lymphocytes, the undegraded ^{3}H-dsRNA in V79 cells was not considered to be associated with dead cells. Because of the nature of fibroblast growth as a monolayer, and the washing and harvesting techniques used, only the viable, adherent cells were examined for analysis of ^{3}H-dsRNA incorporation. Dead cells would have been removed in the culture medium or during the washing procedures. Since these were more lightly labelled than viable cells from the same culture, the primary cause of death was probably not ^{3}H-dsRNA accumulation but serum deprivation.

SECTION IV - PRELIMINARY STUDIES ON THE FATE OF ^{3}H-dsRNA IN VIVO IN THE MOUSE.

1. Acute toxicity of dsRNA in CBA mice

Nine male mice of average body weight 24g were injected intravenously (i.v.) into the lateral tail vein with dialysed dsRNA in 0.15M NaCl at a dosage of 10 mg/kg. After 24 hours, 6/9 mice had died. The survivors lost approximately 10% body weight, an observation associated with a hunched posture, ruffled fur and general inactivity. At 24 hours, the surviving mice were bled from the tail and blood smears examined after Giemsa staining. The smears showed a dramatic fall in the number of white blood cells (WBC), and the presence of large phagocytic cells of the monocyte series, some of which were actively engaged in erythrophagocytosis. On dissection of the dsRNA-treated mice, the most prominent organ damage had occurred to the ileum which was flattened, translucent and yellow in colour. Spleens were of normal size and appearance.

Less toxicity was observed when 10 mg/kg dsRNA was administered intraperitoneally (i.p.) rather than intravenously. Three mice weighing 24g were injected i.p. with either dialysed or undialysed dsRNA at 10 mg/kg. After 18 hours, all six mice were alive but showed the typical symptoms of dsRNA toxicity (see above), with no differences between the group receiving dialysed dsRNA and the group receiving undialysed dsRNA. The mean fall in body weight was 13% ($\pm$ 1.75 s.d.).

2. Effect of dsRNA on WBC levels in the blood

Table 35 shows the peripheral blood leucopenia which follows a single i.v. injection of dsRNA. There was an early dose-dependent fall in total WBC numbers, which was maximal on day 1 and returned to normal by day 6. The most obvious depression involved the lymphocytes. Platelet counts remained normal, the animals showing no tendency towards haemorrhage.

3. Labelling of lymphoid organs after ^{3}H-dsRNA administration

CBA mice were injected either i.v. or i.p. with a sub-lethal dose of ^{3}H-dsRNA (20 µCi, 65 µg per mouse). The mice were bled and sacrificed at 2, 6 and 24 hours,when radioactivity in the serum, spleen, thymus, bone marrow and lymph nodes was measured (see Table 36). Disappearance of label from the serum was not

Table 35 - The effect of a single i.v. injection of dsRNA on circulating white blood cell (WBC) numbers in the mouse.

Time after dsRNA administration	Sex	Total WBC x 10^8/dl.			Lymphocytes x 10^8/dl.			PMNL x 10^8/dl.		
		1	10	100	1	10	100	1	10	100
3 hours	♀	4.86 ±0.45	3.02 ±0.88	2.03 ±0.32	3.88 ±0.29	1.74 ±0.57	1.31 ±0.61	092 ±0.55	1.22 ±0.33	0.95 ±0.19
	♂	4.80 ±1.0	2.72 ±0.76	2.32 ±0.53	3.88 ±0.79	1.47 ±0.13	1.05 ±0.06	0.85 ±0.35	1.17 ±0.61	0.87 ±0.47
1 day	♀	3.98 ±0.63	1.85 ±0.38	0.48 ±0.10	3.19 ±0.64	1.38 ±0.32	0.14 ±0.02	0.70 ±0.18	0.43 ±0.09	0.33 ±0.11
	♂	5.20 ±0.92	3.30 ±1.30	1.53 ±0.66	3.76 ±0.81	2.15 ±1.08	0.45 ±0.17	1.36 ±0.06	0.74 ±0.25	1.04 ±0.50
2 days	♀	5.13 ±0.15	3.81 ±0.65	1.98 ±0.55	4.85 ±0.32	2.70 ±0.13	1.24 ±0.31	0.22 ±0.14	0.97 ±0.57	0.68 ±0.22
	♂	6.25 ±0.04	2.63 ±1.06	2.63 ±1.29	5.02 ±0.44	1.75 ±0.56	1.14 ±0.51	0.94 ±0.44	0.80 ±0.40	1.43 ±0.77
6 days	♀	5.17 ±1.48	6.30 ±1.88	6.75 ±1.64	4.63 ±1.45	5.60 ±1.27	6.32 ±0.14	0.48 ±0.09	0.64 ±0.53	0.39 ±0.31
	♂	7.90 ±1.84	10.50 ±1.01	8.90 ±1.08	6.48 ±1.50	8.35 ±0.22	7.91 ±1.42	1.25 ±0.18	1.57 ±0.15	1.14 ±0.39

CBA mice weighing 20g (♀) or 27g (♂) were injected i.v. into the tail vein with dsRNA at a level of 1, 10 or 100 µg per mouse (3 mice per group). The mice were bled from the retro-orbital sinus while under ether anaesthesia. The blood was mixed with K^+-EDTA and diluted in 1.5% acetic acid containing methyl violet for estimation of total WBC. Blood smears were made for differential counts following Giemsa staining. PMNL - polymorphonuclear leucocyte.

Table 36 - Labelling of lymphoid tissues in the mouse after administration of ^{3}H-dsRNA.

Route of ^{3}H-dsRNA administration	Time after administration (h)	WBC No./dl x 10^{8}	^{3}H-c.p.m./20 µl serum		^{3}H-c.p.m./10^{7} cells			
			Insta-Gel	Disc	Spleen	Bone Marrow	Thymus	Lymph Nodes
i.v.	2	5.5	N.A.	N.A.	590 (470)	360 (380)	50 (40)	230 (250)
	6	1.7	54,100	27,800 (33,300)	1,140 (1,070)	1,970 (1,880)	120 (70)	450 (310)
	24	1.0	12,100	150 (40)	1,680 (1,760)	2,400 (2,740)	70 (70)	540 (390)
i.p.	2	5.1	84,800	48,000 (62,000)	550 (570)	580 (740)	60 (50)	240 (260)
	6	1.8	47,300	28,300 (34,500)	1,040 (940)	1,380 (1,320)	130 (90)	350 (140)
	24	0.7	7,000	100 (40)	510 (510)	470 (480)	80 (50)	310 (320)

3 CBA mice weighing 23g were injected with 20 µCi ^{3}H-dsRNA (310 µCi/mg) in 0.15M NaCl, either intravenously (i.v.) or intraperitoneally (i.p.). One mouse was bled and sacrificed after 2, 6 and 24h. WBC were enumerated in the blood, while radioactivity in 20 µl of serum was estimated both directly in Insta-Gel scintillant, and after drying onto filter paper discs. Samples of spleen, bone marrow (femurs), thymus, and pooled lymph nodes (inguinal and axillary) were disaggregated to form cell suspensions, which were washed twice in Tyrode's solution before estimation of the radioactivity on filter paper discs. ^{3}H-c.p.m. in parentheses represent TCA-insoluble radioactivity. (N.A. data not available.)

associated with increased degradation of ^{3}H-dsRNA to TCA-soluble products until 24 hours, by which time most of the radioactivity appeared to be tritiated water or other volatile materials. At all times after ^{3}H-dsRNA administration, more label was found in spleen and bone marrow cells than in lymph node cells. Thymus cells contained insignificant levels of radioactivity. After an intravenous injection of ^{3}H-dsRNA, TCA-precipitable label accumulated in the spleen and bone marrow cells.

At this dosage of dsRNA, histological damage to the small intestine was greatest at 6 hours with some degree of recovery occurring after 24 hours.

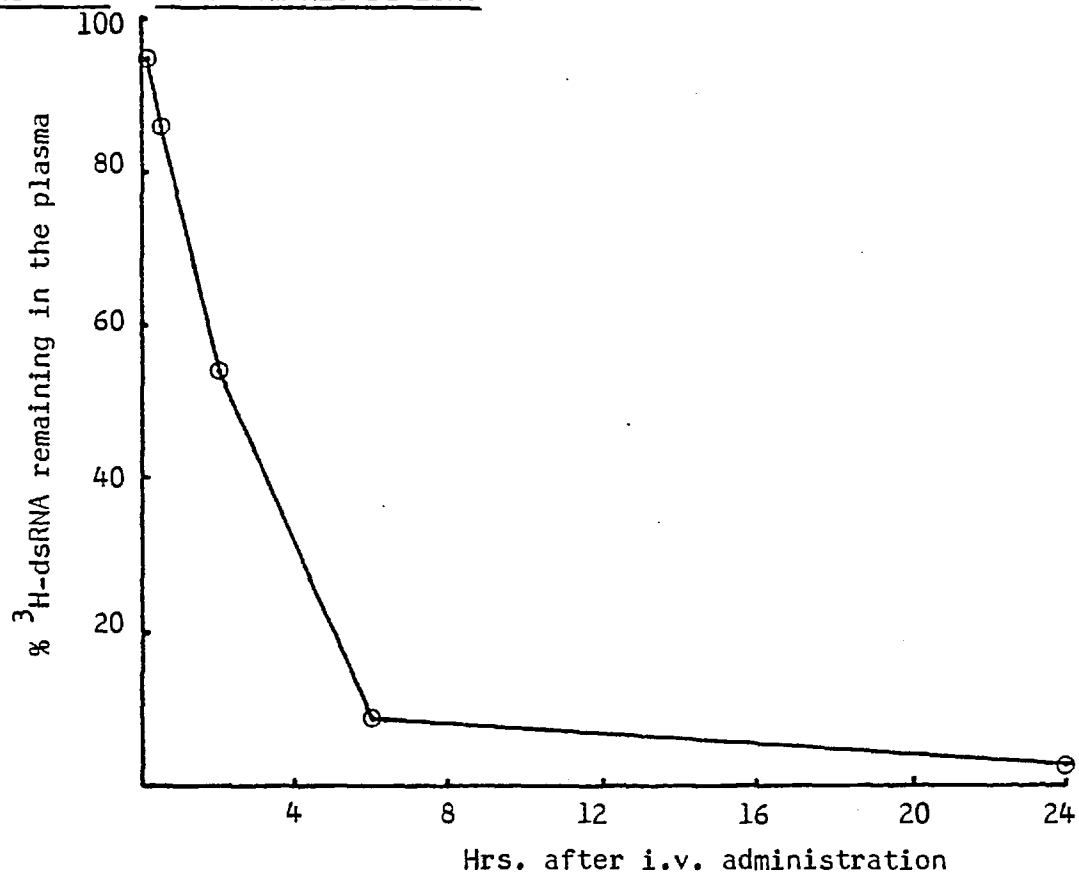
4. Nature of ^{3}H-dsRNA in the blood following i.v. administration

Five weight-matched CBA male mice were injected i.v. into the tail vein with 33 µCi ^{3}H-dsRNA (310 µCi/mg). The mice were bled by cardiac puncture at intervals over 24 hours, and the plasma fractions collected. After mixing the plasma with an equal volume of 0.5% sarkosyl to inhibit ribonuclease activity, aliquots were counted in Insta-Gel or by drying onto paper discs, while the remainder was reserved for sucrose density gradient centrifugation.

The fall in plasma radioactivity with time is shown in Figure 30 and Table 37. The plasma concentration of ^{3}H-dsRNA dropped to 50% after 2 hours and to 10% after 6 hours. Again, by 24 hours, most of the remaining radioactivity was ^{3}H-water or other volatile materials, since it was lost during evaporation of plasma samples from the filter paper discs. The amount of ^{3}H-dsRNA bound to the red blood cells was determined by solubilising 20 µl of washed, packed cells and counting in Insta-Gel. This amount rose to a maximum after 2 hours, at which time it comprised only a small percentage of the total label in the blood. It was obviously not possible to establish a clearance rate from only five measurements, however the results suggest that clearance of P. chrysogenum dsRNA in mice may be considerably slower than that of DNA or poly I.poly C (Chused, Steinberg and Talal, 1972).

The results in Table 37 show that ^{3}H-dsRNA circulating in the plasma after 6 hours remained TCA-insoluble. Its macromolecular nature was confirmed by sucrose density gradient centrifugation; Figure 31 shows the distribution of ^{3}H-dsRNA on sucrose gradients up to 6 hours following i.v. administration.

Figure 30 and Table 37 - Clearance of ^{3}H-dsRNA from mouse plasma following i.v. administration.



Time after ^{3}H-dsRNA administration	^{3}H-c.p.m. per 20 µl undiluted plasma			^{3}H-c.p.m. per 20 µl packed RBC
	Insta-Gel	Disc (-TCA)	Disc (+TCA)	
5 min	338,000	239,000	286,000	1,820
30 min	308,000	192,000	221,000	2,690
2 h	196,000	119,000	136,000	3,100
6 h	31,000	25,100	30,000	2,200
24 h	11,500	250	100	1,370

5 CBA mice weighing 24g were injected i.v. into the tail vein with 33 µCi ^{3}H-dsRNA (310 µCi/mg) in 0.3 ml saline. One mouse was bled by cardiac puncture at each time point shown. The plasma was collected, diluted with an equal volume of 0.5% sarkosyl in 0.15 M NaCl, and the radioactivity estimated by counting aliquots directly in Insta-Gel,or after drying onto filter paper discs, with and without subsequent TCA treatment. ^{3}H-dsRNA associated with washed, packed blood cells (RBC) was determined after solubilisation and counting in Insta-Gel. A plasma volume of 1 ml was assumed in calculating the % of ^{3}H-dsRNA remaining in the plasma (Fig. 30).

Figure 31 - Sucrose density gradient centrifugation of ^{3}H-dsRNA remaining in mouse plasma following i.v. administration.

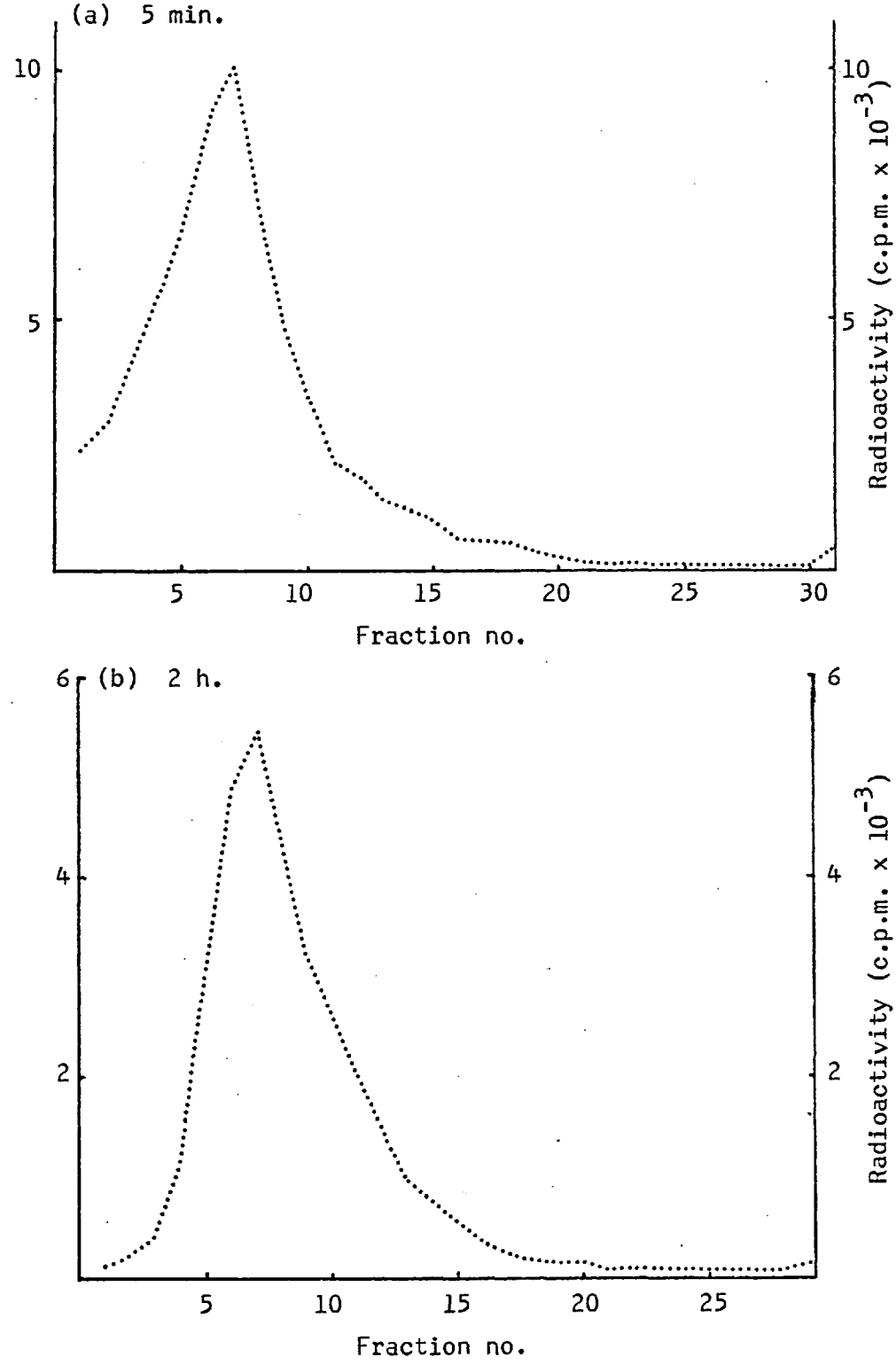


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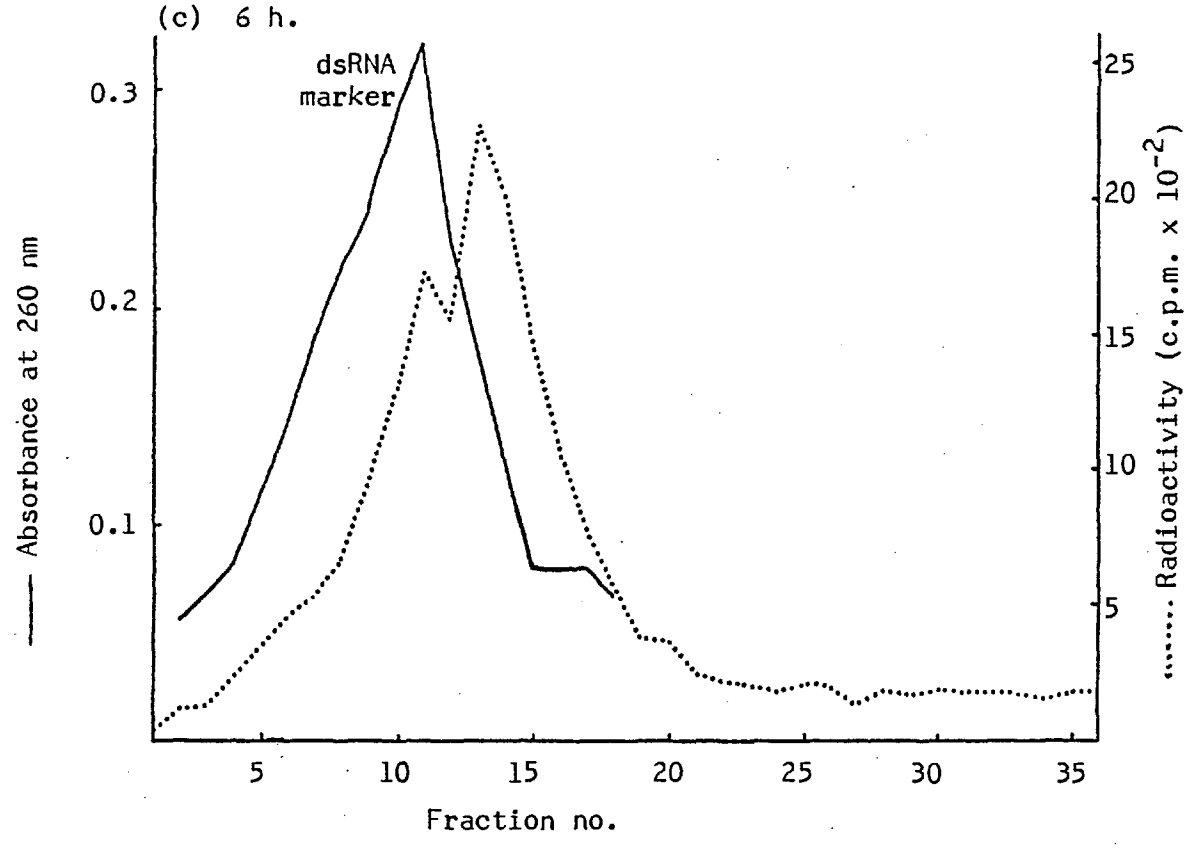
Figure 31 (cont.)



Experimental details were described in Figure 30 and Table 37. Briefly, CBA mice weighing 24g were injected i.v. into the tail vein with 33 µCi ^{3}H-dsRNA (310 µCi/mg) in 0.3 ml saline. After 5 min, 2 h and 6 h, blood was collected and the plasma separated. After mixing the plasma with sarkosyl to prevent ribonuclease activity, aliquots were analysed on 5-20% neutral sucrose gradients. Gradient fractions were counted on filter paper discs.

Unlabelled dsRNA was not included as A_{260} marker for the 5 and 120 min measurements, therefore very small changes in molecular weight would have been undetectable. The skewing of the peak to the low density side of the gradient at 2 hours suggests that a decrease in size was beginning. By 6 hours a fall in molecular weight from 2.35×10^6 to 1.75×10^6 had taken place [Figure 31(c)]. Thus, 6 hours after administration of ^{3}H-dsRNA, material of high molecular weight remained in the blood of the mouse.

Disappearance of ^{3}H-dsRNA from the circulation during the first 6 hours was shown not to result from serum nuclease activity. In a control in vitro experiment, ^{3}H-dsRNA was incubated with fresh pooled mouse plasma. Over a period of 6 hours, no increase in TCA-solubility of ^{3}H-dsRNA took place (data not shown), although a fall in molecular weight occurred which was approximately 12%, and therefore smaller than that which occurred in vivo during 6 hours (see Figure 32).

These results show that ^{3}H-dsRNA is degraded very slowly by mouse plasma nucleases, so that during the 6 hour period following i.v. administration, when most of the label disappears from the circulation, ^{3}H-dsRNA is taken up by the tissues in an intact form. Following this observation, an attempt was made to examine the nature of the ^{3}H-dsRNA in mouse tissues after i.v. administration of a toxic dosage (10 mg/kg). CS density gradient centrifugation was used to compare the fate of ^{3}H-dsRNA in a severely damaged organ, the ileum, to that in a relatively undamaged organ, the spleen.

5. Nature of ^{3}H-dsRNA in the ileum and spleen following i.v. administration

Two CBA mice were injected i.v. with 60 µCi ^{3}H-dsRNA at an acutely toxic dosage (10 mg/kg) previously shown to cause damage to the ileum within 6 hours. One of the mice was injected intraperitoneally 3 days prior to ^{3}H-dsRNA administration with 0.1 ml of a 20% suspension of sheep red blood cells (srbc), in order to stimulate DNA and RNA synthesis in the spleen. 6 hours after ^{3}H-dsRNA administration, the mice were sacrificed and samples of the liver, lung, spleen, lymph nodes (inguinal), sternum and ileum were prepared for histological examination and autoradiography. Cell suspensions were prepared from the spleens for separation into nuclear and cytoplasmic fractions. A section of the damaged ileum from each mouse was cleaned, homogenised and separated directly

Figure 32 - Sucrose density gradient centrifugation of ^{3}H-dsRNA after incubation with mouse plasma in vitro.

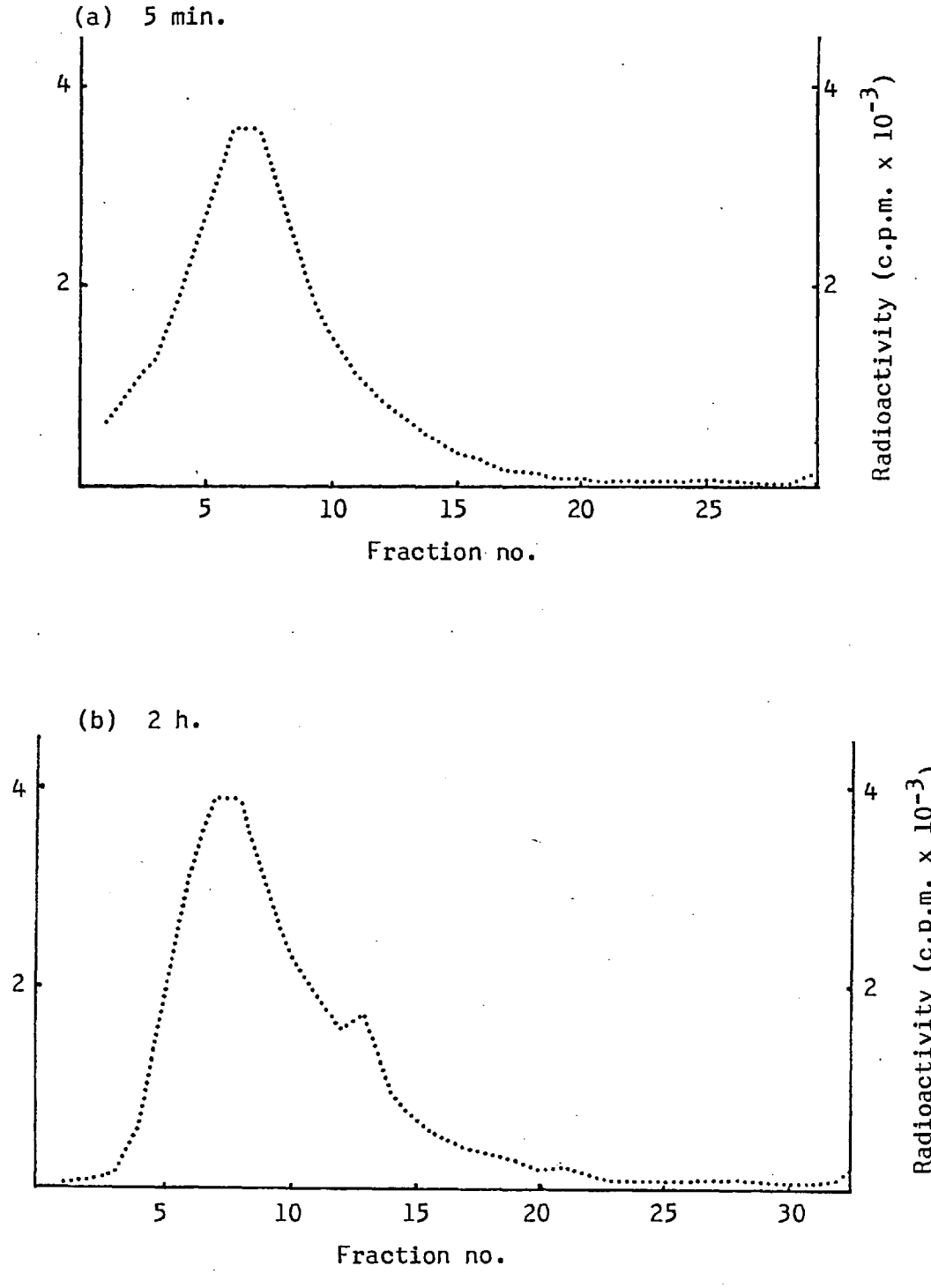


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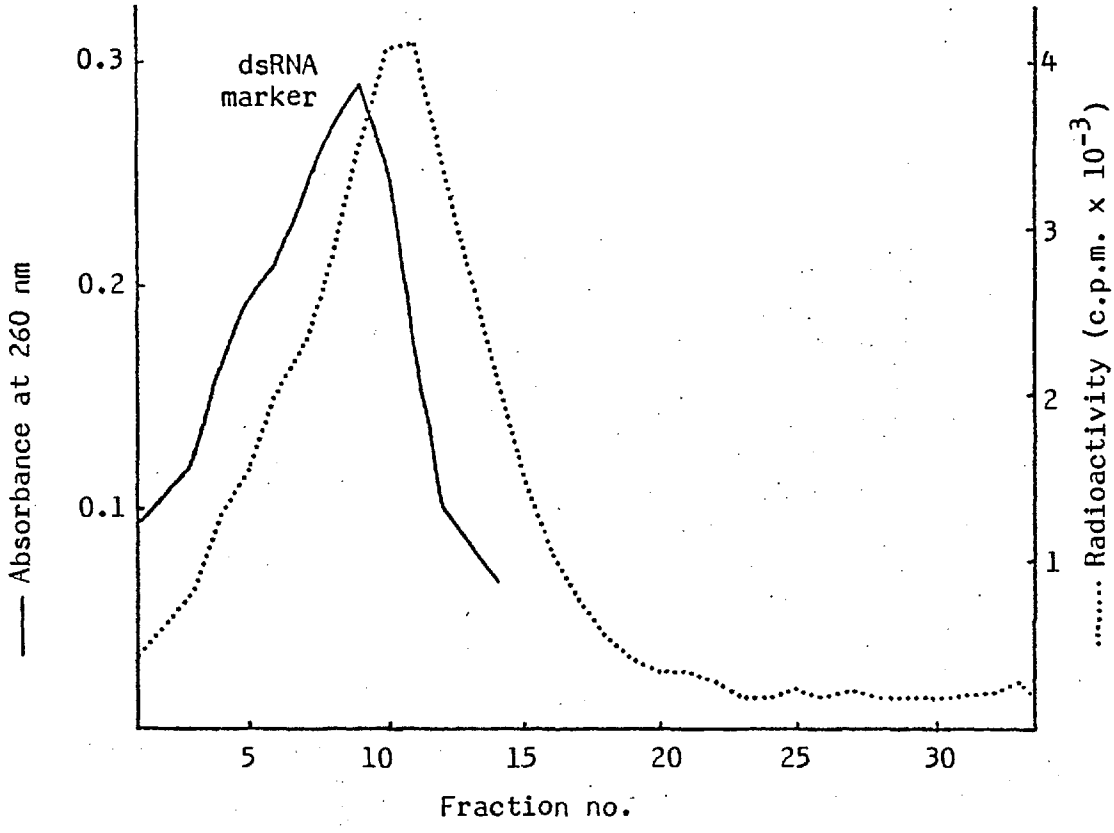
Figure 32 (Cont.)



2 µCi ^{3}H-dsRNA (310 µCi/mg) was incubated at 37° in 0.3 ml freshly drawn, pooled CBA mouse plasma. 50 µl aliquots were withdrawn at 5 min, 2 h and 6 h, and mixed with 50 µl 0.5% sarkosyl in 0.15 M NaCl. 50 µl of the diluted samples were analysed on 5-20% neutral sucrose gradients. Gradient fractions were counted on filter paper discs. ^{3}H-dsRNA remained TCA-precipitable during a 6 h incubation period.

into nuclear and cytoplasmic fractions. Nucleic acids were extracted from the nuclei by the Marmur procedure before analysis on CS density gradients.

Table 38 shows the distribution of radioactivity in the samples of spleen from the two mice. Intracellular incorporation and distribution was quantitatively similar in both spleens. However, most of the label in the tissue was found to be extracellular, that is, recoverable in the cell washings after passing the minced spleens through a 60-gauge mesh to prepare a single cell suspension. The spleen from the mouse undergoing a primary immune response to srbc (II) contained more extracellular radioactivity than the control (I). There was evidence of degradation to TCA-soluble products in the cell cytoplasm, and to a lesser extent in the cell washings, suggesting that catabolism of ^{3}H-dsRNA was in progress.

The CS buoyant density profiles of the nucleic acids extracted from the spleen cell nuclei can be seen in Figure 33. ^{3}H-dsRNA was undergoing degradation and re-utilisation in the spleen cells, as evidenced by the lack of a significant peak of radioactivity at a density of 1.61 and the appearance of label in mouse cell RNA and DNA. Utilisation of label for DNA synthesis was apparently greater in the spleen cells of the mouse undergoing an immune response. This observation is consistent with the increased number of DNA-synthesising lymphocytes in the spleen under these circumstances (Harris and Pelc, 1970).

It was not possible to compare incorporation of ^{3}H-dsRNA per 10^{6} cells in the ileum and spleen, since ileum samples were homogenised directly. A 2 cm section was suspended in 5 ml of cell lysis buffer, and the incorporation per ml into nuclear and cytoplasmic fractions was as follows, with TCA-insoluble c.p.m. given in parentheses:

I. nucleus 27,500 (29,300); cytoplasm 35,400 (33,300),
II. nucleus 41,000 (42,900); cytoplasm 48,000 (47,500).

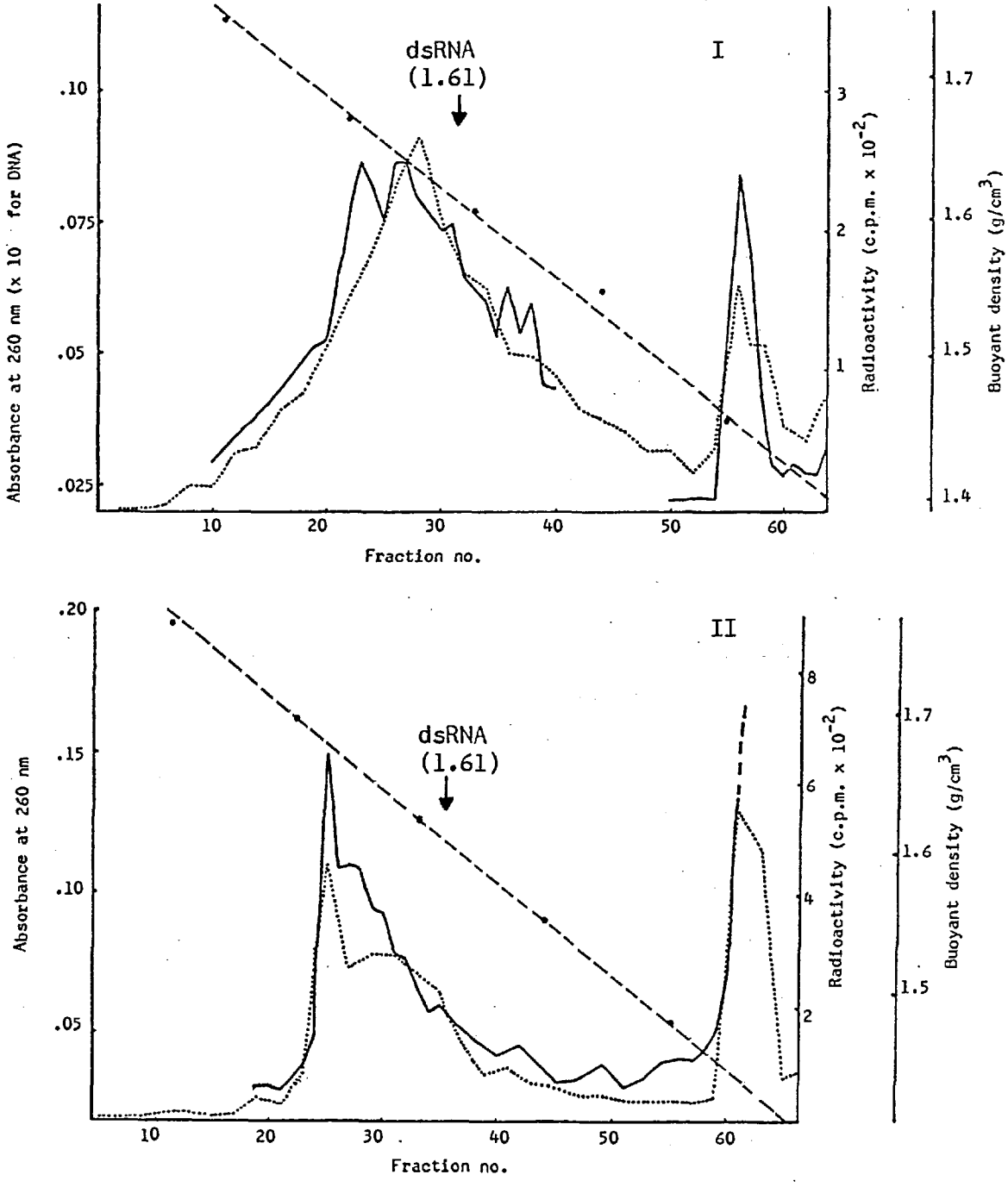
Thus some degradation of ^{3}H-dsRNA may have begun in the cytoplasm, although the effect of lipid, which was present, on quenching of radioactivity was not evaluated. All the label in the ileal cells was hydrolysed by NaOH.

Table 38 - Labelling of mouse spleen cells 6 h following i.v. administration of ^{3}H-dsRNA.

		I	II
Spleen wt. (mg)		70	160
No. of cells in tissue sample		6.5×10^7	5.5×10^7
^{3}H-c.p.m./10^7 cells:	Cells	1,420 (1,380)	1,850 (1,530)
	Nucleus	510 (480)	580 (760)
	Cytoplasm	420 (270)	680 (450)
	Extracellular	11,000 (9,000)	19,600 (17,200)

Two CBA mice weighing 23g were injected i.v. into the tail vein with 60 µCi ^{3}H-dsRNA (310 µCi/mg), 3 days following the administration of 0.1ml sheep red blood cells (II) or an equal volume of saline (I). After 6 hours the mice were sacrificed and cell suspensions prepared from portions of the spleens (containing approximately 6×10^7 cells). The spleen cell washings ("extracellular") were analysed for total and TCA-insoluble radioactivity, together with aliquots from the cells, nuclear and cytoplasmic fractions. TCA-insoluble c.p.m. are given in parentheses.

Figure 33 - CS density gradient centrifugation of the nucleic acids extracted from the spleen cell nuclei of a mouse, 6 h after i.v. ^{3}H-dsRNA administration.



Two 23g CBA mice were injected i.v. into the tail vein with 60 µCi ^{3}H-dsRNA (310 µCi/mg), 3 days after administration of 0.1ml sheep red blood cells (II) or an equal volume of saline (I). After 6 h, the mice were sacrificed and cell suspensions were prepared from portions of the spleens. Nucleic acids were extracted from the nuclear fraction of these cells by the modified Marmur procedure, and centrifuged to equilibrium on CS density gradients.

——— absorbance (260nm); ····· radioactivity; -·-·- buoyant density.

The nucleic acid extracted from the nuclei of the ileal cells, when analysed on CS density gradients, gave the banding profiles shown in Figure 34. Almost all the label equilibrated at a density of 1.61, showing that the ^{3}H-dsRNA was present in the ileum mainly as intact dsRNA. No labelling of DNA occurred in the nuclei of the ileal cells, an observation consistent with the persistence of undegraded ^{3}H-dsRNA.

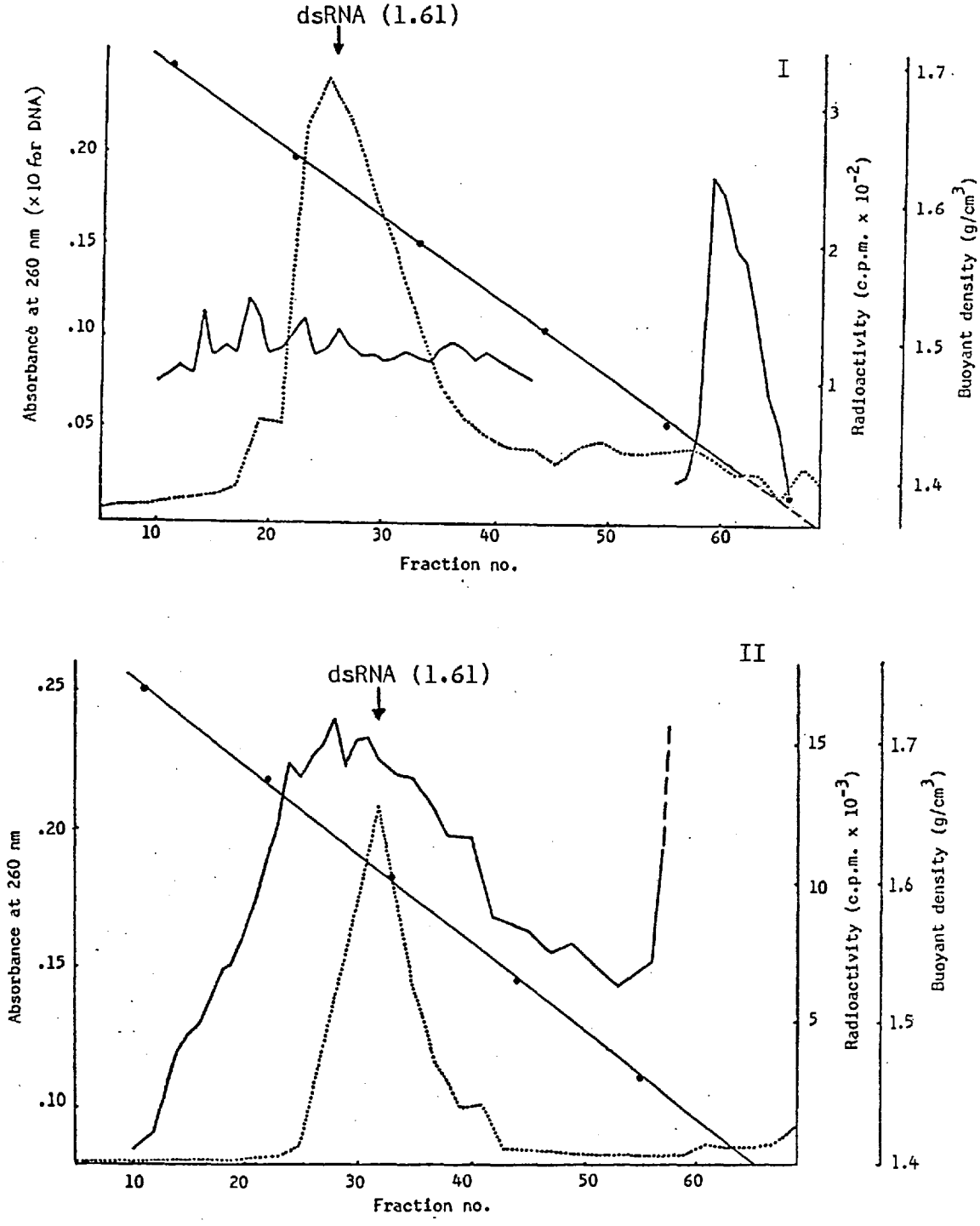
The fractions in the CS gradients containing labelled RNA were pooled together, dialysed and concentrated appropriately for hydroxylapatite chromatographic analysis of the macromolecular label. The results of this analysis, shown in Table 39 , confirm that the ^{3}H-dsRNA was largely converted (approximately 60%) to ssRNA in the spleen cells, while only 2-6% was converted in the cells of the ileum.

6. Autoradiography of tissue sections

Sections of all tissues taken 6 hours after i.v. ^{3}H-dsRNA administration contained label. The liver and spleen contained most radioactivity, and the thymus and sternal bone marrow the least. Increased numbers of WBC, both lymphocytes and polymorphs, were present in the small blood vessels and sinusoids of the liver. Most of the radioactivity in the liver was associated with these WBC in the blood spaces, and with the Kupffer cells. Surrounding parenchymal cells were poorly labelled, if at all. Major blood vessels contained low levels of radioactivity at this time. The labelled WBC appeared to be thrombosed in some of the smaller vessels.

The spleen was well labelled throughout, with a slightly higher grain density in the red pulp than the white. Blood capillary spaces were again heavily labelled, as were hemosiderin-containing macrophages. Hemosiderin deposition was seen in the liver, spleen and lung, being particularly dense around the margins of the splenic nodules in the srbc-immunised mouse. The lung was labelled throughout, particularly in the capillary spaces, although very little radioactivity was seen in the alveolar lining cells and bronchi. Sections of thymus and bone marrow were poorly labelled; lack of label in the sternal bone marrow was surprising since a reasonable level of incorporation of macromolecular material had been found in bone marrow cell suspensions prepared from the femurs (Table 36).

Figure 34 - CS density gradient centrifugation of the nucleic acids extracted from the ileal cell nuclei of a mouse, 6h after i.v. ^{3}H-dsRNA administration.



Injection schedule as for Figure 33. Nuclei were prepared from sections of the damaged ileum 6h following ^{3}H-dsRNA administration. Nucleic acids were extracted (Marmur) and centrifuged to equilibrium on CS density gradients.

——— absorbance (260nm); ····· radioactivity;

•— — —• buoyant density.

Table 39 - Hydroxylapatite chromatographic analysis of the macromolecular ^{3}H-RNA from the nuclei of spleen and ileal cells following i.v. ^{3}H-dsRNA administration.

Tissue	% low mol. wt. ^{3}H-c.p.m.	Macromolecular ^{3}H-c.p.m.	
		% ssRNA	% dsRNA
Spleen I	0.6	60.6	39.4
Spleen II	0.0	56.6	43.4
Ileum I	0.4	5.7	94.3
Ileum II	0.1	2.3	97.7
^{3}H-dsRNA control	0.2	0.9	99.1

For injection schedule see Table 38. RNA-containing fractions from the CS density gradients shown in Figures 33 and 34 were pooled, dialysed against 0.1 x PBS, concentrated by evaporation and redialysed against 0.012 M potassium phosphate buffer, pH 6.8, for hydroxylapatite chromatography.

In the ileum sections, ^{3}H-dsRNA was found in the lamina propria at the base of, and extending into, the villi. Cell-associated label appeared not to be predominantly attached to the dividing cells of the villi, but rather to infiltrating polymorphs and lymphocytes in the mucosa and crypts. Particularly highly-labelled areas were also seen at the tips of the villi, which were flattened and swollen in places. Necrotised cells, with associated label, appeared to be sloughing off from the villi into the gut lumen, which contained much debris.

7. Discussion

From these preliminary observations, a general picture of the fate of dsRNA in mice could be built up which may serve as a useful working hypothesis for future comprehensive investigations. Six hours following a single i.v. injection of 5-10 mg/kg dsRNA in the mouse, the peripheral blood showed an acute and dramatic lymphopenia, and the small intestine was damaged. Only 10% of the injected dsRNA dose remained in the plasma, but this disappearance could not be accounted for by enzymic catabolism since the dsRNA retained a high molecular weight at this time. Urinary excretion should be considered quantitatively in any thorough analysis. It was possible to obtain urine from the bladder of one mouse injected 6 hours previously with ^{3}H-dsRNA; this was found qualitatively to be TCA-insoluble.

^{3}H-dsRNA therefore passed from the blood to the tissues in an intact form during the first 6 hours. Increased numbers of WBC were found in the liver, spleen, lung and intestine, at a time when decreased numbers were present in the blood. Radioactivity was associated largely with these sequestered cells and with tissue macrophages. It seems logical to propose that ^{3}H-dsRNA injected into the circulation became associated with WBC which were then trapped in the blood capillaries and sinuses of the various organs, particularly the reticulo-endothelial system.

Label in the spleen after 6 hours was largely extracellular, and the nature of this label *per se* was not examined. The intracellular label in the spleen was undergoing extensive breakdown and re-utilisation for nucleic acid biosynthesis, particularly in the spleen cells of a mouse undergoing a proliferative response to srbc. Tissue macrophages were

probably the major centres of dsRNA catabolism, as large, active, well-labelled cells were seen in spleen and liver sections. These may degrade ^{3}H-dsRNA which they encounter both in free form and associated with the WBCs, and release the products to the surrounding cells for re-utilisation.

Persistence of intact ^{3}H-dsRNA in the small intestine was temporally associated with severe but transient gut damage. Recovery of the ileum after a 65 µg dose of dsRNA was faster than from a 200 µg dose. It would be interesting to follow the biochemical fate of ^{3}H-dsRNA in the intestine during recovery; the material might be expected to be metabolised as cell repair and proliferation takes place, or as macrophages move into the damaged tissue areas.

DISCUSSION AND CONCLUSIONS

P. chrysogenum dsRNA, as prepared by Beecham Pharmaceutical Research Division (BRL 5907) is both highly purified and very homogeneous, with a well-defined molecular weight and base composition. It is essentially free from protein and DNA, and contains traces of carbohydrate. The dialysable impurity that was found to inhibit the transformation of freshly-prepared lymphocytes must therefore be biologically active in very small amounts. It was not possible to recover any inhibitory material by lyophilisation of the dialysate, and its precise identification was beyond the scope of these studies. The impurity was unstable to heating at 100°C for 20 minutes, since after such treatment, most of the inhibitory effect of undialysed dsRNA was lost.

These studies underline the need for caution in the use of "purified" naturally-occurring materials for tissue culture experiments, and the dangers in drawing conclusions from studies using impure biological preparations. The dsRNA retained both its physical structure and biological activity after dialysis. The removal of the impurity had no effect on the in vivo toxicity of dsRNA in mice, the toxic effects being very similar to those observed following administration of poly I. poly C (Philips et al, 1971). It is not known how far this dialysable impurity contributes to the other in vitro cytotoxic properties of naturally-occurring dsRNAs. For example, Content et al (1978) showed that the inhibition of protein synthesis in rabbit reticulocyte lysates by naturally-occurring dsRNAs, including that from P. chrysogenum, was greater by several orders of magnitude than the inhibition by synthetic double-stranded polynucleotides of similar size. It may be that the relatively poor production of interferon in tissue culture by P. chrysogenum dsRNA compared to poly I.poly C (Thang et al,1977; De Clercq and Torrence, 1977) is due to toxic impurities in the natural materials.

Dialysed dsRNA inhibited DNA synthesis in MS cells stimulated by ConA or by allogeneic cells in serum-supplemented medium. The inhibition of ^{3}H-thymidine incorporation was somewhat variable from one experiment to another, and it was not possible to assess the contribution of competing unlabelled precursors arising as a result of degradation of dsRNA by serum. Poly I.poly C and poly A.poly U at 100 µg/ml were also found to suppress ^{3}H-thymidine incorporation in ConA-stimulated MS cells, without significantly affecting cell yields at 48 hours.

There have been very few reports of the effects of dsRNAs on lectin-stimulated lymphocyte transformation, and the experiments published have all been conducted in serum-supplemented medium. Poly I.poly C was found to inhibit DNA (and RNA) synthesis at 50-150 µg/ml in PHA-stimulated human lymphocytes (Badger, Cooperband, and Green, 1972). However, in this study the poly I.poly C was incubated with the cells for 3-6 days in medium with 10% FCS, and ^{3}H-thymidine incorporation was measured during a 24 hour period; both poly I.poly C and ^{3}H-thymidine would have been degraded in the medium under these circumstances. Chess et al (1972) and Friedman et al (1969) reported that 100 µg/ml poly A.poly U inhibited DNA synthesis in human leucocytes stimulated with PHA. In a very recent publication, O'Malley et al (1979) showed that poly I.poly C and its mismatched analogues, at concentrations of 10^{-4} to 10^{-7}M, had no effect on ^{3}H-thymidine incorporation by PHA-stimulated human peripheral blood leucocytes, but the polymers were not added until 72 hours of transformation and ^{3}H-thymidine incorporation, measured at 75-91 hours, was the only parameter examined.

^{3}H-thymidine incorporation is widely used as a measure of the blastogenic response in stimulated lymphocyte cultures. However DNA synthesis (^{3}H-thymidine incorporation) does not correlate absolutely with cell division in these MS cell cultures; autoradiographic analysis has shown that while 90% of the cells are synthesising DNA, only 25% of them are actually in division cycle (Harris and Olsen, 1976; Rogers et al, 1972). Autoradiographic analysis of dsRNA-treated cells should therefore provide useful information on the mechanism of inhibition of DNA synthesis and cell division.

The protective effect of FCS in these experiments may be ascribed to two factors; first, it degrades dsRNA to ssRNA and smaller fragments which are perhaps less biologically active, and secondly, by improving the viability of lymphocytes in culture it may thereby increase their resistance to cytoxic agents. Cell death may account in part for the lack of an absolute increase in cell number seen in serum-supplemented lymphocyte cultures, especially at high initial cell concentrations (Bernheim et al, 1978), and viability is decreased even further in the absence of serum. In order to preserve molecular integrity of dsRNA, experiments should ideally be conducted in serum-free media. Perhaps canine serum, which has no detectable degradative activity against dsRNA (Douthart and Burgett, 1978; Cunnington and Naysmith, 1975) could be used as a substitute for calf serum in these cultures, provided that the background stimulation due to the presence of foreign antigens was sufficiently low.

In the absence of serum, 100 µg/ml dsRNA (dialysed) was toxic to MS cells if added at the initiation of transformation, but not if added after 30 hours. In the former case, inhibition of DNA synthesis per culture was severe (80%) and was associated with inhibition of cell division, while in the latter, inhibition of DNA synthesis was considerably less (26%) and cell division was unaffected. However, the optimum concentration of ConA for transformation in serum-free medium may be lower than that in serum-containing medium, so that the serum-free cultures may not have been examined under strictly optimal conditions. With this reservation, the results showed that lymphocytes already actively engaged in DNA synthesis were far more resistant to the inhibitory effects of dsRNA than freshly-isolated lymphocytes in a quiescent state.

The growth of V79 and LDV cells, which form more homogeneous cultures of viable, dividing cells than MS cell cultures, was also relatively insensitive to the effects of dsRNA. Following exposure to high concentrations of dsRNA, V79 and LDV cells remained morphologically unchanged and no significant, irreversible effects on cell growth were observed. Therefore cells synthesising DNA may be able to resist the cytoxic effects better than resting cells. There is some evidence in the literature to support this suggestion, from studies on the effect of poly I.poly C on cell division.

Levy et al (1970) found that ^{3}H-thymidine incorporation could be either suppressed (in rabbit kidney and mouse embryo cells) or enhanced (in mouse L cells) by poly I.poly C. The effects occurred without a change in cell growth rate, which attests either to the flexibility of living cells or to the possibility that the measurement reflected changes in permeability or precursor pool sizes, rather than in macromolecular synthesis. Poly I.poly C was reported to increase ^{3}H-thymidine incorporation in HeLa and mouse L cells (Badger, Cooperband, and Green, 1972); however, the increase in ^{3}H-thymidine uptake was directly proportional to the cytotoxic effect of poly I.poly C, and could therefore be interpreted as the consequence of DNA damage and the attempt at repair. Under more rigorous conditions, in synchronised HeLa cell cultures, poly I.poly C at a non-toxic concentration (5-12 µg/ml) actually inhibited DNA synthesis, specifically at the late G_0 and early S phases of the cell cycle. (Teng et al, 1973). In these cells, single-stranded polynucleotides and native DNA were inactive, and poly I.poly C had no effect on RNA and protein synthesis. ^{3}H-thymidine incorporation was inhibited if the poly I.poly C was added immediately after reversal of the thymidine block used to synchronise the cells, but not if addition was delayed until two

hours or more after the beginning of S phase. Thus the effect of poly I.poly C on DNA synthesis was apparently limited to a specific period of the cell cycle.

The observation that MS cells were less sensitive to dsRNA after DNA synthesis had begun than at the beginning of transformation is also supported by the results of studies on the effect of poly I.poly C on DNA synthesis in vivo. Intraperitoneal administration of a single non-toxic dose of poly I.poly C caused dramatic inhibition of isoproterenol-stimulated DNA synthesis in mouse salivary glands, when given from 10 minutes before to 18 hours after the injection of isoproterenol (Serota and Baserga, 1970). If poly I.poly C administration was delayed until DNA synthesis had begun (after 20 hours), no inhibition of DNA synthesis occurred. Poly I.poly C was also reported to inhibit mitosis in liver cells following partial hepatectomy (Jahiel et al, 1971). Since mitosis was also inhibited by other interferon inducers, namely Newcastle disease virus and statolon (dsRNA from P. stoloniferum), the cell growth inhibitory effect, in this study at least, may have been mediated by interferon.

The inhibitory effects of interferon on cellular proliferation and DNA synthesis are well documented. It suppresses the proliferation of transformed mouse and human cell lines (Gresser, et al, 1970; Adams et al, 1975), and inhibits DNA synthesis in normal mouse and human fibroblasts (Sowaka et al, 1977; Lundgren et al, 1979). Sowaka et al (1977) showed that interferon suppressed the G_0-G_1 transition in cycling 3T3 cells. More recently, Lundgren et al (1979) demonstrated an inhibitory effect of interferon on synchronised human fibroblasts in the early G_1 phase, before the cells were committed to DNA synthesis. It had no effect if added after the late G_1 phase. These results suggest that interferon, as well as poly I.poly C, inhibits DNA synthesis at an early regulatory event rather than during the process of DNA replication itself.

If dsRNA were to induce interferon production in MS cell cultures, its inhibitory effect on DNA synthesis in ConA-stimulated MS cells might be mediated by endogenously-induced interferon. The inhibitory effect of interferon on in vitro immune responses and lymphocyte transformation is well known. It inhibits MS cell transformation in response to ConA, PHA, or allogeneic cells (Rozee et al, 1973; Lindhal-Magnusson et al, 1972). Miörner et al (1978) showed that interferon inhibits ConA-stimulated human lymphocytes at an early stage of transformation. Thus it is possible that dsRNA could inhibit the mitogenic response through the early induction of interferon. Such a mechanism

has been shown to account for the inhibition by double-stranded polynucleotides of the primary *in vitro* antibody response to srbc or LPS (Johnson *et al*, 1975); the inhibition was mediated by an effect of the polynucleotide on T-cells, and was abrogated by antibody to interferon, suggesting that the inhibition was brought about by the intermediary production of interferon from T-cells (Johnson and Baron, 1976).

In the absence of ConA, *P. chrysogenum* dsRNA stimulated ^{3}H-thymidine incorporation above background levels in serum-supplemented MS cell cultures. This reflected a true mitogenic response, since the cell number per culture also increased significantly above control values. This finding agrees with previous reports of the mitogenic effect of poly I.poly C on unstimulated spleen cells (Dean, Wallen and Lucas, 1972; Woods *et al*, 1974; Ts'o *et al*, 1976). The increase in ^{3}H-thymidine incorporation was very much lower than that induced by PHA or ConA, but significantly higher than that in unstimulated cultures. Poly A.poly U was shown to be mitogenic for MS cells (Han and Johnson, 1975), and to enhance human lymphocyte activation by soluble antigens or allogeneic cells (Friedman *et al*, 1969). There seems to be general agreement that these polyribonucleotides augment the response of lymphocytes to soluble or cellular antigens, while inhibiting the response to non-specific T-cell mitogens (Friedman *et al*, 1969; Chess *et al*, 1972; Badger, Cooperband and Green, 1972). The difference may be due to the activation of macrophage function by dsRNAs, necessary for the former, but not the latter response. dsRNA has been reported to activate macrophages *in vitro*, by rendering them cytotoxic to tumour cells. *

A primary site of action on the macrophage in spleen cell cultures may also explain differences in the lymphocyte type reported to be stimulated by dsRNAs. Scher *et al* (1973) claimed that poly I.poly C was a specific B-cell mitogen, and indirect immunofluorescence has been used to show that the subset of murine splenic B-cells converted to lymphoblasts by poly I.poly C have specific membrane receptors for poly I.poly C (Diamantstein and Blitstein-Willinger, 1978). On the other hand, poly A.poly U is believed to activate T-cells (Cone and Johnson, 1971, 1972), and has been shown to bind to thymocyte membranes by immunofluorescent techniques (Mutchnik, Han and Johnson, 1977).

There is good evidence for the production of interferon (Type II) in MS cell cultures stimulated by mitogens (Wallen, Dean and Lucas, 1973; Stobo *et al*, 1974), the peak of production occurring 24 hours prior to the peak of DNA synthesis. Induction of interferon synthesis by ConA

* (Alexander and Evans, 1971)

could therefore provide feed-back regulation of the lymphocyte proliferative response, resulting in eventual mitotic arrest and cell death. A similar feed-back role of interferon in regulation of the in vitro and in vivo antibody response has already been proposed (Brodeur and Merigan, 1975; Johnson and Baron, 1976). Heine and Adler (1977) provided some experimental support for a regulatory role of interferon on lymphocyte transformation, by showing that the decrease in response of spleen cells to mitogen with increasing age of mice was accompanied by increased interferon production in the cultures. The cells producing interferon and those undergoing DNA synthesis apparently reside in different lymphocyte subsets; ConA transforms T-cells but induces interferon from B-cells through a T-cell dependent function, while PWM induces and transforms B-cells directly (Wallen, Dean and Lucas, 1973). It is interesting to speculate that the inhibition of ConA-stimulated lymphocyte transformation by dsRNA could be brought about by the dsRNA-induced interferon, perhaps produced from a different cell type than that responding to ConA. This would then hasten the feed-back inhibition of cellular proliferation programmed by the ConA-induced interferon. It would be necessary to measure the kinetics of murine interferon production and the effect of antibody to interferon in the present system, in order to examine this possibility. dsRNA has been shown to induce interferon production in human leucocyte cultures (Taborsky and Dolnik, 1977; O'Malley et al, 1979).

Like the other double-stranded polynucleotides, P. chrysogenum dsRNA inhibited mitogen-stimulated transformation and was mitogenic for unstimulated MS cells, although both effects were not impressive in serum-containing medium and required relatively high in vitro concentrations. Its effect on mitogen-stimulated MS cells has not previously been reported, and no detectable mitogenic effect on lymphocytes was noted in a previous study (Cunnington, 1977), although the dsRNA used in that study was undialysed and caused cytotoxicity at 100 µg/ml.

Further insight into the interaction of dsRNA with lymphoid cells and fibroblasts was gained by examining the uptake and fate of ^{3}H-adenosine-labelled dsRNA in the presence and absence of serum. The method suggested to me for measuring the incorporation of radioactive precursors and nucleic acids by cells was the filter paper disc technique, popularised by Bollum (1968), which allows the rapid batch-wise TCA-precipitation of label in many different samples, avoiding time-consuming centrifugation and washing procedures. Since the discs are counted

directly in scintillant, a certain degree of counting efficiency is sacrificed by the omission of sample solubilisation. The method proved to be efficient and reproducible for measurement of ^{3}H-thymidine incorporation. One disadvantage of this technique was the lack of sensitivity for quantitative assessment of the state of ^{3}H-dsRNA degradation in tissue culture medium or in cells. This was apparently due to both quenching of label by medium components dried onto the discs, and the intramolecular quenching which may be observed in labelled macromolecules. In future work it would be advisable to solubilise the label from TCA-treated and untreated discs using a tissue solubiliser, or perhaps to remove quenching agents by combustion of the discs in a sample oxidiser, and collection of $^{3}H_2O$.

^{3}H-dsRNA was efficiently converted into MS cell DNA and RNA in serum-containing cultures. Conversion to DNA was shown to result from reduction and subsequent polymerisation of the ribonucleotide products of ^{3}H-dsRNA degradation, being inhibited by hydroxyurea. Appearance of very low levels of label in DNA was occasionally observed in cells labelled in serum-free medium. This DNA-associated label was too small to permit digestion and chromatography. Since it was always accompanied by solubilisation of ^{3}H-dsRNA in the cytoplasm, it was likely to occur via the same pathways. The very much slower rate of ^{3}H-dsRNA utilisation in serum-free cultures was a consequence of the inability of the MS cells to degrade dsRNA, since ssRNAs were converted to DNA in the absence of serum.

^{3}H-dsRNA was degraded in the medium of serum-containing cultures to products of approximate molecular weight 1×10^{5} after an 18 hour incubation period. Labelled RNA predominantly of this size or smaller was also found in the cell cytoplasm. This material was largely single-stranded and probably consisted of ^{3}H-dsRNA degradation products and newly-synthesised MS cell RNA. Nuclear label consisted of DNA and MS cell RNA of heterogeneous molecular weight, there being no significant amount of intact ^{3}H-dsRNA present after 24 hours.

Under serum-free culture conditions, where ^{3}H-dsRNA in the medium remained double-stranded, albeit of a slightly diminished size, MS cells took up the material to a far lesser extent than in serum-containing medium. The ^{3}H-dsRNA remained largely double-stranded in the nuclei (87 - 92%) and became further reduced in molecular weight to approximately 1.4×10^{6} after 24 hours. Although membrane binding (estimated by uptake at 0°C) was shown to account for about 50% of the observed cellular uptake (half of which was irreversible), the other 50% was genuine metabolic incorporation since it occurred at 37°C, was shown to be inside cells and

associated with isolated nuclei in autoradiographs, and was sometimes accompanied by partial conversion of label to MS cell DNA.

The uptake of dsRNAs by primary spleen cell cultures has received little attention. Demonstration of the uptake of "immune RNA" (single-stranded) has provided experimental support for the theory of transfer of immunologic information via RNA (Wang et al, 1973). In this study unstimulated rabbit spleen cell cultures took up homologous rabbit lymph node ^{3}H-ssRNA and B. subtilis ^{3}H-ssRNA, during a 15 min pulse in serum-free medium. The homologous RNA was more readily incorporated than the bacterial RNA. In both cases, ^{3}H-ssRNA remaining in the medium was totally degraded after 15 min, as shown by sucrose density gradient centrifugation. Cell-associated B. subtilis ^{3}H-RNA was also degraded, while part of the lymph node ^{3}H-RNA remained of high molecular weight (S >12). Since the same results were obtained in the presence of actinomycin D, the high molecular weight material associated with the cells was felt to be exogenous ^{3}H-RNA rather than RNA synthesised de novo from degradation products.

The problem of substrate degradation has been encountered in many previous studies on the uptake of nucleic acids, especially ssRNA, by mammalian cells in culture (Bhargava and Shanmugam, 1971). Even in the absence of serum, cell-produced enzymes may degrade exogenous nucleic acids. Like the rabbit spleen cells used by Wang et al (1973), the MS cells employed in the present study were found to contain and release an enzyme capable of degrading ^{3}H-ssRNA and ^{3}H-poly C. The nuclease had only limited activity against ^{3}H-dsRNA. Conditioned medium from MS cell cultures caused ^{3}H-dsRNA to band at a slightly lower molecular weight position on sucrose density gradients. This was thought to be due to actual limited enzymic activity, rather than a gradient artefact, since it was dependent on temperature and on the presence of cell lysate or conditioned medium. This cleavage could result from specific nucleolytic attack, analogous to restriction endonuclease activity.

The results in section II show that dsRNA persists in a high-molecular weight, double-stranded form in stimulated lymphocytes for at least 24 hours. Preliminary results of a similar nature have also been obtained in cultures of rabbit spleen explants labelled with ^{3}H-dsRNA (Harris and Olsen, unpublished observations). The characteristics of this explant system have been fully described (Harris, 1973; Harris and Olsen, 1973). Antibody-producing cells undergoing an in vitro immune response to srbc migrate out from tissue explants after 24-48 hours. Such outgrowth cells, incubated with ^{3}H-dsRNA for 24 hours in medium

containing 10% FCS, incorporated label which became converted to cellular RNA and DNA. In serum-free medium, however, ^{3}H-dsRNA uptake was reduced, and the nucleus-associated label was intact ^{3}H-dsRNA, with a buoyant density of 1.61 and a molecular weight of 1.6×10^{6}. Although the degree of membrane binding was not assessed, intracellular localisation of this label was considered to be a real observation because the nucleus-associated label remained largely as intact ^{3}H-dsRNA, with a low level of conversion to DNA, during a chase period in which the labelled cells were washed and re-incubated in medium containing 10% FCS for 48 hours.

These results therefore confirm the findings in MS lymphocytes. The rabbit explant cells apparently remained more viable than MS cells after long periods of labelling with ^{3}H-dsRNA in serum-free medium. Therefore this culture system would provide the better model for examining nuclear penetration, and the relationship between persistence of high molecular weight dsRNA in lymphoid cells with its biological effects, such as interferon induction and modulation of the antibody response.

Fenster et al (1975) reported the penetration of intact radio-labelled poly A.poly U by unstimulated human lymphocytes during a 40 minute labelling period, and its persistence in the cell nucleus for a further 2 hours. However, these results may be criticised on the following grounds. Incorporation of poly A.poly U was measured in the presence of 50 µg/ml DEAE-dextran, because this substance is thought to increase cell penetration of nucleic acids (Colby and Chamberlain, 1969). However, DEAE-dextran forms complexes with dsRNAs (Pitha and Carter, 1971), and these bind extensively and non-specifically to both cell membranes and culture vessel surfaces (see below). It seems likely that such complexes would contaminate cells and nuclei at all stages of the cell fractionation procedure. Nucleus-associated label remained as intact poly A.poly U for 2 hours. No autoradiographic confirmation of nuclear penetration was carried out, no assessment of cell viability was made, and there were no controls for non-specific association of poly A.poly U with nuclei during fractionation. The DEAE-dextran also protected the polynucleotide against ribonuclease degradation, since in its absence, poly A.poly U incorporated into the lymphocyte nuclei was shown to become nearly 50% TCA-soluble after a 30 minute pulse. This rapid catabolism of poly A.poly U by lymphocytes may perhaps explain why poly A.poly U is generally found to be a poorer interferon inducer than poly I.poly C and naturally-occurring dsRNAs.

The use of DEAE-dextran to increase penetration of ^{3}H-dsRNA into MS cells was considered unsuitable because preliminary measurements showed that if DEAE-dextran was present at 50 µg/ml during a 24 hour labelling period, 100% of the input ^{3}H-dsRNA pelletted with the MS cells, whether FCS was present or not. The non-specific association of ^{3}H-dsRNA at 0°C was increased from 0.2% (Table 28) to 12% (data not shown). Therefore it was felt that DEAE-dextran would increase non-specific membrane binding of RNase-resistant ^{3}H-dsRNA, which would mask any low level of real cellular penetration of intact ^{3}H-dsRNA.

In a well-controlled study of the interaction of labelled DNA with fibroblast monolayers, Ehrlich et al (1976) showed that DEAE-dextran dramatically increased the binding of DNA to the monolayers. However, most of the donor DNA was adsorbed to the plastic culture vessels, and remained attached to the surface after removal of cells with trypsin. More than 95% of the cell-associated DNA was bound to the membrane, and upon cell fractionation, the small percentage found in the nuclei was shown to be non-specifically associated, since it was displaced with excess unlabelled DNA. DEAE-dextran forms similar complexes with P. chrysogenum ^{3}H-dsRNA. These adsorb non-specifically to glass and plastic culture vessels (author's unpublished observations), and would thus hinder any analysis of the biochemical fate of dsRNA in cells.

The persistence of foreign genetic information in the form of dsRNA in lymphocyte nuclei poses a potential safety problem in the administration of naturally-occurring dsRNAs to man, if the material is not adequately degraded by serum nucleases. However, there was no evidence for integration of sections of dsRNA into the DNA of the recipient cells, although sensitive hybridisation techniques would be needed to study this problem more thoroughly. Label entering the cellular DNA did so by a process of degradation and re-utilisation. However, it is conceivable that genetic information, if not label, could be transferred to DNA by a process of reverse transcription. The dsRNA would need to be rendered single-stranded then copied by an RNA-dependent DNA polymerase. Such an enzyme, while generally associated with the retroviruses, has been found in normal cells (Nelson, Leong and Levy, 1978).

The persistence of ^{3}H-dsRNA in MS cells may have accounted for the death of a proportion of these cells, as observed in autoradiographs. These studies highlight the importance of carrying out autoradiography in parallel with biochemical analysis of the incorporated label, since it was only by such means that the heterogeniety of labelling and the contamination of nuclear preparations with labelled, dead cells was appreciated. It was not possible to decide whether these cells had died

as a result of the accumulation of non-metabolisable ^{3}H-dsRNA (that is, from a direct cytotoxic effect of the ^{3}H-dsRNA itself) or had died as a result of the sub-optimal serum-free culture conditions and then adsorbed ^{3}H-dsRNA to the cell membranes. Dead cells without any label were also present in both serum-free and serum-containing cultures, therefore unless cells which die at a particular phase of the cell cycle preferentially absorb dsRNA, cell death in culture alone was not sufficient to induce membrane binding of dsRNA. Furthermore, no gross association of grains with cell membranes was seen in autoradiographs of cell sections, and MS cells killed by incubation with 50 µg/ml undialysed dsRNA at 0 hours of transformation did not take up ^{3}H-dsRNA.

Increased binding of RNA to dead cells has been reported in the literature. Juliano and Mayhew (1972) noted a correlation between uptake of ssRNA by Ehrlich ascites tumour (EAT) cells and loss of cell viability in the culture. Detergent-killed EAT cells bound 5-10 times as much RNA as viable cells. Although no autoradiography was performed, this uptake of RNA was assumed to represent genuine incorporation, because the labelled RNA failed to exchange with unlabelled RNA and did not affect the electrophoretic mobility (surface charge) of the cells. It seems likely that detergent treatment, as well as heat-treatment, results in membrane alterations different from those induced by natural cell death. Strong association of ^{3}H-dsRNA with heat-killed MS cells was due to membrane binding only, as although it was not completely displaced by unlabelled RNA, it was seen entirely in a peripheral distribution in autoradiographs. No such labelling was observed in control cultures.

It was therefore concluded that cells lost viability in serum-free medium due primarily to the accumulation of ^{3}H-dsRNA, either inside them or on the membranes. The mechanism of toxicity was not understood, since unlabelled dsRNA, even at 100 µg/ml, was not particularly inhibitory to MS cells when added after 30 hours of culture, (although cell viability *per se* was not measured directly). ^{3}H-dsRNA would not have contained the dialysable impurity, since during its purification, the labelled dsRNA is additionally chromatographed through Sepharose 2B to remove low molecular weight impurities. In any case the amount of ^{3}H-dsRNA used in labelling experiments was always between 3 and 10 µg/ml, at which level even the undialysed dsRNA was not significantly toxic. For the cytotoxicity to occur through radiation damage, ^{3}H-dsRNA would need to localise in the cell nuclei in close association with DNA, where β-particle emission, with its short track length, could influence

nucleic acid turnover. Thus, accumulation of ^{3}H-dsRNA in MS cell nuclei may have induced some radiation damage. In support of this suggestion, it has been found that the ^{3}H-labelled dsRNA is more inhibitory than unlabelled dsRNA to the production of antibody-producing outgrowth cells in rabbit spleen explant cultures undergoing an *in vitro* immune response (G. Harris, personal communication).

It was not possible, in these heterogeneous MS cell cultures, to show the nature of the RNA label in any particular cell or cell type. For example, the large viable transformed cells were probably responsible for the low level of degradation of ^{3}H-dsRNA and conversion to DNA observed in some experiments under serum-free culture conditions, since isolated nuclei from such cells were certainly labelled in autoradiographs. This would be in line with the observations of Olsen and Harris (1974), that the uptake and metabolism of bacterial ^{3}H-DNA by human lymphocytes was greater and more rapid in Con A-stimulated cultures than in unstimulated ones, and that label was confined solely to the blast cells. MS cells at an earlier stage of transformation may have taken up ^{3}H-dsRNA and been unable to degrade it, due to lack of the appropriate enzyme machinery at that time, and thus have been killed by it. More sophisticated techniques would be required to examine the nature of the RNA label in a particular cell. Antibodies specific for dsRNA, but not ssRNA or DNA, may be used in conjunction with a fluorochrome-conjugated antiserum to localise dsRNA in cells or cell sections. Silverstein and Schur (1970) have used such indirect immunofluorescence to demonstrate native dsRNA *in situ* in Reovirus-infected L929 cells. The technique is now sufficiently sensitive to detect the fraction of dsRNA occurring naturally in heterogenous nuclear RNA, situated in the nucleoplasm of normal Vero cells (Stollar *et al*, 1978). The application of such a technique to labelled lymphocytes should now ideally be used to confirm the intranuclear localisation of ^{3}H-dsRNA.

Transformation was necessary for the uptake of ^{3}H-dsRNA by lymphocytes, but DNA synthesis *per se* was not a prerequisite, at least for uptake in the presence of serum, since incorporation and conversion to MS cell RNA took place in hydroxyurea-inhibited cells. It is interesting to note that transformation is also a prerequisite for the replication of viruses in lymphocytes (Hirsch *et al*, 1972; Wheelock *et al*, 1971), and for the production of type II interferon from lymphocytes (reviewed by Epstein, 1977). Because of the association of label with metabolically active cells, it was predicted that rapidly dividing cell lines would incorporate substantial amounts of ^{3}H-dsRNA, and this was borne out by the results obtained in the established cell lines.

The uptake and fate of ^{3}H-dsRNA in V79 and LDV cells differed from that in stimulated MS cells in three major respects. First, the cell lines incorporated, on average, 10-fold more label per 10^{6} cells than the mouse cells in 24 hours. In the presence of serum, MS cells incorporated between 2 and 10% of the available ^{3}H-dsRNA, equivalent to approximately 1 µg dsRNA per 10^{7} cells, while V79 cells took up approximately 10 µg dsRNA per 10^{7} cells. The figure for V79 cells may be a minimum, since the amount of ^{3}H-dsRNA was probably rate-limiting for uptake. Quantitative comparisons were difficult to make in view of the quenching problems discussed earlier. Secondly, the cell lines showed a more homogeneous labelling pattern than MS cells. Autoradiographs showed that every cell incorporated ^{3}H-dsRNA, to approximately the same degree. This observation was predictable given the greater homogeneity and viability of cultures of established cell lines compared with primary lymphocyte cultures. Thirdly, the metabolic fate of ^{3}H-dsRNA in cells cultured without serum was different in the two cell types. V79 and LDV cells rapidly catabolised ^{3}H-dsRNA and re-utilised the products for DNA synthesis, while MS cells catabolised it very slowly, if at all. The greater activity of V79 cells in this respect was due to their higher nuclease activity, present both in the V79 cell cytoplasm and released into the culture medium.

In serum-containing medium, ^{3}H-adenosine and ^{3}H-dsRNA degradation products were re-utilised largely for DNA synthesis in MS cells, and for RNA synthesis in the cell lines. The ratio of DNA:RNA synthesis is therefore higher in stimulated MS cells at 30-48 hours than in unsynchronised fibroblasts and lymphoblastoid cells, although this relatively enhanced level of DNA synthesis is not necessarily associated with cell division (Harris and Olsen, 1976). In the absence of serum, MS cells failed to convert ^{3}H-dsRNA to DNA due to their inability to degrade dsRNA. Lack of conversion to DNA by V79 cells (in confluent cultures during a 4 hour labelling period) was not due to an inability to degrade ^{3}H-dsRNA, since the label was still re-utilised for synthesis of V79-type ssRNA. Lack of DNA labelling was therefore due to the absence of active DNA-polymerisation in the cells at that time.

Because growth becomes contact-inhibited at high cell densities, fibroblasts at stationary phase have a reduced rate of mitosis, and rates of DNA, RNA and protein synthesis are markedly reduced. However, the addition of fresh serum to confluent cells at stationary phase has been shown to initiate them into a further round of cell division (Todaro et al, 1965, 1967). This stimulation occurs in a partial wave of synchrony. The phenomenon of contact inhibition of cell growth

and division appears to be the result of depletion of essential growth-promoting factors from the serum (Holley and Kiernan, 1968), so that the establishment and activity of stationary phase cultures is dependent to a large degree on the concentration of fresh serum.

When confluent V79 cultures were incubated with fresh medium containing serum, further cell division took place in the subsequent 24 hours (increase in cell number was >50%), and utilisation of degradation products from ^{3}H-dsRNA was greater in these than in serum-free cultures. 4 hours after the addition of serum, when DNA synthesis would not yet have commenced, CS gradient analysis of the nuclear label confirmed that no labelling of DNA had occured, while after 24 hours, when DNA synthesis would have taken place, the DNA band was labelled. Therefore the biochemical fate of ^{3}H-dsRNA depends not only on the cell type but also on the patterns of nucleic acid metabolism prevailing in the cell at the time of labelling.

Because the lymphoblastoid cells were able to catabolise dsRNA, the inability of Con A-stimulated lymphocytes (and rabbit spleen explant cells) to do so was not a property of lymphoid cells in general; it may rather be a function of the short-lived and finite nature of primary lymphocyte cultures. Therefore it would be interesting to study the fate of ^{3}H-dsRNA in primary cultures of non-lymphoid cells, and also in cultured macrophages which do not normally divide <u>in vitro</u> but remain metabolically active for several days.

The results demonstrating the degradation and re-utilisation of ^{3}H-dsRNA in V79 and LDV cells are in agreement with those of other studies on the fate of poly I.^{3}H-poly C in cell types other than lymphocytes. Kelly and Levy (1973) measured incorporation of poly I.^{3}H-poly C by rabbit kidney cells in the presence and absence of FCS. The degree of cell-associated label persisting as dsRNA was measured by phenol-extraction and subsequent bio-assay titration in Sindbis virus-infected rat embryo cells. This is a sensitive assay for double-stranded poly I.poly C, capable of detecting 15 ng/ml. During a 6 hour incubation, rabbit kidney cells took up 4 times as much poly I. poly C in the presence,than in the absence,of serum. In both cases the cell-associated label had become partially TCA-solubilised, and had a diffuse banding pattern on polyacrylamide gels, suggesting degradation and re-utilisation. No intact poly I.poly C could be extracted from the labelled cells, as determined by bio-assay.

An electron microscopic study of the uptake of poly I.^{3}H-poly C by rabbit kidney cells was conducted by Prose et al (1970). Following a 1 hour labelling period in the absence of serum, cells were incubated without polynucleotide, and cell sections examined sequentially by emulsion autoradiography. Penetration of label, with some evidence of phagocytosis, was detected after 30 minutes. By 2 hours, labelling over the nucleolus was greater than that over the rest of the cell, after which the grains became redispersed. This pattern was indicative of re-utilisation of hydrolysis products,since de novo RNA synthesis takes place initially in the nucleolus.

Autoradiography of human fibroblasts after exposure to poly I.^{3}H-poly C was also carried out in the study by Bausek and Merigan (1969). During a 60 minute incubation, the polynucleotide became membrane-bound, and was largely removable by treatment of the cells with ribonuclease. Autoradiography showed nuclear labelling by 3 hours, when the grains were predominantly in nucleolar regions. During longer incubation periods, progressively more grains were found in the nucleus and cytoplasm, suggesting a process of degradation and re-utilisation of fragments. This movement of grains from the nucleolus out to the nucleus and cytoplasm was prevented in the presence of actinomycin D, an inhibitor of RNA synthesis. Actinomycin D, severely inhibited total incorporation of poly I.poly C, as was also reported by Levy et al (1970).

In order to distinguish between membrane-bound and intracellular label, many workers have treated poly I.poly C-labelled cells with a high level of pancreatic ribonuclease or snake venom phosphodiesterase (100 µg/ml) for 15-60 minutes, assuming that this treatment removes surface bound, accessible dsRNA (Schell, 1971; Bausek and Merigan, 1969; De Clercq, Wells and Merigan, 1972). The susceptibility of dsRNAs to ribonucleases at physiological salt concentrations is very much lower than that of ssRNAs. P. chrysogenum dsRNA was degraded very slowly by pancreatic RNase in 0.01M tris buffer or tissue culture medium, therefore a brief RNase treatment would not have completely hydrolysed membrane-bound ^{3}H-dsRNA. Indeed, ^{3}H-dsRNA-labelled MS cells lost no label during a 30 minute treatment with RNase. This difference in RNase sensitivity between dsRNA and poly I.poly C, also observed previously (Carter et al, 1976), may be due to the more highly base-paired duplex nature of P. chrysogenum dsRNA compared with most preparations of heat-annealed poly I.^{3}H-poly C, or to the fact that the poly C strand of the latter is hydrolysed considerably more rapidly than the poly I strand (De Clercq, 1979).

Schell (1971) used snake venom phosphodiesterase to remove membrane-bound poly A.poly U and poly I.poly C from EAT cells after a 30 minute incubation period. ^{14}C-poly I.^{3}H-poly C appeared to behave as two separate polymers, since only the ^{14}C-poly I was resistant to removal by the nuclease. However, no data were presented for the comparative susceptibilities of poly I and poly C to the enzyme. ^{14}C-poly A.^{3}H-poly U was taken up by the cells as a duplex entity,as the ^{3}H/^{14}C ratio was preserved during the subsequent cell processing. The triple-stranded complex poly A.2^{3}H-poly U was rapidly taken up by EAT cells. After phenol extraction of the cell-associated label, sucrose density gradient analysis showed it to be intact poly A.2 poly U; it was also resistant to takadiesterase treatment, confirming that it contained no guanosine and therefore had not been re-incorporated into newly-synthesised RNA during a 60 minute labelling period.

Other work on the incorporation of dsRNAs by cell lines has given less clear-cut results, due to the use of DEAE-dextran to enhance RNA uptake. (Pérez-Bercoff et al, 1974; Táborský et al, 1977; Colby and Chamberlain, 1969). These studies are therefore subject to the criticisms discussed earlier. Pérez-Bercoff et al (1974) claimed that the labelled infective replicative form of dsRNA from Mengovirus penetrated L-cell nuclei (48% of the cell-associated label was nuclear), and conserved its double-stranded form for 5 hours during the virus replication cycle. No controls were conducted for non-specific association of dsRNA with nuclei. Colby and Chamberlain (1969) reported that DEAE-dextran increased cell penetration of ^{32}P-poly I.poly C, yet their method of counting cell-associated label would have included label bound to the culture vessel, and there was no direct evidence for cellular penetration of the inducer.

As discussed in the introduction, Volkin et al (1973) used double-labelling and nearest neighbour analytical techniques to examine the integrity of ssRNA taken up by skin fibroblasts. The results suggested that the donor ssRNA retained a high degree of polymeric integrity for 3 hours, after which it became degraded and re-utilised. During the 3 hour incubation, in the absence of serum, 58-70% of the cell-associated label was localised in a purified nuclear fraction, and most of this was further associated with the DNA-protein fraction, although all the label remained alkali-soluble. If ssRNAs are able to persist inside cells for a few hours, then dsRNAs should also retain integrity, by virtue of their lower susceptibility to ribonucleases than ssRNAs.

The difference in buoyant density of mammalian and bacterial DNA on caesium chloride density gradients has been used to follow the incorporation and fate of bacterial DNA in Con A-stimulated HBPLs (Olsen and Harris, 1974). The incorporated label was mainly bacterial-type DNA after 24 hours, but had become converted to mammalian DNA after 72 hours. Although the culture medium contained FCS, the uptake of degradation products, arising from donor bacterial DNA by serum degradation, could not alone have accounted for the rate of appearance of labelled mammalian DNA. Unstimulated HBPLs metabolised the bacterial DNA considerably more slowly. By contrast, V79 fibroblasts degraded and re-utilised ^{3}H-bacterial DNA after only 24 hours of culture. Kao, Regan and Volkin (1973) likewise observed the rapid catabolism of bacteriophage DNA in human skin fibroblasts. These results show that the metabolism of exogenous DNA, like dsRNA, occurs at different rates in different cell types, and depends on the metabolic activity of the recipient cells.

The biological activities of dsRNAs, in particular the induction of interferon and antiviral resistance, depend on the structural integrity of the dsRNA. Molecular requirements for activity include a stable base-paired secondary structure, reflected in a high melting temperature, a high molecular weight, and resistance to ribonuclease degradation (reviewed by Torrence and De Clercq, 1977). For example, a decrease in molecular weight of poly I.poly C reduces it ability to induce interferon and antiviral resistance *in vivo* and *in vitro* (Lampson *et al*, 1970; Niblack and McCreary, 1971; Morahan *et al*, 1972). Modifications to a polynucleotide duplex which increase its resistance to ribonuclease, such as thiophosphate substitution, increase its interferon-inducing capacity *in vitro* (De Clercq *et al*, 1970). Thus the biological activity is limited by the rate at which the dsRNA is degraded intracellularly.

Similarly, there is evidence to suggest that the biological activity of dsRNAs *in vivo* is limited by the rate at which they are degraded by serum nucleases before reaching the target cells. There is an inverse correlation between the degree of enzymic activity against dsRNA in the sera of various species and the ability of these species to respond to dsRNA as an interferon inducer. The rapid hydrolysis of dsRNA by human serum compared with rodent serum may account for the relatively poor *in vivo* interferon response obtained in man (Cunnington and Naysmith, 1975; Douthart and Burgett, 1978). Conferring poly I.poly C with resistance to ribonuclease, by complexing

it with poly-L-lysine, increases its capacity to induce interferon in both mice (Rice et al, 1970) and monkeys (Levy et al, 1975).

It is generally true that the biological and toxic properties of dsRNAs are inseparable (Stinebring and Absher, 1971). Antiviral activity and toxicity of poly I.poly C cannot be dissociated on a molecular weight basis alone (De Clercq et al, 1972; Niblack and McCreary, 1971). Purposeful mismatching of bases in the poly I.poly C duplex, which led to increased susceptibility to ribonuclease, generally produced less biologically active and less toxic analogues than the parent poly I.poly C (Carter et al, 1972, 1976). When poly I.poly C is exposed to the sera from various species, a differential effect is observed on reducing the pyrogenic response in rabbits (Norlund et al, 1970). The variable reduction in toxicity again correlates well with the level of degradative enzyme activity against dsRNA in the different sera (Douthart and Burgett, 1978). The complete lack of hydrolysis of dsRNA in dog serum presumably accounts for its acute and severe toxicity in this species.

Thus the degree and duration of biological responses of dsRNA in vivo appears to be dependent on the half-life of dsRNA as large fragments in the serum, and toxicity is enhanced by the persistence of undegraded material in the serum. This also seems to be true at the cellular level, since under conditions where ^{3}H-dsRNA from P. chrysogenum remained intact in the culture medium, it was more toxic to MS cells, in which it remained largely undegraded, than to V79 and LDV cells, in which it was rapidly catabolised. The fate and effects of dsRNA in mouse lymphocytes in vitro were dependent on the presence of foetal calf serum in the medium. Mouse serum contains much lower levels of enzymic activity against dsRNA than bovine serum, therefore the role played by serum nucleases in limiting the toxicity of dsRNA in the mouse in vivo may be quite small.

As an extension to the in vitro work already discussed, some in vivo experiments in mice were conducted in order to see how the fate of ^{3}H-dsRNA in the whole animal could be related to the toxic effects seen in vivo, and to the biochemical fate observed in cells in culture. The experiments established the timing and nature of the main toxic manifestations in CBA mice, and then examined the integrity of ^{3}H-dsRNA in the blood and tissues at the appropriate time. Due to limitations on the number of animals and the supply of ^{3}H-dsRNA, these in vivo results must be considered to be preliminary.

The toxic effects of dsRNA observed in the mouse, that is, weight loss, ruffled fur, hunched posture, diarrhoea, and acute transient lymphopenia, are in agreement with those previously reported for poly I.poly C and mycophage dsRNA (Leonard et al, 1969; Carter et al, 1976). Degré (1973) observed a biphasic change in WBC numbers in response to a single i.p. injection of poly I.poly C. An initial rise after 6 hours, due to an increase in neutrophils, was followed by a reduction from days 1 to 5, due to a lymphopenia. The kinetics of the lymphopenia were consistent with both a reduced supply of precursor cells to the circulation and a disappearance of mature circulating cells, possibly by a direct lethal effect of poly I.poly C or by their sequestration in the stationary reticulo-endothelial system.

Jullien and De Maeyer-Guignard (1971) noted a dramatic, transient lymphopenia 24 hours after i.p. administration of 10 mg/kg poly I.poly C, followed by a restoration and overshoot at day 5. The lymphopenia was considered to result from the toxic effect of poly I.poly C on the dividing colony-forming cells in the spleen and bone marrow, since these were reduced by 30-40% 6 hours after administration and by 65-85% after 24 hours. Resting cells were apparently spared the cytotoxicity. Both lymphopenia and haemopoietic cell toxicity were properties of the double-strandedness of the polynucleotide, and were not observed following the administration of Newcastle disease virus to produce interferon titres comparable to those induced by poly I.poly C, thus ruling out interferon as the mediator of toxicity. It seems unlikely that a toxic effect of P. chrysogenum dsRNA on haemopoietic cells could by itself account for the rapid fall in WBC apparent as early as 3 hours after dsRNA administration, and the initial leucopenia, at least, must result from sequestration of WBC in the organs. An effect of poly A.poly U on T-cell traffic in the spleen has been proposed to account for the adjuvant effect of this polynucleotide in mice (Moatamed, Karnovsky and Unanue, 1975).

There is a small literature on the clearance of double-stranded RNA from the circulation of mice following i.v. administration. Chused, Steinberg and Talal (1972) observed a rapid clearance of ^{3}H-poly I.poly C (s value = 8.0) in NZB/W mice, such that 75% of the initial radioactivity was lost from the circulation within 5 minutes, localising in the liver, and to a lesser extent in the spleen and kidneys. The disappearance from the blood was not due to rapid hydrolysis by serum nucleases. A slightly slower rate of clearance was observed by Chia et al (1979) for ^{125}I-poly I.poly C (s value = 7.6) in Swiss-Webster mice. 50% of a non-toxic dosage of poly I.poly C was

lost from the circulation in 10 minutes and 90% in 60 minutes. Hepatic uptake reached a maximum at 15 minutes and thereafter declined, while label in the gastric washings increased steadily through 4 hours. In a short communication, Mayer, Stanček and Doskočil (1976) reported the disappearance of approximately 75% of labelled phage f2 dsRNA from the serum of mice 1 hour after i.p. administration. The liver took up more label than the other organs, and a high proportion of the injected dose was excreted in the urine in TCA-precipitable form.

It was not shown, in any of the above-mentioned studies, whether the label that localised in the tissues was extra- or intra-cellular, and whether it was high molecular weight dsRNA or products of metabolism.

In the preliminary studies described in section IV, it was shown that 50% of the ^{3}H-dsRNA injected intravenously remained in the serum after 2 hours,and 10% remained after 6 hours. Serum nucleases played no role in blood clearance up to 6 hours, at which time the molecular weight of the dsRNA in the plasma had been reduced by only 25%. This rate of clearance of dsRNA from the circulation is clearly much slower than that of poly I.poly C, and may in part be accounted for by the greater resistance of the former to serum nucleases (Carter et al, 1976). A slow clearance of P. chrysogenum ^{3}H-dsRNA in the mouse has been observed previously (R. Imrie, personal communication), when the half-life of TCA-insoluble label in the blood was found to be 65-75 minutes, compared to 13-27 minutes and 15-33 minutes in the rabbit and guinea-pig respectively.

The toxic effect of dsRNAs in the mouse intestine has been previously documented. Philips et al (1971) described early gut damage following i.v. administration of 12.5 mg/kg poly I.poly C; the stomach and ileum became distended with fluid, and the histology showed necrosis of the duodenal epithelium with blunting of the villi. Inhibition of cellular proliferation would inevitably lead to shortening of the villi, as cells which are normally shed from the tips are not replaced by new upward-moving cells. Such changes are also seen following the inhibition of cell proliferation induced by acute radiation damage.

Kobus, Sawicki and Korbecki (1977) studied the effect of i.p. administration of a non-toxic level of poly I.poly C (1 mg/kg) on cellular proliferation in the epithelial cells of the ileal crypts, using ^{3}H-thymidine incorporation and autoradiography. The results showed that the polynucleotide had no effect on the spatial distribution of proliferating cells in the crypts, but that after 12-24 hours, numbers

of DNA-synthesising and mitotic cells were reduced (by less than 50%). After 7 days, labelling and mitotic indices in these cells had returned to normal values. The possibility could not be ruled out that this inhibition of DNA synthesis was in fact mediated by poly I.poly C-induced interferon, rather than the dsRNA itself.

The mechanism of toxicity induced in the mouse ileum by P. chrysogenum dsRNA is speculative. The pathogenic changes are accompanied by the persistence of undegraded dsRNA in the mucosa, and by the presence of increased numbers of lymphocytes and polymorphs, which are associated with much of this material. Such cells may thus serve as vehicles for the distribution of undegraded dsRNA to the tissues following i.v. administration. The dsRNA may then affect the surrounding mucosal cells by various mechanisms. The capacity of a tissue to deal with the dsRNA will depend on the type and metabolic activity of the resident cells, for example, a rich supply of macrophages.

The findings are consistent with an indirect effect of dsRNA, which, because of its polyanionic nature, may induce membrane damage and aggregation of circulating, quiescent lymphocytes and polymorphs. The observed leucopenia is due to their subsequent sequestration in the reticulo-endothelial system and the gut mucosa. Local and systemic release of pharmacologically active mediators (endogenous pyrogens, inflammatory mediators, and interferon itself) from the leucocytes and macrophages may ensue, similar to that following endotoxin administration.

Alternatively, the dsRNA may exert a direct cytotoxic effect on the rapidly dividing mucosal cells, perhaps inhibiting DNA synthesis as discussed previously, or by inhibiting protein synthesis—currently the most popular explanation for the cytopathogenicity of dsRNAs at the cellular level. Inhibition of protein synthesis and cytolysis by dsRNA is enhanced in cells previously exposed to interferon (Stewart et al, 1972; Kerr, Brown and Ball, 1974). dsRNA from P. chrysogenum is a potent interferon inducer in the mouse (Sharpe, Birch and Planterose, 1971), producing high serum titres 4-6 hours after administration via the i.v. or i.p. route. Thus interferon would have been present in peak levels in the body fluids at a time when undegraded dsRNA remained in the serum and certain organs (ileum).

As part of a recent publication, O'Malley et al (1979) studied the effect of poly I.poly C on several human cell systems in vitro, which they used as models for comparing the toxic effects of various poly I.poly C analogues. The results showed that poly I.poly C had no effect on DNA synthesis in PHA-transforming lymphocytes, and was only

mildly mitogenic in the absence of PHA (stimulation index 2.7). It had no effect on the proliferation of myeloid colony-forming cells, and inhibited foreskin fibroblast replication only after several days of repeated addition of polymer. These observations seem to rule out a direct cytotoxic effect of dsRNA on cellular proliferation, and further support the suggestion that toxicity *in vivo* is mediated indirectly. However, in view of the foregoing discussion, the *in vitro* cell systems could perhaps be made more relevant by examining the effects of poly I.poly C in the presence of interferon.

The *in vivo* experiments suggest that the peripheral blood leucocytes are the first targets for intravenously-administered dsRNA, as well as the macrophages of the reticulo-endothelial system. These are thought to be the main cells producing interferon *in vivo* (Ho, 1977; Carter *et al*, 1977). The persistence of intact dsRNA in murine lymphocytes may also be correlated with interferon induction in these cells. The linkage between interferon induction and toxicity is observed at the cellular level as well as in whole animals. For example, pre-treatment of cells with interferon before exposure to dsRNA has two effects; first, it "primes" the cells to produce enhanced levels of interferon when subsequently exposed to dsRNA (Rosztóczy and Mecs, 1970), and secondly, it potentiates the cytotoxicity of dsRNA, resulting in increased cell lysis. Perhaps interferon treatment renders the cells less capable of metabolising dsRNA, thereby increasing its persistence in cells and amplifying its biological responses, whether these be desirable (interferon production) or undesirable (cytotoxicity).

If the desirable and undesirable biological effects of dsRNA are triggered by the same biochemical event, the therapeutic potential of the material as an antiviral agent seems limited. If, however, the effects are triggered by different receptors, either recognising different fine structural features of the polymer, or recognising the same features but with different kinetics, it must be hoped that inducer modifications can be made to allow separation of the beneficial and deleterious biological effects. For therapeutic purposes, dsRNA may rather be employed indirectly in cell cultures to induce the production of interferon, which can then be purified and administered to man with far fewer toxic side-effects than dsRNA itself. By separation of the cytotoxic and interferon-inducing abilities of dsRNA, it may be possible to induce very high interferon levels in such cultures. In this respect, the techniques developed in the present work may be usefully employed for investigations into the relationship between persistence of dsRNA inside cells, the induction of interferon, and cytopathogenicity.

Furthermore they highlight the problems arising in work of this kind, such as the effect of serum, the need to conduct proper controls, and the value of parallel biochemical and autoradiographic analysis.

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APPENDIX I

A cytotoxic dialysable impurity in dsRNA from P. chrysogenum

dsRNA, as supplied, was found to have a dose- and time-dependent inhibitory effect on Con A-induced MS cell transformation. This appendix describes the nature of the inhibition, and the studies which led to the conclusion that it was a dialysable impurity in dsRNA which was responsible for the effects observed in serum-containing medium.

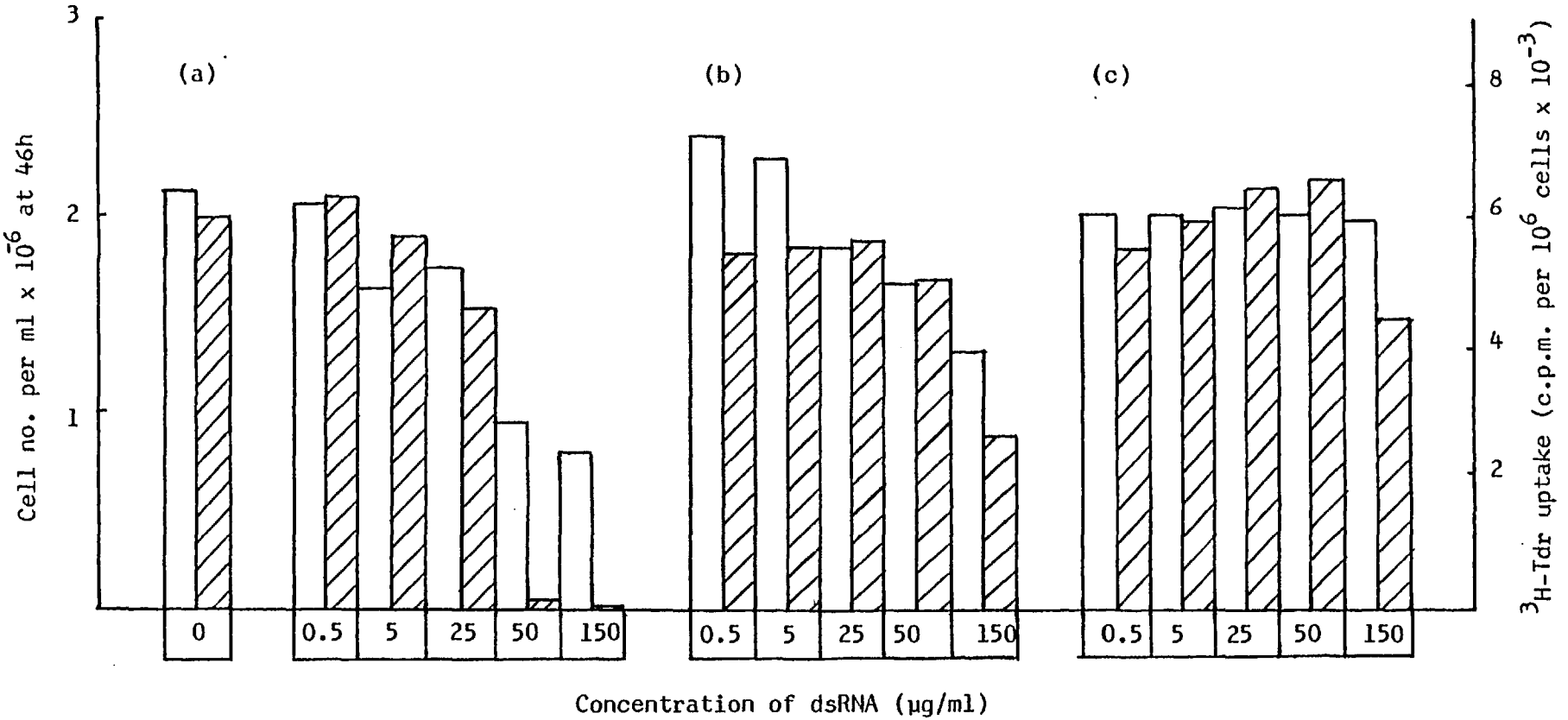
1. Characteristics of dsRNA inhibition

The dose-response effect of dsRNA on Con A-stimulated MS cells in 1 ml culture is shown in Figure 35. When present in the medium from 0 hours, it had a marked inhibitory effect at concentrations >50 µg/ml; the final number of cells per culture at 48 hours was significantly reduced, and the cells showed decreased ^{3}H-thymidine incorporation. At 150 µg/ml dsRNA, no large transformed cells were present at all, and the small lymphocytes remaining were mainly aggregated together, showing signs of gross lysis and nuclear pyknosis.

When addition of dsRNA was delayed until 20 hours, after the cells had become "committed" to transformation, inhibition of ^{3}H-thymidine uptake was less dramatic, and blast cells were present at 48 hours, even at the highest dsRNA concentrations used. When added immediately before the thymidine pulse at 44 hours, a concentration of 150 µg/ml was required to significantly inhibit DNA synthesis.

Figure 36 shows the effect of dsRNA on MS cells stimulated by Con A in 5 ml culture, in the presence of 2-mercaptoethanol. These larger cultures show approximately a 3-fold increase in cell number over a 72 hour period, i.e. more cells are actually in division cycle. This difference may be related to the lower cell: culture surface area ratio as well as to the inclusion, in the larger cultures, of 2-mercaptoethanol. Ling and Kay (1975b) showed that the rate of ^{3}H-thymidine incorporation (c.p.m. per 10^{6} cells) was dependant on the initial cell concentration as well as the shape and size of the culture vessel. 2-mercaptoethanol has been shown to enhance DNA synthesis and blast cell transformation in stimulated lymphocyte cultures (Fanger et al, 1970; Broome and Jeng, 1973), although the mechanism of enhancement remains unclear.

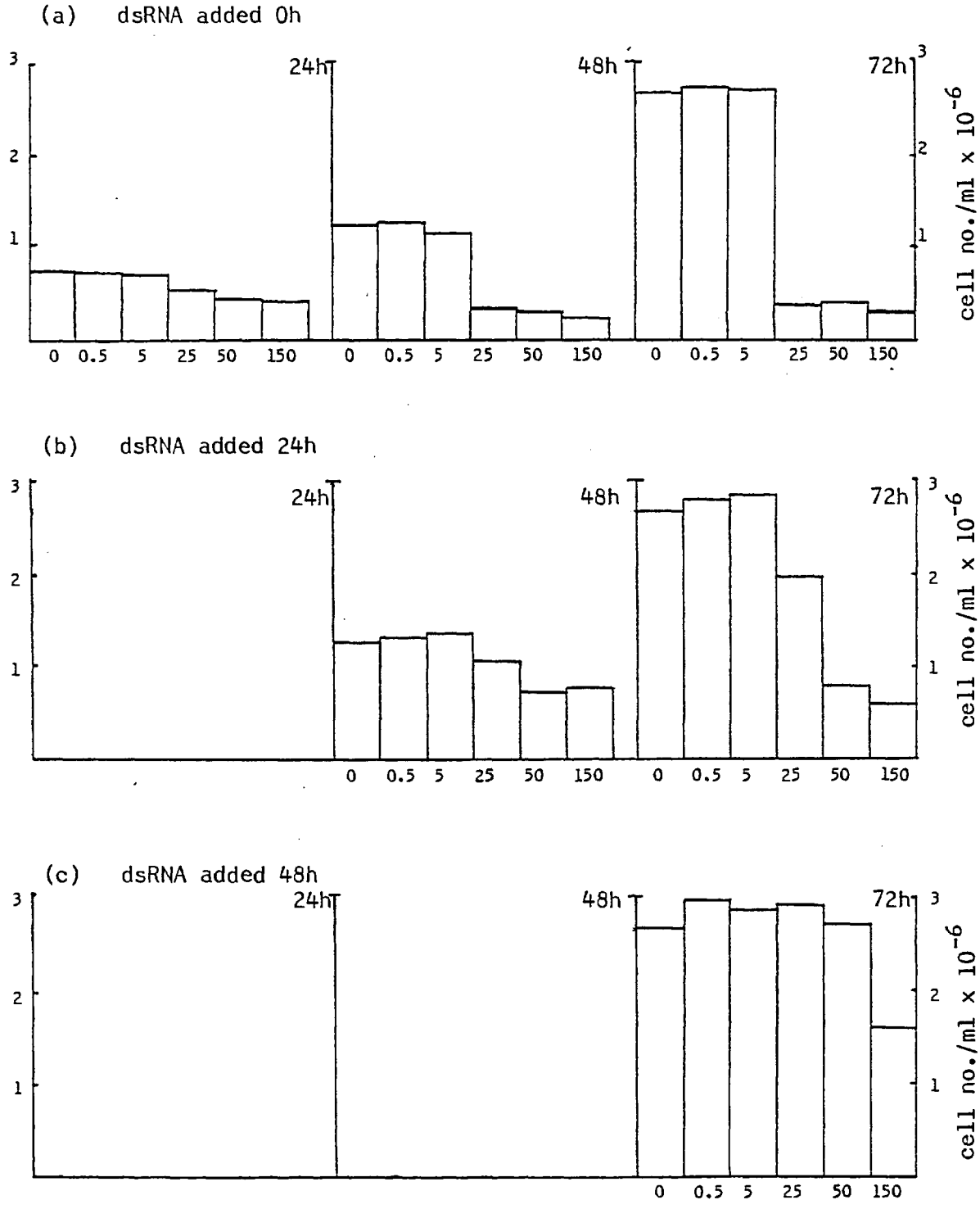
Figure 35 - The effect of dsRNA on Con A-induced transformation of mouse spleen cells in 1 ml culture.



1 ml mouse spleen (MS) cell cultures were prepared at 2.5 x 10^{6} cells/ml in medium containing 5% FCS and 4 µg/ml Con A. dsRNA was added at (a) 0h, (b) 20h or (c) 44h of transformation. Cells were pulsed with 2 µCi/ml ^{3}H-thymidine from 44-46h, when incorporation into TCA-insoluble material was measured in pooled duplicate cultures. Numbers of nucleated cells in the cultures at 46h were estimated microscopically after fixation.

□ Cell no. per ml at 46h; ▨ ^{3}H-Tdr incorporation per 10^{6} cells, 44-46h.

Figure 36 - The effect of dsRNA on Con A-induced transformation of mouse spleen cells in 5 ml culture.



5 ml MS cell cultures were prepared at 1 x 10^6 cells/ml in medium containing 5% FCS, 4 µg/ml Con A and 5 x 10^{-5}M 2-mercaptoethanol. dsRNA was added, at the concentrations shown on the abscissa, at (a) 0h, (b) 24h, or (c) 48h. The numbers of nucleated cells in the cultures were estimated microscopically after 24, 48 and 72h.

The increase in cell number found in Con A-stimulated 5 ml MS cell cultures was prevented by 25 µg/ml dsRNA added at 0 hours. The dose response fell sharply between 5 and 25 µg/ml, suggesting that these cultures were more sensitive to dsRNA than the 1 ml cultures. Again, delayed addition resulted in less inhibition of cell division; 150 µg/ml dsRNA was required to significantly suppress cell replication if added 48 hours after the initiation of transformation.

1 ml rabbit spleen cell cultures, prepared in the same way as MS cells, responded identically to the 1 ml MS cell cultures, transformation being completely prevented by the addition of 150 µg/ml dsRNA at 0 hours (data not shown).

Since dsRNA seemed to affect an early event in transformation, the effect of its addition and removal during the first few hours was investigated. In the experiment reported in Table 40(a), an inhibitory concentration of dsRNA was added at two-hourly intervals and at 24 hours. Delaying addition by as little as 2 hours significantly reduced the inhibitory effect. Thus, dsRNA predominantly affected an early event in transformation, either by a direct effect on the cells, or possibly by interacting with Con A and so inactivating the mitogen.

Table 40(b) shows the results obtained when dsRNA was removed at two-hourly intervals by centrifugation of the cells and resuspension in warm medium which had been incubated with Con A in parallel from 0 hours. The supernatant containing dsRNA was removed carefully with a fine pasteur pipette, and the cell pellets flicked up gently in fresh medium. This technique allowed removal of almost all the original medium (residuum <20µl) containing dsRNA. The data confirm that dsRNA inhibited an early event (0-2 hours) involved in the initiation of lymphocyte transformation. The inhibition was irreversible, the continued presence of dsRNA not being required.

Interaction of dsRNA with Con A itself seemed unlikely, since replenishment of the cultures with fresh Con A after centrifugation and removal of dsRNA failed to restore subsequent transformation. The fact that the material affected primarily the cells rather than the Con A was confirmed by the observation that it inhibited a murine mixed lymphocyte reaction in a similar way (to be discussed in Appendix I. 2, below).

Table 40 - The effect of dsRNA on the initiation of Con A-induced transformation of MS cells

(a) Delayed dsRNA addition

Time of dsRNA addition (h)	% inhibition ^{3}H-Tdr incorporation	
	50 µg/ml	150 µg/ml
0	88	97
2	53	83
4	45	74
6	36	72
24	44	66

dsRNA was added at 50 or 150 µg/ml to 1 ml MS cell cultures at various times after initiation of transformation with 4 µg/ml Con A. Cultures were pulsed with 2 µCi/ml ^{3}H-Tdr from 50-52 h. ^{3}H-Tdr incorporation and cell counts were measured in duplicate cultures separately and the averages taken. Control cultures receiving no dsRNA incorporated 41,500 c.p.m. per 10^{6} cells.

(b) Delayed dsRNA removal

Time of dsRNA removal (h)	% inhibition ^{3}H-Tdr incorporation	
	50 µg/ml	150 µg/ml
2	72	92
4	91	96
6	82	91
24	91.5	97
Not removed	89.5	93

dsRNA was added at 50 or 150 µg/ml to 1 ml MS cell cultures at the initiation of transformation with 4 µg/ml Con A. At subsequent intervals, the cells were centrifuged at 200 g for 10 mins at 37°C and resuspended in fresh medium with Con A, without dsRNA. Control cultures initiated without dsRNA were resuspended in the same way. After pulsing with 2 µCi/ml ^{3}H-Tdr from 50-52h, ^{3}H-Tdr incorporation and cell counts were measured in duplicate cultures as above. Control cultures receiving no dsRNA incorporated 26,000 c.p.m. per 10^{6} cells.

This dramatic inhibition of the initiation of lymphocyte transformation in vitro by dsRNA has not previously been documented. It was suspected that there might be some non-RNA impurity in the dsRNA preparation, arising during the extraction and/or purification process. Attempts were made initially to purify the dsRNA further by CS density gradient centrifugation. During the course of this study, it was found that an inhibitory material was present in the dsRNA preparation, and was removable by simple dialysis.

2. The effect of dialysed and caesium sulphate purified dsRNA on lymphocyte transformation

Figure 2 showed that the major part of the dsRNA banded at a density of 1.61 on CS density gradients, while a small fraction, thought perhaps to contain the inhibitory activity, banded at a density of 1.70. Separation of these two species was achieved by sedimentation equilibrium centrifugation of 1 mg dsRNA in a 5 ml gradient of caesium sulphate. Fractions containing the RNA were pooled, dialysed, concentrated by evaporation and redialysed against 0.1 x PBS. As a control, dsRNA not subjected to CS gradient centrifugation was dialysed against 0.1 x PBS. Both preparations were redialysed against 0.15M NaCl, then tested for their ability to inhibit Con A-stimulated MS cell transformation. Table 41 shows the results obtained.

Both the CS-purified and dialysed dsRNAs lost significant inhibitory activity when present at 100 µg/ml. The satellite RNA band at ρ = 1.70, tested in another experiment (data not shown), was likewise non-inhibitory. This was not removed by dialysis. Thus it seemed that dialysis alone was sufficient to render the dsRNA much less toxic to MS cells. Because of the possibility that this particular batch of dsRNA (B.197) contained a contaminating substance, samples of two other batches were also studied before and after dialysis. All three dsRNA batches showed the same phenomenon—the inhibitory effect on MS cell transformation could be largely, or sometimes completely, removed by dialysis.

Transformation induced by Con A was not the only lymphocyte proliferative response to be inhibited by the dialysable impurity in dsRNA preparations. The effects of dsRNA, before and after dialysis, on a murine mixed lymphocyte reaction (MLR) are shown

Table 41 - The effect of purification and dialysis on the inhibitory activity of dsRNA on MS cell transformation

Treatment of dsRNA	Conc$^{n.}$ of dsRNA (µg/ml)	Cell number x 10^6/ml	^{3}H-Tdr incorporation	
			c.p.m./10^6 cells	% inhibition
None	10	1.75	26,617	9
	100	1.20	1,088	96
Dialysis only	10	1.78	25,752	12
	100	1.42	20,591	30
Cs_2SO_4 purification and dialysis	10	1.76	26,500	10
	100	1.42	29,345	0

The dsRNA preparations were added at 0 h, at 10 or 100 µg/ml, to 1 ml MS cell cultures stimulated with Con A. ^{3}H-Tdr incorporation was measured at 46-48 h with 2 µCi/ml ^{3}H-Tdr. Control cultures receiving no dsRNA incorporated 29,360 c.p.m. per 10^6 nucleated cells.

in Table 42. Spleen cells from CBA and Simpson mice were mixed to initiate such a response. ^{3}H-thymidine incorporation in mixed cultures was three-fold greater than in unmixed control cultures. The effects of dsRNA in this MLR were analogous to those in the Con A-stimulated cultures. The MLR was particularly sensitive to undialysed dsRNA present from 0 hours, since only 10 µg/ml produced definite inhibition of the response, and 50 µg/ml apparently killed the cells. This effect was abrogated by dialysis, therefore, the dialysable impurity inhibits lymphocyte transformation stimulated by histocompatability antigens as well as by Con A. It thus seems to interact directly with the cells themselves, possibly at the time of an early membrane event, preventing subsequent blast cell formation.

A similar pattern of response was seen in Con A-stimulated human peripheral blood lymphocyte cultures, prepared from freshly-drawn,defibrinated blood using a Ficoll-Triosil gradient (Harris and Ukaejiofo, 1970). Transformation was completely prevented by the addition of 100 µg/ml dsRNA at initiation, but was only inhibited 7-15% by 100 µg/ml dialysed dsRNA (data not shown).

The results suggested that dialysis was either removing an inhibitor from the dsRNA preparation, or was bringing about a structural change in the dsRNA itself which rendered it non-inhibitory. This was investigated by attempting to recover the impurity from the dialysate by lyophilisation, and by analysis of the dialysed dsRNA. Experiments testing the effect of the recovered dialysate per se gave inconclusive results, probably due to the difficulty of recovering trace amounts of material from the large volume. The A_{260}/A_{280} ratio of the dialysate was 1.6. No further analysis of the impurity was carried out.

3. Analysis of dialysed dsRNA

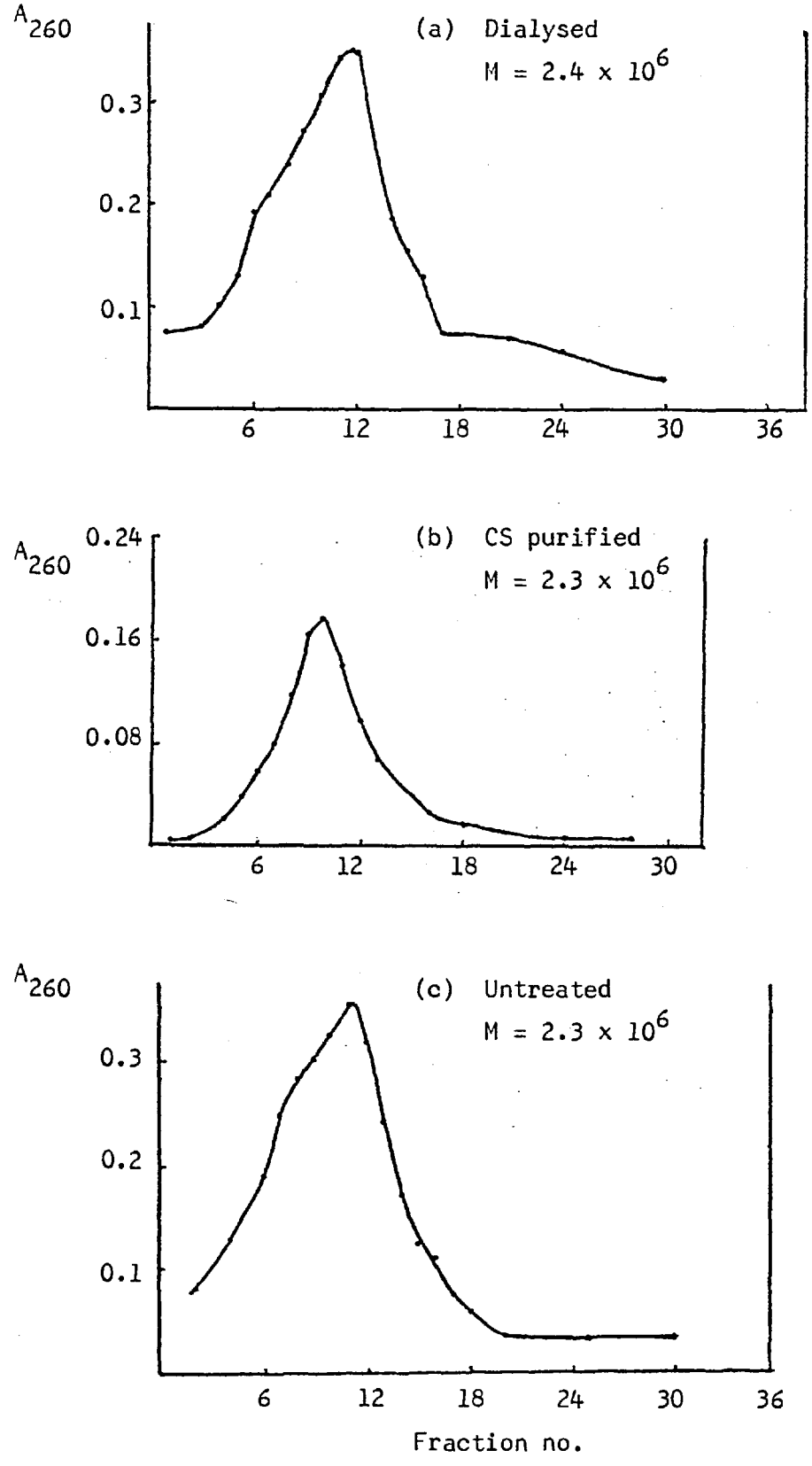
The dialysed and CS-purified dsRNAs were subjected to sucrose density gradient centrifugation in order to determine the molecular weights. The A_{260} gradient profiles are shown in Figure 37, with the estimated molecular weights in Table 43. The similarity in shape and position of the peaks shows that there was no obvious change in the size of the dsRNA as a result of these procedures. All three molecular weights were within the normal range.

Table 42 - The effect of dialysed and undialysed dsRNA on a murine mixed lymphocyte reaction (MLR)

Mouse strain	Conc$^{n.}$ of dsRNA (μg/ml)	Cell number x 10^6/ml	^{3}H-Tdr incorporation	
			c.p.m./10^6 cells	% inhibition
CBA alone	-	1.18	31,054	-
Simpson alone	-	1.06	30,706	-
CBA + Simpson	-	1.26	101,580	-
"	Undialysed 1	1.36	91,349	10
"	" 10	0.46	48,464	52
"	" 50	0.40	0	100
"	" 100	0.38	0	100
"	Dialysed 1	1.22	99,987	2
"	" 10	1.26	94,746	7
"	" 50	1.26	80,851	20
"	" 100	1.12	79,394	22

MS cell suspensions at 2.5 x 10^6 cells/ml were prepared from 2 month old Simpson or CBA mice. Mixed cultures containing 0.5 ml of each cell suspension, or control cultures containing 1.0 ml of a single suspension,were incubated in the presence of dsRNA as above for 72 h. ^{3}H-Tdr incorporation into DNA was measured for 4 hours, beginning at 68 h of culture. Nucleated cells were counted with a haemocytometer at the end of the labelling period.

Figure 37 - Sucrose density gradient centrifugation of dsRNA after dialysis and CS density gradient purification



After the following treatments, dsRNA (50-100 µg) was analysed on 5 ml neutral sucrose density gradients (5-20%):

(a) dialysis against 0.1 x PBS for 48h, then against 0.15 M NaCl.

(b) purification of the 1.61 density band from CS density gradient of dsRNA, then dialysis as in (a).

(c) untreated

Table 43 - The molecular weight of dsRNA after purification and dialysis, determined by sucrose density gradient centrifugation

Treatment of dsRNA	Molecular weight
None	2.35×10^6
Dialysis	2.7×10^6
Cs_2SO_4 purification and dialysis	2.2×10^6

See Figure 37 for experimental details.

Molecular weights are the averages of two determinations.

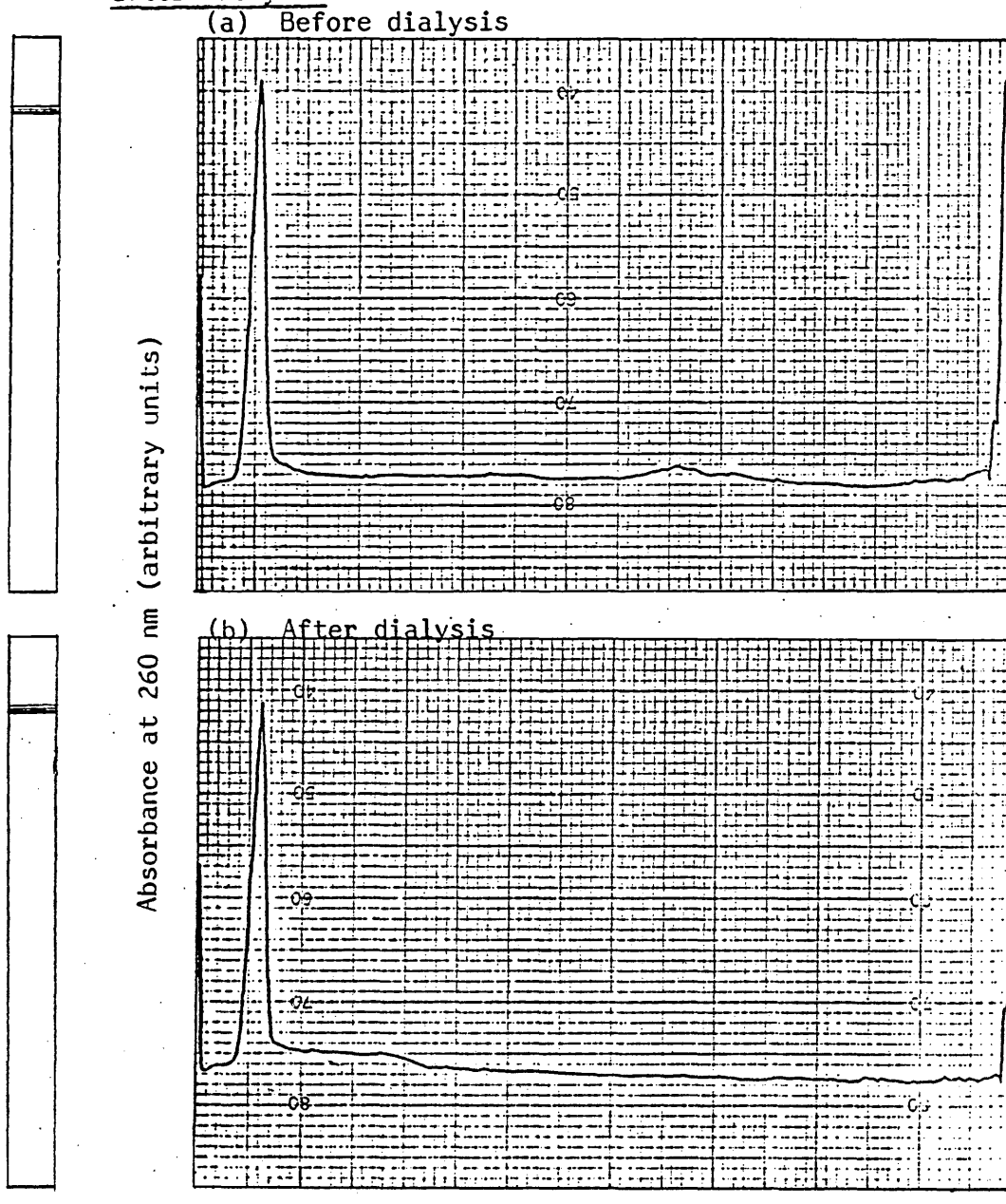
The banding pattern of dialysed dsRNA on polyacrylamide gels was compared to that of untreated dsRNA. Identical results were obtained, both preparations migrating the same distance from the top of the gels to give three barely resolvable bands characteristic of P. chrysogenum dsRNA (Figure 38).

The melting curve of dsRNA after dialysis was checked in order to establish whether or not the material was still double-stranded. The rise in A_{260} with increasing temperature of 40 µg/ml solutions in 0.1 x SSC is shown in Figure 39. Two batch preparations of dsRNA were tested after dialysis. The hyperchromicity of these samples was 143% and 142% compared with 141% for undialysed dsRNA, and the Tm of all three samples was approximately 85°C. It therefore appeared that dsRNA retained its intact double-stranded structure during dialysis.

Biological activity of dsRNA was also retained following dialysis, as evidenced by its ability to induce levels of interferon comparable to those induced by the undialysed preparation in a human fibroblast induction system currently in use at Beecham Pharmaceutical Research Division, Brockham Park, Surrey. (P. Butlin, personal communication).

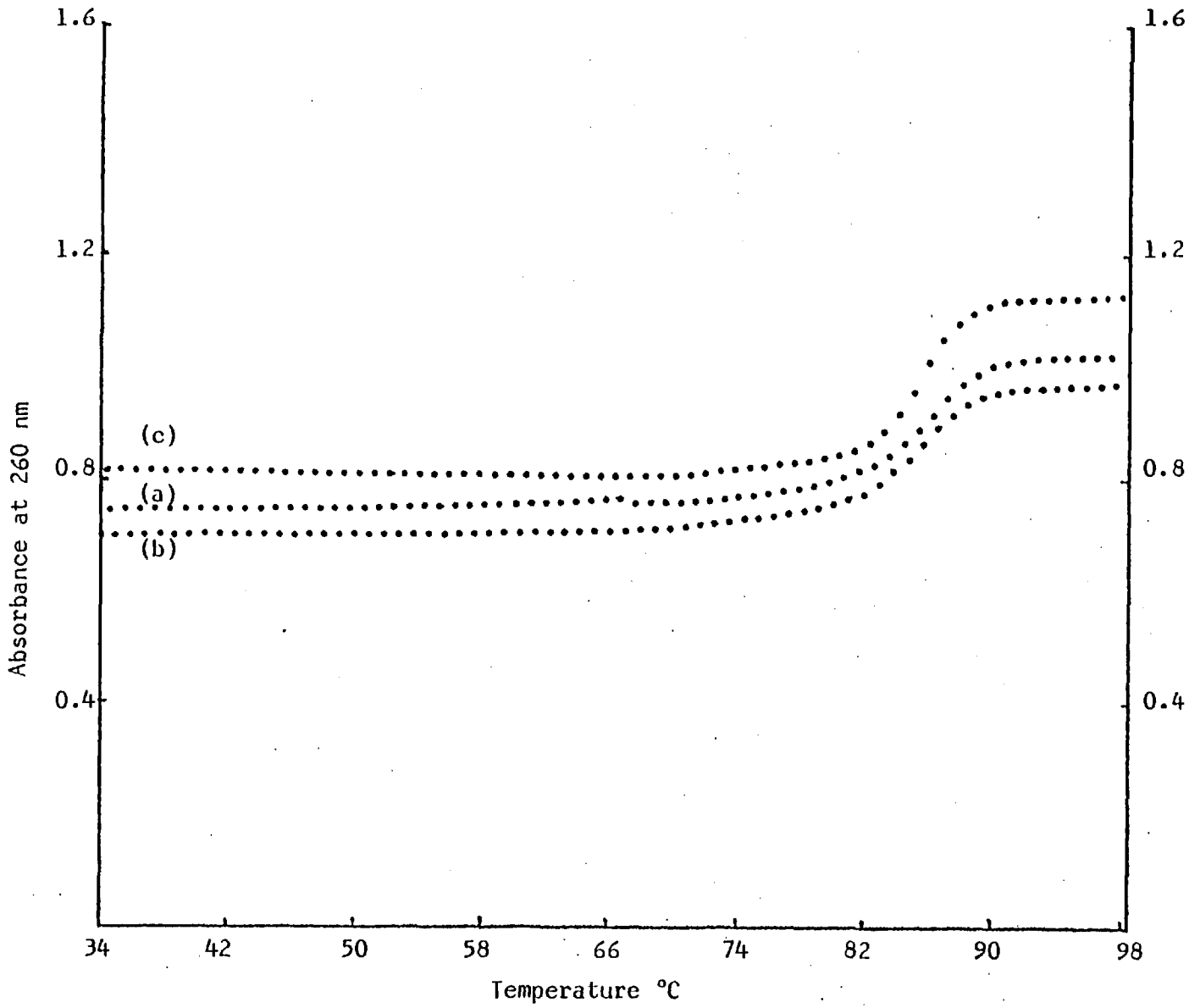
From the results of the foregoing studies, it was concluded that dialysis of dsRNA preparations did not alter the dsRNA molecule itself, but removed a low molecular weight impurity which inhibited the initiation of Con A-stimulated MS cell transformation. The identity of this contaminant was not elucidated. Cetavlon (cetyl trimethyl ammonium bromide) seemed an obvious candidate for this impurity, since it is used in a late stage of the dsRNA purification schedule, and does contaminate the final preparations in trace amounts (CTA^+ usually <0.05% of the total organic material). The effects of low concentrations of Cetavlon and mixtures of Cetavlon with dialysed dsRNA were therefore examined in the Con A-stimulated MS cell system. However, at the level found in dsRNA preparations, Cetavlon alone had no effect, and in combination with dialysed dsRNA produced no synergistic inhibition above that due to the dialysed dsRNA itself.

Figure 38 - Polyacrylamide gel electrophoresis of dsRNA before and after dialysis.



A 2 mg/ml solution of dsRNA (Batch CT3647) was dialysed against 20 vols. water for 48 hours, then adjusted to 0.15M NaCl. 10 µg samples were analysed as described in Materials and Methods 5.2. After fixing in 7% acetic acid, gels were either stained with methylene blue (left) or the absorbance measured at 260 nm (right).

Figure 39 - Melting profile of dsRNA



A 2 mg/ml solution of dsRNA (as supplied) was dialysed against 20 vols. water for 48 hours, then adjusted to 0.15 M NaCl. The solutions were further diluted to 40 µg/ml in 0.1 x SSC before measuring the rise in A_{260} with temperature

(a) dsRNA (Batch CT.3647), undialysed.

(b) dsRNA (Batch CT.3647), dialysed.

(c) dsRNA (Batch 197). dialysed.

The identity of the contaminant was not elucidated. It may be a small protein or carbohydrate that is co-purified with dsRNA from P. chrysogenum cultures. However, the analytical data suggest that if this is the case it must be present only in trace amounts (see Appendix II). Because undialysed dsRNA was considerably less inhibitory if added after the first 2-4 hours of culture, the impurity must suppress an early event after lymphocyte stimulation which commits the cell to blastogenesis. Additional data not presented showed that in freshly-prepared MS cell cultures, ^{3}H-thymidine incorporation was shut off immediately after addition of undialysed dsRNA, both in the presence and absence of Con A, while in transformed cultures (after 40 hours incubation with Con A) the rate of ^{3}H-thymidine incorporation was lowered, but not completely arrested, approximately 4 hours after dsRNA addition. Thus the impurity was unlikely to be a general cytotoxic agent.

APPENDIX II

BRL 5907: CHEMICAL CHARACTERISATION

BRL 5907 is the sodium salt of a double-stranded ribonucleic acid, of molecular weight about two million. Some evidence for this statement is as follows :-

1. BRL 5907 gives a u.v. spectrum showing an absorption maximum at 258.5 nm and a minimum at 232 nm. The absorbance ratio for λmax. to λmin. is 2.05, and the ratio for 260 nm to 280 nm is 2.11. These characteristics are typical of nucleic acids.

2. The absorbance at 260 nm increases by about 50% when the material is fully hydrolysed with alkali. This behaviour is characteristic of nucleic acids.

3. Chromatography on ion exchange resin of alkali-hydrolysed BRL 5907 shows the presence of the four nucleotides typical of ribonucleic acids. These are the 2' and 3' phosphates of adenosine, guanosine, cytidine and uridine.

Combination of the fractions containing each nucleotide, and calculation of the concentrations from the known molar absorbance values of the nucleotides gives a base ratio of 0.97:1.04:0.98:1.00 for adenine, uracil, cytosine, guanine respectively. This result, with similar proportions of adenine, uracil, cytosine, guanine, is consistent with a double-stranded nucleic acid structure.

4. Assay by the orcinol method indicates an available ribose content of approximately 23%. This is in good agreement with the value expected for the sodium salt of RNA with a 1:1:1:1 base ratio. The theoretical ribose content of such an RNA is 43.7%, but only slightly more than half of this will be available in the assay procedure because ribose bound to the pyrimidine bases reacts to only a small extent.

5. The absorbance at 260 nm increases quite sharply with increase of temperature over a narrow temperature range above 80°C. This phenomenon is characteristic of a double-stranded as opposed to a single-stranded RNA.

6. Analytical ultracentrifugation of BRL 5907 in 0.15M sodium chloride with 0.015M trisodium citrate, pH 7, gives a sedimentation coefficient of 14.1. In the same solvent, an intrinsic viscosity value of about 9.5 dl/g is obtained. These values can be substituted in the Mandelkern-Flory equation; giving a molecular weight of about 2×10^6 daltons

$$M = 13{,}200\ (S^\circ_{20W}[\eta]^{1/3})^{3/2}$$

Where S°_{20W} is the sedimentation coefficient;

$[\eta]$ is the intrinsic viscosity.

BEECHAM PHARMACEUTICALS RESEARCH DIVISION, BROCKHAM PARK

CERTIFICATE OF ANALYSIS

Product; BRL 5907 Batch No. 5/129

1.	Description		Slightly off-white fluffy solid
2.	Identification	(a) UV spectrum	Pass
		(b) Tm	84.0°
		(c) Hc	40.6%
		(d) Sepharose	Pass
		(e) Electrophoresis	Pass
		(f) Available ribose	21.6
3.	pH (1 mg RNA/ml solution)		7.5
4.	Toxicity		Pass
5.	Assay	(a) as % of all organics	97.4
		(b) as % of solid	21.8
6.	DNA		0.03
7.	Carbohydrate		0.5
8.	Protein		0.04
9.	Cetyl trimethylammonium ion		0.01
10.	Phosphate ion		3.7
11.	Chloride ion		40.8
12.	Acetate ion		<0.1
13.	Sodium ion		29.9
14.	Potassium ion		0.07
15.	Water		4.4
16.	Antibiotic contamination		Not detected

Assays carried out March / April 1971 except test 16, by MASU (Worthing) July 1974.

Comments: Satisfactory material. Passes specification DMS 83/2 on all tests except 5 (b), 11 and 13; outside specification on these tests only because NaCl was deliberately added to the product before freeze-drying.

Date: 4. 4. 75 Signed:

Head, Physical & Analytical Unit.

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