INTERFERON ACTION IN ANIMAL CELLS

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INTRODUCTION
INTERLUDE

Interferons are proteins secreted by animal cells in response to viral infection and certain other stimuli; these proteins interact with cells of the same species to render them virus-resistant (Isaacs and Lindenman, 1957; Ho, 1973). Interferons also have other effects on responsive cells, such as growth inhibition, regulation of interferon synthesis, and modulation of the immune response (see 'Non-antiviral effects of interferon'). As well as being of enormous potential benefit clinically, interferons are of great interest as examples of inducible cellular systems.

Interferons are active on cells of the same species as that in which they are produced, and, in some cases, on cells of a limited number of other species. Different molecular forms of interferon are produced by different cell types of the same species; thus human fibroblast interferons differ in several respects from human leucocyte interferons (see 'Purification and properties of interferons'). Several different interferons may also be produced by a single cell type (Erikson and Pauker, 1979; Lin et al., 1978), so that the antiviral activity may be spread over a broad range of molecular weights and isoelectric points. This makes purification very difficult by conventional methods such as precipitation by salts, gel filtration, and electrophoresis, though these procedures have been applied to interferons (Fantas, 1973; Knight, 1976). Interferons are also extremely potent, having specific activities in the order of $10^9$ units per milligram of protein (Knight, 1975); a unit of interferon is normally defined as that amount which will inhibit by 50% the yield of virus from a cell which is subsequently infected. Activities are usually expressed as reference units, by comparison with international standards. Because of their high specific activities, only extremely small quantities of the interferon proteins
need produced by cells, adding further to the problems involved in purification.

The events involved in the induction of interferon and in its action are poorly understood despite the efforts which have been made; some of the reasons for this are the minute amounts of interferon which are normally produced, the limitations imposed by the biological assay, and the difficulties involved in the preparation of pure interferon. Many of the studies performed on the interferon system have been carried out on cells (normally fibroblasts) in culture. The present work is a continuation of such studies; mutant cells were utilised in an attempt to circumvent some of the difficulties outlined above.

Interferon Induction

Most cell lines in culture produce interferon in response to viral infections, although the amount synthesised varies widely from one cell-virus interaction to another (Ho, 1975). Virus replication is not essential for interferon induction, and indeed, u.v.(ultra-violet light)-inactivation of some viruses may enhance the production of interferon (Metz, 1975a). Double-stranded RNA (ds RNA) has also been shown to be a potent inducer of interferon synthesis (Field et al., 1968). The use of ds RNA has simplified the investigation of the interferon induction process by eliminating the complexities of viral replication. The most important requirements of ds RNA as an efficient interferon inducer are a stable helical structure, and the presence of the free 2'-hydroxyl groups (Colby and Morgan, 1971). Thus double-stranded DNA and DNA-RNA hybrids are not effective inducers of interferon. ds RNA does not appear to induce an antiviral state without the mediation of interferon (Vengris et al., 1975). As single-stranded RNA viruses replicate via ds RNA intermediates, it has been proposed that these molecules are responsible for the induction of interferon by viruses (Field et al., 1967).

Temperature-sensitive mutants of several viruses have been
employed in the attempt to assess the role of ds RNA in interferon
induction. Temperature-sensitive (ts) mutants of Sindbis virus which
did not make viral RNA at the non-permissive temperature also failed
to produce interferon at this temperature (Lockart et al., 1968); however,
a mutant which made more than 80% of the normal amount of RNA induced
only 10% of the normal amount of interferon. The results of Lomniczi and
Burke (1970) gave some indication that interferon induction may relate
to the synthesis of viral RNA; ts mutants of Semliki Forest virus
which are defective in RNA synthesis do not induce interferon at the
non-permissive temperature (39°C). The anomalous results (in which
interferon was produced at 39°C) obtained at high multiplicities of
infection may have been due to the contamination of virus stocks with
ds RNA. ts mutants of reovirus, a double-stranded RNA virus, have also
been investigated (Lai and Joklik, 1973). Interferon synthesis failed
to correlate with any viral function (including RNA synthesis), except
a very late function. Furthermore, one mutant which neither synthesised
viral RNA nor induced interferon at the non-permissive temperature
became a potent inducer after u.v.-irradiation, yet still did not
synthesize detectable RNA. In these experiments, there is a lack of
correlation between the synthesis of viral RNA and the induction of
interferon. However, only a few molecules of ds RNA per cell appear to
be required for interferon induction, and it is quite possible that such
small amounts may be synthesised by some of these mutants while RNA
synthesis remains undetectable.

Some more recent results using ts mutants of Sindbis virus
have given stronger indications that the induction of interferon by
this virus may require viral RNA synthesis (Atkins et al., 1974; Marcus
and Fuller, 1979). Other work, utilising defective interfering (DI)
particles of Vesicular Stomatitis virus (VSV), has indicated that the
synthesis of a single molecule of ds RNA within the cell may be sufficient
to induce interferon production (Marcus and Sekellick, 1977). The DI particles contained a single strand of self-complementary RNA (\(-\) RNA of the genome covalently linked to the + RNA messenger), which is non-replicating, and is presumed to form ds RNA upon release into the cytoplasm. A further interesting observation from this work is that the maximal interferon yield appears to be obtained from infection at 1 DI particle per cell.

Other agents, such as bacterial endotoxins and specific antigens (those to which the animal has been made immune), which do not induce interferon in cultured fibroblasts, do induce interferon in vivo; these agents also stimulate the production of interferon in cells of the reticulo-endothelial system (Colby and Morgan, 1971). Mouse interferon formed by such cells in response to bacterial endotoxins or polyanions is similar to mouse fibroblast interferon, in that it is stable at pH 2, exhibits a similar cross-species activity, and is inactivated by antibodies to L cell interferon (Youngner and Salvin, 1973; Schultz and Chirigos, 1979). Interferon induced by specific antigens or by T cell mitogens is labile at pH 2, exhibits a different cross-species activity, and is not inactivated by antibodies to fibroblast interferon (Youngner and Salvin, 1973; Schultz and Chirigos, 1979); the pH 2-labile interferon is designated type II, while the acid-stable interferons are classed as type I. The production of interferon in response to endotoxin or a specific antigen is abolished by treatment of spleen cells with cytochalasin D, indicating that a membrane interaction is involved (Ito et al., 1978b). However, cytochalasin D does not significantly decrease the yield of interferon from mouse spleen cells induced with HVJ (Haemagglutinating virus of Japan) unless the virus is previously inactivated by u.v.-irradiation; this indicates that in spleen cells the virus may induce interferon either by a membrane interaction, or by penetration of the viral genome into the cell, a mechanism similar to the induction of interferon in L cells by
HVJ (Ito et al, 1978a and b). This virus-induced interferon from spleen cells is presumed to be similar to leucocyte interferon. Untreated or u.v.-irradiated HVJ appears to induce interferon in both T and B lymphocytes (Ito et al, 1978a). In order to produce type II interferon, it appears that T cells require the cooperation of B cells and/or macrophages (Sonnenfeld, et al, 1979).

It is thus indicated that the dsRNA formed during the replication of many RNA viruses may be the major, and possibly the only, inducing moiety in RNA virus-fibroblast interactions. However, while the virus genome may give rise to the production of interferon in reticuloendothelial cells, other viral components and non-viral substances are also capable of interferon induction in these cells. It is interesting to speculate that the type of interferon produced (fibroblast, leucocyte, type II, etc.) may depend upon the inducer as well as upon the cell (Erikson and Pauker, 1979). The nature of the inducing moiety of DNA viruses in fibroblasts is unknown; it would not seem likely that ds RNA is involved (Metz, 1975a).

The Control of Interferon Induction

The extracted mRNA of induced, but not control, cells can be translated in heterologous cells or in in vitro protein synthesising systems to give interferons characteristic of the donor cell (Lebleu, et al, 1978); the production of such mRNA (as measured by its capacity to promote interferon formation in heterologous cells) is prevented by inhibitors of RNA synthesis (De Maeyer-Guignard, et al, 1972). Furthermore, inhibitors of both RNA and protein synthesis prevent the production of interferon from cells if present throughout the inducing period (Metz, 1975a). These experimental results indicate that transcription of an interferon gene and translation of the mRNA are necessary steps in the production of interferon from cultured cells.

Hybrid cells have been used in the attempt to assign the
gene for human fibroblast interferon to a particular chromosome. Human-rodent hybrid cells gradually lose human chromosomes, and the presence of different chromosomes may be correlated with the production of interferon. However, there are serious difficulties involved in this procedure. The continuous random loss of chromosomes from the cells results in 'clones' of cells which are not identical in their complement of chromosomes: thus a clone may be described as 78% positive for a given chromosome, meaning that the chromosome is present in 78% of the cells analysed. Furthermore, a small part of a chromosome may be translocated onto a different chromosome. It is thus not surprising that the results from different laboratories are not entirely in agreement.

Tan et al (1974) indicated that both chromosomes 2 and 5 were required for the production of human interferon in mouse-human hybrids. However, the results of Meager et al (1979) suggest that chromosome 5 is not required for human interferon production, but that the presence of chromosome 9 is necessary; the additional requirement of human chromosome 2 could not be excluded. It is interesting to note that chromosome 9 was not detected in any of the hybrids examined by Tan et al (1974). Other experiments on Chinese hamster-human hybrids have indicated that the presence of chromosome 2 is not required for the production of human interferon (Morgan and Faik, 1977; Meager, et al, 1979). Morgan and Faik (1977) suggested that the presence of chromosome 5 was necessary for human interferon induction in these cells, and the results of Meager et al (1979) do not exclude this possibility. However, the latter authors' conclusion is that the structural gene for human fibroblast interferon is located on chromosome 9 (and possibly on the short arm of this chromosome). The analysis of results from Chinese hamster-human hybrids is complicated by the fact that the parental Chinese hamster cells produce little, if any, interferon. There
may thus be an additional requirement for a human regulatory protein in order that the hybrids produce significant levels of human interferon (Morgan, 1976a and b; Morgan and Faik, 1977). A further point is that only one group (Meager et al., 1979) characterised the human interferon produced as being fibroblast interferon. It is interesting to note that even hybrids derived from parental human lymphocytes produced human interferon of the fibroblast type. To summarise these experiments, it would appear that the structural gene for human fibroblast interferon may be located on chromosome 9, and that the possible requirement of chromosome 5 for the production of interferon in Chinese hamster-human hybrids may reflect the need for a human regulatory protein.

The results of Morgan and Faik (1977) showed that Chinese hamster ovary (CHO-K1) cells contained the structural gene for interferon but were unable to express it; the product of a human chromosome appeared to rectify the deficiency, indicating that a regulatory protein may be required for the production of interferon. The monkey cell line Vero, which appears to be unable to synthesise interferon, has also been used to investigate the control of interferon induction. These cells are able to translate exogenous mRNA for monkey or mouse interferon, and to secrete an active product (De Maeyer-Guignard et al., 1972), indicating that the defect is an inability to produce functional interferon mRNA. Mouse-Vero hybrid cells have been isolated and tested: the majority of the hybrids produced mouse interferon upon suitable stimulation, but none could be induced to synthesise monkey interferon (Emery and Morgan, 1979). The authors concluded that the defect in Vero cells was an inactive or deleted interferon gene, and not a regulatory protein.

As both the common classes of interferon inducers, viruses and dsRNA, inhibit protein synthesis, Tan and Berthold (1976) suggested that this was the basis of the inducing capacity. They proposed that
a labile repressor of interferon synthesis was continually produced in non-induced cells, and that interferon inducers prevented its formation, thus allowing the transcription and translation of interferon mRNA. Several inhibitors of protein synthesis, other than viruses and dsRNA, were shown to induce low levels of interferon in a sensitive cell line (Tan and Berthold, 1976). In order to explain the shut-off of interferon synthesis which occurs even in the continued presence of the inducer, however, it would be necessary to postulate another repressor protein. Furthermore, the results of Morgan and Faik (1977), discussed above, indicate that a positive control mechanism may be required for derepression of the interferon gene. The low levels of interferon induced by these inhibitors might also suggest that the proposed mechanism is an oversimplification.

Cells induced for interferon produce the protein for a limited period only (Tan, 1970), and become resistant to a second induction; this resistance, termed the refractory state, lasts for several days in non-dividing cells, but for shorter periods in rapidly-dividing cells (Pauker and Boxaca, 1976). Interferon pretreatment also inhibits a subsequent induction of interferon, a phenomenon known as 'blocking' (Pauker and Boxaca, 1976). It has been proposed that the refractory state is the result of interferon action, creating an inhibitory feedback on its own production (Colby and Morgan, 1971). This hypothesis is supported by observations on mutant mouse cells which were resistant to interferon action: the mutant cells were repeatedly inducible for interferon production, unlike the parental cells, and also produced up to ten-fold more interferon at the initial induction (Chany and Vignall, 1970).

Inhibitors of protein synthesis and of RNA synthesis may be used to enhance interferon production ('superinduction') if applied at particular times in relation to the inducer; it has been proposed that these antimetabolites prevent or delay the
formation of a regulatory protein which inhibits interferon synthesis (Vilcek, 1969; Tan *et al.*, 1970). The combination of inhibitors of both protein synthesis and RNA synthesis results in a greater enhancement of interferon yield than either alone (Tan *et al.*, 1970). The procedure commonly used is the inhibition of protein synthesis during the inducing period, followed by removal of the inducer and the inhibitor and the addition of an inhibitor of RNA synthesis. The production of interferon may continue at a high rate for several days after superinduction; if the inhibition of RNA synthesis is reversed during this period, however, there follows a rapid shut-off of interferon production (Sehgal, Tamm and Vilcek, 1975). A wide range of inhibitors are effective in the superinduction of interferon, and the yield of interferon may be increased by 100-fold or more (Atherton and Burke, 1978; Havel and Vilcek, 1972). Dianzani *et al.* (1970) showed that a similar procedure would prevent the establishment of the antiviral state (AVS) in induced cells, but would not prevent interferon production.

It would thus appear that interferon (or a protein co-induced with interferon) normally interacts with the producing cell to promote the synthesis of a regulatory (inhibitory) protein (Sehgal and Tamm, 1976); superinduction might thus be understood as the prevention of the synthesis of such a protein. This hypothesis would also explain the phenomenon of blocking.

*In vitro* protein-synthesising systems have been used to investigate the shut-off of interferon production and superinduction. However, the results of these experiments have not always been interpreted with caution. Thus Greene *et al.* (1978) have suggested that the decrease in activity of interferon mRNA during shut-off of interferon synthesis may be due to a specific inactivation of the mRNA; however, it would appear quite possible that the mRNA for a repressor protein is also translated in the recipient cells, and
subsequently inhibits interferon production (i.e. by inhibiting the translation of the interferon mRNA in the recipient cells). Raj and Pitha (1977) indicated that the activity (not necessarily the amount) of interferon mRNA from superinduced cells was greater than that from control cells when measured in the wheatgerm translation system, but not when measured in *Xenopus* oocytes; in contrast, Señal et al. (1977) demonstrated a greater activity in *Xenopus* oocytes of the interferon mRNA from superinduced cells than that from normally-induced cells. Such an increase may reflect either the altered processing of the interferon mRNA in the superinduced cell before extraction of the mRNA or a difference in the translation system caused, as indicated above, by other mRNAs.

The pretreatment of cells with low levels of interferon may increase the yield of interferon at a subsequent induction; this effect, 'priming', does not appear to require the mediation of protein synthesis, unlike blocking (Stewart, Gosser and Lückart, 1971). Priming enhances the yield of interferon from a range of different cells, and under different conditions, including human fibroblasts induced with poly(rI).poly(rC) (Havell and Vilcek, 1972), mouse L cells induced with virus (Knight, 1974), and endotoxin-induced rabbit lymphoid cells (type II interferon) (Hashimoto et al., 1979). Priming appears to increase the amount of interferon mRNA available for translation (Fujita et al., 1979).

Diethylaminoethyl (DEAE)-dextran appears to enhance the inducing capability of dsRNA (Havell and Vilcek, 1972), and is now routinely used in conjunction with this inducer. DEAE-dextran and other polycations such as poly(L)-lysine may enhance the uptake of the RNA, or protect it from enzymatic degradation (Metz, 1975a). The production of interferon may be further stimulated by the addition of calcium (Booth and Borden, 1978) or ascorbic acid (Siegel et al., 1975) to the
cells at the same time as the dsRNA. It has also been noted that the pretreatment of lymphoid cells with 5′-bromodeoxyuridine (BrdU) very significantly increased the yield of interferon (Tovey et al., 1977); this observation has also been extended to fibroblastoid cells (Baker et al., 1979).

These results all reflect facets of the mechanism which controls interferon production, but their satisfactory interpretation remains to be achieved. However, it has been indicated that part of the control process may involve the interferon-mediated production of a protein which prevents the synthesis of interferon. This hypothesis is supported by the observation that prior u.v.-irradiation of cells may increase the yield of interferon to the level achieved by use of superinduction (Lindner-Primmel, 1974).

The Purification and Properties of Interferons

Fibroblast, leucocyte, and lymphoblastoid interferons are produced from the respective cells by infection with virus or by treatment with dsRNA. Interferons from these types of cells are type I interferons (acid-stable), and are active on all interferon-sensitive homologous cells, though there may be quantitative differences in the cellular response (Gardner and Vilcek, 1979). Leucocyte and lymphoblastoid interferons are also frequently active on heterologous cells, and may be significantly more active on these cells than on homologous cells, while fibroblast interferons tend to be relatively species-specific (Gresser et al., 1974; Pauker et al., 1977; Havell et al., 1977). The use of anti-interferon antibodies has demonstrated that human fibroblast and leucocyte interferons are antigenically distinct (Havell et al., 1975). Such antibodies have also been used to identify the interferons from lymphoblastoid cells as fibroblast (about 13%) and leucocyte (majority) interferons; when separated by chromatography on anti-interferon antibody-Sepharose, the leucocyte interferon portion
exhibited a characteristic cross-species activity, while the fibroblast interferon fraction was relatively inactive in heterologous (bovine and porcine) cells (Havell et al., 1977). Anti-interferon antibody affinity columns have been used successfully as a step in the purification of interferons (Ogburn et al., 1973; Pauker et al., 1977; De Maeyer-Guignard et al., 1978).

Both fibroblast and leucocyte interferons bind to hydrophobic ligands; the fibroblast preparations generally bind more efficiently (Jankowski et al., 1975). Such ligands may be used to purify interferons, though interferon preparations frequently show a heterogeneous response to hydrophobic chromatography (Jankowski et al., 1975; Erikson and Pauker, 1979; Yakobson et al., 1979). Fibroblast interferons also bind to polymucleotides, probably due to hydrophobic interactions (De Maeyer-Guignard et al., 1978).

In general, fibroblast interferons appear to contain large amounts of carbohydrate, in contrast to leucocyte interferons; thus fibroblast interferon has been shown to be sensitive to periodate (Pantes, 1973), and to bind to lectins and gangliosides (Besancon and Ankel, 1974a and b). Human leucocyte interferon is not retained by lectin affinity columns which bind human fibroblast interferon (Jankowski et al., 1975). Furthermore, highly-purified human fibroblast interferon may be visualised after polyacrylamide gel electrophoresis either by protein-specific stains or by stains specific for carbohydrate (Knight, 1976). The carbohydrate component of interferons does not appear to be essential for activity; thus removal of most of the carbohydrate enzymically (Bose et al., 1976) or by treatment with periodate (Stewart et al., 1977a) does not inhibit biological activity. In addition, biologically active interferons may be synthesised, from the extracted mRNA of an induced cell, in an in vitro protein-synthesising system or in frog oocytes (De Maeyer-Guignard et al., 1972;
Lebleu et al., 1978), again indicating that glycosylation is not essential for activity. However, production of both fibroblast and leucocyte interferons is diminished by treatment of the cells with inhibitors of glycosylation (Havell et al., 1975; Cundliffe and Morser, 1979). This indicates that glycosylation is essential for interferon production.

Human fibroblast interferon binds to lectins specific for sialic acid, L-fucose, and D-mannose, indicating that the interferon contains these sugar residues; human leucocyte interferon, in contrast does not bind to any of these lectins (Jankowski et al., 1975). Variations in the amount of terminal sialic acid in fibroblast interferons appear to be responsible for the charge heterogeneity of these molecules; treatment with neuraminidase results in the conversion of the more acidic components to a species with a higher isoelectric point (Dorner et al., 1973; Morser et al., 1978). This conversion appears to be reversible. Isoelectric focusing of leucocyte and lymphoblastoid interferons has shown that these preparations are heterogenous in charge, with the majority of the activity focusing in the acidic part of the gel in several peaks (Havell et al., 1977; Lin et al., 1978). It has been indicated that pretreatment of leucocyte interferon with periodate removes the charge heterogeneity (Stewart et al., 1977b); thus leucocyte interferon, like fibroblast interferon, appears to be variably glycosylated.

Different species of fibroblast interferons may be separated on the basis of molecular weight, often achieved by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE). Mouse L cell interferon has been separated by gel filtration and PAGE into two components of 24,000 and 36,000 daltons (Yamamoto, 1979). These two appear to be antigenically unrelated and to have different cross-species activity. Both species bind partially to mannose-specific lectins (indicating some variation in glycosylation within each sub-group) but
only the higher molecular weight form binds to Wisteria floribunda lectin, which is specific for galactose (Yamamoto, 1979). Apparently pure mouse fibroblast interferon (from C₂⁴₃ cells) has been prepared by sequential affinity chromatography on poly(uridylic acid)-agarose and anti-interferon antibody-agarose, a simple process which gives almost 100% recovery of activity (De Maeyer-Guignard et al., 1978). These highly-purified preparations showed two bands of activity when separated by SDS-PAGE, corresponding to molecular weights of 35,000 daltons (80% of recovered activity as assayed on homologous cells) and 22,000 daltons (about 15%). The 35,000 dalton species appeared to contain large amounts of carbohydrate, and was unaffected by reducing conditions; the smaller component contained little, if any, carbohydrate, and its activity was greatly reduced by incubation under reducing conditions (De Maeyer-Guignard et al., 1978). Less pure preparations of mouse fibroblast interferon from the same source gave similar results upon SDS-PAGE; the higher molecular weight component (about 38,000 daltons) was insensitive to reducing conditions, and was less active on heterologous than on homologous cells; the smaller species (22,000 daltons) was sensitive to reducing conditions, and was found to be three- to ten-fold more active on heterologous cells than on mouse cells (Stewart et al., 1977a). Thus the lower molecular weight component of mouse fibroblast interferon appears to resemble leucocyte interferons, in that it contains much less carbohydrate and exhibits cross-species activity. Although there is broad agreement on the molecular weights of the components of mouse fibroblast interferon, it is apparent that there is considerable heterogeneity within these two components (Erikson and Pauker, 1979). Furthermore, differences have been observed between preparations, even when prepared by the same methods (Erikson and Pauker, 1979; Stewart et al., 1977a).

Leucocyte and lymphoblastoid interferon preparations have
been analysed in similar ways. SDS-PAGE of human leucocyte interferon (and lymphoblastoid interferon) results in two bands of activity, corresponding to molecular weights of approximately 21 000 (range 21 000 to 23 500) and 16 000 (range 13 500 to 17 500) daltons (Havell et al, 1977; Pauker et al, 1977; Lin et al, 1978). In each case the smaller component represented the majority of the antiviral activity as assayed on homologous cells; furthermore, only the smallest component (about 13 500 daltons) had significantly greater activity (100-fold) on bovine cells than on homologous cells (Lin et al, 1978; Pauker et al, 1977).

Thus although preparations of fibroblast and of leucocyte interferons may be broadly distinguished in terms of their immunological, chemical, and biological characteristics, within each of these groups there is considerable diversity. In particular, some species of fibroblast interferons may resemble leucocyte interferons, and both leucocyte and lymphoblastoid interferons have been shown to contain components with similar properties to fibroblast interferons. Because of the large degree of heterogeneity in preparations of fibroblast, leucocyte and lymphoblastoid interferons, it is essential that the purification procedures utilised do not select for particular interferon species. As the carbohydrate portion of interferons seems to be highly variable, procedures which rely on interactions with sugar residues (affinity chromatography using lectins or gangliosides, for example) should be used with great caution. In general, procedures which give high recoveries should be used (for example, the purification of mouse fibroblast interferon by De Maeyer–Quignard et al, 1978). Knight (1976) demonstrated that a preparation of human interferon (from fibroblasts) contained an active glycoprotein of about 20 000 daltons; however, in several of the purification steps less than 50% of the activity was transferred to the subsequent stage, and other
species of interferon may therefore have been lost (for example, a higher molecular weight species may have been lost during fractionation on Bio-Gel P150—see figure 1 of Knight, 1976).

Type II interferons appear to be of higher molecular weight than fibroblast and leucocyte interferons. The major components of both human and mouse type II interferons have a molecular weight of about 40,000 - 45,000 daltons; in each case there is also a larger component (greater than 65,000 daltons) which represents only a small part of the activity (Langford et al., 1979; Osborne et al., 1979). These interferon preparations exhibit a great deal of molecular heterogeneity, including variable glycosylation (Wietzerbin et al., 1979).

Many of the purification procedures developed for fibroblast and leucocyte interferons have recently been used for the characterisation and purification of type II interferon; hydrophobic chromatography, and lectin-affinity chromatography have both proved to be of use in purification, and have also further demonstrated the heterogeneity of type II interferon preparations (Wietzerbin et al., 1979; Osborne et al., 1979; Langford et al., 1979).

Interferon Action

The Membrane Interaction with Interferon

Cells made virus-resistant by treatment with interferon inducers appear to have been acted upon by external interferon, as anti-interferon antibody can abolish the effect (Vengris et al., 1975). It is thus indicated that interferon acts at the cell membrane.

Interferon has been shown to bind to the membranes of sensitive cells, and to be recoverable from the membrane fraction of homogenised cells in amounts proportional to the sensitivity of the cell to interferon (Stewart et al., 1972). No detectable interferon was recovered in a similar fraction from interferon-resistant cells, but was recovered from the parental, interferon-sensitive, cells (Gresser et al., 1974).
Interferon covalently attached to Sepharose beads induces an antiviral state only in adjacent cells, and remains effective even after multiple cell-to-cell transfers (Ankel and Chany, 1973). Although it is possible that the protein becomes detached from the Sepharose, the indication again is that interferon acts at the cell surface.

Studies on mouse–monkey hybrid cells have indicated that components of the cell membrane are responsible for the species specificity of interferons (Chany et al, 1973; Bourgeade, 1974). Primate and mouse interferons have a synergistic antiviral effect in these cells, indicating that after a specific membrane effect, there is a single mechanism common to mouse and monkey cells which is responsible for the induction of the antiviral state (Bourgeade, 1974). The membrane receptor(s) for primate interferon, but not that for mouse interferon, is inactivated by treatment with trypsin (Chany et al, 1973), indicating that, at least in the case of monkey cells, part of the membrane receptor for interferon may be a protein. Tan (1975) showed that human cells trisomic for chromosome 21 were more sensitive to the antiviral effects of interferon than the normal cells (disomic for chromosome 21), and that cells monosomic for this chromosome were less sensitive than normal cells to interferon action. Revel et al (1976) used mouse–human hybrid cells to raise antibodies against cell-surface components coded for by human chromosome 21, and demonstrated that the antibody preparation inhibited the response to interferon of normal human fibroblasts. These authors also showed that the inhibitory effects of the preparation on interferon action were greater in monosomic 21 cells and less in trisomic 21 cells than in the normal disomic 21 cells. These experiments strongly suggest that human chromosome 21 carries a gene for a membrane receptor for interferon.

The action of interferon is blocked by pretreatment of the
protein with certain lectins; the inhibition may be reversed by lectin-specific sugar residues. Similarly, pretreatment of mouse cells with other lectins inhibits interferon action, and may also be reversed by particular sugars (Besancon and Ankel, 1974a). Sepharose-bound interferon is also inhibited after preincubation with glycolipids, and soluble interferon binds to Sepharose-bound gangliosides (Besancon and Ankel, 1974b). This latter interaction is inhibited by phytohaemagglutinin (PHA), and appears to involve a specific interaction of interferon with the carbohydrate moiety of certain gangliosides (Besancon, Ankel and Basu, 1976). Certain cell lines deficient in gangliosides are relatively insensitive to interferon; their response to the protein may be enhanced by prior incubation of the cells with gangliosides (Vengris et al., 1976). Cholera toxin and some of the polypeptide hormones utilise gangliosides on the cell surface as receptors; the effect of the toxin and of one hormone on the antiviral activity of interferon has been examined. Thyrotropin competitively inhibited the effect of interferon (Kohn et al., 1976); and cholera toxin added before the addition of interferon, but not at later times, inhibited the establishment of the antiviral state in mouse L cells (Friedman and Kohn, 1976). These several results indicate that gangliosides may be required for a successful interaction between interferon and its specific receptor.

The Development of the Antiviral State

It has been indicated that interferon need not enter the cell in order to exert its effects, and that the species specificity of interferon is determined by membrane receptors. It would thus appear likely that a secondary messenger molecule is involved to link the membrane interaction with events leading to the development of the antiviral state. Such a messenger, which may be the same molecule for different species (Bourgeade 1974), has remained elusive, although
there has been an indication that cyclic AMP could be indirectly involved (Weber and Stewart, 1975).

Blalock and Baron (1977) showed that while human (WISH) or baby hamster kidney (BHK) cells alone are insensitive to the action of mouse interferon, the co-cultivation of either cell line with mouse L cells in the presence of mouse interferon resulted in a marked decrease in the virus yield from the insensitive cells. Further experiments demonstrated that mouse, hamster or human interferons were not involved in the transfer of viral resistance, and that the process required both cell-to-cell contact and a period of transcription in the recipient cells (Blalock and Baron, 1977; Blalock and Baron, 1979). It would appear likely that the transfer, which is highly efficient, is mediated by secondary messenger molecules. The transfer of the antiviral state also occurs between homologous cells (Blalock and Stanton, 1978). Blalock (1979) further showed that populations of mouse L cells consisted of cells which varied widely in their response to interferon: the cells which were most sensitive to interferon determined the response of the whole population. It would appear likely that this transfer occurs in vivo, and amplifies the response to interferon. It has been reported that the treatment of cells with ammonium salts interferes with the development of the antiviral state in response to interferon (Commoy-Chevalier et al., 1978); it is possible that these salts, which disrupt membrane processes, prevent the transfer of viral resistance between cells, rather than affecting a process within the cell.

Cells require a period of incubation at 37°C in order to develop the antiviral state in response to interferon (Sonnabend and Friedman, 1966). Treatment with actinomycin D, an inhibitor of RNA synthesis, during this period prevents the establishment of the antiviral state (Taylor, 1964); inhibitors of protein synthesis
applied during this period also have the same effect (Sonnabend et al., 1970). Furthermore, enucleation of cells prior to interferon treatment prevents the expression of antiviral activity, whereas cells enucleated after incubation with interferon develop an antiviral state (Radke et al., 1974). It is thus strongly indicated that host macromolecular synthesis is essential for the development of the antiviral response.

**The Effects of Interferon on Virus Replication in vivo**

The first step towards an understanding of the mechanism(s) by which interferon inhibits virus replication is to determine the stage or stages at which replication is blocked. Interferon inhibits viral replication in cells inoculated with infectious RNA from a number of different viruses, indicating that adsorption and penetration of the virus particle into the cell are not the inhibited stages (Vilcek, 1969). It has been shown that both viral RNA (Mecs et al., 1967) and protein (Friedman, 1968) synthesis are inhibited in interferon-treated cells infected with Semliki Forest virus, a single-stranded RNA virus with a genome identical in sequence to the mRNA. The activity of the viral polymerase was inhibited, but no polymerase inhibitor could be detected in vitro (Sonnabend et al., 1967). However, because of the interdependence of RNA and protein synthesis in this system, the inhibition of the polymerase could reflect an effect on the synthesis of the enzyme (via an effect on protein synthesis), on its function (i.e. RNA synthesis), or on both.

Vesicular Stomatitis virus (VSV), a single-stranded virus with a genome complementary to the mRNA, contains an RNA polymerase activity associated with the virus particle. It has been claimed that the polymerase, and thus viral transcription, is inhibited in interferon-treated cells (Marcus et al., 1971; Manders et al., 1972). In order to avoid the possible involvement of protein synthesis,
Marcus and Sekellick (1974) measured the rate of viral RNA synthesis in infected Vero cells in the presence of cycloheximide, and showed that prior interferon treatment decreased this rate four-fold. In the presence of cycloheximide the rate of primary transcription (that which is independent of protein synthesis) is increased, presumably because the synthesis of a control protein is inhibited. Other experiments showed that in VSV-infected human foetal tonsil cells both primary transcription and viral protein synthesis were inhibited by interferon pre-treatment; however, although similar treatment of simian (BGM) cells resulted in an inhibition of viral protein synthesis, there was very little effect on primary transcription (Thacore, 1978). Baxt et al (1977) also noted that interferon treatment of simian LLC-MK and human U cells resulted in a decrease in the extent of primary transcription. However, these authors also reported a preferential decrease in the accumulation of those species of RNA whose synthesis was dependent on protein synthesis, and a much greater inhibition of viral protein synthesis than could be accounted for by a decrease in the amount of viral mRNA. It would thus appear that interferon pretreatment results in an inhibition of viral protein synthesis in VSV-infected cells. There may also be a significant inhibition of viral RNA synthesis (primary transcription), the extent of which appears to be dependent on the host cell. It is also reported that host protein synthesis is not further inhibited by interferon pretreatment of VSV-infected cells (Gupta et al, 1974; Thacore, 1978). Indeed, it has been noted that with increasing concentrations of interferon there appears to be a decrease in the virus-induced inhibition of host protein synthesis (Baxt et al, 1977).

Of the DNA viruses, Vaccinia has been the most studied in interferon-treated cells. The virus loses its outer envelope when penetrating into the cell, releasing the viral core containing the double-stranded DNA and a polymerase into the cytoplasm. Early viral
mRNA is synthesised within the core, exported, and the early viral proteins are made. Subsequently, the genome is released, and DNA replication is initiated. Interferon treatment inhibits DNA synthesis (Joklik and Merigan, 1966) and the release of the genome from the core (Magee et al, 1968). There is, however, an enhancement of early viral RNA synthesis in the interferon-treated cell, an effect which may be mimicked by inhibitors of protein synthesis (Metz and Esteban, 1972). The ability of the viral mRNA to associate with ribosomes appears to be decreased (Joklik and Merigan, 1966) and it has been reported that both initiation and elongation of protein synthesis directed by the viral mRNA are inhibited (Metz et al, 1975). Thus it is apparent that the major effect of interferon on Vaccinia virus replication is to inhibit the translation of viral mRNA.

Simian virus 40 (SV 40), an oncogenic DNA virus, has been studied because, unlike the majority of viruses, it does not significantly alter the gross level of host RNA and protein synthesis for long periods after infection. However, the system has proven to be rather complicated. In the normal lytic infection, viral RNA is transcribed from the double-stranded genome in two stages, the first before ('early' RNA), and the second after ('late' RNA), viral DNA synthesis is initiated. Infection of interferon-treated cells resulted in the inhibition of early viral RNA synthesis, with a resultant inhibition of protein synthesis (Oxman and Levin, 1971). However, when interferon treatment was delayed until after the initiation of DNA synthesis, viral protein synthesis, but not the synthesis of viral RNA, was inhibited (Yakobson et al, 1977).

Interferon appears to inhibit the maturation and release of RNA tumor viruses (Swetly and Ostertag, 1974; Billiau et al, 1975). It has been indicated that the non-infectious particles which are produced contain aberrant structural proteins (Pitha et al, 1980);
these proteins appear to result from a defect in the proteolytic cleavage associated with virion assembly. It has also been demonstrated that interferon pretreatment of cells may prevent infection by these viruses; viral replication in this case is inhibited at an early stage (Morris and Burke, 1979).

Studies of interferon action in single cells have indicated that the effect of the protein is to delay viral replication and to decrease the yield of progeny virus (Fleischmann and Simon, 1973). It also appeared that interferon treatment could not fully protect the infected cell, but could only delay cell death (Stitz and Schellekens, 1980). While this may be true in general, the derivation of virus-resistant mutant cells by selection with temperature-sensitive viruses (Morgan et al., 1973) would indicate that at least a proportion of the infected cells may escape this fate (it has been indicated that the mutant cells are constitutive for interferon production (Jarvis and Colby, 1978) or for the antiviral state (this thesis)).

Most of the experiments on interferon action described above have been carried out on fibroblast interferon, and the work described herein has been to investigate the mechanism of action of this species of interferon. However, cells appear to respond rather differently to different interferons. Thus human fibroblasts respond more rapidly to fibroblast than to leucocyte interferons (Gardner and Vilcek, 1979), and the anticellular effects of fibroblast interferon appear to be greater than those of leucocyte interferon (Lundgren et al., 1979). Cells selected for resistance to either fibroblast or leucocyte interferon are also resistant to the effects of the other species (Kuwata et al., 1979), indicating that there are similarities between the two species. However, cells selected for resistance to type I interferon were sensitive to the antiviral action of type II interferon (Bourgeade et al., 1979), indicating that the mechanisms of
action of type I and type II interferons may differ, at least in some respects. This is also indicated by the observation that type I and type II interferons have a synergistic effect in mouse L cells (Fleischmann et al., 1979).

**In vitro Studies of Interferon Action**

It has been indicated that the major effect of interferon on many virus infections is to inhibit the translation stage of viral replication. There may also be an inhibition of transcription. Cell-free protein synthesising systems have been utilised in order to examine the effect on translation in greater detail. An effect on transcription in vitro has not been widely studied, although it has been reported that the viral polymerase of Semliki Forest virus does not appear to be inhibited by extracts from interferon-treated cells (Sonnabend et al., 1967).

There have been many reports of an inhibition of protein synthesis directed by virus mRNA in vitro (Kerr, 1971; Friedmann et al., 1972; Gupta et al., 1973; Samuel and Joklik, 1974; Content et al., 1975; Sen et al., 1976). Many of these systems showed an indiscriminate inhibition of viral and cellular mRNA translation (Gupta et al., 1973; Gupta et al., 1974; Falcoff et al., 1976; Hiller et al., 1976; Mayr et al., 1978). It has been reported that the addition of certain tRNA (transfer RNA) species may partially overcome the inhibitory effect apparent in extracts from interferon-treated cells (Content et al., 1975; Falcoff et al., 1976; Hiller et al., 1976). However, tRNA species from interferon-treated cells appear to be as effective in this respect as those from untreated cells, and it is also reported that tRNA inactivation occurs only in extracts which have been passed through Sephadex G25 (Sen et al., 1976). Furthermore, some laboratories have failed to show this reversion by tRNA (Samuel, 1976; Mayr et al., 1978), and this effect may be an artifact of the preparations (Hovanessian, 1979).
Some of the cell-free systems from interferon-treated cells have required prior infection of the cells or the addition of dsRNA to the system in order to demonstrate an inhibition of virus-directed protein synthesis (Friedman et al., 1972; Kerr et al., 1974). In most cases, there has also been a similar inhibition of protein synthesis directed by exogenous cellular mRNA, globin mRNA, or synthetic polyribonucleotides (Falcoff et al., 1976; Gupta et al., 1973). This inhibitory system requires ATP as well as dsRNA for its activation (Roberts et al., 1976a). The inhibitor is a novel oligonucleotide, or series of nucleotides, with a novel 2'-5' linkage; the most active component of the series has the structure "pppA'pA'pA'pA" (Kerr et al., 1977; Kerr and Brown, 1978). This low molecular weight inhibitor (LMWI) is effective at nanomolar concentrations, and may operate via the activation of an endonuclease (Clemens and Williams, 1978; Shaila et al., 1977) which degrades mRNA. The LMWI appears to be identical to an inhibitor formed upon the addition of dsRNA to rabbit reticulocyte lysates (Hovanessian and Kerr, 1978; Cooper and Farrel, 1977). The LMWI has also been shown to be active on intact cells, inhibiting both viral and cellular protein synthesis by 50-80%; the mechanism of action appears to involve the stimulation of a nuclease (Hovanessian, 1979).

The kinetics of the inhibition of protein synthesis caused by the addition of dsRNA or LMWI to cell-free extracts are quite characteristic: an initial lag phase of about 15 minutes is followed by a severe reduction in the rate of protein synthesis (Kerr et al., 1974; Hovanessian et al., 1977; Cooper and Farrel, 1977; Clemens and Williams, 1978). Because of the lag phase, the maximum inhibition of protein synthesis observed in these systems is approximately 60-75%.

The incubation of extracts from interferon-treated cells with dsRNA also results in the activation of a protein kinase activity;
extracts from untreated cells are also stimulated in this respect, but to a lesser degree (Roberts et al., 1976b; Lebleu et al., 1976; Shaila et al., 1977). The kinase causes the preferential phosphorylation of histones, a 67 000 dalton (67K) protein and the small subunit of eIF-2 (eucaryotic initiation factor 2) (Lebleu et al., 1976; Roberts et al., 1976b; Cooper and Farrel, 1977). An increase in the phosphorylation of the 67K protein was noted in extracts from interferon-treated parental L1210S cells, but not in extracts from the interferon-insensitive subline (L1210R), or those from untreated cells (Vanderbussche et al., 1978). The kinase(s) and the enzyme responsible for the synthesis of the LMWI (2-5A synthetase) may be separated and purified by the use of poly(rI).poly(rC)-Sepharose affinity columns (Hovanessian and Kerr, 1979). A dsRNA-activated kinase has been partially purified from the ribosomes of interferon-treated cells; the activated kinase inhibits in vitro protein synthesis and causes phosphorylation of the small subunit of eIF-2 (Sen et al., 1978). The inhibitory effects of the activated preparations are destroyed by heat (whereas the LMWI is heat-stable).

The treatment of rabbit reticulocytes with dsRNA also results in the activation of a kinase which phosphorylates the small subunit of eIF-2 (Cooper and Farrel, 1977; De Haro and Ochoa, 1978). The action of unphosphorylated, but not phosphorylated, eIF-2 is stimulated by another factor (ESP) when low levels of eIF-2 are present; thus the inhibitory effects of the kinase may be overcome by the addition of excess eIF-2 (De Haro and Ochoa, 1978). It has been reported that the activity of this initiation factor is reduced in extracts from interferon-treated cells compared to those from untreated cells (Ohtsuki et al., 1977). Furthermore, preparations of initiation factors from interferon-treated cells (high-salt ribosomal washes) also contain greatly increased levels of protein kinase activity compared to preparations from control cells (Johnson and Ohtsuki, 1979). The two
kinase activities may have similar modes of action, though there do appear to be differences between them (Roberts et al., 1976b; Cooper and Farrel, 1977).

It has been proposed that the treatment of cells with interferon results in a latent antiviral state which may be triggered by dsRNA (Roberts et al., 1976a). In the intact cell dsRNA produced during viral replication could act as the necessary stimulus. Although most RNA viruses replicate via a dsRNA intermediate, it would appear unlikely that the production of dsRNA is an obligatory step in the replication of DNA viruses. It has been well-documented that the dsRNA-activated inhibitors from extracts of interferon-treated cells are non-specific in their action, inhibiting cellular and viral protein synthesis in vitro and in intact cells (see above discussion). However, it is also widely reported that the action of interferon in at least some virus-infected cells is to preferentially inhibit the translation of viral mRNAs (Baxt et al., 1977; Gupta et al., 1974; Thacore, 1978).

Mouse embryonal carcinoma (EC) cells neither produce interferon nor respond to it, unlike differentiated cells derived from EC cells; extracts of interferon-treated EC cells do, however, synthesise the dsRNA-dependent LMWI in amounts similar to those produced by extracts of interferon-treated differentiated cells (Wood and Hovanessian, 1979). Furthermore, addition of the LMWI to EC cell extracts resulted in an enhanced nuclease activity. These cells are thus capable of responding to interferon by the synthesis of the LMWI and activation of a nuclease, yet show neither an antiviral nor an anticellular effect of interferon treatment (Wood and Hovanessian, 1979). This indicates that the LMWI system may be irrelevant to the development of the antiviral state. However, extracts from interferon-treated EC cells do not show the enhanced kinase activity which is characteristic of extracts from other interferon-treated cells. It is thus possible that the kinase
may be involved in the generation of the antiviral state, particularly as its apparent dependence on dsRNA may be artefactual (Hovanessian, 1979).

Samuel and Joklik (1974) described a cell-free protein synthesising system from interferon-treated cells which preferentially inhibited the translation of viral mRNAs. This report has frequently been criticised on the grounds that the viral mRNAs used may have contained dsRNA, and that the discrimination is thus artefactual. Such critics have obviously paid scant attention to the facts contained in the report. Firstly, it must be pointed out that the reovirus mRNA used in the study may well have contained traces of dsRNA; however, it would seem unlikely that the mRNA prepared in vitro from Vaccinia virus cores should contain any dsRNA. Secondly, as noted above, the kinetics of inhibition of protein synthesis by the dsRNA-activated inhibitors are quite characteristic, and are entirely different to the kinetics of inhibition observed by Samuel and Joklik: the LMWI does not inhibit protein synthesis for approximately 15 minutes, whereas Samuel and Joklik showed significant inhibition of viral protein synthesis as early as 5 minutes from the start of incubation. Thirdly, the maximum effect of the LMWI over a period of one or two hours is to inhibit in vitro protein synthesis by approximately 70%; Samuel and Joklik (1974) showed a maximum inhibition of 93% over a one hour period. Fourthly, these authors indicated that the inhibitor was associated with the ribosomes from interferon-treated cells, whereas the dsRNA-activated 2-5A synthetase is located in the postribosomal supernatant (see, for example, Roberts et al, 1976a). While it is thus apparent that the LMWI is not involved in the observed inhibition, it is possible that a kinase activity is involved.

In this discriminatory system, the extent of inhibition of in vitro protein synthesis directed by viral mRNAs was increased with the concentration of interferon used to treat the cells before
extraction (Samuel and Joklik, 1974). The inhibitor appeared to be associated with the ribosome, and could be removed by high concentrations of salt. Such ribosomal wash fractions contained a protein of 48 000 daltons which was not detected in the ribosomal washes from untreated cells. Further work indicated that the ribosome-associated inhibitor was not species-specific (Samuel and Farris, 1977). Kortsaris et al (1976) also reported that the translation of added viral mRNA was inhibited in mitochondria obtained from interferon-treated cells, while the translation of added globin mRNA was unaffected. The extent of the inhibition observed makes it appear unlikely that the LMWI was involved. This report also indicates that the prokaryotic-type ribosome may be susceptible to the inhibitor induced by interferon.

Other reports have also implicated the ribosome in the mechanism of action of interferon. Gupta et al (1973) indicated that an interferon-induced inhibitor of protein synthesis was associated with the ribosome; this inhibitor, although not dsRNA-dependent, was non-discriminatory in its action. Ribosomal salt washes derived from interferon-treated mouse L cells contained a factor which suppressed the in vitro plaque-forming cell (PFC) response (Johnson and Ohtsuki, 1979); a similar inhibition of the PFC response has been noted in spleen cells pretreated with interferon (Gisler et al, 1974). These ribosomal preparations from interferon-treated L cells also contained increased levels of two protein kinase activities, one cAMP-dependent and the other cAMP-independent. The cAMP-independent kinase has a molecular weight of about 50 000 daltons (similar to the MW of the protein described by Samuel and Joklik), and inhibits the ability of eIF-2 to form the ternary complex (Johnson and Ohtsuki, 1979). It has also been reported that a dsRNA-dependent kinase activity may be purified from high salt washes derived from interferon-treated cells (Sen et al, 1978).
Non-Antiviral Effects of Interferon

Effects of interferon other than the definitive antiviral response have been difficult to assess, because until the very recent advent of pure interferon preparations (De Maeyer-Guignard et al., 1978), many of the reported effects could have been due to impurities in the interferon preparations. Most of the reported effects occur at interferon concentrations significantly greater than are required to produce a reasonably effective antiviral state. However, priming for interferon induction (discussed above) and the enhancement of the response to interferon by prior treatment with low doses of interferon are two exceptions to this generalisation.

Interferon preparations inhibit the growth of cells in culture (Gresser et al., 1970a and b). An interferon-resistant subline has been isolated by the growth of cells in high concentrations of interferon (Gresser et al., 1970b, 1974); these cells were no longer sensitive to either the anti-growth or the antiviral activities of interferon, indicating that the anti-growth effect is a true property of the interferon. The anti-growth effects, like the antiviral activity of interferon, appears to be dependent on the number of copies of chromosome 21 in human cells; cells trisomic were more responsive to the anti-growth activity than were cells disomic for this chromosome, while cells containing a single copy of chromosome 21 were least sensitive to the anti-growth activity (Tan, 1976).

The antiviral and anti-growth factors remain in the same relative proportions during purification and after partial denaturation (Stewart et al., 1976), and apparently pure preparations of mouse and human interferons inhibit the growth of homologous cells (De Maeyer-Guignard et al., 1978; Knight, 1976).

The effects of fibroblast and lymphoblastoid interferons on events in the cell cycle have been compared. Both types of interferon
inhibit DNA synthesis, and appear to prolong the G1 phase (Lundgren et al., 1979; Watanabe and Sokawa, 1978). The inhibition of DNA synthesis appeared to be accompanied by a significant rise in the levels of intracellular cAMP, while both effects were diminished in a subline which was relatively resistant to interferon. Treatment of the cells with dibutyryl-cAMP led to a similar inhibition of DNA synthesis in both cell types (Fuse and Kuwata, 1978). Fibroblast interferon appears to be more effective than leucocyte interferon in the suppression of cell growth (i.e. when measured in terms of the antiviral activity) (Kuwata et al., 1979; Lundgren et al., 1979).

Interferon also appears to be a natural mediator of the immune response (Youngner and Salvin, 1973; Attallah and Folks, 1979; Huddlestone et al., 1979; Schultz and Chirigos, 1979). The activation of macrophages to cytotoxic activity by a variety of agents including interferon may be inhibited by the simultaneous addition of anti-interferon antibody (Schultz and Chirigos, 1979). The pre-incubation of normal human lymphocytes with interferon results in an increase of the activity of both natural killer (NK) cells and antibody-mediated cytotoxic cells (Attallah and Folks, 1979). However, while low levels of interferon may enhance the plaque-forming cell (PFC) response, this response is inhibited by high concentrations of interferon; the inhibitory effect is due to an action on B cells, and may be effected by the suppression of antibody synthesis (Gisler et al., 1974). It would appear likely that the anti-tumour effects of interferon are mediated by the immune system, and in particular by the activation of NK cells.

Interferon pretreatment of mouse L929 cells enhances the cytotoxic effects of dsRNA (Stewart et al., 1972), an effect which has lent some support to the hypothesis of a latent antiviral state triggered by dsRNA (for, as mentioned above, in many cases the
dsRNA-activated inhibitors appear to inhibit both viral and cellular protein synthesis). Incubation of interferon-treated L cells with dsRNA gives a maximum of 50% cytotoxicity, and this figure cannot be increased by an increase in the concentrations of interferon or dsRNA (Cooper et al., 1979). Cellular extracts from interferon-treated, dsRNA-treated L cells were examined for the presence of the LMAI, but none could be detected (Cooper and Morser, 1979). Furthermore, the interferon-enhanced cytotoxicity of dsRNA has not been found with certain other cell lines, and may thus reflect a response to interferon specific to a few cell lines (Stewart et al., 1972; Emeny and Morgan, 1979b).

Interferon may also affect the induction of cellular enzymes, such as aryl hydrocarbon hydroxylase (Nebert and Friedman, 1973) and glutamine synthetase (Matsuno et al., 1976).

**The Use of Mutant Cells in the Elucidation of Interferon Action**

It is apparent from the previous discussion that mutant cells and cell hybrids have proved to be of enormous value in the study of the interferon system. In particular, such cells have enabled non-antiviral effects of interferon preparations, such as growth inhibition, to be ascribed to the interferon itself. The present study extends the use of such mutants by the investigation of three mutant cell lines and the parental cells.

Mouse L1210 cells are sensitive to the antiviral and cell growth inhibitory effects of mouse interferon (Gresser et al., 1970a). The prolonged culture of these cells in the presence of interferon resulted in the selection of sublines which were insensitive to the inhibition of growth by interferon (Gresser et al., 1970b). The subline(s), L1210R, was also insensitive to the antiviral effects of interferon (Gresser et al., 1974). L1210R cells arise from a sub-population of interferon-insensitive cells already present among L1210 cells, at a frequency of 0.004 - 1% (Gresser et al., 1974).
Investigation of the L1210R cells indicated that the membranes did not bind significant amounts of interferon, unlike the membranes of the parental, interferon-sensitive L1210S cells (Gresser et al., 1974). The membrane interaction with interferon involves both gangliosides and a protein receptor molecule (see previous discussion). The high frequency of occurrence of the L1210R cells would indicate that they are not true mutants, and that the receptor protein is therefore unlikely to be altered. However, cells in culture frequently have alterations in glycolipid metabolism (Critchley and Vicker, 1977), and it thus appeared possible that such an alteration was responsible for the altered response to interferon. The glycolipids of the parental cells and a clone of L1210R cells were therefore extracted and compared. It appeared that there was indeed an alteration in the pattern of glycolipid biosynthesis: in particular, it appeared that one glycolipid, GT$_{1a}$, might be absent, or present only in extremely small amounts. The effect of replacement of GT$_{1a}$ on the interferon sensitivity of L1210R cells was studied; however, no firm conclusions could be drawn from the data which were obtained.

The derivation of mutant sublines of mouse 3T6 cells which were resistant to a wide range of viruses has been described (Morgan et al., 1973); the initial characterisation of these cells indicated that resistance to virus infection was manifested at a stage subsequent to adsorption and penetration of the virus. Because the cells were resistant to a wide range of viruses, it appeared possible that the cells were constitutive for part of the interferon system. Jarvis and Colby (1978) reported that one of the clones (3T6 V$^B$2) was constitutive for interferon production; although interferon could not be detected in medium in which the cells had been incubated, anti-interferon antibody appeared to prevent the manifestation of viral resistance. It must be pointed out, however, that the antibody preparation
was apparently not adsorbed against a parental cell preparation before use, and the effect of this antibody preparation on the antiviral state was much more rapid than would have been expected. It is possible that the observed degeneration of the antiviral state was due to the cytotoxicity of the preparation. These mutant cells were also shown to possess a dsRNA-dependent kinase activity; in order to demonstrate the presence of the enzyme system responsible for the synthesis of the LMWI, however, it was necessary to use the chick embryo cell system, which was about $10^5$ times more sensitive than the Ehrlich ascites tumour cell system first utilised (Jarvis et al., 1978).

A second virus-resistant mutant (3T6 S3 A2) was investigated in the present study, in parallel with the parental cells and, in later experiments, with the V$\beta$2 mutant. Cell mixing experiments indicated that the resistance could not be transferred from A2 cells to the parental cells. The LMWI could not be detected in the L cell system, from extracts of A2 cells or interferon-treated wild-type cells; this system is less sensitive than that used by Jarvis et al., but the result does indicate that the inhibitor is present, if at all, at much lower levels than in similar extracts from mouse L cells. Ribosomal wash fractions from the two mutant cells, parental 3T6 cells, and interferon-treated 3T6 cells were analysed by SDS PAGE: a protein of similar molecular weight to that described by Samuel and Joklik (1974) was detected in the samples from cells displaying an antiviral state, but appeared to be absent (or present at much reduced levels) from the 3T6 extracts (chapter 3).

A detailed hypothetical model of interferon action was constructed (chapter 4), and the predictions of the model tested (chapter 5).
CHAPTER 1

MATERIALS AND METHODS
CHAPTER 1

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MATERIALS

Cells Mouse leukaemia L1210 cells (L1210S and L1210R) were kindly supplied by Dr. I. Gresser (Institut de Recherches Scientifique sur le Cancer, Villejuif, France), and mouse 3T6 $^{T^r}$ B2 cells were provided by Dr. C. Colby (University of Connecticut, U.S.A.). Other mouse cell lines (3T6, A2, 3T3 and L cells) were from stocks kept in this laboratory. Embryo cell cultures were prepared as described below, from embryonated chicken eggs (Cobb strain, Golden Hatcheries, Kibworth, Leicestershire), or from mouse embryos (LACA strain, University of Leicester Animal House).

Viruses The origins of the Sindbis virus, Semliki Forest virus and Newcastle Disease virus used in this study have been previously described (Morgan, Colby and Hulse, 1973).

Interferons Partially-purified mouse interferon was prepared as described below. Mouse reference interferon (catalogue number GO02-904-511) was obtained from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, U.S.A.

Chemicals The following chemicals were obtained for use in this study:

- HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; Hopkin and Williams, Chadwell Heath, Essex);
- Tris(hydroxymethyl)aminomethane (Trizma base reagent, Sigma Ltd., London);
- Nonidet P42 (Shell), dithiothreitol, and water soluble phenol red (British Drug
Houses, Poole, Dorset); neutral red and trypan blue (E.Gurr Ltd., London SW14); agarose (Miles Laboratories Ltd., Stoke Poges, Slough SL2 4LY); polyribocinosin.polyribocytidyllic acid, supplied as a lyophilised powder with physiological salts (PL Biochemicals Ltd., Milwaukee, Wisconsin, U.S.A.); diethylaminoethyl dextran, Sephadex G150, and Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden); tissuesolubiliser, cycloheximide, and polyanethol sulphonate (Koch-Light Laboratories Ltd., Slough, Bucks.); Coomassie brilliant blue G, and Coomassie brilliant blue R (R.A. Lamb, London NW10 6JL); Folin and Ciocalteaus' reagent (Fisons Ltd., Loughborough, Leicestershire); diethylpyrocarbonate (Sigma Ltd., London); gentamicin (Nicholas Laboratories Ltd., Slough SL1 4AU); adenosine triphosphate (ATP), guanosine triphosphate (GTP), phosphoenolpyruvate (PEP), and pyruvate kinase (PK, as a solution in glycerol) (Boehringer, Mannheim).

Reagents for polyacrylamide gels (acrylamide, bis-acrylamide, ammonium persulphate and TEMED (N,N,N',N'-tetramethylethylenediamine)) were from Biorad Laboratories, Richmond, California.

Tenuazonic acid, pactamycin, yeast tRNA, and poly(uridylic acid) were the kind gifts of Dr. E. Cundliffe (University of Leicester).

Radiochemicals were supplied by the Radiochemical Centre, Amersham.

All other chemicals were of the finest grade available.

**Scintillation Fluid** Toluene scintillation fluid consisted of 6g of 2,5-diphenyloxazole (PPO) and 0.5g of 1,4-di-2-(4-methyl-5-phenyloxazoyl) benzene (dimethyl POPOP) dissolved in 1 litre of toluene.

Bray's scintillation fluid contained 60g naphthalene, 4g PPO, 0.2g dimethyl POPOP, 100 ml methanol, and 20 ml ethylene glycol made up to 1 litre with p-dioxane.
**Dialysis Tubing**  Dialysis tubing (Visking tubing, Scientific Instrument Centre, London NW1) was autoclaved for 10 minutes at 15 lb/in$^2$ in 100 mM $\text{Na}_2\text{HCO}_3$ and 20 mM EDTA (ethylenediaminetetra-acetic acid), washed six times with distilled water, and stored in distilled water at 4°C.

**Tissue Culture Vessels**  35 mm, 50 mm, 100 mm, and 160 mm tissue culture Petri dishes were obtained from Corning, New York, or Sterilin (Teddington, Middlesex). 25-well dishes were supplied by Sterilin. Roller bottles were obtained from Bellco, Vineland, New Jersey, U.S.A.

**Media and Solutions for Cell Cultures**

**Pre-treatment of Newborn-calf and Newborn-horse Serum**

Newborn-horse serum and newborn-calf serum (Flow Laboratories, Irvine, Scotland) were heat-inactivated for one hour at 60°C before use.

**Media**

Eagle's minimal essential medium, MEM (Eagle, 1959) supplied by Flow Laboratories, was supplemented with 2% dialysed newborn-calf serum and HEPES buffer (final concentration 25 mM). Dulbecco's modification of MEM, DME (Dulbecco and Freeman, 1959; Gibco-Bicult Ltd.), was supplemented with 6% newborn-calf serum (D6), 10% newborn-calf serum (D10), or 5% newborn-calf serum and 5% foetal bovine serum (Flow Laboratories) (D55). Both MEM and DME were also supplied without leucine from the same laboratories.

RPMI 1640 medium (Flow Laboratories; Morton, 1970) was routinely used with the addition of 20% horse serum.

Ham's F12 medium (Flow Laboratories; Ham, 1965) was supplemented with 10% dialysed foetal bovine serum, and glucose was added to the required concentration.
**Phosphate-buffered Saline (Dulbecco and Vogt, 1954)**

Phosphate-buffered saline (PBSA) contained 136 mM NaCl, 2.7 mM KCl, 8.1 mM Na$_2$HPO$_4$, and 1.47 mM KH$_2$PO$_4$. The pH was adjusted to 7.2 by 1M NaOH, and the solution autoclaved. When required, MgCl$_2$ and CaCl$_2$ were added from a sterile stock solution (100X) to give PBS (final concentrations being 4.6 mM MgCl$_2$ and 4.9 mM CaCl$_2$).

**Versene-trypsin Solution**

A buffered salt solution (137 mM NaCl, 3.4 mM KCl, 1.8 mM KH$_2$PO$_4$, 10.1 mM Na$_2$HPO$_4$, pH 7.2) containing 0.54 mM versene (ethylene-diaminetetraacetic acid, EDTA, disodium salt) and 0.001% phenol red was sterilised, and to it added sterile trypsin solution (Flow Laboratories) to a final concentration of 0.05%.

**DMEM/HEPES/Glycerol**

This solution consisted of D55 containing 10% glycerol and 20 mM HEPES, pH 7.2.

**Overlay Media**

The media were made as required from two-fold concentrated media (e.g. 2X (D6) for chick cells) and 1.4% agarose in a 1:1 ratio. Agarose was melted by autoclaving, and both solutions incubated at 41°C prior to mixing and use.

**METHODS**

**Cell Culture**

L1210 cells were grown as suspension cultures in RPMI medium, incubated at 37°C in a humidified atmosphere of 5% CO$_2$ in air. Cells were passaged by transferring the suspension to centrifuge tubes, and spinning in a bench centrifuge for 5 minutes at 1000 r.p.m.; the medium was removed, and the pellet resuspended in RPMI, aliquots then being
taken to seed new Petri dishes or 25-well dishes containing fresh medium.

Cell numbers were estimated with a haemocytometer. The cell suspension was pipetted up and down to mix thoroughly, and a measured sample (usually 0.5 ml) added to one-fifth of its volume of 0.04% Trypan blue. After mixing, the solution was incubated at room temperature for 3 - 4 minutes, before remixing and introducing a sample under the coverslip of a haemocytometer (improved Neubauer). A minimum number of 200 viable cells was normally counted.

All cells other than L1210 cells were grown as monolayers. 3T6, 3T6V^B2 and 3T6S3A2 cells, L cells and mouse embryo cells (MEC) were maintained in D55, while 3T3 cells were grown in D10 and chick embryo cells (CEC) in D6. Cells growing in Petri dishes and 25-well dishes were incubated at 37°C in a humidified atmosphere of 10% CO₂ in air. Bottles were gassed with 5% CO₂ in air, after seeding, and incubated at 37°C. Cultures were passaged by trypsination. The medium was removed, and the cell sheet carefully washed twice with warm PBSA. A small amount of trypsin-verseine solution was then added, washed gently over the whole surface, and the excess removed by aspiration. After incubation at 37°C for 2 - 3 minutes, the cells were suspended in growth medium, and an aliquot used to seed fresh medium in a Petri-dish as a stock culture. For experiments, the remaining cells were counted by Coulter counter, and seeded into appropriate tissue culture vessels containing fresh medium.

Preparation of Embryonic Cell Cultures

1) **Chick Embryo Cells** All operations were carried out under sterile conditions. Eleven-day old embryonated eggs were wiped with Wescodyne solution, and the shell surrounding the air-sac removed. The membranes were removed and the embryos transferred to a Petri dish containing PBSA. The head and limbs were removed, and the torsos
placed in a dry Petri dish and finely minced. After transfer to a
conical flask, the tissue was washed twice with 100 ml PBSA to remove
red blood cells. The tissue was incubated in 100 ml of 0.025% trypsin
in PBSA at 37°C with stirring. After ten minutes the incubation was
stopped, the larger pieces of tissue allowed to settle, and the
supernatant poured through sterile gauze into a beaker. Fresh trypsin
solution was added to the tissue in the conical flask, and the procedure
repeated twice more from the incubation step. 10 ml of newborn-calf
serum was added to the collected cell solution to inhibit trypsin action,
and the cells were pelleted by centrifugation. After resuspending in
D6, an aliquot was taken, diluted and counted by Coulter counter, and
the required number of cells dispensed into Petri dishes. Chick embryo
cells were normally used as primary cultures.

ii) Mouse Embryo Cells Pregnant LACA mice were anaesthetised
4-5 days before term, and the embryos aseptically removed. After mincing
the embryos, a procedure essentially the same as the preparation of chick
embryo cells was followed, the mouse cells being dispensed into Petri
dishes or roller bottles containing D55.

Cloning of 3T6 and A2 Cells

Approximately 100-500 cells were seeded into a Petri dish
containing D55 and incubated for 10-14 days without moving the dish.
Colonies were visualised by microscopic examination, or by direct
observation in oblique light, and their positions marked on the bottom
of the dish. The medium was removed, and individual, well-separated
colonies trypsinised using sterile cloning cylinders. Colonies were
seeded into a compartment of a 25-well dish containing fresh medium.
Cultures were normally cloned twice in succession.
Viruses

Preparation of Stock Sindbis Virus

The medium was removed from confluent monolayers of chick embryo cells, and the cell sheets washed gently with warm PBS. 0.25 or 0.3 ml of virus suspension in DME (approximately 1 plaque-forming unit per cell) was added to each Petri dish, and the dishes rocked to evenly disperse the suspension. The dishes were incubated at 37°C for 30 minutes, again being gently rocked at 5 and 10 minutes after incubation. The excess virus suspension was removed, fresh medium added, and the cells incubated at 37°C until a cytopathic effect was evident. The medium was then harvested, centrifuged to remove cell debris and stored at -70°C until assay and use.

Virus was occasionally grown on L cells, or taken from laboratory stocks grown on BHK cells (baby hamster kidney cells), as the chick-grown virus proved to be only poorly infective in L cells.

Plaque Assay of Viruses

Virus preparations to be assayed were serially diluted tenfold in cold DME. The medium was removed from confluent monolayers of cells in 50 mm Petri dishes, the cell sheets washed with warm PBS and duplicate dishes infected with 0.25 or 0.3 ml of each virus dilution, as described above in the preparation of Sindbis virus. After incubation and the removal of excess virus suspension, 4 ml of overlay medium was added to each dish, and allowed to set. The Petri dishes were incubated at 37°C until plaques were evident by viewing the plates in oblique light, or by microscopic examination. 24-48 hours of incubation were necessary to give good plaques, the time being dependent upon the cells used and the virus being assayed. 2 ml of a 0.01% solution of neutral red in PBS was added to the overlay, and the cells incubated for 2 hours. After
pouring off the excess solution, the clear plaques were counted using a Kodak Coldlight illuminator (Model 3).

Sindbis virus and Newcastle Disease virus were routinely assayed on chick embryo cells.

**Plaque Purification of Viruses**

A procedure essentially similar to the plaque assay was employed, the monolayer being infected with 2-10 plaque-forming units (p.f.u.) of virus. Plaques were visualised with a sterile neutral red solution (0.01% in PBS) and dishes containing only a few plaques, and preferably only a single plaque, were chosen. The virus was isolated by inserting a Pasteur pipette through the overlay medium, and removing cells and cell debris from the plaque area. The mixture was resuspended in growth medium, and used to infect a confluent plate of cells, virus being harvested when a gross cytopathic effect was evident, and stored at -70°C.

Viruses were normally plaque-purified twice successively.

**Interferon**

**Preparation of Mouse Interferon**

Mouse embryo primary or secondary cultures were grown in roller bottles, and allowed to remain at confluency for 3-4 days. The medium was removed, and the monolayers washed with warm PBS. The cells were then infected with ultraviolet-irradiation (U.V.)-inactivated Newcastle Disease virus (approximately 2 p.f.u. per cell in 5 ml of DME), and incubated at 37°C for 30 minutes on a roller machine. Excess virus suspension was removed and 100 ml of fresh medium (DME + 2% calf serum) added to each bottle, which was then gassed with 5% CO₂ and incubated on a roller machine. One roller bottle was mock-infected
with 5.0 ml of DME in place of the virus suspension, and was subsequently treated in the same way as the infected cultures. After 48 hours, the medium was removed and stored at -20°C until partial purification.

Partial Purification of Mouse Interferon

1. **Perchloric Acid Precipitation** 2M perchloric acid was added dropwise to the chilled (4°C) interferon and mock-interferon preparations to a final concentration of 0.15M. The solutions were left for 2-3 days at 4°C to allow protein to precipitate, centrifuged at 10,000 x g, and the supernatant removed. The preparations were neutralised by the dropwise addition of 2M KOH, with stirring, and left for 18 hours at 4°C. The precipitate of potassium perchlorate was removed by centrifugation.

2. **Zinc Acetate Precipitation of Interferon (Lampson, 1963)**

1 M zinc acetate was added, with stirring, to the cold preparations, to a final concentration of 20 mM. The preparations were allowed to stand at 4°C for 18 hours, and then centrifuged for 1 hour at 3,000 x g. The precipitate was dissolved in 0.2M HCl, and dialysed against four changes of PBS (100 ml, minimum time of 4 hours per dialysis).

Interferon Assays

1. **Growth Inhibition of L1210 Cells - A Screening Procedure**

This procedure relies on the fact that interferon inhibits the growth of the interferon-sensitive (L1210S) parental cells. By use of the interferon-resistant (L1210R) subline, any effects on growth other than those due to interferon may be detected.

The interferon and mock-interferon preparations to be tested were diluted in RPMI medium to give final dilutions in the range 1:10 - 1:100. Four samples (0.9 ml) of each dilution were pipetted into separate compartments of 25-well dishes. A further four compartments
contained 0.9 ml each of medium alone. L1210 cells were counted as previously described, harvested by centrifugation and resuspended in a suitable volume of growth medium. Two compartments of each set of four were seeded with 0.1 ml each of L1210S cells, and the remaining wells seeded with 0.1 ml each of L1210R cells. The initial cell density in each well was normally 5 x 10^4 cells ml^-1. The cells were usually incubated for between two and four days and cell numbers estimated once or twice each day for each sample, as previously described (with the exception that only 0.1 ml of each duplicate sample was removed for estimation of cell numbers).

Growth curves were constructed for each sample. Only those interferon preparations which inhibited the growth of L1210S cells and did not inhibit the growth of L1210R cells were regarded as suitable for experimental usage.

2. **Virus Yield Reduction Assay** Serial tenfold dilutions of the interferon preparation were made in the required medium. The medium was aspirated from confluent monolayers of L cells or 3T6 cells in 50 mm Petri dishes and replaced with the interferon dilutions, in duplicate. Fresh medium was added to four control monolayers of cells. The cells were incubated for 18 hours, the medium removed, and the monolayers washed gently with warm PBS. Two of the control plates were mock-infected with 0.25 or 0.3 ml of DME, while all the other dishes were infected with Sindbis virus (2-4 p.f.u. per cell in 0.25 or 0.3 ml of DME), as described above. After removal of the excess virus suspension, the monolayers were washed with PBS and fresh growth medium was added to each Petri dish. The cells were incubated until the control virus-infected plates began to show a cytopathic effect (24-28 hours), when the medium from each well was removed and stored at -20°C. Duplicate samples were combined and the number of infectious particles estimated by a virus plaque assay on
chick embryo cells.

The results were graphed as percentage virus yield (compared to control samples) against the logarithm of the interferon dilution. One unit of interferon in this assay was defined as the amount per ml required to inhibit virus yield by 50%. Thus the potency of the preparation was the reciprocal of the dilution required to give this effect.

3. **Plaque Reduction Assay** Serial dilutions of the interferon sample were made in the appropriate medium, and added to duplicate confluent monolayers of cells from which the growth medium had been aspirated. Four control plates were replenished with fresh medium. After incubation for 18 hours, the medium was removed, and the monolayers washed with PBS. Two control plates were mock-infected, and the other plates infected with approximately 100 p.f.u. of Sindbis virus. The plates were washed with PBS and overlay medium added, as previously described. Plaques were visualised by neutral red uptake after a suitable incubation time, and counted.

The results were expressed as percentage plaque numbers (compared to control infected monolayers) against the logarithm of the interferon dilution. The activity of the interferon preparation was defined as the reciprocal of the dilution which would have given a 50% inhibition of plaque numbers.

4. **Inhibition of Viral Cytotoxicity (Neutral Red Uptake)** The medium was aspirated from confluent monolayers of cells in 25-well dishes, and duplicate dilutions of the interferon preparations in medium added to the wells. Controls consisted of four wells containing growth medium only, and wells containing suitable duplicate dilutions of a mock-interferon preparation. After incubation for 18 hours, the medium was removed, and the cells washed with PBS. All the monolayers, with the
exception of two control monolayers, were infected with 1-10 p.f.u. per cell of Sindbis virus in 0.1 or 0.2 ml of DME. The control monolayers were mock-infected.

After washing the cells with PBS, fresh growth medium was added to each well, and the dishes were incubated until the control infected monolayers showed gross cytopathic effect. The medium was removed and stored at -20°C until assay of the virus. 1 ml of 0.01% neutral red in PBS was added to each well, and the dishes incubated for two hours. The cell sheets were washed with PBS, and 2 ml of a 1:1 mixture of industrial methylated spirits (IMS) and 0.1M NaH$_2$PO$_4$ added to each to extract the dye. After mixing, the absorbance at 540 nm of each sample was measured, using a Pye-Unicam SP6 spectrophotometer. The absorbance of the control mock-infected samples was defined as 100% antiviral, and that of the control infected samples as 0% antiviral. The absorbance at 540 nm ($A_{540}$) is proportional to the concentration of neutral red, and the uptake of the dye is proportional to the number of viable cells present over the ranges used (J.M. Emeny, 1977). Thus, an inverse linear relationship between cytopathic effect and $A_{540}$ was presumed. The results were graphed as percentage antiviral effect versus the logarithm of the interferon dilution. The activity of the interferon preparation (units ml$^{-1}$) was defined as the reciprocal of the dilution which would have given 50% anti-viral effect.

**Standardisation of Interferon Preparations**

Interferon preparations were standardised by the simultaneous assay of the preparations and the mouse reference interferon (fig. 1). All interferon concentrations are expressed in reference units.
Interferon preparations and the mouse reference interferon were assayed on L cells by the inhibition of virus yield. The yield of virus from control cells was $1.13 \times 10^3$ p.f.u. per cell. Symbols: interferon preparation purified by perchloric acid precipitation (X); interferon preparation further purified by zinc acetate precipitation (O); mouse reference interferon (■). The dashed lines (---) indicate the dilution of each preparation which would give 50% inhibition of virus yield (1 unit in this system). The potency of the reference interferon is 18000 i.u./ml, equivalent to 2000 internal units. One internal unit, therefore, is the equivalent of 9 i.u.
Treatment of Cells with Interferon

The treatment of cells with interferon followed the procedures described for the assay of interferon. Cells were washed with warm PBS after interferon treatment, and prior to any subsequent treatment.

The Induction of Interferon with Poly(rI),poly(rC)

Cells in monolayer were washed with warm PBS, and warm medium containing poly(rI),poly(rC) and, usually, DEAE dextran (diethylaminoethyl dextran) was added. Cells in suspension were pelleted by centrifugation, washed in warm PBS, and resuspended in warm medium containing poly(rI),poly(rC) and DEAE dextran. After a suitable incubation period, the cells were washed twice in warm PBS, and fresh medium was added.

Protein Estimation

1. Lowry Protein Estimation (Lowry et al., 1951)

Stock Solutions
- 1% CuSO$_4$·5H$_2$O
- 2% sodium potassium tartrate
- 2% anhydrous Na$_2$CO$_3$ in 0.1M NaOH
- Folin-Ciocaltean's reagent (Fisons, Loughborough).

Method

0.1 ml each of 1% CuSO$_4$·5H$_2$O and 2% NaK tartrate was added to 10 ml of the 2% Na$_2$CO$_3$ solution, and the solution vortexed. 1 ml of the mixture was added to 0.2 ml of each sample. After 10 minutes incubation at room temperature, 0.1 ml of dilute Folin-Ciocaltean's reagent (50% in water) was added to each sample and the solutions mixed. After a further 30 minutes at room temperature, the absorbance at 750 nm was measured. A standard curve was constructed using bovine serum albumin, and the protein concentration estimated from this.

2. Protein Estimation using Coomassie Brilliant Blue G250 (Sedmak and Grossberg, 1977)

Coomassie Brilliant Blue G250 was prepared
as a 0.05% solution in 0.2M perchloric acid, and filtered to remove undissolved dye. If necessary, the absorbance at 465 nm \( A_{465} \) was adjusted to 1.3 - 1.5 by the addition of 0.2M perchloric acid. This solution is stable and may be stored indefinitely. The reagent was added to an equal volume of each sample, and the absorbances at 620 nm \( A_{620} \) and at 465 nm measured in the SF6 spectrophotometer. The ratio \( \frac{A_{620}}{A_{465}} \) was proportional over the range used to the amount of protein present. The amount of protein present in a sample was estimated from the standard curve constructed by use of bovine serum albumin.

This assay is simple and sensitive, and is less subject to interference by other chemicals (e.g. Tris buffer, high concentration of ions such as potassium, and amino acids) than is the Lowry protein estimation. However, the standard curves vary widely, depending upon the standard protein chosen, and it is thus advisable to calibrate the assay against the Lowry protein assay, using a mixture of proteins such as a cellular extract.

Polyacrylamide Gel Electrophoresis (PAGE) of Proteins (Laemmli, 1970)

Discontinuous sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was employed for the analysis of proteins from ribosomal salt washes. The running gel consisted of 12.5% acrylamide, 0.3% bis-acrylamide, 0.1% SDS, 0.05% TEMED and 0.05% ammonium persulphate in 0.37M Tris buffer (pH 8.8). The stacking gel contained 5% acrylamide, 0.12% bis-acrylamide, 0.1% SDS, 0.05% TEMED and 0.05% ammonium persulphate in 0.12M Tris buffer (pH 6.8). In each case, the required amount of a freshly-made 10% ammonium persulphate solution was added to a deaerated mixture of the other components immediately before casting the gel.

Samples in a small volume of sample buffer (0.125M Tris, pH 6.8, 2% SDS, 0.1M mercaptoethanol, 0.001% bromophenol blue, and 20%
sucrose) were heated at 90°C for 3 minutes and applied to the sample wells. The samples were carefully overlaid with running buffer (0.025M Tris, 0.019M glycine, 0.1% SDS, pH 8.8), and the top and bottom tanks of the electrophoresis equipment filled with the same buffer. Electrophoresis was carried out at 50 volts until the samples had penetrated to the running gel. Further electrophoresis was at 100 V, for approximately 8–10 hours.

After electrophoresis, gels were washed in 10% acetic acid, and incubated for 1 hour at room temperature in the same solution. The gels were stained in a solution of 0.05% Coomassie brilliant blue R in 25% propan-2-ol and 10% acetic acid for 2 hours, with a change of staining solution after 1 hour. Destaining was carried out in a solution containing 10% propan-2-ol and 10% acetic acid, with several changes of solution over approximately an 18-hour period.

The stained gels were photographed with a polaroid camera using Ilford FP4 film; the gel was illuminated from beneath (using a Kodak Coldlight illuminator, model 3), and an orange filter was employed to enhance contrast.

If required, gels were prepared for autoradiography or fluorography, as described below.

Radioactive Procedures

Estimation of Radioactivity

Samples dried onto glass fibre filter papers (Whatman GF/C) were placed into scintillation vials (or into plastic inserts in scintillation vials), and 5 ml (3 ml when using inserts) of toluene scintillation fluid added. The radioactivity was estimated by a Packard Scintillation Counter (Model 3330 or 3385). Aqueous samples (10–500 μl) were added to Bray's Scintillation fluid in vials or inserts,
and the radioactivity estimated as above.

**Incorporation of Radioactive Precursors into Cells**

Cells were labelled by the addition of a small volume of the radioactive substrate to the medium in which they were growing. Alternatively, the growth medium was replaced by fresh medium containing the radiolabelled component. After incubation, cells in suspension were mixed and an aliquot filtered through glass fibre discs. The filter discs were washed with PBS, dried under an infra-red lamp and the radioactivity estimated. A further sample was mixed with an equal volume of ice-cold 10% trichloroacetic acid (TCA) and left at 4°C for at least 30 minutes. This preparation was then filtered through glass-fibre discs and the discs washed with cold 5% TCA. The discs were dried and the TCA-insoluble radioactivity estimated as previously described.

Cells grown in monolayer were labelled with a radioactive substrate, as previously described. The medium was removed after incubation and the monolayers washed three times with ice-cold PBS, three times with ice-cold 5% TCA, and twice with ice-cold 95% ethanol. The residual ethanol was evaporated by standing the Petri dishes at room temperature for 30 minutes. The cellular material was solubilised by the addition of 2 ml of 0.5M NaOH, and incubation at room temperature for 1 hour with occasional agitation. A 0.5 ml sample was mixed with 10 ml of Bray's fluid in a scintillation vial, and the radioactivity determined as described.

Alternatively, cells were grown in scintillation vials which had been pre-incubated with 0.5% gelatin solution for 30 minutes. After washing the monolayers with PBS, 5% TCA and 95% ethanol, as described above, the residual material was solubilised by addition of 0.25 ml of tissue solubiliser (Koch-Light) in toluene (30% solution). Toluene scintillation fluid was added, and the radioactivity determined as previously.
Autoradiography

Thin-layer chromatography (TLC) plates, and polyacrylamide gels dried under vacuum onto filter paper, were taped to X-ray film (Kodak 'Kodirex') and left for an exposure time of between 1 day and 5 weeks. The film was then developed (Kodak D19 developer, 3 minutes), fixed ('Amfix') until the film cleared, washed and air-dried.

Fluorography

Polyacrylamide gels of radio-labelled samples ($^3$H- or $^{14}$C-labelled) were incubated for 4 hours at room temperature in dimethylsulphoxide (DMSO). The solution was changed after 1 hour and after 2½ hours of incubation. The DMSO was then replaced with a solution of 22% PPO (diphenyloxazole) in DMSO, and the incubation continued for 1 hour. The excess solution was removed, and distilled water added to the gel, causing the precipitation of the scintillant throughout the gel. The water was replaced with fresh distilled water after 1 hour and the incubation continued for a further minimum period of 2 hours. After removal of the water, 1% glycerol solution was added, and the gel left in this solution for 2 hours.

The gel was placed on filter paper, and dried down under vacuum at room temperature. Autoradiography was carried out on the dried-down gel, as described previously.

Extraction and Identification of Gangliosides

Preparation of Media

D-[$^{14}$C]-galactose (100 μCi/ml, 95 mCi/mmole) was added to Ham's F12 medium containing 10% dialysed foetal calf serum, to give a solution of 0.4-5 μCi/ml. Glucose (1M solution) was added to the required concentration.

The solvent was aseptically evaporated from a solution of
[1-^{14}C]-palmitic acid (1 Ci/mmole, 250 μCi) or [U-^{14}C]-palmitic acid (928 mCi/mmole, 50 μCi) in benzene. 1 ml of horse serum was added, and the palmitate redissolved by 5 minutes incubation in a sonicating water bath. The resultant solution was added to 9 ml of RPMI medium (10% horse serum), to give final solutions of 25 μCi/ml and 5 μCi/ml respectively.

**Extraction of Glycolipids**

L1210 cells were grown in Ham's medium or RPMI medium containing the desired radioactive substrate for a period of 24 - 48 hours. Samples were taken when required to estimate the uptake of the substrate by radioactive estimation.

Cells were harvested by centrifugation in a glass centrifuge tube when they had reached a density of 1-2 x 10^6 cells/ml. The cells were washed with PBS, and resuspended in 0.5 ml methanol. Cells were disrupted by sonication in short pulses (30 seconds) using a sonicating probe. The samples were cooled on ice between pulses. 10 μl of each cell extract were taken for estimation of radioactivity. The samples were dried down at 37°C under a stream of nitrogen. 3 ml of cold chloroform : methanol solution (2 : 1 v/v) was added to each sample, and the extracts incubated for 2 hours at 4°C. The samples were centrifuged in the bench centrifuge at 1000 r.p.m. for 3 minutes to pack loose protein, and the supernatants transferred to clean conical centrifuge tubes, and stored at 4°C. The pellets were re-extracted at 4°C overnight with 3 ml of the chloroform : methanol solution, and insoluble material removed by centrifugation. The supernatants from both extractions were combined, 0.2 volumes (1.15 ml) of water added, and the mixture vortexed for 2 minutes. The phases were then left to separate. The aqueous phase was removed to a clean graduated tube, the meniscus
diluted off each interface with chloroform : methanol : water (3 : 48 : 47 v/v/v), and added to the aqueous phase. A sample of each aqueous solution was removed for estimation of radioactivity. The volume of the remaining solution was noted, and the solution dried down at 37°C under a stream of nitrogen.

The extracts were saponified by the addition of methanolic NaOH (methanol : 1M NaOH, 9 : 1) and incubation for 6 hours at 37°C, or for 18 hours at 20°C. The samples were neutralised by the addition of 50 mM HCl; the pH was monitored by the use of wetted indicator paper. The extracts were dried overnight under vacuum and in the presence of phosphorous pentoxide, and dissolved in chloroform : methanol (1 : 1 v/v) solution for analysis, as described below.

Analysis of Glycolipids

Samples and standard glycolipids (Sigma, Ltd., 1 mg/ml in chloroform : methanol, 1 : 1 v/v) were spotted onto precoated 0.25 mm silica gel plates (Merck, Darmstadt). The chromatogram was developed in chloroform : methanol : water (60 : 35 : 8), or in chloroform : methanol : water : concentrated ammonia solution (60 : 35 : 7 : 1). The plate was allowed to dry overnight at room temperature, and autoradiographed as previously described. The standard glycolipids were visualised by standing the chromatogram in iodine vapour for several minutes; the positions of the resultant yellow bands were marked in pencil. Some of the lipids present in the radioactive samples could also be detected by this method.

Isolation of Glycolipid Fractions

The positions of the radioactive bands determined by autoradiography were marked on the thin layer plate, and areas of the gel which corresponded to separate radioactive fractions were scraped off
the glass plate into test tubes. Standards were similarly fractionated by reference to the iodine vapour stain. Unlabelled glycolipids were isolated by comparison with the positions of radioactively-labelled samples on the same chromatogram. 1 ml of chloroform : methanol (1 : 1 v/v) was added to the silica gel in each tube, the mixture vortexed and the solid material allowed to settle. The supernatants were transferred to clean tubes, and an aliquot (100 μl) taken where necessary for estimation of the radioactivity.

The solutions were dried down at 37°C under a stream of nitrogen, and the samples stored at -20°C.

Replacement of Glycolipids

Standard glycolipids, or L1210 glycolipid fractions isolated as described above, were resuspended in RPMI medium. L1210 cells were incubated in the presence of glycolipids at 37°C for 1 hour or overnight, washed twice with PBS, and resuspended in growth medium with or without interferon.

Preparation of Cellular Extracts

**Method 1  Homogenisation**

The medium was removed from confluent monolayers of cells in roller bottles or 150 mm Petri dishes, and the cell sheets washed twice with ice-cold PBS. While washing the monolayers, Petri dishes were placed on ice, and roller bottles were rotated in a shallow ice-water bath in order to ensure rapid cooling. All subsequent operations were carried out at 4°C. Cells were scraped off the surface of the bottle or dish into extraction buffer (10 mM Tris HCl, pH 7.4, 90 mM KCl, 10 mM MgCl₂ (TKM)), and pelleted by centrifugation. The pellet was resuspended in 1 ml of hypotonic buffer (10 mM Tris HCl, pH 7.4, 15 mM KCl, 5 mM MgCl₂), and the cells allowed to swell for 5 minutes. The cells were
disrupted by 10-30 strokes of a Dounce homogeniser, and breakage monitored by microscopic examination. The homogenate was rapidly adjusted to 90 mM KCl and 10 mM MgCl₂, and centrifuged at 5000 x g for 5 minutes to remove nuclei and other debris. The supernatant (S5) was used for analysis of polysome profiles, or was centrifuged at 10,000 x g for 20 minutes. The resulting supernatant was termed the S10.

In some experiments TKM and the hypotonic buffer described above were replaced with HKMD (20 mM HEPES, pH 7.5, 90 mM KCl, 5 mM Mg(0Ac)₂, and 1 mM dithiothreitol, DTT) and hypotonic HEPES buffer (10 mM HEPES, pH 7.5, 15 mM KCl, 1.5 mM Mg(0Ac)₂, 1 mM DTT) respectively. After homogenisation, the buffer was adjusted to HKMD.

Method 2 Detergent Treatment

Cells in monolayer were chilled and washed as described in Method 1. The cells were then washed with TKM containing 0.05% diethylpyrocarbonate (DEPC), or with HKMD, 1 ml of the respective buffer containing 0.5% Nonidet P40 was added to each 150 mm dish, and the dishes rocked. After 5 minutes, the solution was removed to a conical centrifuge tube, and centrifuged at 5000 x g for 5 minutes to remove nuclei and cell debris. The supernatant (S5) was analysed, or was centrifuged at 10,000 x g for 20 minutes, and the supernatant (S10) saved.

Cells grown in roller bottles were washed and chilled in a similar manner, and scraped from the glass surface into buffer. After centrifugation, the cell pellet was resuspended in 1-2 ml of HKMD containing 0.2% Nonidet P40, and the suspension vortexed. The extract was centrifuged as previously described to give the S5 or S10 fractions.

L1210 cells were washed and chilled, and pelleted by centrifugation. The cell pellet was extracted as described above in
TKM containing 0.05% DEPC and 0.5% Nonidet P40, and the S5 fraction analysed for the polysome profile.

**Analysis of Polysomes**

Cellular extracts were prepared by homogenisation (Method 1) or detergent treatment (Method 2). 0.5 ml of the S5 fraction was immediately layered onto ice-cold linear 8 - 45% sucrose gradients in TKM containing 0.05% DEPC. The sucrose gradients (13.7 ml) were freshly prepared in MSE 15 ml polycarbonate centrifuge tubes. The samples were centrifuged at 25,000 r.p.m. in a 6 x 15 ml swing-out rotor (MSE 65 centrifuge) for 90 or 100 minutes at 4°C. The gradients were analysed by upward displacement with 50% sucrose in TKM through an ISCO gradient analyser. The absorbance at 254 nm was recorded on a linked chart recorder.

**Preparation of Ribosomal Salt Washes**

In the following preparations, a series of buffers containing 20 mM HEPES, pH 7.5, 5 mM Mg(OAc)$_2$, and 1 mM DTT was employed. Each buffer also contained KCl, the concentration of which (mM) is indicated by the suffix associated with K in the abbreviation (thus HK$^{90}$MD buffer contains 90 mM KCl in addition to 20 mM HEPES, pH 7.5, 5 mM Mg(OAc)$_2$, and 1 mM DTT). All procedures were carried out at 4°C unless otherwise stated.

**Method 1 Separation by Use of Centrifugation**

The S10 fraction of the cellular extract (2-3 ml in HK$^{90}$MD) was centrifuged at 100,000 x g for 2½ hours in the 10 x 10 ml fixed angle rotor, using 3 ml tubes and the required adaptor (MSE 65 centrifuge). The supernatant was removed and stored at -70°C, and 1 ml of HK$^{200}$MD added to the pellet. After overnight incubation, the ribosomes were gently
resuspended with the rounded tip of a thin glass rod, and the suspension was centrifuged at 10,000 x g for 20 minutes. The supernatant was centrifuged at 100,000 x g for 2½ hours, and the ribosomal pellet was resuspended as described above (in HK^{200} MD). The suspension was clarified by centrifugation (10,000 x g for 20 minutes), and the ribosomes again pelleted (100,000 x g for 2½ hours). After resuspending the pellet in HK^{300} MD, the suspension was clarified and the ribosomes pelleted as described above. The supernatants from each 100,000 x g centrifugation (S100) were stored at 4°C. The ribosomes were resuspended in buffer containing 600 mM KCl (HK^{600} MD), incubated for 30 minutes, and the solution centrifuged (100,000 x g for 2½ hours). The S100 salt washes were exhaustively dialysed, in pretreated dialysis tubing, against distilled water and were freeze-dried.

Method 2 Molecular Sieving

The S10 fraction of cellular extracts (2 ml approx. in HK^{90} MD or HK^{90} MD containing 0.2% Nonidet P40) was applied to a 6 ml column of Sepharose 6B equilibrated with HK^{90} MD. The eluate was collected in fractions, and the fractions containing ribosomes were pooled. The column was calibrated by radiolabelled extracts. The ribosomal fraction was dialysed overnight against 100 volumes of HK^{200} MD, and the column was washed with 30 ml of the same buffer. The ribosomal fraction was re-applied to the column, and the eluate fractioned as previously. After washing the column with 30 ml of HK^{200} MD, the pooled ribosomes from the second fractionation were re-applied to the column, and the eluate again separated into ribosomal and non-ribosomal fractions.

The final ribosomal fraction was dialysed overnight against 100 volumes of HK^{300} MD, and the solution centrifuged at 100,000 x g for 3 hours. The supernatant, the 0.2 - 0.3 KCl ribosomal wash, was removed and stored, and 2 ml of HK^{600} MD was added to the pellet of ribosomes.
After overnight incubation, the ribosomes were gently resuspended with the rounded tip of a thin glass rod, and incubated for a further 30 minutes. The washed ribosomes were pelleted by centrifugation (100,000 x g for 3 hours) and the supernatant, the 0.3 - 0.6M KCl ribosomal wash, was removed. The two ribosomal washes were exhaustively dialysed against HK\(^{10}\) MD or against distilled water. The fractions in distilled water were freeze-dried, and analysed by polyacrylamide gel electrophoresis. Fractions in HK\(^{10}\) MD were applied to Sephadex gels, as described below.

**Fractionation of Ribosomal Salt Washes**

A Sephadex G150 column (50 cm) was equilibrated with HK\(^{10}\) MD, and the volume of eluate calibrated against standard proteins. 0.5 - 1.0 ml of the ribosomal salt washes in HK\(^{10}\) MD were applied to the column and eluted in five fractions, using the same buffer. Fractions were concentrated by use of the 'Minicon' concentrators (Amicon Ltd.), or were dialysed against 1000 volumes of distilled water overnight at 4°C and freeze-dried. Sample buffer was added to each extract, and polyacrylamide gel electrophoresis carried out on each.

**Cell-free Protein Synthesis and the Assay of dsRNA-dependent Inhibitors**

Two cell-free systems were utilised in this work: the 3T6 cell-free system, and the L cell-free system (Roberts, Clemens and Kerr, 1976) which was used in investigations carried out at the National Institute for Medical Research, under the direction of Dr. I. Kerr.

**3T6 Cell-free System**

3T6 cells were extracted by homogenisation as described, and the S10 fraction centrifuged for 30 minutes at 30,000 x g. The supernatant (S30) was dialysed overnight against HK\(^{90}\) MD buffer, and stored in small aliquots at -70°C.
The following stock solutions were prepared, and stored at -20°C:

**HKM** 200 mM HEPES (pH 7.5) containing 500 mM K(OAc) and 35 mM Mg(OAc)$_2$

**IV - phe** 40 μM each of 19 amino acids (minus phenylalanine), 4 mM GTP, 50 mM PEP, 25 mM ATP; the pH was adjusted to 7.0 by addition of KOH.

The final assay mixture contained:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T6 S30</td>
<td>50</td>
</tr>
<tr>
<td>HKM</td>
<td>10</td>
</tr>
<tr>
<td>IV - phe</td>
<td>10</td>
</tr>
<tr>
<td>$^{14}$C-phenylalanine (100 μCi/ml)</td>
<td>10</td>
</tr>
<tr>
<td>pyruvate kinase (10 mg/ml)</td>
<td>5</td>
</tr>
<tr>
<td>whole yeast tRNA (2 mg/ml)</td>
<td>5</td>
</tr>
<tr>
<td>poly (U) (1 mg/ml)</td>
<td>5</td>
</tr>
<tr>
<td>H$_2$O or other addition</td>
<td>5</td>
</tr>
</tbody>
</table>

**FINAL VOLUME** - 100 μl

**Assay Procedure**

The mixture was made up at 4°C, and poly(U) added last to the other components before immediately transferring the assay system to 30°C. 10 μl aliquots were taken at suitable times, and added to 0.5 ml cold 5% TCA (trichloroacetic acid). For estimation of the incorporation of the radioactive amino acid into protein, the samples were heated at 90°C for 15 minutes, and filtered onto glass fibre discs; after washing with excess 5% TCA, the discs were dried, and the radioactivity estimated as described. Estimation of the incorporation of the radioactive amino acid into protein and aminoacyl-tRNA species was achieved by a similar
process which omitted the heat treatment.

Some experiments used EMC virus RNA (0.5 µg per assay) in place of poly(U); in these cases, phenylalanine (final concentration 40 µM) was added to the IV-phe solution, $^{14}$C-phenylalanine was replaced by $^{14}$C-amino acid mix (50 µCi/ml, 50 mCi/matom carbon), and the concentration of Mg(OAc)$_2$ in the final mixture was adjusted to 1-3 mM.

L Cell-free System

The following solutions were prepared, and stored at -20°C:

**Energy Mix** 5 mM HEPES containing 10 mM ATP, 1 mM GTP, 6 mM CTP, and 100 mM creatine phosphate. The pH was adjusted to approximately 7 with KOH.

**Creatine kinase** 3.2 mg/ml in 10 mM HEPES, pH 7.5.

**Amino Acid Mix** All non-radioactive amino acids, 1 mM each.

The following solutions were made up immediately prior to the assay:

**Solution A** 180 mM HEPES, pH 7.5, containing 900 mM KCl, 20 mM Mg(OAc)$_2$, 1.5 mM spermidine, and 70 mM 2-mercaptoethanol.

**Solution B** (5 µl per 12.5 µl assay)

- Solution A 2 parts
- Energy Mix 2 parts
- $^{14}$C-amino acids (CFB 104, 50 µCi/ml) 2 parts
- Creatine Kinase 1 part
- Amino Acid Mix 1 part

**Solution B'** Add 4 parts of L cell S10 (preincubated and dialysed) to Solution B.

7.5 µl of Solution B' and 0.5 µl of EMC RNA (400 µg/ml) was added to each assay tube, leaving 4.5 µl for the addition of the materials under test.
and sterile distilled water. For routine assays of inhibitory activity, EMC RNA and the appropriate volume of distilled water were added to solution B', and 11.5 µl of the resulting solution dispensed into tubes. 1 µl of each solution to be tested was then added to the individual assay tubes, and the mixtures thoroughly vortexed. The tubes were incubated at 30°C for 120 minutes with gentle shaking.

After the incubation period, a 10 µl sample was removed from each tube, spotted onto a filter disc, and allowed to dry. The discs were incubated in 5% TCA at room temperature for 30 minutes, and further incubated for 10 minutes at 90°C in 5% TCA. The discs were dried, and the radioactivity estimated as previously.

Synthesis of the dsRNA-dependent Inhibitor in Cell Extracts

S10 or S100 fractions of cellular extracts in dialysis buffer (20 mM HEPES, pH 7.5, 90 mM KCl, 1.5 mM Mg(OAc)₂, 7 mM 2-mercaptoethanol) were incubated for 60 minutes at 30°C in the presence of 3 mM ATP and 1 µg/ml of poly(rI).poly(rC). The samples were heated at 90°C for 10 minutes and centrifuged to remove denatured protein. 1 µl samples of appropriate dilutions (in 10 mM HEPES, pH 7.5, 90 mM KCl, 1.5 mM Mg(OAc)₂) were then assayed for inhibitory activity in the L cell-free system.

The Use of Poly(rI).poly(rC) Attached to Solid Supports in the Synthesis of the dsRNA-dependent Inhibitor

The enzyme responsible for the synthesis of the dsRNA-dependent inhibitor will bind to dsRNA attached to a solid support, and may thus be concentrated and partially purified (Hovanessian et al., 1977). Low levels of the inhibitory system may thus be detected by this method.

50-200 µl of cellular extracts in dialysis buffer/glycerol (dialysis buffer containing 20% glycerol (v/v)) were applied slowly to a column of poly(rI).poly(rC)-agarose (50 µl). The flow-through fraction was re-applied three times to the column. Each column was then washed
thoroughly with 15 ml of dialysis buffer/glycerol. Each poly(rI).poly(rC)-agarose sample was then transferred to a small tube, and 50 µl of dialysis buffer/glycerol containing 3 mM Mg(OAc)₂ and 1 mM ATP was added. The samples were incubated overnight at 30°C in a shaking water bath. After centrifugation to remove the agarose, appropriate dilutions of each sample were made, and assayed for inhibitory activity in the L cell-free system.

dsRNA covalently attached to paper was also used in the attempt to detect low levels of the inhibitory system. The dsRNA-paper (prepared by I. Kerr and R.E. Brown) was washed thoroughly in dialysis buffer/glycerol, and cut into 0.1 cm² pieces (0.5 x 0.2 cm). A piece of the paper was added to each of the extracts (20 µl of L cell extracts and 100 µl of 3T6 and A2 extracts, in dialysis buffer/glycerol), and incubated for 60 minutes at 30°C. Each piece of paper was removed from the extract and washed six times with 1 ml of dialysis buffer/glycerol. Each paper was then incubated for 60 minutes at 30°C in the same buffer, and the buffer discarded. 50 µl of dialysis buffer containing 8 mM Mg(OAc)₂ and 2 mM ATP was added to each piece of paper, and the samples incubated overnight at 30°C. Appropriate dilutions of the buffer were then made, and tested for inhibitory activity in the L cell-free system.

**Mycoplasma Detection and Elimination**

Mycoplasma infection of animal cells in culture may have profound effects upon their metabolism. Such infections have been reported both to enhance and to reduce the ability of viruses to replicate (Stanbridge, 1971). Thus it is essential that mycoplasma should be eliminated from cells used for experiments on interferon. Infection of some cell lines with mycoplasma was suspected when the cell cultures deteriorated in appearance, and grew more slowly. The infection was confirmed and eradicated, and has not re-occurred.
Detection of Mycoplasma

1. Ratio of Uridine : Uridine Uptake (Schneider, Stanbridge and Epstein, 1974) Animal cells do not appear to utilise exogenous uracil to any significant extent, whereas mycoplasma can efficiently incorporate this substrate into RNA. The ratio of the incorporation into TCA-insoluble material of uridine to that of uracil is thus high for uninfected animal cells, and low for those infected with mycoplasma.

Cells were radioactively labelled, as previously described, for 18 hours in medium containing 0.5 μCi each of $^3$H-uridine and $[2-^{14}C]$-uracil (each adjusted to 61 mCi/m mole). The TCA-insoluble radioactivity due to $^3$H and to $^{14}C$ was estimated, as described previously, by use of the automatic settings [$^3$H($^{14}C$) and $^{14}C($$^3$H)] on the Packard 3330 scintillation counter. Cells grown in scintillation vials were normally used for these estimations. After correcting the figures for the different counting efficiencies, the ratio of uridine-uracil incorporated was estimated.

2. Scanning Electron Microscopy (SEM) Mycoplasma may be detected by SEM, appearing as rounded blebs on the cell surface with a diameter of 0.3 - 0.8 μm, and having a characteristic dimple.

Cells were grown as monolayers on glass cover-slips pre-treated with 0.5% gelatin for 30 minutes. The cells were washed three times with PBS, and fixed for 1 hour in 1% glutaraldehyde in PBS. Fixation was continued by overnight incubation in 3% glutaraldehyde in PBS.

The cells were dehydrated by incubation for 15 minutes successively in each of 30%, 50%, 70%, 80%, 90%, 95% and 100% ethanol. The samples were then incubated for 15 minutes in each of ethanol : amylacetate (1 : 1 v/v) and amylacetate.

The cover slips were dried in the critical point drier in an atmosphere of CO$_2$. The samples were mounted on microscope studs,
Mycoplasma may be detected as dimpled blebs on the surface of cells. The photographs of 3T6 cells below show such blebs of the characteristic size.
<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Treatment</th>
<th>Udr:U ratio(^{(b)})</th>
<th>Detection by SEM(^{(c)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210S</td>
<td>None</td>
<td>104</td>
<td>- Not done</td>
</tr>
<tr>
<td>L1210R</td>
<td>None</td>
<td>117</td>
<td>- Not done</td>
</tr>
<tr>
<td>3T6</td>
<td>None</td>
<td>1.9, 2.4</td>
<td>+</td>
</tr>
<tr>
<td>A2</td>
<td>None</td>
<td>1.3, 2.3</td>
<td>+</td>
</tr>
<tr>
<td>3T6</td>
<td>Antibiotics</td>
<td>5.3</td>
<td>+</td>
</tr>
<tr>
<td>A2</td>
<td>Antibiotics</td>
<td>4.9</td>
<td>+</td>
</tr>
<tr>
<td>3T6</td>
<td>5-F-U</td>
<td>4.7</td>
<td>+</td>
</tr>
<tr>
<td>A2</td>
<td>5-F-U</td>
<td>4.6</td>
<td>+</td>
</tr>
<tr>
<td>3T6</td>
<td>Gentamycin (^{(a)})</td>
<td>71</td>
<td>-</td>
</tr>
<tr>
<td>A2</td>
<td>Gentamycin (^{(a)})</td>
<td>76</td>
<td>-</td>
</tr>
<tr>
<td>3T6</td>
<td>Polyanethol sulphonate</td>
<td>91</td>
<td>-</td>
</tr>
<tr>
<td>A2</td>
<td>Polyanethol sulphonate</td>
<td>90</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^{(a)}\) Cells were incubated in Gentamycin (200 µg/ml) for 3 weeks after having been treated with Gentamycin and Kanamycin for 2 weeks.

\(^{(b)}\) Uridine : uracil (Udr:U) ratio is the ratio of the incorporation of each substrate into TCA insoluble material, as described.

\(^{(c)}\) Scanning Electron Microscope (SEM)
vacuum coated with gold in the sputter-coater, and examined in the Scanning Electron Microscope. Photographs were taken when required.

Elimination of Mycoplasma

Cells were grown in the presence of 200 \( \mu \text{g/ml} \) gentamicin and 200 \( \mu \text{g/ml} \) kanamycin over a 2-3 week period. Initially, this treatment did not appear to give mycoplasma-free cultures, but further treatments with gentamicin (200 \( \mu \text{g/ml} \)) were successful.

Cells were also passaged in the presence of 6-fluoro-uracil, in an attempt to selectively disrupt mycoplasma metabolism. This method was unsuccessful.

The best results were obtained by the use of 10% polyanethol sulphonate in growth medium over a 48 hour period. This method appeared to be completely successful.

In order to detect contamination of cell cultures, which may lead to mycoplasma infection, all the cells used in this study have been grown in medium without antibiotics.
CHAPTER 2

INVESTIGATION OF THE RESISTANCE TO INTERFERON

OF L1210 CELLS
CHAPTER 2 INVESTIGATION OF THE RESISTANCE TO INTERFERON OF L1210 CELLS

Mouse L1210 cells (Law et al., 1949) are sensitive to the antiviral and cell growth inhibitory effects of mouse interferon (Gresser et al., 1970a). The prolonged culture of L1210 cells in the presence of high concentrations of interferon results in the selection of a subline of cells which is insensitive to inhibition of growth by interferon (Gresser et al., 1970b). This subline, L1210R, is also insensitive to the antiviral effects of interferon (Gresser et al., 1974). L1210R cells arise from a sub-population of interferon-insensitive cells already present among L1210 cells, at a frequency of approximately 0.004 - 1% (Gresser et al., 1974). Investigation of the L1210R cells indicated that the membranes did not bind significant amounts of interferon, unlike the membranes of the parental, interferon-sensitive cells L1210S (Gresser et al., 1974).

Glycolipids, components of the cell membrane, have been shown to bind to interferon, and have been implicated in the response of cells to mouse interferon (Introduction). Thus the response to interferon of cells which are deficient in glycolipids may be enhanced by the artificial insertion of certain glycolipids (Vengris et al., 1976). As L1210R cells do not appear to bind interferon, a possible explanation of their insensitivity to interferon is that they lack the necessary glycolipid receptor. Cells in culture frequently have alterations in glycolipid metabolism (Critchley and Vicker, 1977), which may account for the high frequency of occurrence of interferon-insensitive cells among the parental L1210 cells. Therefore, after characterisation of the response of the cells to interferon and poly(rI).poly(rC), the glycolipids of the parental
cells and a clone of L1210R cells were analysed and compared. The effect of preincubation of the cells with glycolipids on their response to interferon was also studied.

Characterisation of the Response of L1210 Cells to Interferon and Poly(ri).poly(rc)

In the following experiments, the conditions were chosen so as to maximise the response to interferon, in order that a limited response by L1210R cells might be more easily detected. Such a response may be expected in L1210R cells pretreated with glycolipids.

The Effect of Interferon on Cell Growth

In RPMI medium (RPMI + 20% horse serum), both L1210S and L1210R cells had a doubling time of about 10 hours. The doubling time (Td) of the interferon-sensitive cells increased to 24 hours in the presence of 500 units/ml of interferon, and 2000 units/ml of interferon reduced growth still further (Td = 40 hours). In contrast, neither concentration of interferon had any effect upon the growth of L1210R cells (Fig. 3). The cell growth inhibitory effect on L1210S cells is a property of apparently pure mouse interferon (De Maeyer-Guignard et al., 1978), and, as noted above, selection of cells resistant to this effect also results in the selection of cells insensitive to the antiviral activity of interferon. Thus the cell growth inhibition appears to be a true effect of interferon, and may therefore be used to assay the potency of interferon preparations. This was the basis of the screening procedure for interferon preparations (Methods) which was devised. L1210R cells served as a control for non-interferon effects.
Cells were resuspended at time 0 in RPMI medium alone, or RPMI containing interferon. Samples were taken at the indicated times for estimation of viable cell numbers. Each point is the average of duplicate suspensions of cells. Open symbols, L1210S cells; closed symbols, L1210R cells; cells grown in the absence of interferon (△, △); cells grown in the presence of 500 units/ml of interferon (○, ○); cells grown in the presence of 2000 units of interferon per ml (□, □).
The Effect of Interferon Pretreatment of L1210 Cells on Viral Infection

Interferon pretreatment of L1210S cells resulted in a dramatic decrease in virus yield from cells infected with Sindbis virus (Table 2). There was no significant effect of interferon on the virus yield from infected L1210R cells.

The effect of interferon on the growth of L1210S cells has been reported to be enhanced by a reduction of the serum content of the medium (Gresser et al., 1970b). It was thus of interest to determine whether the antiviral effects of interferon could similarly be enhanced.

Interferon assays (virus yield reduction assays) performed in medium containing 20% horse serum (RPMI-20) were more sensitive than those carried out in RPMI-5 (medium supplemented with 5% horse serum) (Table 3). This was largely because the virus yield from untreated cells was very much reduced by the decrease in the serum content from 20% to 5%. Virus yield reduction assays of interferon in these cells were thus carried out in the normal high-serum medium (RPMI-20).

Interferon pretreatment of L1210S cells also inhibited the virus-induced cytopathic effect; high concentrations (100 U/ml) of interferon abolished this cytopathic effect (Fig. 4).

The Effect of Pre-treatment with Poly(rI).poly(rC) on Virus-induced Cytoxicity in L1210S and L1210R Cells

Poly(rI) poly(rC) is an effective inducer of interferon in many cell lines and the anti-viral effects of this dsRNA have been ascribed to its capacity to induce interferon (Introduction). It might thus be expected that poly(rI) poly(rC) would induce an antiviral state in L1210S cells, and not in the interferon-sensitive L1210R cells.

L1210 cells were incubated with poly(rI) poly(rC) for 12 hours as previously described (Chapter 1), washed and resuspended in fresh medium. After incubation for 12 hours, cells were infected or mock-infected,
Table 2 The Effect of Interferon Pretreatment on the Yield of Sindbis Virus from Infected L1210S and L1210R Cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Pretreatment</th>
<th>Virus Yield (p.f.u./ml x 10^-5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210S</td>
<td>None</td>
<td>27</td>
</tr>
<tr>
<td>L1210S</td>
<td>+ interferon (100 U/ml)</td>
<td>0.02</td>
</tr>
<tr>
<td>L1210R</td>
<td>None</td>
<td>36</td>
</tr>
<tr>
<td>L1210R</td>
<td>+ interferon (100 U/ml)</td>
<td>30</td>
</tr>
</tbody>
</table>

10^4 cells/ml incubated with or without interferon for 18 hours, and infected with Sindbis virus (3 p.f.u./cell). Virus yield estimated at 36 hours p.i.
Table 3

The Effect of Serum Concentration and Interferon Pretreatment on the Yield of Sindbis Virus from Infected L1210S Cells

<table>
<thead>
<tr>
<th>Medium</th>
<th>Pretreatment (for 18 hours preinfection)</th>
<th>Virus Yield (p.f.u./ml) at 22 hours post-infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI-20</td>
<td>None</td>
<td>$4.5 \times 10^7$</td>
</tr>
<tr>
<td>RPMI-20</td>
<td>+ interferon (100 U/ml)</td>
<td>$2 \times 10^3$</td>
</tr>
<tr>
<td>RPMI-5</td>
<td>None</td>
<td>$2.5 \times 10^5$</td>
</tr>
<tr>
<td>RPMI-5</td>
<td>+ interferon (100 U/ml)</td>
<td>$10^3$</td>
</tr>
</tbody>
</table>

Cells (initial density of $2 \times 10^5$/ml) were infected, after incubation for 18 hours, with Sindbis virus (approximately 4 p.f.u. per cell).
L1210S cells were incubated in RPMI medium, or in RPMI containing 100 μ/ml of interferon. After incubation for 15 hours, the medium was removed, and the cells were washed, and infected with Sindbis virus (2 p.f.u./cell). Excess virus was removed, the cells resuspended in RPMI medium, and viable cell concentrations ascertained at the times indicated. Symbols: no interferon, mock-infected (□); no interferon, infected (♦); interferon, mock-infected (■); interferon, infected (▲).
incubated in fresh medium, and virus-induced cytotoxicity followed by
estimation of viable cell numbers (Fig. 5). Poly(rI) poly(rC) in the
absence of DEAE-dextran did not protect L1210S cells from the viral
cytotoxic effect (data not shown). Poly(rI) poly(rC) in the presence of
DEAE-dextran gave considerable protection to L1210S cells, but did not
protect L1210R cells (Fig. 5). The data support the hypothesis that the
antiviral state induced by poly(rI) poly(rC) is mediated by interferon
production.

The antiviral activity of the supernatants was assayed on
mouse L cells. The activities were low, equivalent to 10 U/ml of
interferon from L1210S cells treated with 5 or 20 μg/ml of poly(rI) poly(rC)
in the presence of 10 μg/ml of DEAE-dextran, and equivalent to 5 U/ml of
interferon from L1210R cells similarly treated.

It is also of interest to note that under the particular
experimental conditions employed, untreated L1210S cells are less
susceptible to the cytotoxicity induced by Sindbis virus than are untreated
L1210R cells. It has also been noted that the yield of virus from
L1210S cells was consistently lower than the yield from L1210R cells
(e.g. Table 5). These results presumably reflect the role of interferon
which is induced by the infecting virus.

The Recovery of Interferon from the Media of Interferon-treated L1210S
and L1210R Cells

Interferon may be recovered from the membrane fraction of
interferon-treated L1210S cells, but not from L1210R cells similarly
treated (Gresser et al., 1974). It is possible that this result is due
to the presence of an interferon receptor on the membranes of L1210S cells
which is absent from L1210R cell membranes. Another possible explanation
is that cultures of L1210R cells inactivate interferon before it can
LI210 cells were treated with poly(rI).poly(rC) and DEAE dextran, or untreated, for 12 hours from time 0. The cells were then washed and resuspended in fresh medium. Cells were infected with Sindbis virus (2 P.F.U. per cell), or mock-infected, at 24 hours after the commencement of treatment. Symbols: (▼,▼) control cells, mock-infected; (○,○) control cells, infected; (□,□) cells treated with 10 µg/ml poly(rI).poly(rC) and 50 µg/ml DEAE dextran, and infected; (▲,▲) cells treated with 20 µg/ml poly(rI).poly(rC) and 50 µg/ml DEAE dextran, and infected; open symbols, LI210S cells; closed symbols, LI210R cells.
exert its effects. To distinguish between these two possibilities, the two cell lines were incubated with interferon, and the residual antiviral activity of the medium from each incubation was assayed. The results (Table 4) show that interferon is not inactivated by incubation with L121OR cells. There is an indication that interferon may be removed from the medium by L1210S, and not L1210R cells; however, no firm conclusion may be drawn, as the differences are not large enough to be significant (because of the inaccuracies involved in the assay of interferon).

Investigation of the Role of Glycolipids in the Action of Interferon on L1210 Cells

The majority of mammalian glycolipids are glycosphingolipids (Fig. 6) and occur predominantly on the cell surface (Critchley and Vicker, 1977). The biosynthetic pathways of the main gangliosides (sialic acid-containing glycolipids) are shown in Figure 7.

Gangliosides have been implicated in the response of cells to interferon (Introduction). Gangliosides, particularly GM₂ and GT₁, bind to mouse interferon and inhibit its action, a process which may be reversed by sialyl lactose (Besancon, Ankel and Basu, 1974). Replacement of glycolipids may increase the sensitivity to interferon of some transformed mouse cell lines which are ganglioside-deficient; the most efficient gangliosides in this replacement again appear to be GM₂ and GT₁ (Vengris et al., 1976).

Interferon-insensitive L121OR cells appear to have lost the ability to bind interferon. As this may be due to the lack of the necessary gangliosides, the glycolipids of L121OR cells were analysed, and compared to those from the interferon-sensitive parental cells.
Table 4 The Recovery of Interferon Activity from the Media of Interferon-Treated L1210S and L1210R Cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Final Dilution of Sample</th>
<th>Interferon Titre (U/ml)</th>
<th>Estimated Total Interferon (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210S</td>
<td>80</td>
<td>12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>960</td>
</tr>
<tr>
<td></td>
<td>160</td>
<td>7.5</td>
<td>1200</td>
</tr>
<tr>
<td>L1210</td>
<td>80</td>
<td>18</td>
<td>1440</td>
</tr>
<tr>
<td></td>
<td>160</td>
<td>11</td>
<td>1760</td>
</tr>
</tbody>
</table>

1400 Units (U) of mouse interferon was incubated for 18 hours in 10 ml of RPMI containing L1210S or L1210R cells (initial cell density $4 \times 10^5$ cells/ml). Antiviral activity of the supernatants was assayed in duplicate (except for <sup>a</sup>, which is a single measurement) on mouse L cells by the neutral red uptake assay.
FIGURE 6  THE STRUCTURE OF A TYPICAL GLYCOLIPID (CL2)

Galactose  Glucose  Ceramide
Symbols: Cer = ceramide; Glu = glucose; Gal = galactose; Gal Nac = N-acetylgalactosamine; SA = sialic acid.

**Figure 7** Biological Pathways of the Main Gangliosides

- **CERAMIDE**
  - β-Glc-Cer
  - β-Gal-Cer

- **β-Gal(1-4)Glc-Cer**
  - (GL1)
  - β-Gal(1-3)Gal-Glc-Cer (GL3)
  - β GalNac(1-3)Gal-Gal-Glc-Cer (GL4)

- **Gal-Glc-Cer**
  - SA
  - (GM3)
  - Gal-Glc-Cer (GD3)
  - SA

- **GalNac(1-4)Gal-Glc-Cer**
  - SA
  - (GM2)
  - GalNac-Gal-Glc-Cer (GD2)
  - SA

- **Gal-GalNac-Gal-Glc-Cer**
  - SA
  - (GML)
  - Gal-GalNac-Gal-Glc-Cer (GD1b)
  - SA

- **Gal-GalNac-Gal-Glc-Cer**
  - SA
  - (GD1a)
  - Gal-GalNac-Gal-Glc-Cer (GT1a)
  - SA

- **Gal-GalNac-Gal-Glc-Cer**
  - SA
  - (GT1b)
  - Gal-GalNac-Gal-Glc-Cer (GTET)
The Uptake of Radioactive Precursors of Glycolipids into L1210 Cells

Palmitic acid is readily taken up by cells in culture, and incorporated into glycolipids (Critchley and Vicker, 1977); in the experiments to be described, 40-60% of the total radioactive palmitate applied to the cells was taken up by them in 48 hours. Galactose, a component of most glycolipids, may not be readily transported into the cell, particularly in the presence of high concentrations of glucose. Cells were therefore grown in medium containing various concentrations of glucose, and the incorporation of radioactive galactose measured over a two-day period (Fig. 8). Cells grew at the same rate in medium containing 20 mM, 10 mM, and 5 mM glucose, but poorly in medium containing 2.5 mM glucose. The rate of incorporation of galactose into the cells was highest in those cells grown in medium containing 5 mM glucose; this concentration of glucose was therefore used in the medium when labelling the glycolipids of L1210 cells with radioactive galactose.

Analysis of the Glycolipids of L1210S and L1210R Cells

The glycolipids were extracted from L1210S and L1210R cells which had been grown in the presence of \(^{14}C\)-palmitic acid or \(^{14}C\)-galactose. The glycolipids were analysed by TLC and autoradiography (Figures 9 and 10), as described (Chapter 1). The portions of the chromatograms above the GD\(_{1a}\) standard appear to be similar in L1210R extracts to the samples from L1210S cells. However, some differences are apparent in the regions around and below GD\(_{1a}\). In the \(^{14}C\)-palmitate-labelled samples, a component below GD\(_{1a}\) (possibly GD\(_{1b}\)) appears to migrate faster in the extracts from L1210R cells than in those from L1210S cells. It is possible that this is the result of a slightly faster-migrating component, present only in the L1210S samples, which slows down the movement of its neighbour. This part of the chromatogram from \(^{14}C\)-galactose-labelled
The glycolipids were extracted and analysed as previously described (Chapter 1).
cells is obscured by a large spot, probably due to the intermediate UDP-galactose, which migrates at a similar rate to GD_{1a} (D. Critchley, personal communication). This chromatogram (Figure 10) clearly shows the absence from L1210R extracts of two components which are present in the L1210S samples. One of these components migrates as the GT_{1a} standard, and the other has a mobility between those of GT_{1a} and GD_{1a}. An increased incorporation of radioactivity into a component just below GT_{1a} is evident in L1210R extracts compared to samples from L1210S. This component may be C_{TET}. The top of the chromatograms contain the glycolipids GL1, GL2, etc., and other lipids; other lipids show up only in the chromatogram shown in fig. 9, for they do not contain galactose. The double band labelled 'B' at the top of fig. 9 probably represents GL1, which has an analogue containing glucose in place of galactose; as these glycolipids are only faintly detected in fig. 10, it would appear that L1210R cells contain less of the galactose analogue. This may be due to enhanced catabolism of GL1, or decreased synthesis.

To aid further the comparison of the gangliosides from L1210S and L1210R cells, bands of radioactivity were scraped from the gel by reference to the autoradiogram. The radioactivity was eluted and estimated as described (Chapter 1). The results (Fig. 11) indicate that in L1210R cells, relatively more radioactivity is incorporated into the rapidly migrating gangliosides at the expense of glycolipids in the area between GT_{1} and GD_{1a}.

It would thus appear that both cell types have a functional biosynthetic pathway to GD_{1a}, including the formation of the intermediates GM3, GM2, and GM1. One of the unidentified components may also be GT_{1b} (possibly 'A' of figure 10). However, GT_{1a} and a second unidentified ganglioside (possibly GD_{1b}) which are present in the parental cells, appear to be absent from the interferon-insensitive subline. It is possible that the interferon-insensitivity of L1210R cells is due to a deficiency in one of these gangliosides, in which case, replacement of gangliosides may restore interferon sensitivity (see Introduction).
The chromatogram shown in Figure 10 was divided into the sections indicated, and the radioactivity from each section was eluted and estimated as previously described (Chapter 1). Total radioactivity: L1210S, 24,700 c.p.m. L1210R, 14,050 c.p.m. Symbols: L1210S (—); L1210R (—–).
The Effect of the Replacement of Glycolipids on the Response of L1210 Cells to Interferon

L1210 cells were pretreated with gangliosides for one hour, as described (Chapter 1), incubated with or without interferon for 18 hours, and infected with Sindbis virus. The yield of virus from the cells (Table 5) indicated that preincubation with standard glycolipids, or glycolipids extracted from L1210S cells, did not render L1210R cells sensitive to interferon.

If glycolipids are involved in interferon action, as has been indicated (Introduction), these results may indicate that the conditions used were unsatisfactory. The glycolipids from L1210S cells were at a very low concentration (estimated at less than 1 µg/ml), and the standard glycolipids may not contain the required ganglioside. However, the standards do contain GT^la, which appears to be absent in L1210R cells, and has been reported to increase the sensitivity to interferon of some ganglioside-deficient cells (Vengris et al., 1976). It is possible that any effect of glycolipid replacement may have lasted for a short period only, and thus have been undetectable after the 18 hours of interferon treatment.

The assay was therefore repeated over a shorter period: cells were pretreated for one hour with standard glycolipids (100 µg/ml) as described, washed, and incubated in medium with or without interferon (1000 U/ml) for 6 hours, the cells were infected with Sindbis virus (3 p.f.u./cell), and the yield of virus determined 16 hours post-infection. The cytoxic effect of the viral infection was also monitored. The results (Table 6) indicate that interferon may have some effect in L1210R cells which have been pretreated with standard glycolipids; the yield of virus was decreased in L1210R cells treated with glycolipids and interferon, although only slightly, and the viral cytopathic effect was also decreased.
Table 5 The Effect of Preincubation with Glycolipids on the Yield of Sindbis Virus from Interferon-Treated and Untreated L1210 Cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Pretreatment</th>
<th>Virus Yield (p.f.u./ml x 10^{-5})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>glycolipids</td>
<td>interferon (150 U/ml)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1210S</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L1210R</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Cells were infected with approximately 2 p.f.u./cell of Sindbis virus.
(a) In Experiment 1, the glycolipids were those extracted from L1210S cells, initial cell density was 4 x 10^5 cells/ml, virus yield estimated at 24 hours p.i.; in Experiment 2, glycolipids were standard glycolipids (100 µg/ml), initial cell density was 10^5 cells/ml, virus yield estimated at 30 hours p.i.
### Table 6

The Effect of Glycolipid Pretreatment on the Response of L1210R cells to Interferon

<table>
<thead>
<tr>
<th>Cells</th>
<th>Pretreatment</th>
<th>Virus Yield (p.f.u./ml x 10^-5)</th>
<th>Estimation of Cytotoxicity (cells/ml x 10^-4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>glycolipids</td>
<td></td>
<td>viable cells</td>
</tr>
<tr>
<td></td>
<td>(100 μg/ml)</td>
<td></td>
<td>non-viable cells</td>
</tr>
<tr>
<td></td>
<td>interferon</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(1000 U/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1210S</td>
<td>-</td>
<td>39</td>
<td>93 (60%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>61 (40%)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>5.4</td>
<td>164 (82%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>36 (18%)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>32</td>
<td>96 (66%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>49 (34%)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>14.2</td>
<td>120 (8%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24 (17%)</td>
</tr>
</tbody>
</table>

Cells were treated with glycolipids for 1 hour where indicated, washed, and resuspended in RPMI-20, with or without interferon. After 6 hours, the cells were washed, infected with Sindbis virus (3 p.f.u./cell), and resuspended in fresh medium. The virus yield was estimated at 16 hours post-infection, and the cytotoxicity was estimated at 26 hours post-infection by the estimation of viable and non-viable cell densities. The initial cell density was 9 x 10^5 cells/ml.
It is noted that although high concentrations of interferon were used, only a small antiviral effect is apparent in L1210S cells, possibly a reflection on the brief period of interferon treatment. These results do no more than give an indication that glycolipid pretreatment may restore interferon sensitivity to L1210R cells. Therefore, the less sensitive but more immediate assay of growth inhibition by interferon was used to monitor the effects of glycolipid replacement.

Cells were treated in duplicate with the standard glycolipids (100 μg/ml) in medium, or with medium alone for one hour, washed, and resuspended in medium, or in medium containing interferon (150 U/ml). The cells were incubated, and growth monitored by estimation of cell densities. The results (Fig. 12; Table 7) indicate that there may be an effect of glycolipid pretreatment on the sensitivity of L1210R cells to interferon; however, further experimental observations are required before this result may be confirmed. It is noted that the growth of L1210R cells pretreated with glycolipids and incubated with interferon is as rapid as the control cells after 26½ hours. Pretreatment of L1210S cells with gangliosides appears to inhibit the action of interferon; this is presumably due to traces of gangliosides remaining after washing the cells, and may be expected, as such glycolipids have previously been shown to inhibit interferon action (Besancon, Ankel and Basu, 1971)]. It thus appears that one or more of the components present in the standard glycolipids (GM₄, GD₃a, GD₃b, and GT₃a) may affect the response of L1210S and L1210R cells to interferon.
L1210R cells were incubated for 1 hour in 1 ml of RPMI medium alone, or in 1 ml of medium containing standard glycolipids (100 μg/ml). The medium was then removed, the cells washed in PBS, and resuspended in 2.5 ml of fresh medium. 0.5 ml of each cell suspension was seeded into duplicate wells containing 0.5 ml medium alone, or medium plus interferon (1000 U/ml). As a control, L1210S cells were seeded in the same manner into wells containing medium alone, or medium plus interferon. Cell concentrations were determined at the indicated times.

Symbols: L1210S cells (□); L1210S cells plus interferon (○); L1210R cells (■); L1210R cells plus interferon (●); L1210R cells treated with glycolipids (▲); L1210R cells treated with glycolipids and interferon (▼).
The error bars indicate the standard error of the mean (SEM).
<table>
<thead>
<tr>
<th>glycolipid pretreatment (100 μg/ml)</th>
<th>interferon (150 U/ml)</th>
<th>Td(a) (hours)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>8½</td>
<td>9</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>25</td>
<td>9½</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>16</td>
<td>17</td>
</tr>
</tbody>
</table>

(a) Td is the doubling time between 1½ hours and 26½ hours after the commencement of treatment with glycolipids.
The response to interferon of L1210S cells and an interferon-insensitive subline (L1210R) were characterised. Previous work had indicated that glycolipids were involved in the interaction of interferon with the membrane, and that the defect in L1210R cells may be located at the membrane. The glycolipids of the two cell types were therefore analysed and compared. It appeared that L1210R cells were deficient in at least two gangliosides compared to the parental cells; one of these gangliosides was identified as GT1a by comparison with the standard glycolipids. It appears that pre-incubation with gangliosides (including GT1a) inhibits the response of L1210S cells to interferon; it is also indicated that such treatment may restore interferon-sensitivity to L1210R cells, but this observation requires confirmation.
CHAPTER 3

CHARACTERISTICS OF CONSTITUTIVELY VIRUS-RESISTANT CELLS
Chapter 3  CHARACTERISTICS OF CONSTITUTIVELY VIRUS-RESISTANT CELLS

The derivation of a mutant subline of 3T6 cells which is resistant to a wide range of viruses has been described (Morgan, Colby and Hulse, 1973). Initial characterisation of this subline (A2) showed that resistance to virus infection was manifested at a stage subsequent to the adsorption and penetration of virus. It was also demonstrated that a persistent infection of the cells was unlikely (Morgan, Colby and Hulse, 1973). Interferon could not be detected in the medium in which the cells were incubated, even after concentration of the medium (M.J. Morgan, personal communication). A possible explanation of the virus-resistance is that the cells are constitutive for the antiviral state normally induced by interferon.

A second virus-resistant subline of 3T6 (3T6 V'B2), which had been derived by the same procedure, was investigated in later experiments. This cell line has recently been reported to be constitutive for interferon production (Jarvis and Colby, 1978).

By use of these mutants, it was hoped that the essential components in the response of cells to interferon could be identified.

Characteristics of Parental Cells and the Virus-Resistant Subline, A2

A2 cells grew more slowly (doubling time, Td, of 12-14 hours) than the parental 3T6 cells (minimum Td of 10 hours) (Fig. 13). A2 cells are also contact-inhibited, unlike 3T6 cells. Contact inhibition and a slower growth rate are also characteristic of 3T6 cells grown in high concentrations (500 U/ml) of interferon (Fig. 13). The yield of Sindbis virus from infected cells is greatly reduced in the mutant and in interferon-treated 3T6 cells compared to the parental cells (Table 6). A2 cells consistently gave virus yields which were 10-100 times lower.
Figure 13: The growth of 3T6 cells in the presence and absence of interferon, and of the virus-resistant subline (A2).

Cells were seeded into 50 mm Petri dishes at time 0. Cell numbers were estimated at the times indicated by trypsinising duplicate dishes. Symbols: ▲, 3T6 cells; ○, 3T6 cells plus interferon (100 U/ml from time 0); □, A2 cells.
Table 8  The Yield of Sindbis Virus from Confluent Monolayers of 3T6 Cells, Interferon-treated 3T6 Cells and Virus-resistant A2 cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Input Multiplicity (p.f.u./cell)</th>
<th>Time of Harvesting (hours p.i.)</th>
<th>Virus Yield (p.f.u./ml x 10^-6)</th>
<th>3T6</th>
<th>3T6+IF</th>
<th>A2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2</td>
<td>18</td>
<td></td>
<td>38</td>
<td>3.2</td>
<td>1.7</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>18</td>
<td></td>
<td>21</td>
<td>5.0</td>
<td>4.3</td>
</tr>
<tr>
<td>3</td>
<td>2.0</td>
<td>44</td>
<td></td>
<td>25</td>
<td>2.4</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Where indicated, 3T6 cells were treated with 40 U/ml of interferon (IF) for 18 hours.
than the yield from untreated 3T6 cells. Thus the mutant cells possess some of the major characteristics of interferon-treated cells.

A2 cells continue to express virus-resistance, contact inhibition, and a reduced growth rate for up to about 100 continuous passages. After this period, the cells appear to gradually lose virus-resistance and contact inhibition.

Cell-Mixing Assay for Transfer of Resistance

Although A2 cells do not produce detectable amounts of interferon, it is possible that very small amounts continuously synthesised by the cells are sufficient to induce a significant antiviral effect. In this respect, it is noted that small amounts of interferon inducers may produce no detectable interferon, and yet render the treated cells resistant to virus infection (Vengris, Stoller and Pitha, 1975). Furthermore, 3T6 V'B2 cells have been reported to be constitutive for interferon production (Jarvis and Colby, 1978).

Mixed cultures of L cells and A2 cells in various ratios were infected, after co-cultivation for 48 hours. In this way it would be expected that any interferon produced by the virus-resistant cells would protect adjacent L cells. The yield of virus from the mixed cultures were, however, as would be expected if no transference of resistance occurred (Fig. 14). It appears, therefore, that interferon is not constitutively produced by these cells.

The Cytotoxic Effects of Double-stranded RNA

Stewart et al. (1972) reported that interferon pretreatment of mouse L929 cells greatly enhanced the cytotoxic effects of double-stranded RNA (dsRNA). This observation is of particular interest because of its possible relationship to the dsRNA-activated inhibitor of protein synthesis.
A2 cells and L cells were mixed in various proportions, and seeded into Petri dishes. After 40 hours, the cells were infected with Sindbis virus which had been grown on BHK cells. The yield of virus (x) was determined 24 hours post-infection. (@) average of duplicate samples; prediction of virus yield from non-interacting mixed cell cultures (---); predicted virus yield from interacting mixed cell cultures (...).
present in extracts from interferon-treated cells (Introduction; Roberts, Clemens and Kerr, 1976).

3T6 and A2 cells and L cells, interferon-treated or untreated, were incubated with poly(I).poly(C) for 1 hour. The cells were washed twice in warm FBSA, and incubated in DME. After 48 hours, some cytotoxicity was observed in L cells treated with interferon and poly(I).poly(C), and the cytotoxicity was measured as previously described (Chapter 1). It may be seen (Table 9) that while interferon-pretreatment enhances the cytotoxic effects of dsRNA in L cells, there is no such effect in 3T6 or A2 cells. Furthermore, untreated A2 cells are not significantly affected by concentrations of poly(I).poly(C) up to 100 µg/ml. Other reports (Emeny, 1977; Cooper and Morser, in press) have also indicated that many cell lines do not show a significant interferon-induced enhancement of cytotoxicity due to dsRNA.

Analysis of the Ribosome-associated Proteins of 3T6 Cells, Interferon-treated 3T6 Cells and Two Virus-resistant Cell Lines

The induction of the antiviral state by interferon appears to be mediated by production of a novel protein or proteins (Introduction). A ribosome-associated protein which is absent from untreated cells has been detected in interferon-treated cells; ribosomal wash fractions containing this protein have been reported to selectively inhibit the translation of viral mRNAs in a cell-free protein synthesising system (Samuel and Joklik, 1974; Samuel, 1976). If the virus-resistant cells are constitutive for the antiviral state or for interferon production, such a protein might be expected to be permanently expressed in these cells.

Confluent monolayers from six roller bottles each of 3T6 cells, A2 cells and 3T6 cells pretreated for 18 hours with interferon (150 U/ml),
**Table 9** The Effect of Interferon on the Cytotoxicity of dsRNA

<table>
<thead>
<tr>
<th>Poly(I).poly(C) (µg/ml)</th>
<th>L cells</th>
<th>3T6</th>
<th>A2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-IF</td>
<td>+IF</td>
<td>-IF</td>
</tr>
<tr>
<td>0</td>
<td>300</td>
<td>330</td>
<td>236</td>
</tr>
<tr>
<td>10</td>
<td>260</td>
<td>240</td>
<td>210</td>
</tr>
<tr>
<td>100</td>
<td>268</td>
<td>182</td>
<td>220</td>
</tr>
</tbody>
</table>

α = plates were pretreated with gelatin

Poly(I).poly(C) treatment was for 1 hour at the indicated concentration. Where indicated, cells were pretreated for 18 hours with interferon (IF) (200 U/ml). Cell viability was estimated by neutral red uptake.
were extracted by homogenisation as described. Ribosome-associated proteins were obtained by salt-washing the ribosomes, separation of the wash-fractions and the ribosomes being achieved by centrifugation (Chapter 1). Analysis of the salt-wash fractions was by SDS polyacrylamide gel electrophoresis (Fig. 15). Because of the difficulty of resuspending pelleted ribosomes, this procedure gave low yields of protein and also resulted in an inefficient fractionation. A large number of proteins were present in each fraction, many present in both washes (Fig. 15). One protein (P) appears to be present in the interferon-treated 3T6 and the A2 0.2 - 0.3M KCl salt-washes, and not in either salt-wash from untreated 3T6 cells. The poor quality of the fractionation, however, precludes any firm indications.

A second procedure for the separation of ribosomes and salt-wash fractions was developed. This separation, by the use of column chromatography (molecular sieving), is demonstrated by the elution profile from Sepharose-6B (Fig. 16). Cell extracts for use in this fractionation procedure were obtained by detergent-treatment (NP40), as this increased the yield of ribosomes. This method of preparing ribosomal salt washes gave greater yields (Table 10) and an efficient fractionation (Fig. 17).

Analysis of the salt washes indicates the presence of a protein (P1) in the 0.2 - 0.3M KCl ribosomal wash in A2 cells, 3T6 Vβ2 cells and interferon-treated 3T6 cells, which is not present in untreated 3T6 cells, or present only at much lower levels than in the other cells (see figure 17). This difference is clearly seen in the densitometric traces obtained from the photographic negative of the gel (Fig. 18). The molecular weight of this protein, estimated from the position of standard proteins by a graphical method (Fig. 19), is 56,000 daltons; the protein appears to be similar in molecular weight to the novel protein reported
The S10 fraction was prepared from 3T6 cells which had been radioactively labelled with $^{14}$C-leucine, and was applied to a 6 ml column of Sepharose 6B. The column was eluted, fractions collected, and the radioactivity from each fraction was estimated.
Table 10  Comparison of the Yield of Ribosome-associated Proteins from Salt Washes obtained by Different Methods

<table>
<thead>
<tr>
<th>Source of Ribosomes</th>
<th>Amount of Protein in each salt-wash (μg)</th>
<th>0.2-0.3M KCl wash</th>
<th>0.3-0.6M KCl wash</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Method 1</td>
<td>Method 2</td>
</tr>
<tr>
<td>3T6</td>
<td></td>
<td>140</td>
<td>290</td>
</tr>
<tr>
<td>A2</td>
<td></td>
<td>40</td>
<td>120</td>
</tr>
<tr>
<td>3T6 + interferon</td>
<td></td>
<td>-</td>
<td>75</td>
</tr>
<tr>
<td>3T6 + interferon</td>
<td></td>
<td>160</td>
<td>225</td>
</tr>
</tbody>
</table>

Method 1 was homogenisation and centrifugation

Method 2 was detergent-treatment and molecular sieving

The amount of protein is that obtained by extraction and further treatment of six roller bottles of cells (1-2 x 10^8 cells). Protein estimation was by Coomassie brilliant blue G.
FIGURE 16  DENSITOMETRIC TRACES FROM POLYACRYLAMIDE GELS OF RIBOSOMAL
The molecular weight of the novel ribosome-associated protein shown in Figure 17 was estimated from the position of molecular weight markers (●). The molecular weight of the similar protein reported by Samuel and Joklik (1974) was also estimated, by reference to their molecular weight markers (■). The molecular weight markers used were bovine serum albumen (67,000), ovalbumen (45,000), and lactate dehydrogenase (34,000).
by Samuel and Joklik (1974) (Fig. 19), although the reported molecular weight was 48,000 daltons. Samuel and Joklik first reported that this protein was to be found in the 0.3 - 0.6M KCl wash, but later reports indicated that the protein was removed from the ribosome by 0.3M KCl (Samuel, 1976; Samuel and Farris, 1977).

There appear to be no other significant differences between the 0.2 - 0.3M KCl wash from interferon-treated 3T6 cells and that from untreated 3T6 cells. The ribosomal salt washes from A2 cells are also very similar to those obtained from 3T6 V7B2 cells. Some proteins (P2 and P3, for example) are, however, present in greater amounts in the extracts from 3T6 and interferon-treated 3T6 cells.

Other techniques have been applied to the analysis of the ribosomal salt washes. Two such techniques were fluorography of pulse-labelled extracts after electrophoresis of the ribosomal washes, and the further fractionation of the 0.2 - 0.3M salt washes on a Sephadex G150 column. Unfortunately, no additional information was obtained by these methods.

Assay for a dsRNA-activated Inhibitor of Protein Synthesis in Extracts of 3T6 and A2 Cells

Incubation of extracts from interferon-treated L cells with dsRNA and ATP results in the production of a potent inhibitor of protein synthesis (Clemens, Brown and Gilbert, 1976). It has been proposed that this low molecular weight inhibitor (LMWI) is synthesised in the interferon-treated cell in response to viral dsRNA. The activation of such an inhibitor upon viral infection would inhibit viral replication.

It has been suggested that the virus-resistant cell line (A2) is constitutive for part of the interferon system. If the production of the inhibitor (LMWI) in response to dsRNA is an important part of the
interferon system, it might be expected that this capacity is permanently expressed in the virus-resistant cells. Extracts from 3T6 cells, A2 cells, and interferon-treated 3T6 cells were therefore assayed for the production of a dsRNA-dependent inhibitor of protein synthesis.

Cell extracts were incubated with dsRNA and ATP as described (Chapter 1). 1 μl additions of suitable dilutions were made to the L cell-free systems, and after incubation at 30°C for 2 hours, the TCA-insoluble radioactivity was estimated. The results (Table 11) indicated that the extracts from A2 cells and interferon-treated 3T6 cells do not synthesise significant amounts of heat-stable inhibitor, unlike extracts from interferon-treated L cells. In this cell-free system, the maximal inhibition by the dsRNA-activated inhibitor was approximately 60%.

The enzyme system responsible for the synthesis of LMWI may be purified and concentrated by use of its affinity for dsRNA (Kerr and Brown, 1978). Extracts from various cells were applied to poly(rI).poly(rC)-agarose columns, or to poly(rI).poly(rC)-paper in attempts to detect low levels of the inhibitor-synthesising system (Chapter 1). The results (Table 12) suggested that the use of poly(rI).poly(rC)-agarose diminished the sensitivity of the assay for the inhibitory system compared to the addition of dsRNA to the extracts. The use of poly(rI).poly(rC)-paper increased the sensitivity of the assay. Even the most sensitive assay, however, failed to demonstrate any dsRNA-activated inhibitory activity from extracts of A2 cells or interferon-treated 3T6 cells.

As the LMWI inhibits both viral and cellular protein synthesis (I. Kerr, personal communication), it would be expected that interferon pretreatment should enhance the cytotoxicity of dsRNA for cells which produce the inhibitor, as has been shown in L cells (Stewart et al., 1972).
Table 11  The Capacity of Cell Extracts to Form the dsRNA-dependent Inhibitor of the L cell-free System

<table>
<thead>
<tr>
<th>Sample (1 μl)</th>
<th>Concentration of Added Sample</th>
<th>TCA-insoluble Radioactivity (c.p.m. x 10^{-3})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>13.5, 11.7</td>
</tr>
<tr>
<td>LMWI</td>
<td>10^{-7}M</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 x 10^{-8}M</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10^{-8}M</td>
<td></td>
</tr>
<tr>
<td>dsRNA/ATP-activated samples</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L cell S10</td>
<td>10^{-1}</td>
<td>14.3, 12.9</td>
</tr>
<tr>
<td>L cell + IF S10</td>
<td>10^{-1}</td>
<td>5.5, 5.2</td>
</tr>
<tr>
<td></td>
<td>10^{-2}</td>
<td>6.9, 4.4</td>
</tr>
<tr>
<td></td>
<td>10^{-3}</td>
<td>12.3, 8.6</td>
</tr>
<tr>
<td>3T6 S10</td>
<td>10^{-1}</td>
<td>15.1</td>
</tr>
<tr>
<td></td>
<td>10^{-2}</td>
<td>14.5</td>
</tr>
<tr>
<td>3T6 + IF S10</td>
<td>10^{-1}</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td>10^{-2}</td>
<td>8.1</td>
</tr>
<tr>
<td>3T6 + IF S100</td>
<td>10^{-1}</td>
<td>14.1, 13.8</td>
</tr>
<tr>
<td></td>
<td>10^{-2}</td>
<td>14.9, 12.2</td>
</tr>
<tr>
<td>A2 S10</td>
<td>10^{-1}</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>10^{-2}</td>
<td>10.1</td>
</tr>
<tr>
<td>A2 S30</td>
<td>10^{-1}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10^{-2}</td>
<td></td>
</tr>
</tbody>
</table>
Table 12  Comparison of the Ability of dsRNA-purified Components from Different Cell Extracts to Produce an Inhibitor of the L Cell-free System

<table>
<thead>
<tr>
<th>Sample (1 μl)</th>
<th>Concentration of Added Sample</th>
<th>TCA-insoluble Radioactivity (c.p.m. x 10^{-3})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td>--------------</td>
</tr>
<tr>
<td>H₂O</td>
<td>-</td>
<td>10.6, 11.0</td>
</tr>
<tr>
<td>dialysis buffer</td>
<td>-</td>
<td>12.8, 13.6</td>
</tr>
<tr>
<td>LMWI</td>
<td>10^{-7} M</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>3 x 10^{-8} M</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>10^{-8} M</td>
<td>11.5</td>
</tr>
<tr>
<td>Poly(rI),poly(rC)-Agarose Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L cell + IF S10</td>
<td>10^{-1}</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>10^{-2}</td>
<td>8.9</td>
</tr>
<tr>
<td>3T6 + IF S10</td>
<td>10^{-1}</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>10^{-2}</td>
<td>9.4</td>
</tr>
<tr>
<td>A2 S10</td>
<td>10^{-1}</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>10^{-2}</td>
<td>6.8</td>
</tr>
<tr>
<td>Poly(rI),poly(rC)-paper Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L cell S10</td>
<td>10^{-1}</td>
<td>12.9</td>
</tr>
<tr>
<td>L cell + IF S10</td>
<td>10^{-2}</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>10^{-3}</td>
<td>9.4</td>
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<td></td>
<td>10^{-4}</td>
<td>15.8</td>
</tr>
<tr>
<td>3T6 S10</td>
<td>10^{-1}</td>
<td>13.9</td>
</tr>
<tr>
<td></td>
<td>10^{-2}</td>
<td>13.9</td>
</tr>
<tr>
<td>3T6 + IF S10</td>
<td>10^{-1}</td>
<td>14.7, 14.3</td>
</tr>
<tr>
<td></td>
<td>10^{-2}</td>
<td>14.2, 13.7</td>
</tr>
<tr>
<td>A2 S10</td>
<td>10^{-1}</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td>10^{-2}</td>
<td>9.4</td>
</tr>
<tr>
<td>A2 S100</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>10^{-2}</td>
<td>9.4</td>
</tr>
</tbody>
</table>
However, this enhancement of dsRNA-mediated cytotoxicity was not detected in virus-resistant cells or interferon-treated 3T6 cells. It would thus appear that the LMWI does not have a major role in the expression of the antiviral state in these cells.

Summary

A virus-resistant subline of 3T6 (A2) was investigated in parallel with the interferon-treated parental cells. Initial characterisation of A2 cells had indicated that they permanently expressed the antiviral state. Interferon could not be detected in the medium from A2 cells, and co-cultivation of the cells with mouse L cells did not lead to transfer of virus-resistance. Neither A2 cells nor interferon-treated 3T6 cells showed an enhanced response to the cytotoxic effects of dsRNA. The dsRNA-dependent inhibitor from extracts of interferon-treated L cells could not be detected in extracts of A2 cells or interferon-treated 3T6 cells. A protein from the 0.2 - 0.3M KCl ribosomal washes, which has a similar molecular weight to that described by Samuel and Joklik (1974), was detected in two virus-resistant cell lines and in interferon-treated 3T6 cells. The protein was not present, or present at much lower levels, in the untreated parental cells. It is thus indicated that these mutant cells are constitutive for part of the interferon system, most likely for the antiviral state. It would also appear that a protein of about 56 000 daltons may be involved in the antiviral state; it is possible that this protein is the ribosome-associated kinase which has been reported by others (see Introduction).
CHAPTER 4

MODELS OF INTERFERON ACTION AND INDUCTION
Despite the efforts which have been made to elucidate the mechanism of action of interferon, there has been no widely-accepted hypothesis to explain the effects of the protein in molecular terms. The introduction of one or more models of interferon action would serve to focus attention on a few pertinent questions. The central element of such a model must be an explanation of the selective inhibition of viral replication, but many other observations such as the effects of interferon on its own synthesis must also be taken into account. As interferon treatment of cells appears to inhibit mainly the translation step of viral replication (Introduction), the basic postulate of the proposed mechanism is that interferon action is effected by some modulation of protein synthesis. The confirmation of the presence of a novel ribosome-associated protein in interferon-treated cells, and the detection of a similar protein in virus-resistant cells (Chapter 3), lend support to such a postulate.

Cells can clearly distinguish host mRNA from viral mRNA. Thus interferon treatment selectively inhibits viral protein synthesis, and the opposite effect, an inhibition of the translation of cellular, but not viral, mRNAs, occurs in many virus-infected cells. In order to determine how such discrimination may occur, it is necessary to appreciate the effect of various conditions on the rate of translation of any mRNA.

The Kinetics of Protein Synthesis

Several factors determine the rate at which a mRNA molecule is translated; apart from alterations in the overall rates of initiation and elongation, different mRNAs initiate protein synthesis at different rates (Lodish, 1974). The better mRNAs, those which have a high initiation rate
constant ($K_i$), are termed high-affinity mRNAs, as they may be regarded as having a greater affinity for the [met-tRNA^*_{40S ribosomal subunit}] complex. The poorer mRNAs are described as low-affinity mRNAs. Lodish (1974) developed a kinetic model to describe variations in the rate of protein synthesis; the predicted rates of translation of different mRNAs as functions of the initiation rate and of the elongation rate are shown in Figures 20 and 21. It may be seen that a general inhibition of the initiation rate would be expected to preferentially inhibit the translation of low-affinity mRNAs. This prediction has been verified (Lodish, 1974; Kabat and Chappell, 1977). However, the validity of the model is dependent upon the even distribution of ribosomes along the mRNA (appendix), and this criterion will not be fulfilled when the rate of elongation is small compared to the rate of initiation.

A model for protein synthesis which does not have this limitation was therefore constructed. The model is described in the appendix. This new model is empirical, in that the computer-simulated process follows the passage of each ribosome along the mRNA. The rate of protein synthesis is estimated, after the system has stabilised, from the time taken for a given number of ribosomes to terminate protein synthesis. The predicted variations in the rate of protein synthesis as different parameters are varied are shown in Figures 22 and 23. The variation in the average number of ribosomes per mRNA, as a percentage of the possible maximum, is also shown (Fig. 22). It may be seen that the model closely follows that of Lodish when the initiation rate is high compared to the rate of elongation, that is, under conditions in which the Lodish model is likely to be valid. The anomalous predictions of the Lodish model at low elongation rates are avoided (Fig. 21). The model predicts the preferential inhibition of the translation of low-affinity mRNAs when the rate of initiation is lowered, and, conversely, the preferential inhibition of protein synthesis directed
The rate of protein synthesis was calculated from the Lodish equation (Appendix) for mRNAs with specific initiation rates (i.e. "affinity") of 1.0 (●), 0.25 (▲), and 0.05 (■). The elongation rate constant, $K_e$, was 1.0.
The rate of protein synthesis was calculated from the Lodish equation (Appendix) for combined initiation rate constants (the products of $K_i$, the specific initiation rate, and $R$, the general initiation rate) of 0.24 (●), 0.04 (▲), and 0.01 (■).
FIGURE 22  THE PREDICTED RATE OF PROTEIN SYNTHESIS AND RIBOSOME RATIO AS A FUNCTION OF THE GENERAL INITIATION RATE (COMPUTER-SIMULATED MODEL)

(a) The predicted ribosome ratio (the ratio of the average number of ribosomes on a mRNA to the maximum number of ribosomes on that mRNA) as a function of the general initiation rate (R). The specific initiation rate is 0.25.

(b) The predicted rate of protein synthesis for mRNAs with specific initiation rates of 1.0 (●), 0.25 (▲), and 0.05 (■) was estimated. (a) and (b) Error-bars indicate the standard error on the mean of 8 determinations. K_e is 1.0.
FIGURE 22  THE PREDICTED RATE OF PROTEIN SYNTHESIS AND RIBOSOME RATIO AS A FUNCTION OF THE GENERAL INITIATION RATE (COMPUTER-SIMULATED MODEL)
The rate of protein synthesis was estimated for mRNAs with combined initiation rates ($K_i R$) of 1.0 (●), 0.5 (●), 0.1 (■), 0.05 (■), 0.025 (▼), and 0.01 (X). Error-bars indicate (where significant) the standard error on the mean of 8 determinations.
by high-affinity mRNAs when the elongation rate is lowered. The kinetic predictions of this model suggest that discrimination in the translation of various mRNAs may be achieved by a non-specific regulation of protein synthesis.

**Viral Protein Synthesis**

Viral mRNA can completely outcompete cellular mRNA in protein synthesis in a cell-free system (Abreu and Lucas-Lenard, 1975), thus indicating that the viral mRNA is of high affinity. It has also been demonstrated that translation of viral mRNA is resistant to inhibition of the initiation of protein synthesis in vivo (Madore and England, 1975; England et al., 1975; Nuss et al., 1975), and in a cell-free system (Carrasco and Smith, 1976), again indicating that viral mRNA is of high-affinity. Viral infection frequently results in an inhibition of host, but not viral, protein synthesis; this inhibition is effected at the initiation step of protein synthesis (Carrasco and Smith, 1976) as may be demonstrated by the rapid breakdown of host polysomes (Fig. 24; Metz, 1974). It has been indicated that the viral-induced inhibition may be due to an increase in the intracellular concentration of sodium ions - in effect, an inhibition by hypertonic conditions (Carrasco and Smith, 1976); the effect appears to be mediated by the coat proteins of picornaviruses. Viral protein synthesis, as indicated above, is resistant to such inhibition, and is even likely to benefit from an enhanced supply of components (ribosomal subunits, charged tRNA species, and enzymes), no longer required for host protein synthesis. Thus the demonstration that viral mRNAs are of high-affinity leads to a likely explanation of the discrimination between translation of host and viral mRNA observed in virus-infected cells.
An Hypothesis of Interferon Action

The result of interferon action appears to be the preferential inhibition of viral protein synthesis. Whatever the characteristics of viral mRNA which are recognised by the antiviral mechanism, it is likely that they are essential to viral replication; they would otherwise have been eliminated under the selective pressure of the interferon system.

The high-affinity characteristic of viral mRNA would appear to be essential to viral replication, in order that viral protein synthesis might effectively compete against the large number of cellular mRNAs. It has been suggested that the high-affinity characteristic is the element which is recognised, and discriminated against, by the interferon system (Metz, 1975). It is further suggested that interferon action inhibits the expression of the high-affinity characteristic - in other words, that all mRNAs are translated with the same frequency in a cell displaying a full antiviral state. Viral mRNA would thus be translated much more slowly, and viral protein synthesis would no longer be resistant to the virus-induced inhibition of translation. As cellular mRNAs have a range of affinities (Kabat and Chappel, 1977; Nuss and Koch, 1976), some inhibition of the synthesis of particular proteins would be expected in an interferon-treated cell. In general, however, host protein synthesis would not be grossly affected by interferon treatment. A less efficient discrimination against high-affinity (e.g. viral) mRNAs would result from an inhibition of elongation. Inhibitors of elongation might thus to some extent mimic the effects of the proposed mechanism of interferon action, the inhibition of the expression of the high-affinity characteristics of mRNA.

The Inter-relationship between the Induction and the Action of Interferon

Interferon pretreatment of cells may result in an enhanced production of interferon at a subsequent induction; under different
conditions, treatment with the protein may inhibit interferon production (priming and blocking, Introduction). Furthermore, interferon may regulate its own production, as interferon-resistant cells produce greater yields of the protein than wild-type cells (Chany and Vignall, 1970), a phenomenon which may be related to the process of superinduction (Introduction). Thus any detailed model of interferon action must attempt to explain the effects of the protein on its own synthesis.

A possible Mechanism of Interferon Induction

Tan and Berthold (1977) proposed that interferon inducers had a common mechanism of action - the inhibition of cellular RNA and protein synthesis. Certainly viruses and dsRNA, the two major classes of interferon inducers, inhibit protein synthesis. Furthermore, other inhibitors of RNA and protein synthesis were shown to be capable of inducing interferon, although at much lower levels (Tan and Berthold, 1977). This theory postulated the existence of a labile repressor protein to prevent transcription of the interferon gene. However, neither the shut-off of interferon synthesis in the continued presence of inducer, nor the hyporeactivity to a second induction could be explained on this basis.

It has been noted above that viral infection frequently results in the inhibition of the initiation of protein synthesis; furthermore, dsRNA is a potent inhibitor of this same step in translation (Hunter, 1975). It is thus proposed that the induction of interferon results from the inhibition of initiation of protein synthesis. It is envisaged that the effect is mediated by a preferential inhibition of the synthesis of a repressor protein (r). Thus it is further postulated that the repressor mRNA is of low-affinity, and its translation inhibited under these conditions, while the mRNA for an inducer protein (i) is of high-affinity,
and consequently resistant to inhibitors of initiation. This proposed mechanism is further described below.

A Proposed Model of Interferon Induction and Action

The proposed mechanisms of interferon action and induction, discussed above, are developed and combined to give an integrated model of induction and action.

The normal levels of a repressor (r) and an inducer (i) in the non-induced cell ensure that transcription of the interferon gene does not occur. Inhibitors of the initiation of protein synthesis, for example, viral infection or dsRNA, preferentially inhibit the synthesis of the repressor protein, so that the ratio r : i gradually decreases to a level at which transcription may occur. It is postulated that the rate of transcription of the interferon gene will increase as the ratio r : i decreases, within a certain range. Removal of the inhibitor of protein synthesis will allow the gradual return of the repressor molecule to its original concentration, preventing further transcription (Fig. 25). As translation of the interferon mRNA occurs in the virus-infected cell, under conditions which severely limit host protein synthesis, it is reasonable to assume that this mRNA is of high-affinity. Interferon is thus synthesised, glycosylated, and exported even under adverse conditions (i.e. in the continued presence of the inducer molecule). External interferon then interacts with neighbouring cells and also with the producing cell.

The membrane receptor for interferon appears to be a glycolipid (Introduction; Chapter 2), as is the case for several polypeptide hormones (Critchley and Vicker, 1976). It is likely that the membrane-bound interferon stimulates or represses the production of a secondary messenger molecule(s); the polypeptide hormones mentioned above stimulate
The predicted effect of various conditions on the synthesis of an inducer protein (•••) and repressor protein (---) is shown. The inducer protein (i) is envisaged to be translated from high-affinity mRNA, unlike the repressor molecule (r).

The inhibition of initiation (I) causes the preferential inhibition of the translation of the repressor mRNA, and a gradual decrease in the ratio r : i. When a particular low level of the ratio r : i is reached, transcription of the interferon gene occurs (T). The removal of the inhibitor (R) allows a gradual increase in the ratio r : i, and transcription ceases. Interferon released by the cell leads to the development of an antiviral state (A), and thus a decreased synthesis of the inducer. A subsequent treatment with the inhibitor of initiation (I2) will inhibit the synthesis of both the inducer and repressor proteins, and the low ratio r : i required for interferon induction will not be attained.
adenyl cyclase, thus raising the intracellular concentration of cyclic AMP (cAMP) (Critchley and Vicker, 1976). It is possible that interferon has a similar effect, since interferon treatment causes a rise in intracellular cAMP levels (Weber and Stewart, 1975; Fuse and Kuwata, 1978). Furthermore, treatment of cells with dibutyryl-cyclic AMP, or with hormones which increase the intracellular level of cAMP, can potentiate the antiviral effect of interferon (Weber and Stewart, 1975). However, this report also indicated that an increase in cAMP alone was insufficient to induce the antiviral state.

Although transcription and translation appear to be necessary for the establishment of the antiviral state (Introduction), protein synthesis does not seem to be required to mediate other effects of interferon, such as cell growth inhibition (Fuse and Kuwata, 1978) and priming for interferon induction (Stewart, Gosser and Lockart, 1971). It has been indicated that the interferon-induced rise in cAMP levels is responsible for cell growth inhibition, as this effect may be mimicked by an increase in intracellular cAMP (Fuse and Kuwata, 1978).

It is assumed that a particular level of secondary message(s) is required for transcription of the gene(s) responsible for the development of the antiviral state. Treatment of cells with low concentrations of interferon may result in levels of secondary message below this threshold; such intermediate levels would be expected to potentiate the effects of subsequent interferon treatment. It is proposed that the mRNA which has been transcribed under the direction of the secondary message is translated to give a protein which acts to block the expression of the affinity characteristics of mRNAs. There are several possible mechanisms by which such a protein could act, and the synthesis of a novel gene product is not necessarily required (see Discussion). The result of such a protein would be the inhibition of the translation
of high-affinity mRNAs, including viral mRNAs. The majority of cellular mRNAs, being of low-affinity, would continue to be translated at the same rate; however, the synthesis of certain host proteins, those translated from high-affinity mRNAs, would be inhibited. Thus the production of the inducer protein required for interferon induction will be depressed, resulting in a raised r : i ratio, and providing an explanation of the refractory state and the phenomenon of blocking. At the same time, the translation of interferon mRNA will be inhibited if, as has been suggested, the mRNA is of high affinity. Thus the interaction of interferon with the induced cell will inhibit the transcription of the interferon gene, and the translation of interferon mRNA. The interruption of this feedback mechanism by inhibitors of RNA and protein synthesis (superinduction), or by use of interferon-resistant cells, will result in enhanced production of interferon. Immunoglobulin mRNA is of high affinity (Nuss and Koch, 1976), and thus antibody synthesis may be expected to be inhibited in the interferon-treated cell, as has been indicated (Gisler, Lindhal, and Gresser, 1974). The inhibitory effects of interferon on the induction of tyrosine amino transferase (Matsuno, Shirasawa and Kohno, 1976) and glutamine synthetase (Beck et al., 1974) may also be explained if their mRNAs are of high-affinity, as has been indicated in one case (Matsuno, Shirasawa and Kohno, 1976).

The inhibition of the initiation of high-affinity mRNAs would result in a large decrease in the density of ribosomes on these mRNA, and might thus enhance their susceptibility to ribonucleases.

This model of interferon induction and action appears to be able to accommodate most of the major observations of the interferon system, with the possible exception of some in vitro studies on interferon action. The model, summarised in Figure 26, is further discussed in Chapter 6.

Several predictions based on this model may be made: these include the
Normal protein synthesis
Low-affinity mRNA for repressor (r)
High-affinity mRNA for inducer (i)

Ratio i:r ensures continued repression of interferon (IF) gene

virus infection or artificial inducer

Specific inhibition of initiation of protein synthesis

Differential inhibition of protein synthesis; preferential translation of high-affinity mRNA

Facilitated virus replication

Decrease in synthesis of repressor relative to inducer; ratio i:r increased, leading to transcription of IF gene

Preferential translation of IF mRNA (high-affinity)

Glycosylation and export of IF

Feedback inhibition on IF synthesis

IF-membrane interaction

Derepression of anti-viral protein (AVP) gene
Production of AVP
Binding of AVP to ribosome

Inhibition of preferential translation of high-affinity mRNA

Inhibition of protein synthesis; high-affinity mRNA preferentially inhibited

Inhibition of virus replication

Decrease in synthesis of inducer relative to repressor; ratio i:r decreased, leading to an inhibition of the transcription of IF mRNA

Inhibition of translation of IF mRNA

Refractory State

Decrease in synthesis of inducer relative to repressor; ratio i:r decreases

Blocking

FIGURE 26 SUMMARY OF THE MODEL OF INTERFERON INDUCTION AND ACTION
induction of interferon by inhibitors of initiation; the enhanced production of interferon by any interferon-resistant cell compared to the wild-type cell; the inhibited translation of any high-affinity mRNA in interferon-treated cells; and the partial mimicry of the interferon system by inhibitors of elongation. Some of these predictions were tested in Chapter 5.

Summary

A kinetic model of protein synthesis is presented which predicts the preferential translation of particular mRNAs by regulation of non-specific factors. The model satisfactorily accounts for the preferential inhibition of host protein synthesis in the virus-infected cell. This kinetic model was used to develop an integrated model of interferon induction and action, which is in agreement with most observations on the interferon system, and makes several testable predictions.
CHAPTER 5

THE INVESTIGATION OF THE PROPOSED MECHANISMS OF INTERFERON

ACTION AND INDUCTION
CHAPTER 5  THE INVESTIGATION OF THE PROPOSED MECHANISMS OF INTERFERON ACTION AND INDUCTION

It has been proposed that the major effects on cells of interferon treatment are due to the production of a protein which causes the preferential inhibition of the translation of high-affinity mRNAs (Chapter 4). Some effects of interferon, those which do not require the mediation of protein synthesis, may be due to increased levels of a secondary message (possibly cAMP). The induction of interferon was postulated to be effected by an inhibition of the initiation of protein synthesis, via a differential effect on the synthesis of an inducer protein and a repressor protein (Chapter 4). This chapter is concerned with the investigation of some of the predictions which result from the proposed mechanisms of interferon induction and action.

The Effect of Interferon on Cellular Protein Synthesis

It is envisaged that the translation of all high affinity mRNAs will be inhibited in the interferon-treated cell; it may be possible, therefore, to detect some inhibitory effect of interferon on cellular protein synthesis. The effect of interferon on protein synthesis in L1210 cells was studied by the analysis of polysome profiles; interferon-resistant L1210R cells were used as a control for non-interferon effects.

L1210 cells were treated with interferon (300 U/ml) or untreated for 18 hours prior to the extraction and analysis of polysomes (Chapter 1). The results (Fig. 27) indicated that interferon treatment did not significantly alter the distribution of polysomes. It was thus inferred that neither the initiation of protein synthesis alone, nor elongation alone, had significantly altered. However, a similar inhibition of both initiation and elongation would also result in an unchanged polysome profile.

The initiation of protein synthesis is rapidly inhibited as the
FIGURE 27 THE EFFECT OF INTERFERON TREATMENT ON THE POLYSOME PROFILES OF L1210 CELLS

L1210S and L1210R cells were untreated, or treated with 300 U/ml of interferon for 18 hours before extraction and analysis of the polysomes (Chapter 1). Symbols: L1210S cells, untreated (---), and interferon-treated (—); L1210R cells, untreated (-----), and interferon-treated (----).
temperature is lowered, whereas elongation is more resistant to this treatment. Rapid chilling of samples is therefore essential in order to obtain representative polysome profiles. These properties may be utilised to gain an indication of the relative rates of elongation in different cell extracts.

L1210 cells were treated with interferon (300 U/ml) or untreated as described above. Instead of their being rapidly chilled to 4°C, however, the samples were cooled to 25°C, left at this temperature for 30 seconds, and chilled rapidly to 4°C. This treatment allowed some of the ribosomes to 'run off' the mRNA (Fig. 28). It may be seen from these results that interferon-treated L1210S cells were more resistant to the breakdown of polysomes than the untreated cells, indicating a reduced rate of elongation in interferon-treated cells. From the previous analysis of polysomes (Fig. 27), it may thus be inferred that interferon treatment results in a small but significant inhibition of both the initiation and the elongation steps of protein synthesis. Interferon treatment of cells has previously been shown to inhibit Vaccinia virus replication at the steps of initiation and elongation (Metz et al., 1972). It is proposed that the inhibitory mechanism is the same in each case, but that the effects on virus replication are more severe (because of a non-specific discriminatory effect, as discussed in Chapter 4).

A Comparison of the Effects on cells of Interferon and an Inhibitor of Elongation

It has been indicated that a reduction in the rate of elongation of protein synthesis will result in the preferential inhibition of the translation of high-affinity mRNAs (Chapter 4). Thus inhibition of elongation may mimic, to a limited degree, those effects of interferon which are postulated to involve the selective inhibition of protein synthesis.
FIGURE 28  THE EFFECT OF DELAYED COOLING ON THE POLYSOME PROFILES FROM UNTREATED AND INTERFERON-TREATED L1210 CELLS

L1210S cells were untreated, or treated with interferon (300 U/ml) for 18 hours prior to extraction. Instead of being rapidly chilled, the cells were left for a period of 30 seconds at 25°C, to allow ribosomes to run off the mRNA. The polysomes were then extracted and analysed as previously described. Symbols: L1210S cells, untreated (---), and interferon-treated (---).
Tenuazonic acid, one of the few specific inhibitors of the elongation step of eukaryotic protein synthesis in vivo (Vasquez, 1974), was used to test this proposal. This inhibitor has previously been reported to inhibit the replication of a range of viruses (Miller et al., 1963).

The antiviral effects of the drug and of interferon were compared. L929 cells were pretreated with interferon for 18 hours, or with tenuazonic acid for 6 hours, and infected with Sindbis virus. After infection, tenuazonic acid was added to the medium of the cells which had been pretreated with the drug. Estimations of the yield of virus and of the viral cytotoxic effects were carried out. The results (Table 13) indicated that tenuazonic acid was a weak antiviral agent under the conditions employed, particularly when compared to interferon. The inhibitor (5 μg/ml) increased the doubling time of L929 cells from 18 hours to 36 hours.

Compared to the parental 3T6 cells, the virus-resistant A2 cells grow more slowly and adhere more firmly to the surface on which they are grown; A2 cells are also contact-inhibited, and have an altered morphology (personal observations). Treatment of 3T6 cells with interferon leads to a similarly-altered morphology, and renders the cells contact-inhibited. Some of these effects, such as increased adhesion, slower growth, and altered morphology, are similar to those observed in cells treated with dibutyryl cyclic AMP (Pastan and Willingham, 1978). Thus the observed alterations could be due to elevated levels of cyclic AMP, which are reported to occur in interferon-treated cells (Fuse and Kuwata, 1978; Weber and Stewart, 1975). It is also possible that these changes may be caused by the differential inhibition of most protein synthesis; an alteration of the relative amounts of different cellular proteins may lead to changes in metabolism and morphology. It is interesting to note, in this context, that the inhibition of protein synthesis in chick lenses by hypertonic medium results in an alteration of the relative amounts of two different
# Table 13: A Comparison of the Antiviral Effects of Interferon and Tenuazonic Acid in L929 Cells Infected with Sindbis Virus

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Virus Yield Assay</th>
<th>Assay of Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Assay (p.f.u./ml x 10^-6)</td>
<td>Antiviral Effect (%)</td>
</tr>
<tr>
<td>None</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Interferon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4 U/ml</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>4 U/ml</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>40 U/ml</td>
<td>0.75</td>
<td>97</td>
</tr>
<tr>
<td>160 U/ml</td>
<td>0.02</td>
<td>99.9</td>
</tr>
<tr>
<td>Tenuazonic Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 µg/ml</td>
<td>7.5</td>
<td>70</td>
</tr>
<tr>
<td>20 µg/ml</td>
<td>10</td>
<td>60</td>
</tr>
</tbody>
</table>

The virus yield was estimated at 20 hours post-infection. The assay of cytotoxicity was carried out at 40 hours post-infection, by the estimation of neutral red uptake. All figures are the average of duplicate determinations. The confluent monolayers were treated as indicated, and infected with Sindbis virus (3 p.f.u./cell).
cf-crystallin proteins; this differential synthesis may be associated with cataract formation (Shinohara and Piatigorsky, 1977).

It has been proposed that inhibition of elongation may mimic some of the effects of interferon. The effect of tenuazonic acid on the morphology of 3T6 cells was therefore studied. 3T6 cells were grown in the presence of tenuazonic acid (5 μg/ml); at this concentration, the drug increased the doubling time of the cells from 15 hours to 34 hours. Treatment with the inhibitor altered the morphology of the cells, so that they resembled A2 cells, and also rendered the cells contact-inhibited (Fig. 29). Removal of the inhibitor at any stage resulted in a reversion to the 3T6 morphology, and the loss of contact inhibition (data not shown). Two inhibitors of initiation, pactamycin and hypertonic medium, similarly inhibited the growth of the cells, but did not render 3T6 cells contact-inhibited, or result in the change of morphology. Thus, the treatment of 3T6 cells with tenuazonic acid results in alterations which are similar to those resulting from interferon treatment and those associated with the virus-resistant A2 cells. The inhibitor has also been reported to have antiviral activity (Miller et al., 1963); however, results from this laboratory indicated that the compound was a poor antiviral agent under the conditions used. Tenuazonic acid thus mimics some of the effects of interferon on cells, but does not effectively discriminate against viral protein synthesis.

The Effect of Cellular Extracts and Ribosomal Salt-washed Fractions on Cell-free Protein Synthesis

A cell-free protein-synthesising system from 3T6 cells was constructed (Chapter 1) in order to analyse the effect of various cellular fractions on protein synthesis. The 3T6 cell-free system incorporated radioactivity from 14C-phenylalanine into TCA-insoluble material when
3T6 cells in Petri dishes were treated with tenuazonic acid (5 µg/ml) or untreated for the course of the experiment. Photographs of the cells were taken at various times after the commencement of treatment, as indicated.
poly(U) was used as mRNA (Fig. 30). However, the system failed to incorporate radioactive amino acids into TCA-insoluble material when directed by EMC mRNA. It thus appeared that the system was defective in the normal initiation of protein synthesis. This cell-free system, therefore, could not be used in the analysis of the role of the novel protein present in salt-washes from virus-resistant cells.

The L cell-free system of Kerr, Brown and Ball (1974) was therefore used to examine the effect of fractions from various cells on translation. The results (Table 14) indicated that the S10 fraction from virus-resistant A2 cells inhibited the translation of EMC mRNA much more severely than did the same fraction from 3T6 cells. However, the 0.2 - 0.3M KCl ribosomal washes from both 3T6 and A2 cells also severely inhibited protein synthesis. The inhibiting activity was due to the presence of a thermo-labile component, as it was abolished by heat-treatment (Table 14). The salt-washes from interferon-treated 3T6 cells did not show the drastic inhibition found with the same fractions from untreated 3T6 cells and A2 cells. Some inhibition of protein synthesis was observed with the addition of the 0.2 - 0.3M KCl ribosomal wash from interferon-treated 3T6 cells. It is possible that the A2 and 3T6 salt-washed fractions were contaminated with ribonuclease during their preparation; these two cellular extracts were prepared at the same time, while the salt-washes from interferon-treated cells were prepared at a later date. Unfortunately, no conclusions concerning the effects of the different ribosomal washes on protein synthesis may be drawn from these experiments.

Interferon Induction

The model developed in Chapter 4 proposed that interferon may be induced by inhibitors of the initiation of protein synthesis. Hypertonic medium was used as an inhibitor of initiation in order to test this
A cell-free system was set up from extracts of 3T6 cells, as described. The incorporation of $^{14}$C-phenylalanine into hot TCA-insoluble material in the presence (■) and absence (○) of poly(U) is shown.
Table 14  The Effect of Cellular Extracts and Ribosomal Salt-washed Fractions on Cell-free Protein Synthesis

<table>
<thead>
<tr>
<th>Sample</th>
<th>Heat-treatment of sample-minutes at:</th>
<th>TCA-insoluble Radioactivity (c.p.m. x 10^-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50°C</td>
<td>70°C</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialysis buffer</td>
<td>-</td>
<td>-</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extracts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T6 S10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A2 S10</td>
<td>-</td>
<td>-</td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>0.2 - 0.6M KCl Ribosomal Washes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(2 µl)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>-</td>
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<tr>
<td></td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>A2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(2 µl)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>3T6 + IF</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(2 µl)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>(2 µl)</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>0.3 - 0.6M KCl Ribosomal Wash</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T6 + IF</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(2 µl)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>(2 µl)</td>
<td>-</td>
<td>5</td>
</tr>
</tbody>
</table>
hypothesis. This inhibitor was chosen because of its ready availability, and the ease with which it may be removed from the cells. The effect of the pre-incubation of cells with hypertonic media on the yield of Sindbis virus was measured, as a sensitive indicator of the possible production of interferon. The media of cells was also assayed for the presence of interferon.

3T6 cells were treated with hypertonic media for various times, washed, and infected with Sindbis virus (approximately 3 p.f.u./cell). The yield of virus was determined at 24 hours post-infection (Table I).

Pretreatment with the lower concentrations of excess NaCl appeared to enhance the virus yield. There is an indication that the pretreatment of cells for longer periods of time with high concentrations of excess NaCl may inhibit virus replication. No detectable interferon was present in the dialysed samples of hypertonic media.

Mouse L cells, which are more sensitive to the antiviral effects of interferon inducers, were therefore assayed for their response to treatment with hypertonic medium. The time between the initiation of treatment and infection was extended, in order to enable the cells to respond more fully to any interferon produced. The cells were incubated with excess NaCl for various times, washed, and incubated in isotonic medium. 29 hours after the commencement of the experiment, the medium from each dish was removed and stored, and the cells infected with Sindbis virus (approximately 2 p.f.u./cell). The virus yield was estimated at 24 hours post-infection by titre on L cells. The results (Table 16) indicate that the pretreatment of cells with hypertonic medium may result in a pronounced antiviral effect. The media from the treated cells were assayed for the presence of interferon; the assay system failed to detect any interferon which may have been present. The assay system is sensitive to concentrations of interferon from 10 U/ml upwards; however, the lowest dilution assayed was 1 : 2. Thus, up to 20 U/ml of interferon may be
Table 15  The Effect of Hypertonic Media on the Yield of Sindbis Virus from 3T6 Cells

<table>
<thead>
<tr>
<th>Excess NaCl (mM)</th>
<th>Length of Pretreatment (hours)</th>
<th>Virus Yields (p.f.u./ml x 10^-7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>28.5</td>
</tr>
<tr>
<td>50</td>
<td>3</td>
<td>36</td>
</tr>
<tr>
<td>50</td>
<td>6</td>
<td>40</td>
</tr>
<tr>
<td>100</td>
<td>3</td>
<td>51.5</td>
</tr>
<tr>
<td>100</td>
<td>6</td>
<td>33.5</td>
</tr>
<tr>
<td>150</td>
<td>3</td>
<td>28.5</td>
</tr>
<tr>
<td>150</td>
<td>6</td>
<td>12.5</td>
</tr>
<tr>
<td>150</td>
<td>9</td>
<td>9.0</td>
</tr>
</tbody>
</table>

The virus yields are the average of duplicate samples.
### Table 16 The Effect of Hypertonic Medium on the Yield of Sindbis Virus from L Cells

<table>
<thead>
<tr>
<th>Excess NaCl (mM)</th>
<th>Time of Treatment (hours from commencement)</th>
<th>Virus Yield (p.f.u./mL x 10^{-7})</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>5.0</td>
</tr>
<tr>
<td>100</td>
<td>6 - 24</td>
<td>0.9</td>
</tr>
<tr>
<td>100</td>
<td>0 - 6</td>
<td>0.004</td>
</tr>
</tbody>
</table>

The virus yields are the average of duplicate samples.

Note: the virus-induced cytotoxicity was less in cells treated for 6 hours with hypertonic medium than in the control cells (as estimated by microscopic examination).
present, but undetectable in the media from the treated cells.

**Summary**

Some of the predictions of the model proposed in Chapter 4 were tested. It was shown that both the initiation and elongation steps of protein synthesis may be inhibited by the treatment of L1210S cells with interferon. Tenuazonic acid, an inhibitor of elongation, mimics some of the effects of interferon, but is a poor antiviral agent compared to interferon. Hypertonic medium was assayed for its predicted ability to induce interferon synthesis; although no detectable interferon was produced, cells pretreated with this inhibitor were rendered significantly virus-resistant. The effect of various cell extracts and salt-wash fractions on L cell-free protein synthesis was investigated, but no conclusions could be drawn from these experiments.
CHAPTER 6

DISCUSSION
CHAPTER 6  DISCUSSION

This study has been concerned with the characterisation of cell mutants which have alterations in the interferon system, and their use, in conjunction with the parental cells, in the analysis of interferon action. A model of interferon action and induction was developed, and some of the predictions of the model tested. In the following discussion, the model serves as a suitable framework for the ordered evaluation of the effects of interferon and its possible modes of action.

The Induction of Interferon

It has been proposed that interferon production is controlled by two proteins, an inducer (i) and a repressor (r) (Chapter 4). It is envisaged that the inducer protein is translated from high-affinity mRNA, while the repressor protein is translated from low-affinity mRNA. Inhibition of protein synthesis initiation would result in a decrease of the ratio i : r (Chapter 4), in a manner analogous to the decrease in the ratio $\alpha$ globin : $\beta$ globin (Lodish, 1974). The decreased ratio $r : i$ then allows the transcription of the interferon gene(s). The ratio $r : i$ which allows the production of interferon mRNA may be different for different interferon genes. Thus some treatments may result in the transcription of a single gene, while a more pronounced reduction in the $r : i$ ratio could lead to the production of more than one interferon species.

The model predicts that agents which partially inhibit cellular protein synthesis at the stage of initiation will induce the synthesis of interferon. It is important to note that all events following the binding of the mRNA to the 40S subunit are treated as elongation steps.
by the model, as all these steps preclude another initiation event (Lodish, 1974; Appendix).

The two common classes of interferon inducers, double-stranded polyribonucleotides and viruses, are inhibitors of initiation (Introduction). Hypertonic medium, an inexpensive and reversible inhibitor of protein synthesis initiation, was assayed for its ability to induce the synthesis of interferon. Although interferon was not detected in the media from cells treated with this inhibitor, the pretreatment of cells with hypertonic media resulted in a pronounced anti-viral effect (Chapter 5). It is unlikely that this effect is due to the direct action of the inhibitor, as the cells were washed and incubated in isotonic medium. Indeed, the most pronounced anti-viral effect was observed in cells which had been incubated in isotonic medium for 18 hours after treatment with the inhibitor. It is suggested that this effect, like that of low concentrations of interferon inducers, is mediated by the production of interferon (Introduction). The use of a more sensitive assay for interferon, and adjustments in the conditions of treatment with hypertonic media, may result in the production of detectable interferon. Anti-interferon antibody could be used to test for the mediation of interferon in the development of the anti-viral response provoked by hypertonic media.

The production of interferon may limit its own synthesis by its interaction with the producing cell (Introduction). Thus super-induction may be understood as the prevention of the feedback mechanism. The production of maximal amounts of interferon will thus occur when this inhibitory feedback is diminished or prevented, as in interferon-insensitive cells. Other ways of increasing interferon yields could be the induction of interferon synthesis in the presence of compounds which inhibit its action, such as gangliosides or anti-interferon antibody; it would then
be necessary to devise purification techniques to separate interferon from such molecules. Under conditions in which interferon no longer limits its own production, it is possible to predict, from the model, the optimal conditions for interferon induction. These would be the treatment of cells with the concentration of inducer which most rapidly led to the transcription of the interferon gene (that is, the required low ratio $r:i$); and the subsequent maintenance of the cells in a low concentration of the inducer (or a different inhibitor of protein synthesis initiation). This treatment would keep the ratio $r:i$ low, so that transcription continues, yet will not greatly inhibit the translation of interferon mRNA.

The control of interferon is proposed to involve repressor and inducer proteins. It should thus be possible to derive mutant cells which no longer express one of these proteins. A mutation in the gene for the inducer protein might result in cells which, like Vero cells, are unable to produce interferon (Emeny and Morgan, 1979). Chinese Hamster ovary (CHO) cells, although unable to produce interferon, contain an interferon gene which may be expressed in hybrid cells (M.S. Morgan, personal communication). A mutant cell which is unable to synthesise a functional repressor protein would constitutively produce interferon; it has been indicated that 3T6 $V^B_2$ cells are constitutive for interferon synthesis (Jarvis and Colby, 1978). Thus the postulated existence of these two proteins is consistent with the cell mutants which have been described.

Interferon Action

The Membrane Interaction of Interferon

It is very likely that the interaction of interferon with a cell occurs at the membrane; this event appears to be mediated by glycolipids (Introduction). The species-specificity of interferon appears
to be determined by membrane components (Bourgeade, 1974; Samuel and Farris, 1977). The membranes of interferon-insensitive L1210R cells do not bind interferon, unlike the membranes of the parental cells (Gresser et al., 1974). Analysis of the glycolipids of these cells showed that L1210R cells lacked two of the glycolipids which are present in L1210S cells. The gangliosides GM$_1$ and GD$_{1a}$ appear to be synthesised by both cell types, while GT$_{1a}$ and another glycolipid which is possibly GD$_{1b}$ seem to be present only in the parental L1210S cells (Chapter 2). It thus appears that the synthesis of complex gangliosides is blocked between GM$_1$ and GT$_{1a}$ in the interferon-insensitive cells (Figure 7).

Treatment of the interferon-insensitive cells with a mixture of gangliosides (GM$_1$, GD$_{1a}$, GD$_{1b}$, and GT$_{1a}$) before treatment with interferon resulted in a slight decrease in virus yield and viral cytotoxic effects; in order to demonstrate this effect, it was necessary to reduce the period of interferon treatment (Chapter 2). It is likely that if glycolipid pre-treatment does indeed restore interferon-sensitivity to these cells, this effect will be shortlived. Therefore, the effect of glycolipid replacement on the cell growth inhibitory effects of interferon was studied, as this is a more immediate assay, although less sensitive. The results indicated that interferon-sensitivity may be restored to L1210R cells for a brief period by pretreatment of the cells with glycolipids (Chapter 2). It is also seen that pretreatment of L1210S cells with the glycolipid mixture inhibits the effect of interferon treatment (Table 7).

Other reports have shown that a range of gangliosides, including GM$_2$ and GT$_{1a}$, bind to interferon, and may also enhance the interferon-sensitivity of ganglioside-deficient cells. The results presented in this report suggest that GT$_{1a}$ (and possibly GD$_{1b}$) is an important factor in the response of mouse cells to interferon.
Both cholera toxin and thyrotropin can inhibit the action of interferon (Kohn et al., 1976). These two substances utilise gangliosides as membrane receptors, and act by the stimulation of adenylcyclase (Critchley and Vicker, 1977). Interferon treatment also appears to result in an increase in the level of intracellular cAMP, and an artificial increase in cAMP seems to mimic the effects of interferon on cell growth (Fuse and Kuwata, 1978). An increase in the cellular cAMP level alone does not lead to the development of the antiviral state, but such an increase may enhance the antiviral effect of treatment with interferon (Weber and Stewart, 1975).

The interaction of cholera toxin and GM₁ in artificial membrane bilayers appears to result in the formation of ion channels (Tosteson and Tosteson, 1978); this interaction does not occur between cholera toxin and GM₂. The stimulation of adenylcyclase by cholera toxin may thus be mediated by alterations in the intracellular concentrations of certain ions. Similarly, it is possible that the interaction between interferon and the cell membrane results in the alteration of the levels of some molecular species, and consequently leads to the stimulation of adenylcyclase. However, because many interferons are relatively species-specific, it would appear unlikely that glycolipids alone act as interferon receptors; it is more likely that the glycolipid stabilises the interaction between interferon and a protein receptor. It is anticipated that such an interaction would alter the concentration of some molecular species. This unknown messenger molecule may activate both the antiviral state and those effects of interferon which appear to be mediated by cAMP; alternatively, both the messenger molecule and the increased levels of cAMP which it has caused may be required for activation of the antiviral state.
The Antiviral State

The development of an antiviral state in a cell results in the inhibited replication of viruses in that cell; it appears that translation of viral mRNA is inhibited in most cases, although transcription may also be affected (Introduction). As RNA and protein synthesis are required for the development of the antiviral state, it is reasonable to assume the existence of a novel protein (or proteins) in the interferon-treated cell. Samuel and Joklik (1973) reported the detection of a novel ribosome-associated protein in interferon-treated cells; ribosomal salt-wash fractions containing this protein selectively inhibited the translation of viral mRNA in a cell-free protein-synthesising system. A ribosome-associated protein of similar molecular weight was detected in interferon-treated 3T6 cells and in two constitutively virus-resistant mutants; this protein appeared to be absent from untreated 3T6 cells (Chapter 3). One of the mutant cell lines (3T6 V^B2) has been reported to be constitutive for interferon production (Jarvis and Colby, 1978); the other mutant (A2) may be constitutive for the antiviral state (Chapter 3). Thus this novel protein appears to be present in all cells displaying an antiviral state, and absent from cells which are not in an antiviral state. It would thus appear that this protein is important, if not essential, in the development of the antiviral state.

An Evaluation of the Role of dsRNA in the Development of the Antiviral Response

Many cell-free systems from interferon-treated cells do not show an inhibition of virus protein synthesis unless the cells have been infected, or unless dsRNA is added to the system (Introduction). It was thus proposed that treatment of cells with interferon resulted in a latent antiviral state which could be triggered by dsRNA (Roberts, Clemens and Kerr, 1976). In the intact cell, dsRNA produced during viral
replication could act as the necessary stimulus. Extracts from interferon-treated L cells have been shown to produce a potent inhibitor of protein synthesis when incubated with minute amounts of dsRNA; the structure of the inhibitor has been determined (Kerr and Brown, 1978).

Extracts from interferon-treated 3T6 cells and virus-resistant cells were assayed for their ability to form the dsRNA-dependent inhibitor. These extracts did not appear to synthesise significant amounts of the inhibitor, although extracts from L cells produced large amounts of the inhibitor when treated in the same manner. Taken with the indications outlined above it was thus felt that the low molecular weight inhibitor did not play a major role in the interferon-mediated inhibition of viral replication.

The Mechanism of Interferon Action

It has been suggested that the antiviral state results in the inhibition of virus protein synthesis, and that this effect is mediated by the production of a novel protein, such as that described by Samuel and Joklik (1973), and the protein detected in virus-resistant cells (Chapter 3). It is presumed, for the purpose of this discussion, that these two proteins are identical. The protein is associated with the ribosome, indicating that it may be involved in the control of protein synthesis. It has been proposed that the mechanism of interferon action is to inhibit the high-affinity characteristics of mRNAs, which will result predominantly in the inhibition of viral protein synthesis (Chapter 4).

The initiation of protein synthesis is a complex process,
involving a large number of initiation factors, RNA species, and co-factors (Figure 31). Some of the initiation factors are messenger-discriminatory, having a higher affinity for some mRNA species than for others; in particular, eIF-4A and eIF-4B appear to preferentially select certain mRNAs (Kabat and Chappell, 1977; Golini et al., 1976). When the discriminatory factors are present in excess, no discrimination between different mRNAs will occur (Kabat and Chappell, 1977). eIF-4B appears to recognise the cap structure (\(\text{m}^7\text{G}^\text{5'p} \ldots\)) which is a feature of most mRNA species; however, some viral mRNAs lack the cap structure, yet are effectively bound to eIF-4B (Shafritz et al., 1976). The differing affinities of mRNA species for these initiation factors is presumably related to the sequence of the 5' end of the mRNA. It is possible that base-pairing between ribosomal and messenger RNAs also has a role in the selection of the messenger, perhaps in a similar way to that postulated for prokaryotic mRNA (Steitz and Jakes, 1975). The only recognisable sequence which might have a role in mRNA selection is the AUG initiator codon (Barralle and Brownlee, 1978); however, internal base-pairings may alter the mRNA so that a particular sequence or arrangement is produced. The addition of ribosomal RNA to a cell-free system has been shown to potentiate the translation of haemoglobin mRNA (Kabat, 1975); it is thus possible that ribosomal RNA acts as a former upon which the mRNAs and initiation factors are better able to interact. The possible ways in which the preferential translation of high-affinity mRNAs could be abolished are: the production of a novel protein (or other molecule) which interferes with the selective mechanism; the production of an increased amount of the discriminatory initiation factors; and a modification of these initiation factors such that they no longer recognise the high-affinity determinants of mRNAs. The novel protein
from interferon-treated cells has an apparent molecular weight of 
55,000 daltons, although according to a previous report the molecular
weight was 48,000 daltons (Samuel and Joklik, 1973). As there is a
single novel polypeptide band on the gels, the protein is unlikely to be
a subunit of a normal initiation factor (for example, eIF-2). Because
of the discrepancy in the estimated molecular weight, it is possible that
the protein represents an increased amount of eIF-4A (molecular weight
of 50,000 daltons, Schreier et al., 1977). However, as the protein is
not detected in extracts from untreated 3T6 cells, it would seem unlikely
that it is one of the normal initiation factors. Minor modifications to
an initiation factor, such as phosphorylation, would not be expected to
alter the migration of the protein upon SDS polyacrylamide gel electrophoresis.
It would seem most likely, then, that the 56,000 dalton protein is indeed
a novel protein. Such a protein could act by recognition of the high-
affinity determinants, and binding to mRNA, thus preventing the binding of
the normal initiation factors. If ribosomal RNA were involved in the
selection of mRNAs, the protein could bind to the ribosome and inhibit the
interaction of ribosomal and messenger RNAs. Attempts were made to
estimate the effect on cell-free protein synthesis of fractions containing
the 56,000 dalton protein, but the experiments were unsuccessful (Chapter 5).

Certain initiation factors have a high affinity for viral mRNAs
(Golini et al., 1976). As some RNA viruses use the mRNA species in the
replication of their genome, it is possible that these same initiation
factors are utilised in transcription. It would thus be possible for a
single protein which disturbed the association of mRNA and initiation
factors to inhibit both viral RNA and viral protein synthesis. Similarly,
a protein which bound preferentially to high-affinity mRNA, preventing
translation, could also inhibit transcription.
Further Experiments

A major advance in the study of interferon action would be the introduction of a fractionated cell-free protein synthesising system for the analysis of various components from interferon-treated cells (or constitutively virus-resistant cells). The 55,000 dalton protein from ribosomal salt-washes could be purified, and its effects on such a system studied. The role of the low molecular weight inhibitor could also be more closely defined in such a system.

Mutant cells have previously been invaluable in the investigation of the interferon system. Further studies of the mutants used in this work could yield much valuable information. In particular, the virus-resistant cells could be used, in conjunction with interferon-treated parental cells, for the investigation of effects which could be due to impurities in interferon preparations.

Another area of study would be the induction of interferon, in particular the predictions made by the model concerning inhibitors of initiation. The examination of hypertonic medium as a possible interferon inducer would take first place in this area. The model also has some possible clinical implications. The differential effect of the translation of different mRNAs under various conditions may be important during certain periods of growth and development. Thus the required amounts of the various cellular proteins may be determined in part by the affinities of their mRNAs; under different conditions, such as interferon treatment, the wrong relative amounts of the various proteins may be produced. This view is strengthened by the feedback inhibition of interferon on its own synthesis (Introduction). We should thus be wary of the possible effects of prolonged interferon therapy, particularly during periods of rapid growth and development.
**APPENDIX**

**LODISH'S MODEL**

Lodish (1974) derives rate equations for the initiation and elongation steps of protein synthesis in terms of the probability of a given codon being occupied by a ribosome (MacDonald and Gibbs, 1969). By adopting several simplifying assumptions, the equations may be solved by assuming that the rates of initiation and of elongation are equal, eliminating the probability terms, and obtaining a general equation in the form:

\[ q = R^* \frac{K_i}{K_e + L-1} \]

WHERE:
- \( q \) is the number of new polypeptide chains initiated per unit time per mRNA molecule,
- \( R^* \) represents the concentration of all factors necessary for initiation (i.e. the Met-tRNA\(_i\)-40S ribosome complex, initiation factors, etc.),
- \( K_i \) is the specific initiation rate constant for a given mRNA — that is, it directly reflects the affinity of the message for the ribosome,
- \( K_e \) is the elongation rate constant,
- \( L \) is the number of codons covered by a ribosome, taken here, as suggested by Lodish, to be 12.

The graphs presented were calculated from the above equation.
COMPUTER-SIMULATED MODEL

This is derived in a similar way to Lodish's, with the exception that the empirical movement of 'ribosomes' along the 'message' is followed, instead of translating the movement into a probability.

Each ribosome is represented by a storage location, identified by a number (1 to n). For convenience, the ribosomes associate with the mRNA in sequence, number 1 before number 2, and so on. The position of a ribosome is indicated by the number in the storage location, being 0 for any free ribosome, and between 1 and m for bound ribosomes, where m is the number of codons in the message.

A random number between 1 and n is generated, to refer to the particular ribosome with that number; if this ribosome is free (i.e., if the number stored is 0), it is passed to the initiating sequence, otherwise it is directed to the elongation sequence. In either case, the ribosome is not allowed to move unless the ribosome before it is at least 12 codons away (i.e., the stored number of the location immediately below it must be greater by at least 12).

The rates of initiation and elongation are represented by two other random number generating systems; a number between 1 and x (for initiation, where 1/x is equivalent to the probability of initiation, or a rate constant) or between 1 and y (for elongation) is generated. If the random number equals 1, the value in the storage location is increased by one; otherwise, the programme returns to choose another ribosome for consideration, in the process adding 1 to a clock which counts the number of cycles gone through, and represents time. When the ribosome reaches the end of the message, this fact is recorded on a counter, and the ribosome removed from the message. The programme calculates the rate of protein synthesis for a given $K_e$ and $K_iR^*$, and also estimates the number of ribosomes on a message. The Computer Programme is listed below.
155 PROGRAM RIB (INPUT, OUTPUT, TAPE2 = INPUT, TAPE7 = OUTPUT)
156 DIMENSION N(100), PRATE(20, 20), AKI(20, 20), AKE(29, 20)
157 DIMENSION JJ(10), G(20), H(20)
158 WRITE (7, 15)
159 15 FORMAT (45H INDEPENDENT VARIABLE GT 2.0 WILL CHANGE LINE)
160 WRITE (7, 16)
161 16 FORMAT (44H DEPENDENT VARIABLE GT 2.0 WILL CHANGE GRAPH)
162 WRITE (7, 17)
163 17 FORMAT (33H X- OR Y-AXES GT 1 WILL TERMINATE )
164 WRITE (7, 12)
165 12 FORMAT (30H IF KI 13 TO BE X-AXES WRITE 1)
166 IF (L.EQ.1) GO TO 23
167 3 FORMAT (10H)
168 4 J=J+1
169 IF (L.EQ.1) GO TO 23
170 READ (2, 3) L
171 IF (L.EQ.1) GO TO 23
172 5 FORMAT (2)  IM = IN + IZ
173 6 FORMAT (212)
174 K = IM + 10
175 DO 7 1 = 1, IM
176 7 N(I) = 0
177 JX = 0
178 JY = 0
179 R = (JZ + (50 * RANF(1))))
Ik^kR-IH

IF (N(I).LT.1) GO TO 10
T=QH+RANF(1)
IF (T.LE.1,) GO TO 11
GO TO 13
10 S=A*RANF(1)
IF (S.LE.1,) GO TO 11
GO TO 13
11 IF (IR.EQ.1) GO TO 12
LCC=IR-1
IF (N(LCC)-12.GT.N(IR)) GO TO 12
GO TO 13
12 IF (N(IR).EQ.K) GO TO 23
N(IR)=N(IR)+1
GO TO 13
23 N(IR)=N(IR)+100
JZ=JZ+1
13 JX=JX+1
IF (JX.LT.100) GO TO 6
JY=JY+1
JX=0
JP=JZ+1

IF (JP.EQ.10) PL=JP
IF (JP.EQ.10) YL=JP
IF (JP.EQ.1IN) GO TO 8
IF (JP.EQ.1IN) P=JP-PL
IF (JP.EQ.1IN) Y=JP-YL
PRATE(J,IC)=P/Y
IMP=JP+IM
10=0
DO 75 I=JP,IMP
IF (N(I).GE.1) IQ=IQ+1
75 CONTINUE
IF (JL.EQ.1) GO TO 75
WRITE (7,77) AKE(J,IC),PRATE(J,IC),IQ
77 FORMAT (2F10.5,110)
GO TO 54
76 WRITE (7,78) AKE(J,IC),PRATE(J,IC),IQ
78 FORMAT (2F10.5,110)
57 TO 59
18 STOP
END

12.42 UCLP, 22, 2.163X.LIS.
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In an attempt to clarify the mechanism(s) by which interferon acts, mutant mouse cells with possible alterations to the interferon system were studied. The cell cultures used in this work included interferon-sensitive and interferon-resistant L1210 cells, and two virus-resistant mutants of 3T6 cells, which were studied in conjunction with the parental cells.

It had previously been indicated that glycolipids were involved in the interaction between cell and interferon. The glycolipids of the parental L1210S cells and of the interferon-resistant subline (L1210R) were thus analysed and compared. It was shown that L1210R cells lacked some of the glycolipids which were present in the parental cells; furthermore, L1210R cells could be shown to respond to interferon after pre-incubation of the cells with certain glycolipid mixtures.

It had previously been proposed that a virus-resistant mutant of 3T6 cells, A2, was constitutive for the anti-viral state or for interferon production. A novel ribosome-associated protein had previously been shown to be present in interferon-treated cells, and extracts containing the protein had inhibited viral protein synthesis in vitro. A similar protein was shown to be present in two virus-resistant mutants and in the interferon-treated parental cells. This protein was not detected in untreated parental cells. The experiments presented indicate that an alteration to the interferon system is responsible for the characteristic virus-resistance of the mutants, and that an important component in the response of cells to interferon is a novel ribosome-associated protein.

A model of interferon action which proposes a selective inhibition of viral translation was constructed. An extension of this model includes proposed mechanisms of interferon induction and action. The results of experiments which were designed to test the model are presented.