SOME ASPECTS OF PICORNAVIRUS CHEMOTHERAPY

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ABSTRACT

The present state of rhinovirus chemotherapy is confused, mainly due to the fact that reports by different workers have involved the use of different techniques or the same techniques under different conditions, so that it is difficult to compare the reported activities of individual rhinovirus inhibitory compounds. The aim of this study was therefore to compare different methods for detecting and quantitating rhinovirus inhibitory activity, with respect to their ease of use, sensitivity, reliability and reproducibility. The methods used were the disc plate plaque inhibition and tube culture tests for the detection of activity and the disc plate plaque inhibition, gradient plate plaque inhibition, plaque reduction, tube culture and yield reduction tests for the quantitation of activity. The activities of 4 different compounds (Abbott 36683, SK&F 40491, SK&F 30097 and Wellcome 71-123) as determined by each of the techniques were compared.

Experimentally it was found that for the detection of activity, both the plaque inhibition and tube culture tests had advantages. The plaque inhibition test was easier, more sensitive and less time consuming than the tube culture test, but could only be applied to serotypes which would plaque consistently. The tube culture test, however, could be used with any rhinovirus serotype.

For the quantitation of rhinovirus inhibitory activity it was concluded that the tube culture technique was best used if a large number of compounds or virus serotypes were under study. However, more accurate results were obtained with the plaque reduction

and yield reduction techniques and these should therefore be used when studying a smaller number of compounds or viruses in more detail.

Before this study was carried out it was not known with any certainty whether the different rhinovirus serotypes would exhibit any variation in their sensitivity to rhinovirus inhibitory compounds, of the type which is seen in types 1 and 2 strains of herpes virus, which might cause difficulties in clinical use. The variation in sensitivity of 8 rhinovirus serotypes to each of the compounds used was therefore studied. A narrow but fairly consistent variation in sensitivity was apparent for these serotypes. Hence it appeared that sensitivity variation was not wide enough to constitute a problem in possible future therapeutic use.

Finally, the mode of action of Abbott 36683, SK&F 30097 and Wellcome 71-123 was briefly investigated.

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1.1 ANTIVIRAL CHEMOTHERAPY

General

The history of the development of the antiviral agents has been reviewed by Bauer (1972, 1973).

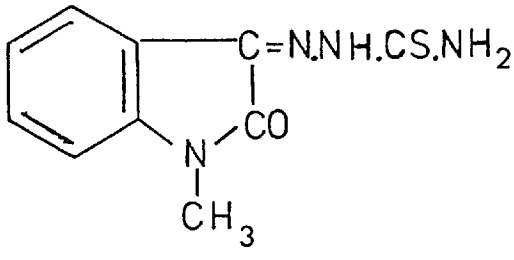
The first true antiviral agents to be found were the thiosemicarbazones. In 1950 p-aminobenzaldehyde thiosemicarbazone was found to be active against vaccinia infection in fertile eggs and in mice (Hamre, Bernstein and Donovick, 1950), and this compound was thus the first compound to be shown to possess true antiviral activity. The first compound to be shown to be effective in the prevention of a virus disease was 1-methylisatin 3-thiosemicarbazone, (Fig.1.1) found to be effective in the prevention of smallpox (Bauer et al., 1963).

The second major group of compounds found to possess antiviral activity were the benzimidazoles. In 1952 2,5-dimethylbenzimidazole (Fig.1.1) was found to inhibit the multiplication of types A and B influenza virus in the chorioallantoic membrane (Tamm, Folkers and Horsfall, 1952). However further trials among the benzimidazoles led to the important discovery of the picornavirus inhibitory compound 2-(α-hydroxybenzyl) benzimidazole (Fig.1.1). The benzimidazoles as antiviral agents will be discussed in detail later in this section.

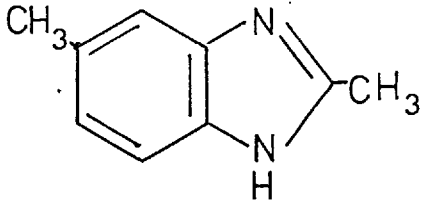
Nearly ten years after the discovery of the antiviral activity of the thiosemicarbazones 5-iodouracil was observed to have antiviral activity. Prusoff (1959) synthesized 5-iodo-2'-deoxyuridine (idoxuridine) (Fig.1.1) and it was found to have activity against vaccinia and herpes in vitro (Herrmann, 1961). Subsequently the activity of idoxuridine against experimental herpetic keratitis in rabbits (Kaufman, 1962) and against herpetic keratitis in man (Kaufman, Martola and Dohlman, 1962) was demonstrated. Idoxuridine is at present the standard treatment for herpetic keratitis.

Fig.1.1

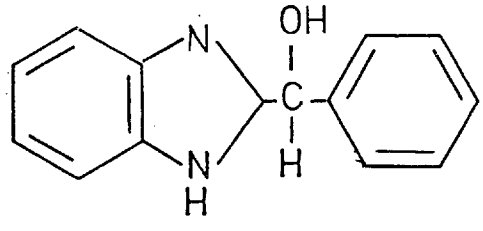
Structural formulae of the first antiviral compounds



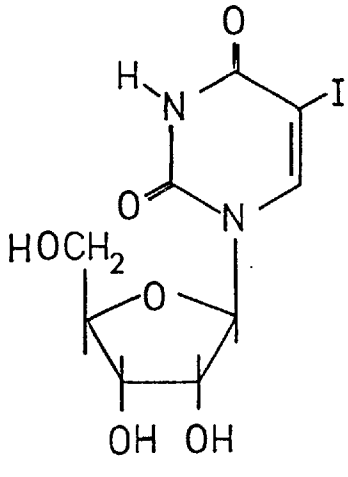
1-methylisatin-
3-thiosemicarbazone



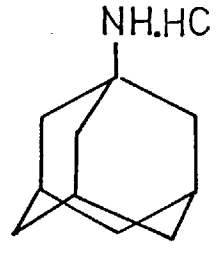
2,5-dimethylbenzimidazole



2-(α-hydroxybenzyl)-
benzimidazole (HBB)



5-iodo 2'-deoxyuridine (IDU)



1 adamantanamine-
hydrochloride

In the search for new groups of antiviral compounds amantadine (1-adamantanamine hydrochloride) (Fig.1.1) was shown to inhibit the growth of some strains of influenza and other RNA viruses in tissue culture (Davies et al., 1964; Maassab and Cochran, 1964). Amantadine will inhibit the multiplication of types A, A1 and A2 and C influenza viruses in cultures of chick embryo cells. Rhinoviruses are not inhibited by this compound. Although its activity in tissue culture is not of a very high order it has been tested widely in man. Results of in vitro and in vivo trials of amantadine made before 1967 have been reviewed (Sabin, 1967). Sabin was of the opinion that it was not highly effective and that the case for its general use in man had not then been proven. In October 1966 the U.S. Food and Drug Administration approved the antiviral compound for prescription use. It is marketed under the name of 'Symmetrel'. However, since its only use is in the prophylaxis of influenza due to A2 strains (it has no therapeutic properties) and since it has many adverse side effects, the licensing of 'Symmetrel' has been criticised.

Chemically synthesized compounds are not the only compounds to have received attention as possible antiviral agents. Interferons, a group of antiviral proteins produced by cells in response to virus infection or to some nonviral substances, confer upon cells a resistance to subsequent viral multiplication.

Interferon was discovered by Isaacs and Lindenmann (1957), but the phenomenon of viral interference can be traced back as far as 1804 when Jenner observed that herpes may prevent the development of vaccinia lesions. The discovery of interferon gave rise to the first reasonable hope of the development of a general prophylactic agent for virus infections. The possible use of interferon as an agent in the prophylaxis of upper respiratory tract infections has received much attention. Some workers feel that it has a future here, although trials so far have not been conclusive.

However, no further review of interferon will be made here since it is not a synthetic chemotherapeutic agent.

This then outlines the main groups of agents found to be effective against virus infection in vitro and/or in vivo in the early search for antiviral compounds. Although the use of some of these compounds in the prophylaxis and treatment of human infection is limited, their discovery is of major importance in establishing antiviral chemotherapy as a feasible prospect for the future. Such compounds, in particular the benzimidazoles, have also been of great importance in the study of the mechanisms of biosynthesis and of the genetics of certain viruses.

There has for a long time been a pessimistic view of the specific treatment of virus infections by drugs. One reason is that the course of virus infection is often acute and self-limiting, therefore making little time available for instituting treatment. Secondly the multiplication of the virus is dependent upon cellular biosynthetic mechanisms and hence antiviral drugs are far more likely to have toxic side effects than antibacterial agents. An additional problem is that treatment cannot be started until diagnosis is made. Upper respiratory tract infections present a formidable problem here in the wide variety of possible causative agents. Since the infection is rarely serious a diagnosis is usually not worth while unless the infection spreads in epidemic proportions. Hence a broad-spectrum antiviral agent would be required here before any real progress could be made.

However acute viral upper respiratory tract disease does appear to offer an attractive target for chemotherapy. Its economic importance is immense since its incidence in the United States alone is nearly a thousand million cases a year. It is prevalent annually, present in all age groups, easily recognised and often spreads epidemically in the community.

A wide variety of agents are associated with acute upper respiratory tract infection. They include influenza, parainfluenza, rhino, types 4, 7 and 14 adeno, Coxsackie B and type A21, types 1,3,6, 11 and 20 Echo, respiratory syncytial and corona-viruses. Thirty per cent of all upper respiratory tract infections are thought to be caused by rhinoviruses and they are the agents most frequently isolated from individuals with common colds (Hamre, 1968). Using organ cultures for virus isolation Tyrrell and Bynoe (1966) obtained agents from 19 of 33 persons with colds, and 14 of these were rhinoviruses.

Chemotherapy of the Rhinoviruses

Rhinoviruses would appear to lend themselves more to control by drugs rather than by vaccines. The large number of serotypes and the lack of epidemiologically predominant serotypes make their control by immunological methods unlikely in the foreseeable future. Results of early attempts to immunize volunteers by rhinovirus vaccine administered by either intramuscular, nasal or oral inoculation have been reviewed by Hamre (1968). The individual trials did generally show that a vaccine which provoked a high level of antibody to a rhinovirus would protect against illness due to that virus serotype. However protection is not afforded against heterologous rhinovirus serotypes. A subsequent study showed that inactivated rhinovirus 13 vaccine given intranasally induced significant protection to challenge with live homologous virus whereas vaccine administered intramuscularly did not (Perkins et al., 1969). Other workers have found parenterally administered vaccine to have only a minimal effect (Douglas and Couch, 1972). Subcutaneous administration of inactivated rhinovirus type 13 vaccine was ineffective in preventing infection and illness caused by challenge with live homologous virus but did reduce significantly virus shedding, severity of illness and duration of illness in the vaccinated persons. It is thought that the protective effect of

intranasal vaccination is associated with the development of local respiratory tract antibody (Perkins et al., 1969). Douglas and Couch (1972) believe that inactivated nasal vaccines or attenuated live vaccines may offer more promise than parenterally administered inactivated vaccine. In view of the apparent importance of nasal secretory antibody in connection with resistance to infection and illess, Buscho and co-workers (1972) investigated further the role of this nasal antibody in protection against experimental rhinovirus type 13 infection. Intranasal vaccination was found to induce resistance to challenge which lasted at least 6 months. Local neutralizing antibody which developed was specific for type 13 virus and this antibody persisted for at least 330 days following vaccination. The major effect of nasal secretory antibody appeared to be suppression of rhinovirus type 13 replication in the nasopharynx of the vaccinated persons after challenge. Therefore it does seem that successful vaccination of persons against rhinoviruses is a possibility, but a very small one, in view of the large numbers of serotypes and the lack of epidemiologically predominant serotypes.

Administration of viral antigen by intranasal inoculation has been demonstrated to be of value. It would seem that application of drug by this route might also prove to be effective. Intranasal administration of drug could be advantageous in that lower doses of drug would be required than for systemic use with fewer resulting problems of toxicity and drug metabolism.

A detailed description of those drugs which have received attention as picornavirus-inhibiting compounds (especially against rhinoviruses) will be given in a later section (1.7). Of prime importance are the triazinoindoles (a group of compounds related to the thiosemicarbazones), and the benzimidazoles.

1.2.1 RHINOVIRUSES - History, isolation and cultivation

Initial research into the common cold was hampered by the difficulty in isolation of the aetiologic agent. Sixty years ago it was suggested that the causative agent for the common cold might be a virus, since it was found to be filterable (Kruse, 1914). However, by 1930 work had progressed little further when similar experiments were performed and Kruse's results were confirmed (Shibley et al., 1930; Long et al., 1931). Shibley and co-workers had already studied the bacterial flora of the nose and throat during colds and could find no bacteria to which an aetiological role could be attributed (Shibley et al., 1926). However, even as late as the 1940's certain workers still believed that pathogenic bacteria were the main cause of colds (Brown, 1945).

Research work was hampered by the lack of an experimental animal in which to study the cold. Colds were first produced experimentally in chimpanzees in 1923 by Olitsky and McCartney but their work was not repeated again until 1929 (Dochez et al., 1929, 1930). The clinical picture of a cold in chimpanzees was found by these workers to be strikingly similar to that in man (Dochez et al., 1930). This in fact casts doubt as to whether the infecting agent was indeed a rhinovirus, since rhinoviruses are now found to produce an asymptomatic infection in chimpanzees. Ferrets were also found to be susceptible to common cold virus (Noble and Brainard, 1935). However, to study the nature of the agent more fully a suitable tissue culture system in which to grow it was required.

The first reports of the cultivation of common cold virus in tissue culture were from Dochez and co-workers (Dochez et al., 1931, 1936). The virus agent was found capable of multiplying in cultures of chick embryo tissue in Tyrodes solution, and the medium from such cultures was capable of infecting humans although the results were irregular.

The growth of the virus in the chorioallantoic cavity of eggs was also reported (Kneeland et al., 1936; Ward and Procter, 1950; Pollard and Caplovitz, 1947) Infected allantoic fluids were found to contain virus particles which did not agglutinate chick red blood cells and which under the electron microscope were found to be unlike influenza virus particles although of similar size (Topping and Atlas, 1947). It must be assumed, however, that in these first attempts at the cultivation of the common cold virus in tissue culture viruses other than rhinoviruses were involved, since the latter will grow only in tissue of human or simian origin. It is well known that viruses other than those of the rhinovirus group will cause symptoms typical of a common cold.

Tissue culture

The first successful attempt at propagating a rhinovirus in tissue culture was not made until 1953 (Andrewes et al., 1953). This was the DC strain, later identified as a strain of rhinovirus type 9 (Conant et al., 1968b). The virus was passaged serially in human embryonic lung cultures and growth was detected by inoculating culture fluids into human volunteers. Later other agents, JH and 2060, originally classified as echovirus 28 (Pelon, 1961; Hamre and Procknow, 1961) were isolated (Price, 1956; Pelon et al., 1957; Price et al., 1959) by inoculating specimens from patients with typical common colds into monkey kidney cell cultures. JH and 2060 are now typed as strains of rhinovirus 1A (Kapikian et al., 1967)

The isolation and propagation of viruses from common colds became easier when it was shown by Tyrrell and Parsons (1960) that these viruses would grow and produce cytopathic effects in cultures of human embryonic kidney cells when they were maintained at 33°, rather than 36°, with a lower bicarbonate concentration and therefore a lower pH than was usually employed (Tyrrell and Bynoe, 1961; Johnson and Rosen, 1963; Ketler et al., 1962; Taylor-Robinson and Tyrrell, 1962a; Connelly and Hamre, 1964; Webb et al., 1964).

The cytopathic effects produced by the growth of rhinoviruses in monkey kidney (MK) or human embryonic kidney cells (HEK) are the same as those produced by enteroviruses, including poliovirus.

Semi-continuous lines of human embryonic lung fibroblasts were then developed (Hayflick and Moorhead, 1961) which would also support the growth of rhinoviruses. Early attempts to grow rhinoviruses in HeLa and similar transformed cell lines were unsuccessful (Tyrrell et al., 1960; Hamparian, Ketler and Hilleman, 1961). Later, however, certain rhinoviruses were adapted to these cells (Mogabgab, 1962; Ketler, Hamparian and Hilleman, 1962; Johnson and Rosen, 1963; Taylor-Robinson et al., 1962), and now adapted strains can be grown in HeLa, L132, KB and HEp-2 cultures, of which HeLa is the preferred line (Mogabgab, 1962; Conant and Hamparian, 1968; Votava and Tyrrell, 1970). Adapted strains of rhinoviruses can also be grown in human embryonic liver, intestine, skin and full term amnion diploid cell lines but these are obviously of little use in routine laboratory work (Jackson and Muldoon, 1973). Human aorta cells and gingival cells have also been used to replicate rhinoviruses (Behbehani et al., 1965; Philips et al., 1965a; Fletcher and Platt, 1968).

The semi-continuous cell lines of human embryonic lung, mainly Wistar-26 and Wistar-38 cells (WI-26 and WI-38) and kidney fibroblasts (Stott and Walker, 1967) are probably the most sensitive cells for the primary isolation of rhinoviruses (Hayflick and Moorhead, 1961; Hamparian, Ketler and Hilleman, 1961; Brown et al., 1963; Phillips et al., 1965a; Stott and Walker, 1967) and the pH of the medium in these cells is not so critical as in primary cell cultures (Johnson and Rosen, 1963).

Stott and Tyrrell (1968) observed cytopathic effects in rhinovirus sensitive HeLa cells infected with 70 different rhinovirus strains (including at least 30 different serotypes) which had not been adapted to HeLa cells. However, they still maintained that semi-continuous strains of human diploid cells should be used in primary isolation attempts, but

once a cytopathic effect has been seen further study of the virus can be carried out in HeLa cells.

Most rhinoviruses when propagated in stationary monolayers of human diploid cells or monkey kidney cells reach titres ranging from $10^{3.0}$ to $10^{5.0}$ $TCID_{50}$/ml, with a very few titres of $10^{6.0}$ $TCID_{50}$/ml being obtained (Holper et al., 1963; Johnson and Rosen, 1963; Connelly and Hamre, 1964; Webb et al., 1964; Douglas et al., 1966a; Kapikian et al., 1966; Mascoli et al., 1966). Higher titres of up to $10^{7.0}$ $TCID_{50}$/ml were obtained by Fenters and co-workers (1967) using WI-38 cell monolayers in rolling bottles. Rhinovirus cultures are now found to give consistently high titres when grown in such rolled cultures and these are therefore routinely used for the production of virus stocks. However, higher titres are generally obtained in HeLa cells. In normal laboratory work to obtain titres of about $10^{6.0}$ to $10^{8.0}$ $TCID_{50}$/ml., rhinoviruses may be propagated in roller cultures of HeLa cells (or the cell line to which the strain is adapted) at 33°C, in the presence of a low bicarbonate concentration (not greater than 0.35 g/litre), 30 mM $MgCl_2$ and a low serum concentration 1 - 2%). It appears that the rolling of cultures at 33°C produces conditions similar to those in the nasal epithelium, which are optimal for rhinovirus growth. In the first single-cycle growth experiments of rhinoviruses it was found that high concentrations of $MgCl_2$ (30 mM) enhanced adsorption and increased virus release (Fiala and Kenny, 1967).

Further attempts however were made to obtain higher yields for physicochemical and immunological studies. Cell suspension cultures are found to be the best for precise one step growth cycle studies and give very high rhinovirus titres. Although Rosenthal (1965) was unable to propagate rhinovirus 2 in HEp-2 cell suspension cultures, Stott and Tyrrell (1968) were able to culture rhinovirus 2 in spinner cultures of HeLa cells. Suspension cultures of L 132 cells were used to study factors affecting the growth of rhinovirus 2 (Stott and Heath, 1970), and

Thomas and co-workers (1970) used HeLa cell suspension cultures to propagate rhinoviruses with titres of up to $10^{9.0}$ $TCID_{50}$/ml resulting. They also used this system for the study of rhinovirus replication.

More detailed reports on some aspects of the isolation and cultivation of rhinoviruses can be found in reviews by Tyrrell (1968), Stott and Killington (1973) and Jackson and Muldoon (1973).

Organ Cultures

Organ cultures of various embryonic tissues have proved advantageous for the isolation of fastidious rhinoviruses (Hoorn and Tyrrell, 1966; Tyrrell and Bynoe, 1965; Almeida and Tyrrell, 1967). Embryonic nasal epithelium, trachea, oesophagus and adult nasal epithelium have been employed, and virus was found to be produced to the highest titre in nasal and tracheal epithelium. Organ cultures greatly enhance the isolation rate of rhinoviruses (Votava and Tyrrell, 1970; Tyrrell and Bynoe, 1965; Tyrrell et al., 1968; Higgins, 1966). Incubation of organ cultures on rotating plates was found to increase viral replication (Tyrrell and Blamire, 1967) but not virus yield. (However, the rotating procedure was in some way detrimental to the cells.) The observable cytopathic effect obtained in virus infected organ cultures is a reduction in ciliary activity. A few days after virus replication has reached a peak ciliary activity is reduced and the indication of virus replication generally used is the cessation of ciliary activity.

Scanning electron microscope observations of rhinovirus infected ciliated epithelium has revealed the extrusion of degenerating ciliated and non-ciliated cells from the surface (Reed and Boyde, 1972). The denuded epithelial surface remaining is smooth. The cells liberated into the medium show a pyknotic nucleus and cytoplasmic vacuoles but no inclusion bodies (Tyrrell, 1968).

Rhinovirus type 2 (but not type 3) has been grown in cultures of human fallopian tubes but ciliary activity is not reduced.

A technique for the demonstration of cell viability and cell injury on the respiratory surface of trachea explants has been developed (Herbst-Laier, 1970a). This technique is far more sensitive than purely microscopic observations to deduce cell viability and it may therefore be applied to the evaluation of antiviral drugs (Herbst-Laier, 1970b).

Animal hosts

Apart from man, natural infection by human rhinoviruses has been reported only in the chimpanzee (Dick and Dick, 1968). Experimentally born gibbons and chimpanzees have been infected (Haff, 1969; Dick, 1968; Pinto and Haff, 1969; Boyle et al., 1970; Pinto et al., 1972). Gibbons have been found to have two advantages as a test system in that they are easier to handle and less expensive than chimpanzees (Haff, 1969). Neutralising antibodies and virus are produced during the course of infection although symptoms are absent. Attempts to infect small laboratory animals and chick embryos with human rhinoviruses have failed (Hamparian et al., 1961; Johnson and Rosen, 1963; Phillips et al., 1970).

1.2.2 POLIOVIRUSES - History, isolation and cultivation

The aetiologic agent of poliomyelitis was first shown to be a virus in 1909 when Landsteiner and Popper (1909) transmitted the disease to monkeys by intracerebral inoculation of a bacteria-free filtrate of spinal cord from a human patient. However, monkeys are both expensive and cumbersome for experimentation and the absence of an easier system in which to study the virus hampered research in this field. It was not until 1939 that poliovirus was adapted to another animal, the cotton rat (Armstrong, 1939), and ten years later it was discovered that the virus could be isolated and propagated in cultures of non-neural human or monkey tissue (Enders et al., 1949) which made extensive study of the disease possible. Although polioviruses were initially found difficult to work with, they now serve as a model for the investigation of many other animal viruses. The growth of polioviruses in tissue culture has been reviewed

by Plotkin and co-workers (1962). The tissue cultures now used routinely for the isolation, identification and propagation of polioviruses include primary rhesus monkey kidney, African green monkey kidney, rabbit kidney and human amnion cultures; semi-continuous diploid cell lines such as Wistar 26 and Wistar 38 and continuous heteroploid cell lines such as HeLa, KB, Vero and HEp-2. Hence cell lines derived from primates are generally susceptible to infection with intact poliovirus since they possess specific receptors for the virus. Non-primate cells lack these receptors and are therefore normally resistant to infection. However, by incubating infectious RNA rather than the whole virus with the cells, normally insusceptible cells may be infected (Holland et al., 1959; Prinzie et al., 1960). One cycle of replication only will take place in these cells since the complete virus produced cannot infect new cells.

1.3 CLASSIFICATION AND NOMENCLATURE

The rhinoviruses and polioviruses are members of the family Picornaviridae. Table 1.1 shows the position of these viruses within the group as decided by the Vertebrate Virus Subcommittee of the International Committee on Nomenclature of Viruses (Wildy, 1971). In this, the most recent classification, the rhinoviruses constitute a separate genus of the virus family whilst polioviruses are species of the genus Enterovirus.

Table 1.1 Classification of Picornavirus Group

Taxonomic Status	Current Name	Main Characteristics
Family	Picornavirus Group	Contain single-stranded RNA of molecular weight 2.5×10^6 daltons. The RNA makes up 30% of particle mass. Capsid has cubic symmetry, is naked and has overall diameter of 20 - 40 nm. Ether resistant. Virus synthesis and maturation is in the cytoplasm with the occasional formation of crystalline arrays of particles
Genus	Enterovirus (type-genus)	
	Calicivirus	
	Rhinovirus	

A list of the criteria upon which admission to the enterovirus genus is based has been made (Wenner and Behbehani, 1968).

The picornaviruses were so named by an International Enterovirus Study Group (1963), and members of this family share four basic properties. The nucleic acid of the core is ribonucleic acid (RNA) and the virus particles are ether stable, therefore indicating the absence of an outer envelope. The hexagonal shape of the virion suggests cubical symmetry of the icosahedral type (although this has not been unequivocally determined for all members of the group), and the virion diameter is between 15 and 40 nm. The derivation of the name picorna is based on these properties: pico indicating very small viruses and RNA indicating the type of nucleic acid in the genome. Alternatively the initial letters may be taken to refer to the polioviruses, insensitivity to ether, Coxsackie viruses, orphan viruses (later named echoviruses) and rhinoviruses.

Within the Picornavirus group the distinction between enteroviruses and rhinoviruses is fairly clear, when based upon physical, chemical and biological properties. However, within the enteroviruses distinction between subgroups is not at all clear (e.g. between the echoviruses and Coxsackie viruses), since it is based largely on the host range of the virus and these are not clear cut in every case. Some workers therefore held the view that because of the fuzzy boundary lines between accepted subgroups of the then Enterovirus family, they should be classified on an antigenic basis in a single numerical system and not be put into subgroups (Committee on Enteroviruses, 1962; Rosen, 1965). Others disagreed with this (International Enterovirus Study Group, 1963; Virus Subcommittee of the International Nomenclature Committee, 1963), and subgrouping was finally decided upon.

Rhinoviruses are kept separate from the enteroviruses on the basis of certain special characteristics. The first is that rhinoviruses are

labile below pH 5 (Ketler et al., 1962; Dimmock and Tyrrell, 1962; Dimmock and Tyrrell, 1964), a characteristic which they share in common only with the feline picornavirus (Bürki, 1965), vesicular exanthema virus (Wawrzkiewicz et al., 1968) and foot-and-mouth disease virus (Plummer, 1965). The former two viruses are also placed separately from the enteroviruses in the genus Calicivirus, and foot-and-mouth disease virus is now placed in the rhinovirus group. Complete or almost complete inactivation of rhinoviruses occurs at pH 3.0, whereas enteroviruses are stable even at pH 2.0 (Robinson, 1950; Loring and Schwerdt, 1944; Bachrach and Schwerdt, 1952). For this reason rhinoviruses are usually inactivated on passage through the gut and are rarely found in the faeces (Tyrrell and Bynoe, 1961; Mufson et al., 1966b), in contrast to enteroviruses which are primarily inhabitants of the intestine (although they may also be found in the nasopharynx). Coxsackie virus A21 can be recovered from faeces of patients and volunteers although it seems to grow best in the respiratory tract (Johnson et al., 1962).

Secondly, rhinoviruses cause upper respiratory tract infections, colds, by infecting and multiplying in the nose, again in contrast to the enteric infections caused by enteroviruses. Other picornaviruses can cause infections similar to colds. Coxsackie A21 virus (commonly called Coe virus) produces symptoms typical of a cold but unlike rhinoviruses is associated with outbreaks in closely knit communities. Certain echoviruses, notably types 1, 3, 6, 11 and 20, have also been found to cause respiratory symptoms but less typical of a cold (Matsumoto, 1963). As has already been described rhinoviruses grow best in vitro at a temperature of about 33° with a plentiful supply of oxygen and in slightly acid medium, thus reflecting their natural environment in vivo.

Rhinoviruses (and Coxsackie A viruses) also differ in general from enteroviruses in their resistance to inhibition by 2-(α-hydroxybenzyl) benzimidazole (HBB), (Eggers and Tamm, 1961a,b, 1962). Half the first 55

rhinovirus serotypes are resistant to inhibition by HBB (Gwaltney, 1968), whereas most enteroviruses are susceptible. However, since this characteristic is variable within the rhinoviruses it is of little value in classification. The hydrochloride of HBB, (D-(-)-2-(α-hydroxybenzyl) benzimidazole HCl, HBB-HCl) is on the other hand found to inhibit all strains of rhinoviruses, except type 1A and subtype 1B, at a concentration of 574μM or less.

Other characteristics also serve to distinguish rhinoviruses from enteroviruses. The buoyant density of rhinoviruses (in caesium chloride) is generally higher than that of enteroviruses, 1.38 - 1.41 g/cm^3 compared with 1.34 - 1.35 g/cm^3. Rhinoviruses are, in addition, unique among the picornaviruses in having an extremely high adenine content (26 - 34% of the total nucleotide base content).

It can therefore be seen that the separation of rhinoviruses into a single subgroup is a reasonable and useful division. However, Newman and co-workers (1973) disagree with the present subgrouping of the picornaviridae into three subgroups, and present data to support the desirability for a division of the family into at least six subgroups. These authors find that on the basis of buoyant density in CsCl five subgroups are distinct (See Table 1.2), namely those with densities of 1.34, 1.37, 1.40, 1.43 and 1.45 g/ml. Then within the group of density 1.34 g/ml a distinct difference in base composition shown between entero- and cardioviruses suggests the need for a sixth subgroup. Both foot-and-mouth disease and equine rhinovirus differ sufficiently from human rhinovirus in base composition to warrant their position as separate subgroups. Additionally in the calicivirus group containing vesicular exanthema virus and feline picornavirus analyses showed the base compositions of the two viruses to be different although only one strain of feline picornavirus was analysed. The authors also report and discuss data of other physico-chemical analyses of

Table 1.2 Buoyant density in caesium chloride of picornaviruses of mammals

Virus	Range of density reported (g/ml)	Average density (g/ml)
Polio	1.34	1.34
Echo	1.33 to 1.35	1.34
Coxsackie	1.33 to 1.34	1.34
Bovine entero	1.33 to 1.34	1.34
Porcine entero	1.34	1.34
Teschen	1.35	1.35
Encephalomyocarditis	1.33 to 1.34	1.34
Maus-Elberfeld	1.33 to 1.34	1.34
Vesicular exanthema	1.36 to 1.38	1.37
Feline picorna	1.36 to 1.39	1.37
Human rhino	1.38 to 1.41	1.40
Foot-and-mouth disease	1.42 to 1.51	1.43
Equine rhino	1.45	1.45

from Newman *et al.*, 1973

picornaviruses to support their proposed division of picornaviridae.

Within the Rhinovirus subgroup distinction has been made between H and M strains. H strains (the majority) are those which will grow only in human kidney cells, and M strains are those which will in addition grow equally well in monkey kidney cells if the pH is kept low (Tyrrell and Parsons, 1960; Tyrrell and Bynoe, 1966). However, this property can now be overcome by adaptation and M and H strains may also exist within a single serotype (Philips et al., 1965b; Stott, 1967). Further differentiation into O strains, those which can only be cultivated in organ culture of human respiratory epithelium, has also been suggested, but again this property may be overcome by adaptation.

1.4 RHINOVIRUSES - Pathogenesis, immunity and antigenicity

The pathogenesis of rhinovirus infection has been reviewed by Tyrrell (1968). Here it will briefly be mentioned that rhinoviruses are primarily inhabitants of the respiratory tract. Virus infection is initiated in nature by attachment of virus particles to cells of the nasal epithelium. Experimentally virus may be administered to volunteers intranasally or *via* the conjunctival route (Bynoe *et al.*, 1961). In susceptible subjects virus can be detected in the nose and throat on the second day after infection. The first symptoms also appear at about this time, and maximum concentration of the virus in nasal secretion is usually found when a profuse nasal discharge develops (Tyrrell, 1963a; Douglas *et al.*, 1966b). The concentration of virus then rapidly declines but may remain at a low level for some time.

Infections caused by rhinoviruses constitute 30% of all upper respiratory tract infections. The frequency of incidence of colds caused by rhinoviruses is due largely to the existence of a very large number of serotypes. New rhinovirus serotypes are being isolated continuously. In 1967 55 numbered rhinovirus serotypes were known to exist (Kapikian *et al.*, 1967); in 1971, 89 serotypes (Kapikian *et al.*, 1971) and in 1972 this number had risen to 105 (Douglas and Couch, 1972). Complete immunity to colds can therefore rarely be achieved in a lifetime, although acquired immunity to a single serotype may itself be lifelong.

Very early in the work on the common cold it was found that specific immunity to colds existed, which is mediated by antibody (Jackson *et al.*, 1958; Jackson and Dowling, 1959; Tyrrell *et al.*, 1960). Later Tyrrell and Bynoe (1961) obtained evidence that different agents causing colds would induce production of specific neutralizing antibody. It has been found that the severity of a cold depends on the level of specific antibody that the subject already has, in that the degree of infection and severity of

symptoms increases as the antibody titre decreases (Bynoe et al., 1961; Mufson et al., 1963; Cate et al., 1964)

Antigenicity in the rhinovirus group has been reviewed fully by Stott and Killington (1973), and will therefore not be dwelt on in great detail here. Antigenic drift and change do not seem to occur in the rhinoviruses in nature (Stott and Walker, 1969). It is not known if passaging in man can produce antigenic change but it seems likely that passage in tissue culture in the presence of antibody can produce such a change with some serotypes (Acornley et al., 1968).

In vitro rhinovirus infectivity may be neutralized by specific antisera. This reaction, which is the basis of classification within the rhinovirus group, may be measured by the reduction of cytopathic effects in tissue culture (Taylor-Robinson et al., 1963; Conant and Hamparian, 1968; Stott and Tyrrell, 1968; Gwaltney, 1966; Kriel et al., 1969) or by macroplaque (Fiala, 1969) or microplaque (Taylor-Robinson and Tyrrell, 1962b) reduction. Combined specific antisera in a series of pools have been used to identify unknown rhinoviruses (Stott and Tyrrell, 1968; Gwaltney, 1966; Lim, 1960; Kenny et al., 1970). Recently the use of specific antiserum in the immune electron microscopy of rhinoviruses has been found to facilitate clear observation of particles and also to show new morphological features (Kapikian et al., 1972).

Within the rhinovirus group types 32 and 9 cross-react (the former may be a sub-type of the latter) (Cooney and Kenny, 1970), and some strains of sub-type 1A cross-react with sub-type 1B strains in reciprocal neutralization tests. However, in neutralization tests no group antigen has been found for the rhinovirus group.

In contrast to this most human sera are found to be positive when tested against type 2 (HGP) virus by the complement-fixation test, although such sera have no neutralizing antibody (Chapple et al., 1967). It appears therefore that in addition to producing a strain-specific antigen the

HGP strain of rhinovirus produces a broadly reacting antigen, antibody to which may be induced by another rhinovirus.

Until recently a haemagglutinin from rhinoviruses had not been found although Stott and Killington (1973) reported that over 100 strains of at least 40 different serotypes had been tested with erythrocytes from human group 'O' adult and cord blood, guineapigs, rats, chickens (both vaccinia-agglutinable and non-agglutinable) and rhesus monkeys at 4°C, room temperature and 36°C (Hamparian et al., 1961; Johnson and Rosen, 1963; Stott, 1967; Mufson et al., 1965).

However, rhinovirus types 3, 4, 5 and 6 have now been shown to agglutinate sheep erythrocytes at 4°C (Stott and Killington, 1972).

1.5 METHODS USED TO DETECT REPLICATION OF AND TO TITRATE RHINOVIRUSES

A test for antiviral activity must depend on a good system in which virus replication can be detected. Such methods are usually based on a visual effect of virus replication or on some other effect which can be detected by some other means. Methods most commonly used to detect rhinovirus replication depend on the appearance of cytopathic effects (CPE) in cell cultures, or on plaque formation.

1.5.1 Detection of CPE in Tissue Culture; Infectivity Titrations in Tube Cultures

The cytopathic effects produced by rhinoviruses, first reported by Tyrrell and Parsons (1960), have been described by many workers, and these reports have been summarized (Jackson and Muldoon, 1973). In cultures of human or monkey diploid cell strains the cytopathic effect has been described (Tyrrell and Parsons, 1960). The first observable cytopathic effect is the appearance of foci of degenerating cells, 2 to 7 days after inoculation of virus. The foci consist of a few cells in which the cytoplasm becomes refractile; cells draw away from surrounding cells and become irregular in outline, later becoming rounded and finally falling off the glass. "The changes therefore resemble those of a very slowly progressive cytopathic effect produced by poliovirus" (Tyrrell and Parsons, 1960).

Titration of rhinoviruses may therefore be carried out by the end-point dilution method. A series of replicate tissue cultures are inoculated with serial dilutions (usually 10 fold or 3.2 fold) of virus stock, and after incubation at 33°C in a roller drum such cultures are observed for cytopathic effects. The titre of the virus may then be calculated from the number of cultures exhibiting cytopathic effect, using the methods described by Kärber (1931) or Reed and Muench (1938).

1.5.2 Plaque Assay

The plaque assay of viruses is a more precise method than the tube dilution method of measuring the titre of a virus sample. A plaque assay for viruses was first described by Dulbecco (1952).

1.5.2.(a) Microplaques

The first plaque assay technique described for rhinoviruses was a microplaque assay (Parsons and Tyrrell, 1961) carried out in roller tube cell cultures. Parsons and Tyrrell took advantage of the fact that the first stage in rhinovirus induced CPE in diploid cell strains is the formation of degenerative foci, which can readily be observed at a magnification of 30 - 40X. It was these foci, or microplaques, which were therefore counted in order to estimate the virus titre. The authors observed countable plaques with two strains of virus (P.K and H.G.P), and they found that the uniformity of monkey kidney cell monolayers made these cells very suitable for plaque assay. The numbers of foci counted by two observers usually agreed quite closely, but Parsons and Tyrrell (1961) reported that difficulties arose at times because of the lack of experience of the observer in searching the cell sheet systematically. The number of plaques observed after three days incubation was proportional to the concentration of virus inoculated and the pH of the medium affected the plaque count, being optimal at pH 7.0 to 7.4. However, in conclusion the authors did note certain disadvantages of this test system. The first is that the end-point of the test changes with time since virus released from cells infected initially will infect further cells, giving rise to secondary foci. The tubes may often be unreadable because of the plaques becoming confluent. Secondly, the authors state that the results may be unsatisfactory due to ill adapted or uneven batches of tissue culture. Additionally the technique is tedious since it involves examination of the

entire cell sheet of a number of replicate tubes for every specimen being tested.

1.5.2.(b) Macroplaques

The problem of the production of secondary plaques obtained with the microplaque technique just described may be overcome by the use of a solid or semi-solid overlay. Parsons and Tyrrell (1961) were unsuccessful in their attempts to produce plaques under agar, but this was later achieved by Porterfield (1962) with HGP virus (rhinovirus type 2) using monkey kidney cell monolayers. However, Porterfield (1962) reported that different batches of monkey kidney cells varied considerably in their sensitivity, and even in single batches of cells there was no constant relationship between virus dose and plaque counts.

Attempts to obtain plaques with the HGP, B632 and Norman strains in cultures of human embryonic lung cells were therefore made and were successful. With virus passaged in monkey kidney cells clearly defined plaques of up to 3mm in diameter were obtained after twelve days incubation. Virus which had been passaged once in human embryonic lung fibroblasts produced well developed plaques at 48 hours, which increased in size and number upon further incubation. Porterfield also noted that it was essential to use densely packed monolayers since otherwise areas of glass not covered by cells might be mistaken for plaques.

Later successively more serotypes were found to plaque in diploid cells. Kisch et al.,(1964) obtained plaques with strains 353, 1059, 1734, 11757 and 33342 of rhinovirus in WI-26 cells using an overlay medium containing nutrient agar. Plaques, which were produced between 2 to 6 days, were of varying sizes depending on the virus serotype. These workers found the titre of virus lower when assayed by this method than when assayed in roller tubes. They also found that in all instances sodium dextran sulphate (NaDS) and DEAE dextran increased plaque sizes.

The plaque enhancing effect of DEAE dextran (25µg/ml) was also reported by Abo-Ahmed et al., (1967), who obtained plaques with 16 strains of rhinovirus and coryzavirus in WI-38 cells using an overlay containing Ionagar. DEAE dextran was found to increase the plaque size of small plaque formers but had no effect on the plaque size of other virus strains. In no case did DEAE dextran have an effect on plaque number.

A plaquing system for rhinoviruses was subsequently described which employed methylcellulose in the overlay (Dolan et al., 1968). The use of methylcellulose avoids the inhibitory effects of agar and the authors reported that the plaques formed by this method were far more distinct than those produced under an agar overlay. The plaque sizes and counts of types 1A and 13 rhinovirus were found to be equal when tested at 26°, 31° and 36° and were not affected by varying pH from 6.9 to 7.5. Magnesium chloride (30mM) was found to increase plaque counts 2 to 4 times when using a methylcellulose overlay although plaque size was not affected. Under an agar overlay plaque counts were increased far more significantly (20-fold) and plaque size was also increased by the addition of magnesium chloride (30mM). Similar titres were obtained with methlycellulose without magnesium chloride or agar with magnesium chloride.

Rhinovirus plaques were first obtained in heteroploid cells by Fiala and Kenny (1966). These workers tested a great number of heteroploid cell lines and initially found Calf-HeLa cells to be optimal for plaque formation. Later they tested the WISH and M-HeLa cell lines and found these more suitable for plaquing. Of 12 virus strains tested, all but 4 gave reproducible plaques of 2 to 4mm diameter in M-HeLa cells. Later Conant et al., (1968) and Fiala (1968) were able to plaque the first 55 rhinovirus serotypes in M-HeLa cells. Fiala (1968) reported plaques of up to 7mm in diameter.

The rhinovirus plaquing system employing HeLa cells is a more reproducible system than that using fibroblast cells. The macroplaques produced are much more clearly defined than those produced in the thin fibroblast monolayers and there is seldom a need for magnification. Hence counting plaques does not require experience (Fiala, 1968).

Fiala and Kenny (1966) found that plaques could be obtained with methylcellulose, ionagar (0.4%), agarose (0.3%), or agar in the overlay although with the latter DEAE dextran (100µg/ml) was required and plaque sizes with a methylcellulose overlay were smaller than with other overlays. DEAE dextran was also found to potentiate plaque size when agarose was used. Conant et al., (1968) found protamine sulphate (50µg/ml) to have a similar plaque enhancing effect.

Magnesium chloride (30 to 40mM) was found to potentiate the plaque size of all rhinoviruses which produced plaques (Fiala and Kenny, 1966) although Conant et al., (1968) found that plaques produced by 18 small plaquing strains were not affected by such a concentration of magnesium chloride when under a starch gel overlay and 10 of these were also not affected when under an agar overlay. The remaining strains produced larger plaques in the presence of 30mM magnesium chloride.

The potentiating effects of DEAE dextran were found initially to be additive (Fiala and Kenny, 1966). However, Fiala (1968) reported later that DEAE dextran alone had a greater enhancing effect on plaque formation by types 14 and 16 rhinoviruses than DEAE dextran and magnesium chloride.

Additionally Fiala (1968) found that plaque formation by rhinovirus types 4, 5, 7, 20, 48 and 53 could be enhanced by the treatment of cell monolayers with actinomycin D (0.05 to 0.1 µg/ml) for 30 minutes before infection.

Fiala and Kenny (1966) and Conant et al., (1968) studied the effect

of the age of the cell monolayers on plaque formation, with conflicting results. Fiala and Kenny found that the sensitivity of monolayers to virus declined rapidly following the second day after seeding. Also plaque sizes generally exhibited an inverse relationship with the number of cells initially seeded per dish. Conant and co-workers, however, infected cell monolayers from the 2nd to the 6th day after seeding and found that the age and density of the cell monolayer had no affect on plaque size.

Conflicting results were also obtained by these two groups of workers in their studies on the effect of the volume of the virus inoculum on the plaque count. Fiala and Kenny reported an inverse relationship between inoculum volume (from 0.1 to 1.0ml) and the number of plaques but Conant and co-workers found the same change in inoculum volume to have no effect on the plaque number.

Both groups of workers found that the plaque assay in M-HeLa cells was more sensitive than the tube assay. Fiala and Kenny found virus titres to be 6 to 7 times greater by the plaque assay although Conant and co-workers found the pfu:$TCID_{50}$ ratio to be an average of 1.03 for the many serotypes tested.

Fiala and Kenny reported that virus stocks propagated in cell cultures other than M-HeLa would produce plaques in monolayers of these cells.

The general method now used to produce rhinovirus macroplaques should be described here. The use of diploid cell lines has many disadvantages, including the long doubling time of the cells and the relative difficulty in producing plaques in these cells as compared with plaque formation in HeLa cells. HeLa cells are therefore now most commonly used for plaquing. Confluent monolayers of susceptible cells contained in plastic tissue culture dishes are infected with a virus inoculum sufficient to give the required plaque count. Virus is adsorbed at 33°C for about one hour, and

the inoculum then replaced with a suitable volume of maintenance medium containing the chosen solidifying agent (methyl cellulose, Ionagar, Noble agar or some purified agar such as agarose). The cultures are then incubated at 33°C to allow plaques to develop. This may take from 2 to 7 days depending on the cell line and the virus strain used. Plaques may then be rendered visible by pouring on a second overlay containing neutral red or tetrazolium (Abo-Ahmed et al., 1967; Conant et al., 1968, Dolan et al., 1968) which upon further incubation will stain the living cells and show up the plaques by contrast. Alternatively the cell sheet may be fixed by adding a volume of formalin to the cultures. The solid overlay is then removed and the uninfected cells, which remain attached to the tissue culture dish, are stained with methyl violet (Fiala, 1968).

It may be concluded that plaque formation is most easily and reproducibly obtained in monolayers of M-HeLa cells with an overlay containing a nutrient portion with the addition of $MgCl_2$ (30 mM) and DEAE dextran (30 µg/ml) solidified by the use of a purified agar.

1.5.3. Other methods

Rhinovirus replication may also be detected by haemagglutination (Stott and Killington, 1972), by the detection of cytopathic effects in organ cultures (see Section 2.1 for references) and by the production of infection in susceptible animals. These methods do not lend themselves to the detection of rhinovirus inhibitory compounds. Haemagglutination has not been found to occur with all rhinovirus serotypes and the organ culture technique has no advantages over normal tissue culture techniques since very few rhinovirus strains have been found which cannot be adapted to grow in tissue culture. Moreover the organs required for organ culture are not freely available. There is no small laboratory animal which is susceptible to rhinoviruses and hence no simple in vivo testing

system for rhinovirus inhibitory compounds. Chimpanzees and gibbons are susceptible to rhinoviruses (Dick, 1968; Haff, 1969), but because of the difficulty in handling these animals and their high cost they cannot be used for initial screening of compounds. Chimpanzees and gibbons have however been used for limited in vivo testing of compounds which have been found to exhibit promising rhinovirus inhibitory activity. This is obviously of great importance, since such compounds may thus be tested for in vivo efficacy before putting them through the procedure required to license them for experimental use in humans (Dick, 1968).

1.6 METHODS USED TO SCREEN COMPOUNDS FOR RHINOVIRUS INHIBITORY ACTIVITY AND TO QUANTITATE THIS ACTIVITY

Screening compounds for rhinovirus inhibitory activity is carried out by tissue culture methods based on the inhibition of virus-induced CPE, the inhibition of virus plaque formation and the inhibition of virus multiplication as determined by the reduction in virus yield.

1.6.1 Inhibition of CPE in Tissue Culture

Rhinovirus inhibitory activity may be detected by the inhibition of virus-induced CPE in tissue culture. The test may be carried out by the end-point dilution method. Two series of replicate tissue cultures (tubes or flasks) are infected with serial 10-fold (or 3.2-fold) dilutions of virus; virus is allowed to adsorb and the inoculum then replaced by maintenance medium. One set of cultures receives normal maintenance medium (control) and the other receives maintenance medium containing the test compound at a suitable concentration (test). The cultures are then incubated and observed at intervals for evidence of CPE, or microplaques may be counted where suitable cell cultures are used.

Evidence of antiviral activity may be determined by a delay in virus-induced CPE in the test cultures, for example a delay in the time taken for test cultures to reach 50% destruction of the cell sheet, or by a depression in the titre of the virus as determined by the Kärber or Reed-Muench methods.

Alternatively antiviral activity may be determined by the reduction in the number of microplaques in the test cultures. (The microplaque reduction technique as applied to rhinoviruses was first described by Taylor-Robinson and Tyrrell (1962a) as a method to determine the titre of rhinovirus antibody in human and animal sera.) A simple test of significance may then be applied to any differences between the test and control series. This method is therefore a simple and convenient

way to detect rhinovirus inhibitory activity and can be applied to any rhinovirus. It has been used widely for the general screening of compounds (Gwaltney, 1968; Gladych et al., 1969; Roderick et al., 1971; Schleicher et al., 1972; and Gladych et al., 1972), most workers preferring to use the diploid cell lines WI-26 and WI-38 for their studies.

The method does, however, suffer from a few disadvantages. Firstly, antiviral activity detected may be due to intracellular activity or to direct inactivation of the virus by the compound, since virus released from infected cells is in direct contact with the compound. To determine which of the two mechanisms is responsible for antiviral activity a separate test must be carried out to detect whether direct virus inactivation is occurring. Secondly, separate uninfected compound treated cultures must be included in the test in order to detect compound cytotoxicity. Toxic compounds will often appear to be antiviral since their effect on cell metabolism may indirectly result in inhibition of virus replication. Thirdly, a false negative result can be obtained by this method if the virus population (largely sensitive to inhibition by the compound) contains a proportion of resistant virions.

There are a number of ways in which this simple method of detecting antiviral activity may be modified in order to quantitate antiviral activity.

Unless one is observing and counting microplaques virus-induced cell damage may be expressed in terms of the proportion of cells showing marked changes, using a scale from 0 to 4, with 1 corresponding to 25%, 2 to 50%, 3 to 75% and 4 to 100% CPE involvement in the monolayer. The compound for test may be employed at a number of concentrations (usually serial 10-fold or 3.2-fold dilutions) and the cytopathic effects observed in the cultures can be expressed as a percentage value of the cytopathic effects in untreated infected controls. This value is then plotted against the concentration of the compound. From the graph

the ED_{50}, the concentration of compound which produces 50% inhibition of virus-induced cytopathic effects, or the ED_{75}, may be determined (Tamm et al., 1953). If the maximal subtoxic concentration has also been determined the selectivity of the compound may also be determined.

The selectivity of the compound was defined by Tamm and co-workers (1953) as the maximal subtoxic concentration (MSC) divided by the ED_{75}. However, problems arise when trying to compare the antiviral activities of compounds when studies have been carried out by different workers.

For example, different groups of workers have used different scales for CPE rating. In work by Gwaltney (1968; 1970) screening tests in tube cultures of WI-38 cells infected with rhinoviruses were considered complete when the untreated control cultures showed 75% or greater involvement of CPE. At this time the CPE in treated cells was observed, and differences of 75% or more between the CPE in the control and test cultures were graded as '+', 75-50% as '±', 50 - 25% as trace, and less than 25% as '-' inhibition.

Secondly, different workers used different formulae from which to calculate the selectivity or therapeutic index of compounds.

Tamm defines selectivity as $\frac{MSC}{ED_{75}}$

Other definitions of selectivity have been :-

(a) Selectivity $= \frac{MSC}{MIC}$

where MIC is the minimal inhibitory concentration.

(b) Therapeutic Index $TI = \frac{WTD}{MEC}$ (Gladych et al., 1972)

where WTD = well tolerated dose, the highest concentration giving no indication of cytopathology,

and MEC = minimal effective concentration, the lowest concentration which provides 50% or less destruction compared with control cultures.

(c) Chemotherapeutic Index.

$$CTI = \frac{\text{highest non-toxic concentration}}{\text{lowest antiviral concentration}}$$

(Schleicher et al., 1972)

where the highest non-toxic concentration is equivalent to the MSC and the lowest antiviral concentration is the lowest concentration to produce 100% inhibition of CPE.

Another system used to quantitate antiviral activity is the virus rating system (Ehrlich et al., 1965). This system does appear to be a little arbitrary in deciding what is considered to be an active compound, but one can argue that all criteria used to define activity here are arbitrary.

In the virus rating system the effect of the compound on the host cell and on the virus-induced cytopathic effects at two virus levels, 100 $TCID_{50}$ and 10 $TCID_{50}$, is evaluated microscopically and expressed again using the scale from 0 to 4. The virus rating is a measure of the inhibition of the virus-induced CPE by the compound at concentrations non-toxic or partially toxic to the host cells. The value assigned to the treated infected cells (T) is subtracted from the value assigned to the untreated but infected cells (C) and these differences (C - T) are added. At the higher virus input level (100 $TCID_{50}$) the value (C - T) is doubled. The virus rating is then calculated by totalling $(C - T)_{10\ TCID_{50}}$ and $2\ (C - T)_{100\ TCID_{50}}$ and dividing this by 10. A virus rating of 1.0 or more was considered to be a basis for further study of the compound (Ehrlich et al., 1965).

The virus rating system and modifications of it have been used by certain workers for the study of antiviral compounds (Sidwell and Huffmann, 1971; Burnham et al 1972).

There are numerous other ways in which different workers have quantitated the activity of various compounds. For example, the time

taken for a treated infected culture to reach a 50% involvement of CPE can be plotted against the size of the virus input inoculum. Using the same series of virus inocula and various different drugs, the activities of these drugs may be compared from the curves obtained (O'Sullivan et al., 1969).

Hence it is clear that comparisons of virus inhibitory activity cannot be made where workers have used different criteria to define this activity.

Also it is well known and has been proved experimentally (O'Sullivan and Ludlow, 1973) that the higher the input of virus inoculum given to a cell culture the higher will be the concentration of compound required to inhibit CPE. A study of the methods used by different workers shows that the size of the infecting inoculum used varies widely. For example, Gladych et al., (1969) used 4 $TCID_{50}$/tube, Boyle et al. (1970) 10 $TCID_{50}$/tube, Schleicher et al. (1972) 32 and 100, Gwaltney (1968) 3, 10, 30 and 100, and Gladych et al. (1972) did not even state the virus inoculum used. Similarly the time at which the tests are considered completed has an effect on the activity determination (O'Sullivan and Ludlow, 1973), since the longer the incubation period the greater is the chance that virus-induced CPE will occur in treated cultures (Haff, 1969).

Hence a meaningful quantitation of antiviral activity using the tube culture system is difficult to achieve, as has been noted by other workers (Haff, 1969).

1.6.2 Inhibition of Plaque Formation

1.6.2.(a) Disc Plate Plaque Inhibition

Rhinovirus plaque inhibition is in many ways a more simple and reliable test system for the detection of antiviral activity.

The disc method of plaque inhibition was first described by Herrmann and co-workers (1960), and by Rada and co-workers (1960). For this system suitable cell cultures, usually in plastic tissue culture dishes, are infected with virus and overlaid as for the normal plaquing

procedure. A filter paper disc impregnated with the test compound is then placed centrally on top of the overlay and the culture then incubated at 33°C to allow plaques to develop. The test compound diffuses from the disc through the agar, and if it possesses antiviral activity the formation of plaques will be suppressed in an area surrounding the disc where the compound is present in sufficient concentration. Beyond this area plaques will develop. The plaques are then visualized by methods already described.

The first report of this type of test applied to rhinoviruses was by Porterfield (1962) who used this as a test for neutralizing antiserum. Porterfield used fish spine beads instead of filter paper discs to take up the antiserum. More recently the disc plaque inhibition method has been used by many workers as a routine procedure for the screening of compounds for rhinovirus inhibitory activity (Haff, 1969; Stanfield et al., 1967; Haff et al., 1972; Boyle et al., 1970; Gladych et al., 1972).

The plaque inhibition test has many advantages over the tests for inhibition of CPE in tissue culture as a method for the detection of antiviral activity. No control culture to test for compound cytotoxicity is necessary, since the appearance of the stained cell sheet will indicate if the compound is toxic by showing a clear zone of cell lysis spreading from the centre of the monolayer. Secondly, if antiviral activity is demonstrated by this method it is unlikely to be due to direct virus inactivation since under the solid overlay virus spread is mainly cell to cell. Solubility of compounds is a problem and active compounds of low solubility may escape detection. However, even compounds of very low solubility should be able to diffuse through the overlay as a result of serum binding. Another great advantage of the plaque inhibition test is that the resulting stained culture plate may be kept and filed for future reference.

The disc plaque inhibition method will in itself give a rough estimation of antiviral activity, since compounds of equal activity will give zones of protection of similar diameter. However, the solubility of the compounds must be taken into account. The titre of the virus inoculum is also important. The dose must never be high enough to destroy the whole cell sheet, since examination of the cell sheet is required for the determination of compound cytotoxicity. It has been found that zone diameters obtained for particular virus/compound concentrations are not very reproducible (Kucera and Herrmann, 1966). This might be due to rapid diffusion of the drug from the filter paper disc, resulting in a steep concentration gradient and hence a narrow area of optimal drug concentration surrounding the disc (Bondi et al., 1947). Other factors also found to affect diffusion of the compound from the disc are the diffusion rate of the compound and the tendency of paper discs to retain compound (Bondi et al., 1947).

1.6.2.(b) Plaque reduction

Modifications of the plaque inhibition method have been used to make more quantitative studies of antiviral compounds.

A quantitative estimation of the antiviral activity of a compound may be made by counting the reduction in the number of plaques produced when the test compound is included in the overlay at varying concentrations. Obviously the input inoculum of virus is more critical here than for the disc plaque inhibition method, since a countable number of plaques is required in the control plates. Using this plaque reduction technique it is possible to determine the minimum concentration of compound needed to suppress plaque formation (the minimal inhibitory concentration, MIC). Also graphs of the number of plaques produced against the concentration of compound in the overlay may be plotted, and from this dose-response curve the ED_{50} or ED_{75} can be determined. Titration of rhinovirus neutralizing antiserum by the plaque reduction assay has been used by Fiala (1969) to replace the end-point tube assay.

Although this technique is an accurate and simple method of quantitating antiviral activity, it is fairly costly in materials used since at each concentration of drug to be tested replicate cultures must be set up. Hence the test is best left as a final quantitative assay for antiviral activity when the approximate concentration range required for the dose-response curve has been estimated. The gradient plate plaque reduction technique is an ideal method by which to estimate this concentration range.

1.6.2.(c) Gradient Plate Plaque Reduction

The gradient plate plaque reduction technique, first described by Kucera and Herrmann (1966), is a modification of the plaque reduction technique. It applies the gradient plate technique first described for use in bacteriology by Szybalski (1952) to antiviral studies. Kucera and Herrmann used cell monolayers grown in 8.5cm plastic tissue culture dishes. The monolayers are infected with 0.5ml of virus inoculum and after adsorption this was removed by washing the monolayers in Hanks balanced salt solution. To obtain gradient plates the monolayers were then overlaid with two layers of agar medium (9 ml). The first layer was added while the plates were slanted (about $\frac{1}{8}$th of an inch) so that the agar medium just covered the uppermost portion of the monolayer. This layer is then allowed to set and the second layer, containing a suitable concentration of the test compound, is added after the plates have been returned to a horizontal position. Plates were then incubated as usual to allow plaques to form. The concentration of compound in the overlay therefore varies continuously and uniformly across the dish and at any particular point is proportional to the ratio of the thickness of the two wedge-shaped layers of normal and compound-containing overlay. Kucera and Herrmann determined the plaque inhibitory concentration of many compounds against a variety of viruses by means of this technique. The plaque inhibitory concentration (C_I) is expressed as the concentration of antiviral compound present at the point of complete plaque inhibition (see Fig.1.2) in which

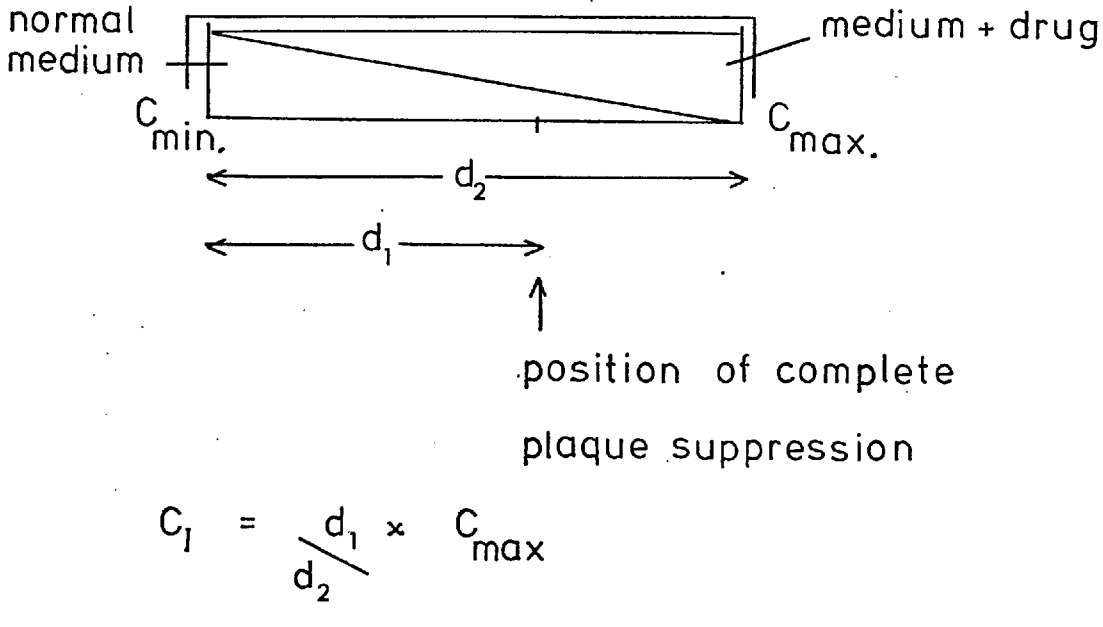
D_1 is the distance (in cm) from the point of complete plaque inhibition to the minimum of the gradient

D_2 is the internal diameter (in cm) of the tissue culture dish

and C is the initial concentration of the compound in the second overlay.

If the compound is used at a sufficiently high concentration in the overlay the maximal subtoxic concentration of the compound may also be determined from the gradient plate and the selectivity index can be subsequently determined (Shindarov et al. 1971; Galabov et al. 1972 a,b).

Fig. 1.2. Diagrammatic Representation of Gradient Plate Plaque Inhibition



$$C_I = \frac{d_1}{d_2} \times C_{max}$$

The results obtained by Kucera and Herrmann using this technique agreed with those obtained using the disc plate plaque inhibition method. However, the results were far more reproducible than with the latter method, possibly because the gradient plate technique allows a gradual concentration gradient to be set up in the medium covering the entire cell monolayer. A much steeper concentration gradient of compound is set up in the disc plate method. Kucera and Herrmann concluded that the data obtained from the gradient plate method were a more accurate assay of antiviral activity than was possible by the tube dilution or plaque inhibition methods. Also, although they had used 3 replicates in each of their tests, this was unnecessary since each plate represents a wide number of concentrations of the compound.

One special use to which this technique can be put is the isolation of resistant mutants. Kucera and Herrmann observed resistant plaques in idoxuridine gradient plates and isolated and cultivated virus from these plaques. These isolated strains were then found to be far more resistant to idoxuridine than the parent strains. The gradient plate plaque reduction technique would therefore seem a reliable method of detecting antiviral activity even when resistant mutants are present in the virus population. The tube dilution test can, however, give false results of negative activity when resistant mutants are present, since these will multiply and produce gross cytopathic effects in the culture. This was also found by Gwaltney when screening for rhinovirus inhibitory activity of D-(-)-2-(α-hydroxybenzyl)benzimidazole.HCl. In tube cultures of human embryonic lung cells (WI-38) a strain or strains of all the first 55 rhinovirus serotypes except type 1A and subtype 1B showed inhibition by the compound at 574μM. However, using the gradient plate technique type 1A showed inhibition at a 184μM concentration of D-HBB.HCl and the method demonstrated a relatively high proportion of compound-resistant virions in the virus strains used.

The gradient plate plaque inhibition technique has been widely used for the determination of rhinovirus inhibitory activity (Gwaltney 1968; Gwaltney 1970). It has proved to be a relatively simple and quick method of obtaining quantitative data of antiviral activity.

1.6.3 Yield Reduction Assay

Another technique for determining antiviral activity is measurement of the reduction in virus yield obtained when cultivating the virus in the presence of varying concentrations of compound. Replicate tissue cultures are infected with virus and after adsorption has taken place the inoculum is replaced by normal maintenance medium or maintenance medium containing the test compound at different concentrations. The cultures are then incubated for the length of a single virus growth cycle, usually 12 to 16 hours. The yield of virus is harvested by freezing and thawing the cultures three times and titrated either by the end-point dilution method or by plaque assay. A dose-response curve of virus yield against compound concentration may then be plotted. The sensitivities of different viruses to one compound or one virus to different compounds may then be compared using the dose-response curves.

Yield reduction assays have been used by several workers to quantitative rhinovirus inhibitory activity (Haff, 1969; Haff et al., 1972; Matsumoto et al., 1972). The procedure proved to be more accurate, sensitive and objective for quantitating compound effect than the more commonly utilised test for inhibition of CPE in tube cultures. It is not yet known whether variables such as the virus multiplicity of infection have an effect on results obtained in the yield reduction assay and this must be studied. However, it does seem that there must be a greater chance of variation in conditions in the multicylce test for inhibition of CPE than in the single cycle yield reduction assay and one would therefore expect results from the latter test to be more comparable.

1.7 GROUPS OF RHINOVIRUS INHIBITORY COMPOUNDS

1.7.1 The Triazinoindoles

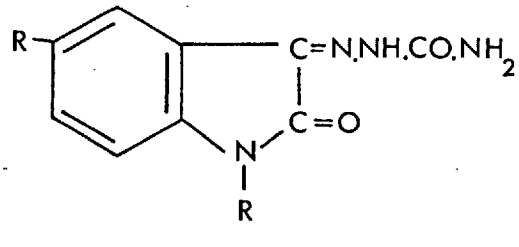
History, Detection of Antiviral Activity in Tissue Culture

Certain thiosemicarbazones, in particular methisazone, are well known as drugs which inhibit the multiplication of pox viruses (Thompson et al., 1953, Bauer and Sadler, 1960; Bauer et al., 1962). The antiviral spectrum of thiosemicarbazones is now known to include poliovirus (Lwoff and Lwoff, 1964), adenovirus (Bauer and Apostolov, 1966) and rhinovirus (Gladych et al., 1969).

Gladych and co-workers (1969) tested the effect of isatin 3-thiosemicarbazone, 1-methylisatin 3-thiosemicarbazone (methisazone) (see Fig.1.3) and 3 triazinoindoles on the replication of 4 rhinovirus serotypes in tube cultures of human embryo lung cells. All compounds were found to be active against at least 1 rhinovirus type.

The triazinoindoles are compounds related to isatin 3-thiosemicarbazone and are prepared by cyclizing the side chain of the methisazone molecule. Since the above finding of antirhinovirus activity in this series, numerous derivatives have been screened for antiviral activity (Gwaltney 1970; Gladych et al., 1972).

Fig.1.3 Structural formulae of thiosemicarbazones

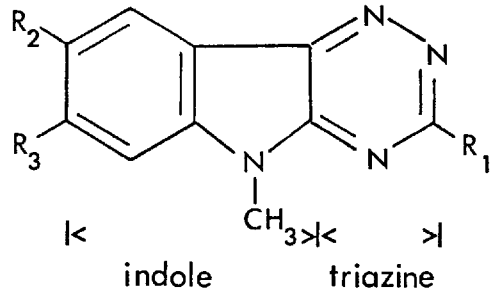


Isatin 3-thiosemicarbazone R = H

5-methylisatin 3-thiosemicarbazone

R = CH_3

Fig.1.4 Structural formulae of triazinoindoles



2,2,-dimethyl-3- [(5-methyl-5H-as-triazino [5,6-b] indol-3-yl)amino]-1-propanol

$R_1 = NH.CH_2-C(CH_3)_2-CH_2OH$

SK&F 28938

2-methyl-4 [(5-methyl-5H-as-triazino [5,6-b] indol-3-yl)amino] -2-butanol

$R_1 = NH.CH_2CH_2-C(CH_3)_2-OH$

SK&F 30097

3-[(5-methyl-5H-as-triazino[5,6-b]indol -3-y)amino] -1-propanol

$R_1 = NH.CH_2CH_2-CH(CH_3)-OH$

SK&F 21687

4- [(8-amino-7-chloro-5-methyl-5H-as-triazino(5,6-b)indol-3-yl) amino] -2 methyl-2-butanol

$R_1 = NH.CH_2CH_2-C(CH_3)_2-OH$

SK&F 40491

$R_2 = NH_2$

$R_3 = Cl$

The report by Gwaltney (1970) describes the activity of 6 different 3-substituted triazinoindoles* against 32 rhinovirus strains. The tests were carried out in test tube cultures of WI-38 cells employing virus inputs ranging from 3 to 100 TC ID_{50} per ml and drug concentrations ranging from 20 to 100 µg per ml. Of the 6 compounds tested, 2 (designated SK&F 28938 and 30097) with paired methyl groups in the side chain, were found to be most active. These 2 compounds and SK&F 21687 inhibited all the rhinovirus strains tested: their chemical names and formulae are given in Fig.1.4.

Gwaltney used the gradient plate plaque reduction technique as an additional method to demonstrate activity and to determine the minimal inhibitory concentration of SK&F 30097 against certain rhinovirus strains The minimal inhibitory concentration for six strains ranged between 21 and 32 µM(6 - 9 µg/ml), which is well below the gross cytotoxic concentration. (SK&F 30097 at a concentration of 350 µM (100 µg/ml) showed no toxicity for HeLa cells as judged by gross morphologic appearance).

Studies on the antirhinovirus activity of SK&F 30097 have been made by other workers (Haff, 1969). Haff reported that the maximum well-tolerated dose of SK&F for 4 to 6 days maintenance of WI-38 cells is 252 µg/ml and that normal growth of HeLa cells was obtained in the presence of at least 50 µg/ml. However, he found that the test for inhibition of rhinovirus CPE in WI-38 cells (as used by Gwaltney, 1970) was insensitive and variable. Sensitivity of the virus to the drug diminished with increasing amounts of virus used for infection, and with successive days of incubation after the viral CPE first became evident

* These compounds were prepared at the Research & Development Division, Smith Kline and French Laboratories, Philadelphia, Pennsylvania, USA.

less inhibition was observed. From tube test experiments minimal inhibitory concentrations in the range of 20 to 50 µg/ml were obtained against 10 $TCID_{50}$ of virus but in view of his doubts on the reliability of the tests Haff investigated the use of other techniques in order to better define the minimal inhibitory concentration. Gradient plate plaque reduction tests and yield reduction assays were employed. Haff reported that the yield reduction assays 'proved to be more accurate, sensitive and objective for quantitating compound effect than the more commonly utilised test for inhibition of CPE in tube cultures.' By this procedure a concentration of less than 10 µg/ml was found to inhibit all of 4 viruses tested.

Yield reduction assays and plaque inhibition tests were also used to study the in vitro antiviral activity of the triazinoindoles designated SK&F 40491 (Fig.1.4), which was found to possess picornavirus inhibitory activity higher than any other triazinoindole. (Haff et al., 1972). Activity against a range of picornaviruses was now observed at 0.8 to 3.5 µg/ml in yield reduction assays whereas in comparable tests SK&F 30097 was found to be as much as 21-fold less active against the same infections.

Antiviral Spectrum

The in vitro spectrum of activity of SK&F 30097 has been found to be very broad (Boyle et al., 1970); all of 19 rhinovirus strains and strains of adeno, Coxsackie, cytomegalo, herpes simplex, polio, pseudorabies, Semliki Forest and vaccinia viruses were inhibited.

The in vitro spectrum of activity of SK&F 40491 has not been studied as extensively as that of SK&F 30097. However, it has been found to be active against all of 8 strains of human rhinovirus, equine rhinovirus type 1, poliovirus type 1 and strains of Coxsackie virus (Haff et al., 1972). No picornavirus tested was insensitive but herpes simplex, parainfluenza type 1 (Sendai) and respiratory syncytial viruses were insensitive.

Structure Considerations

Gladych and co-workers (1972) attempted to establish structure-activity relationships for 5H-as-triazino (5,6-b)-indoles and reported that the most promising compounds against rhinovirus infections were those with hydroxyalkylamino substituents in the C-3 position. The compounds described previously (i.e. SK&F compounds 28938, 30097, 21687 and 40491) have such a group in the C-3 position.

In the paper by Gwaltney (1970) it was reported that certain of the rhinovirus serotypes sensitive to triazinoindoles had previously been tested against D - (-)-2-(α- hydroxybenzyl)benzimidazole. HCl(D-HBB.HCl.) by the plaque reduction technique and had shown maximum sensitivity to this compound. In his discussion Gwaltney notes that all of the 3-substituted triazinoindoles reported contain a =N-C=N- sequence at the site of side-chain attachment, which is similar to the >N-C=N- sequence found in HBB and guanidine (both antipicornavirus compounds).

It had previously been proposed that one (or both) of these nitrogen atoms might be essential for binding HBB to its target (Tamm and Caliguiri, 1972) and it would therefore be interesting to discover whether these atoms in the triazinoindoles have such a role.

A detailed physico-chemical evaluation of SK&F 30097 has been carried out (Ravin et al., 1970). Physico-chemical properties would appear to be of special importance in this group of low solubility compounds.

Mode of Action

The mechanism of action of SK&F 30097 has been studied (Boyle et al., 1970; Matsumoto et al., 1972). The drug was found to be non-virucidal and to have no effect on adsorption of poliovirus (Matsumoto et al., 1972), or rhinovirus (Boyle et al., 1970) to WI-38 monolayer cell cultures. SK&F 30097 is not an interferon inducer (Boyle et al., 1970) which complies with its selective antiviral activity. Similar results have been obtained

for SK&F 40491 (Haff et al., 1972)

The effect of SK&F 30097 on events subsequent to virus adsorption has been reported (Matsumoto et al., 1972). Results of experiments with poliovirus and rhinovirus suggested that a late stage or stages in viral synthesis was subject to inhibition. SK&F 30097 was found to inhibit viral RNA synthesis and this effect was therefore postulated as being responsible for the antiviral activity of the compound. The inhibition of poliovirus by busatin, 1-methylisatin 3-(4',4'-dibutyl)thiosemicarbazone was studied by Pearson and Zimmerman (1969). They found that busatin blocks poliovirus replication also by blocking viral RNA synthesis.

However, the inhibition of vaccinia virus by isatin 3-thiosemicarbazone (Fig.1.1) is reported to be due to the blocking of RNA translation (Woodson and Joklik, 1965). The synthesis of the viral DNA genome (Woodson and Joklik, 1965; Easterbrook, 1963) and RNA (Woodson and Joklik, 1965) is not inhibited. It would seem natural to presume that the inhibition of poxvirus synthesis by methisazone is by a similar mechanism.

Hence in view of the postulated mechanism of action of triazinoindoles and busatin against RNA viruses and of thiosemicarbazones against DNA viruses it seems quite likely that methisazone inhibits sensitive RNA and DNA viruses by different mechanisms.

In Vivo Antiviral Activity

The results of in vivo tests of SK&F 30097 have been reported (Boyle et al., 1970; Haff, 1969). Boyle and co-workers tested the drug in mice against 15 different viral infections and 2 tumours, and in chimpanzees both prophylactically and therapeutically against rhinovirus infections. Results of their experiments are shown below (Table 1.3). Activity against rhinovirus infection was studied by monitoring virus shedding and sero-conversion and a suggestion of activity was obtained against infection with rhinovirus type 14 but not with rhinovirus type 1A.

Table 1.3 EFFECT OF SK&F *In Vivo*

VIRUS	HOST	INFECTION	ACTIVITY
Coxsackie B1/GP	mouse	encephalitis	Neg.
Friend Leukemia	mouse	leukemia	Neg.
Herpes Simplex	mouse	encephalitis	Neg.
Influenza (A, A2, B)	mouse	pneumonitis	Neg.
Mengo	mouse	encephalitis	Neg.
Pseudorabies/ Aujeszky	mouse	encephalitis	Neg.
Rhino (1A, 14)	chimpanzee	virus isolation and seroconversion	±
Vaccinia	mouse	tail lesions	+
	Rhesus monkey	dermatitis	Neg.
Western Equine Encephalitis	mouse	encephalitis	Neg.
L.1210 Lymphoid Leukemia	mouse	leukemia	Neg.
W.256 Carcinosarcoma	rat	solid tumour	Neg.

from Boyle *et al.*, (1970)

The authors thought it possible that the effect of the compound may have been obscured by the fact that large virus inocula were used to initiate infection. SK&F 21687 was ineffective in the treatment of rhinovirus type 44 infection in human volunteers (Togo et al., 1973) but this failure may have been due to the insolubility of the drug.

SK&F 40491 was found to have no activity against Coxsackie A21, B1 and B3, herpes simplex, influenza A2, Mengo, pneumonia virus of mice, pseudorabies or vaccinia in mice when administered systemically. Equine rhinovirus infections in rhesus monkeys were not affected by oral or aerosol prophylactic treatment (Haff et al., 1972). Similarly oral administration to gibbons infected with rhinovirus type 1B did not significantly alter the course of infection (Pinto et al., 1972). However, SK&F 40491 was found to be effective in reducing viral multiplication and serum and nasal antibody response when administered intranasally to gibbons infected with rhinovirus type 2. Haff and co-workers (1972) suggest that a single antiviral dose to the nasal mucosa might show a prolonged effect, since they found that the drug bound tenaciously at antiviral concentrations to cultured cells. They also explain the lack of systemic activity on the basis of the metabolic profile of the drug.

1.7.2 Benzimidazoles

A detailed review of 2-(α-hydroxybenzyl)benzimidazole (HBB) and related compounds has been made by Tamm and Caliguiri (1972). The finding that vitamin B_{12} contains a benzimidazole moiety, 5,6-dimethyl-1-β-D-ribofuranosyl benzimidazole, led to the testing of benzimidazoles as possible antiviral compounds. Vitamin B_{12} is involved in the biosynthesis of nucleic acid and it was therefore thought that inhibitors of virus multiplication might be found in compounds containing related structural features. In 1952 2,5-dimethylbenzimidazole was found to inhibit the multiplication of influenza type A and B viruses in the chorioallantoic membrane (Tamm et al., 1952) and later derivatives of benzimidazoles with a sugar moiety, which thus resembled vitamin B_{12} more closely, were also found to be effective against influenza viruses. Subsequent trials of benzimidazoles led to the discovery of the picornavirus inhibitory compound HBB.

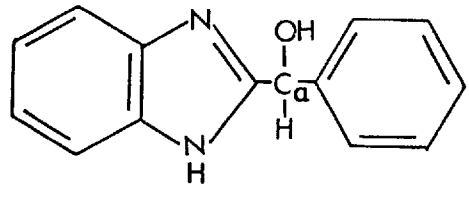


Fig.1.5 Structural Formula of 2-(α-Hydroxybenzyl)benzimidazole (HBB)

The picornavirus inhibitory activity of the benzimidazole derivative 2-(α-hydroxybenzyl)benzimidazole (HBB) was first demonstrated by Hollinshead and Smith (1958) and Tamm and Nemes (1959). Both groups of workers showed that the compound inhibits multiplication and cytopathic effects of poliovirus in primary cultures of monkey kidney cells and it was also found to be slightly protective against experimental poliovirus infection in the mouse (Hollinshead and Smith, 1958). However, HBB is not inhibitory to all picornaviruses and has no effect on viruses belonging to other major groups (Tamm and Nemes, 1959; Tamm et al., 1961; Eggers and Tamm, 1961 a,b).

One exception to this rule is lymphocytic choriomeningitis (LCM) virus. Three strains of this virus were found to be sensitive to HBB (Pfau and Camyre, 1968). Lymphocytic choriomeningitis is the prototype of the arenavirus group (Rowe et al., 1970; Wildy, (1971). Tamm and Caliguiri (1972) note that LCM is not inhibited by 700μM guanidine, which is of interest since no enterovirus has been found which is sensitive to inhibition by HBB but not by guanidine.

The virus inhibitory activity of HBB has been evaluated on the basis of inhibition of virus yield and reduction of virus-induced cytopathic effects. Tamm and co-workers (1961) showed a relationship between the concentration of compound and the inhibition of virus yield or the reduction of virus-induced cytopathic effects. These dose-response lines are approximately parallel but it was found that the concentration of HBB required to inhibit virus-induced cytopathic effects by 75% was 1.55 times greater than that required to reduce virus yield by 75%. The concentration (56μM) which inhibits virus-induced cytopathic effects by 75% reduces the virus yield by 95%. Tamm and Caliguiri (1972) note that these results are in agreement with the finding, obtained with guanidine and poliovirus, that less than maximal virus multiplication leads to maximal virus-induced cytopathic effects (Bablanian et al., 1965).

Therefore the demonstration of inhibition of virus-induced cytopathic effects by HBB or guanidine can be taken as strong evidence of significant inhibition of virus multiplication.

Many studies have been made to determine the antiviral spectrum of HBB (Eggers and Tamm 1961, 1,b; Tamm and Eggers, 1962).

Table 1.4 (Tamm and Caliguiri, 1972) shows a summary of results obtained for the inhibition of picornavirus cytopathic effects by HBB. (Eggers and Tamm 1961, a,b; Tamm and Eggers, 1962). In most cases the cells used were primary cultures of rhesus monkey kidney cells but human embryonic lung cells and other types were also used. 30 to 500 $TCID_{50}$ of virus was generally inoculated per culture tube and challenged with HBB, usually at 98, 219 and 493 µM. Virus-induced cytopathic effects were evaluated in terms of the percentage of cells affected in drug treated and control cultures. It was proposed then (Eggers and Tamm, 1961a) that a virus should be considered sensitive to HBB 'if in the presence of 219µM HBB the extent of virus-induced cell damage is less than 25% of that in untreated control cultures on any day after infection.' This was to allow for the fact that some virus types could be inhibited by 98µM HBB for only a period of time after which escape or breakthrough occurs. Some but not all of such viruses will be completely suppressed by 219 or 493µM. Each virus tested was therefore placed into 1 of 4 groups on the basis of its sensitivity to HBB.

In this study none of the 3 rhinoviruses tested was found to be sensitive to inhibition by HBB. In subsequent studies by other workers of a total of 27 rhinovirus types tested only types 14 and 26 were found to be sensitive to HBB (200µM). The results of these studies and later a fuller investigation made by Gwaltney (1968) were summarised by Tamm and Caliguiri (1972, Table 1.5). Results of Gwaltney's experiments were mainly in agreement with previous work although he found partial or

Table 1.4 Inhibition of Virus Cytopathic Effects by HBB [a]

A Complete inhibition by 98 μM HBB	B Complete inhibition by 219 μM HBB, but not by 98 μM HBB	C Incomplete inhibition by 219 or 493 μM HBB	D No inhibition by 219 or 493 μM HBB
Coxsackie B4 (Powers)	Coxsackie A9 (Woods)	Coxsackie A21 (Coe)	Coxsackie A7 (AB IV Habcl)
Echo 4 (Pesascek)	Coxsackie B1 (P.O. Dalldorf)	Echo 20 (JV-1)	Coxsackie A11 (D 52148)
Echo 6 (D'Amori)	Coxsackie B2 (Ohio 1)	Polio 1 (Mahoney)	Coxsackie A13 (D 5359)
Echo 9 (Hill)	Coxsackie B3 (Nancy)	Polio 2 (MEF1)	Coxsackie A16 (D 52109)
Echo 13 (11-4-1D)	Coxsackie B5 (Faulkner)	Polio 3 (Saukett)	Coxsackie A18 (D 52112)
Echo 14 (Tow)	Coxsackie B6 (1-51-21)		Echo 22 (Harris)
Echo 15 (Charleston 96-51)	Echo 1 (Farouk)		Echo 23 (Williamson)
Echo 17 (CHHE-29)	Echo 2 (Cornelis)		Rhino 1A (2060)
Echo 18 (Metcalf)	Echo 3 (Morrisey)		Rhino 1B (B632)
Echo 24 (de Camp)	Echo 5 (Noyce)		Rhino 2 (HGP)
Echo 26 (11-3-6)	Echo 7 (Wallace)		Reo 1 (Lang)
	Echo 8 (Bryson)		Reo 2 (Jones)
	Echo 11 (Gregory)		Reo 3 (Dearing)
	Echo 12 (Travis 2-85)		Arbo B (West Nile)
	Echo 16 (Harrington)		Arbo C (Marituba; Oriboca)
	Echo 19 (Burke)		Influenza B (1760)
	Echo 21 (Farina)		Parainfluenza 2 (Greer)
	Echo 25 (JV-4)		Parainfluenza 3 (C-243)
	Echo 27 (1-36-4)		Mumps (Utz)
			Adeno 2 (Ind-2)
			Adeno 3 (JF)
			Adeno 4 (RN)
			Herpes simplex (RE)
			Vaccinia

[a] Generally, primary cultures of rhesus monkey kidney cells were used, except that coxsackievirus types 11, 13, and 18 were studied in ERK and HEL cells; rhinovirus types 1A, 1B and coxsackievirus A21 in HEL cells; rhinovirus types 1A and 2 were also studied in monkey kidney cells; herpes virus was examined in HeLa cells. The following media were used: monkey kidney cells: protein-free basal or minimum essential Eagle's medium; ERK cells: Earle's solution with 5% calf serum, 5% lactalbumin hydrolysate, and 5% tryptose phosphate broth; HEL cells: reinforced Eagle's medium with 1% fetal calf serum; HeLa cells: Eagle's medium with 5% chicken serum.

Based on data in Eggers and Tamm, 1961a, b; Tamm and Eggers, 1962.

from Tamm and Caliguiri, 1972

Table 1.5 Comparison of Results of Determinations of Sensitivity of Rhinoviruses to 200-223 μM HBB (Based on Inhibition of Virus Cytopathic Effects)

Type	Strain	Inhibition	Conc. of HBB, μM	Cell type	Reference	Inhibition by 223 μM HBB in WI-38 cells (Gwaltney, 1968)
1A	2060	−[a]	223	HEL	Tamm and Eggers (1962)	−
1B	B632	−	223	HEL	Tamm and Eggers (1962)	−
2	HGP	−[a]	219	MKC	Eggers and Tamm (1961b)	−
3	FEB	−[a]	219	HeLa	Hamparian *et al.* (1963)	+
4	SF748					−
6	SF1349					−
7	11	−	219	HeLa	Hamparian *et al.* (1963)	
11	SF747					±
13	353	−	200	WI-26	Kisch *et al.* (1964)	±
14	1059 or	±	200	WI-26	Kisch *et al.* (1964)	
	101-1	+	200	HEL	Hamre *et al.* (1964)	±
15	1734	−	200	WI-26	Kisch *et al.* (1964)	−
16	11757	−	200	WI-26	Kisch *et al.* (1964)	Inconsistent
17	33342	−	200	WI-26	Kisch *et al.* (1964)	−
18	5,986-CV 17					+
21	CH51					±
22	203-F	−	200	HEL	Hamre *et al.* (1964)	
23	SF1322					−
24	147-H	−	200	HEL	Hamre *et al.* (1964)	
26	127-1	+	200	HEL	Hamre *et al.* (1964)	+
27	SF274					+
28	6,101-CV 29	−	200	HEL	Hamre *et al.* (1964)	
29	179-E	−	200	HEL	Connelly and Hamre (1964)	−
30	106-F	−	200	HEL	Connelly and Hamre (1964)	± or trace
31	140-F	−	200	HEL	Connelly and Hamre (1964)	−
32	363	−	200	WI-26	Webb *et al.* (1964)	trace
33	1200	−	200	WI-26	Webb *et al.* (1964)	−
34	137-3	−	200	HEL	Hamre *et al.* (1964)	+
35	164-A	−	200	HEL	Hamre *et al.* (1964)	−
36	342-H	−	200	HEL	Hamre *et al.* (1964)	
37	151-1	−	200	HEL	Hamre *et al.* (1964)	
38	201-33	−	200	HEL	Hamre *et al.* (1964)	
39	SF299					Inconsistent
40	184-E	−	200	HEL	Hamre *et al.* (1964)	
41	137-F	−	200	HEL	Hamre *et al.* (1964)	±
51	313-G	−	200	HEL	Hamre *et al.* (1964)	

[a] Hamparian *et al.* (1963) obtained variable results in WI-26 cells with types A1, 2, 3, and also 12, but in HeLa cells consistent results were obtained showing no inhibition of types 2, 3, and 7.

The following media were used: HEL cells: reinforced Eagle's medium with 1% fetal calf serum or equal volumes of Eagle's minimum essential medium and medium 199 with 1% calf serum; monkey kidney cells: protein-free Eagle's minimum essential medium; HeLa cells; Eagle's medium with 5% calf serum or less; WI-26: Eagle's minimum essential medium with 2% calf serum; WI-38: equal volumes of Eagle's minimum essential medium and medium 199 with 2% fetal calf serum.

from Tamm and Caliguiri, 1972

inconsistent HBB sensitivity for previously reported insusceptible strains (i.e. those of types 3, 13, 16, 30, 34, 39 and 41). Differences in the conditions of the experiments and in criteria of inhibition are thought to be possible reasons for these discrepancies. In all Gwaltney found that 13 of the 26 strains tested showed some degree of sensitivity to the drug.

Gwaltney also tested the more active hydrochloride of the D-(-) isomer of HBB (D-HBB,HCL) for rhinovirus inhibitory activity. He found that a strain or strains of all the first 55 serotypes, except type 1A and subtype 1B, showed some degree of inhibition by a 574μM concentration or less of the compound in WI-38 cells. This suggests that inhibition by this compound is a common characteristic of the classified rhinovirus types. Also strains of the same serotype showed similar, but not completely uniform, response to compound exposure.

The cell type in which the test is carried out may well be of importance in determining the sensitivity of a virus to a compound especially if this sensitivity is low. Hamparian and co-workers (1963) found that WI-26 cells gave variable results where HeLa cells gave more consistent results. Gwaltney (1968) tested 36 rhinovirus strains against D-HBB.HCl in HeLa and in WI-38 cells and found agreement in 75% of the strains regardless of whether or not they were tested simultaneously.

Activity in vivo

There have been no reports of any trials of benzimidazoles in man. This must be ascribed to the fact that the concomitant development of effective poliomyelitis vaccines has virtually eradicated the disease and thus removed the opportunity for testing chemotherapeutic agents in its control and treatment, but this circumstance in no way detracts from the importance of the discovery of the benzimidazoles as a milestone in the history of antiviral chemotherapy.

Resistance to and Dependence upon HBB

Some virus types may be temporarily inhibited by certain concentrations of HBB but upon prolonged incubation with the compound may escape from this inhibition. The virus population which emerges may be resistant to or, less frequently, dependent upon HBB. Hence screening techniques which do not determine the susceptibility of indivudual virus particles to the test (for example the test for inhibition of cytopathic effect in tube cultures) may falsely show that a virus strain containing predominantly susceptible particles is resistant. However, techniques such as the plaque inhibition or plaque reduction tests will demonstrate the susceptibility of sensitive virus particles in a population of sensitive and resistant particles.

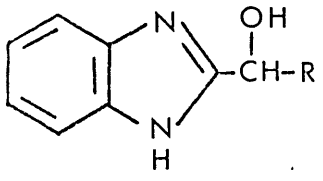
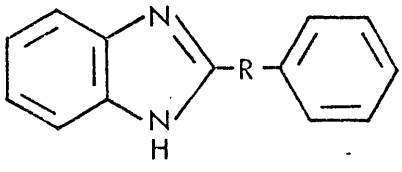
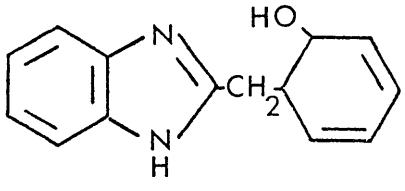
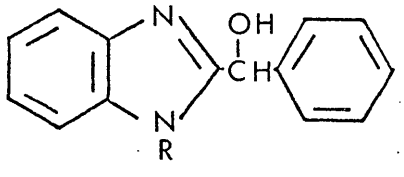
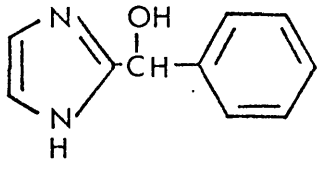
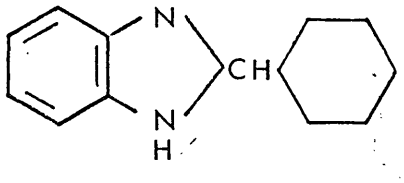
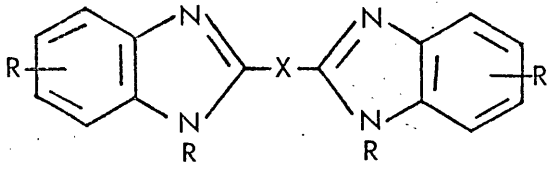
This point may be emphasised by the results of experiments carried out by Gwaltney (1968) on the sensitivity of rhinovirus strains to D-HBB.HCl. Using the test tube culture technique rhinovirus 1A was found to be insensitive to 574μM D-HBB.MCl. However, using the gradient plate plaque reduction technique rhinovirus 1A showed inhibition at 184μM. At this concentration a relatively high proportion of the virions were resistant to D-HBB.HCl, which explains the failure of the tube culture method to demonstrate virus sensitivity.

Drug resistance and dependence is extensively covered in the review by Tamm and Caliguiri (1972).

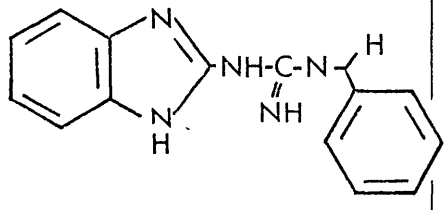
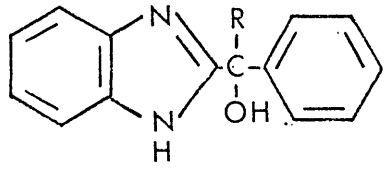
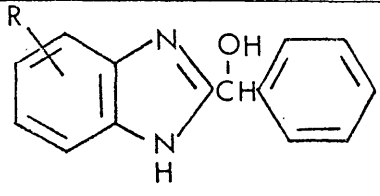
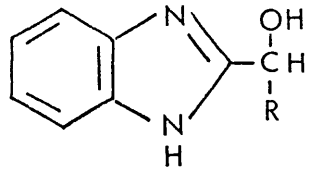
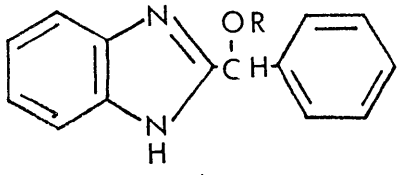
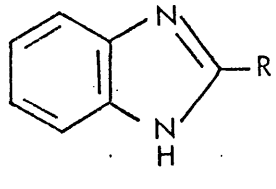
Structure-Activity Relationships

The structure-activity relationships of HBB and related compounds have been reviewed in detail by Tamm and Caliguiri (1972). This therefore will not be repeated but a summary of their review is presented here in tabular form to provide a brief but comprehensive picture of this subject (Table1.6).

Table 1.6 Structure-Activity relationships of derivatives of 2-(α-hydroxybenzyl)benzimidazole

Structural Change	Structural Formula	Example
Replacement of phenyl group of hydroxybenzyl grouping by alkyl group		2-hydroxymethyl- 2-(α-hydroxyethyl) benzimidazole
Replacement of CHOH group by CH_2 or C=O		2-Benzoyl- 2-Benzylbenzimidazole
		2-(o-Hydroxybenzyl)-benzimidazole
Substitution of N-1 hydrogen by alkyl or aryl group		1-methyl- 1-β-methylpropyl- 1-Propyl- 1-Phenyl- 1-Butyl- 1-Benzyl-2-(α-hydroxy benzyl)benzimidazole
Replacement of entire benzimidazolyl nucleus with monocyclic imidazole		2-(α-Hydroxybenzyl)-imidazole
		1,3-dialkylbenzimidazolium iodides
		Parent compound, 1,2-bis (2-benzimidazolyl)-1,2-ethanediol

	Activity	Conclusion	Reference
	Inactive	Hydroxybenzyl group in position 2 of the nucleus is of fundamental importance to activity.	Tamm et al., 1961 Tamm and Eggers, 1963a,b
	Reduced activity		Tamm and Eggers, 1963b
	Reported to be as active as HBB against PV 2 by 1st authors but to be less active against PV 2, Coxsackie A9 and echo 6 by 2nd authors		O'Sullivan and Sadler, 1961 Eggers and Tamm, unpublished observations
	1-methyl deriv. similar activity to HBB. 1-propyl more active 1-phenyl more active than 1-propyl Generally more active than HBB	Results suggest that activity of HBB and its analogues is not dependent upon their biosynthetic conversion to nucleosides by condensation at the N-1 position with ribose or deoxyribose	O'Sullivan and Wallis, 1963a O'Sullivan et al., 1964 a,b O'Sullivan et al., 1967 Eggers and Tamm, 1961a
	Reduced activity	Benzo moiety of benzimidazole nucleus in HBB also critically important	Tamm and Eggers, 1963a Tamm et al., 1969
	Reduced activity to polioviruses	Effect of quaternization is to reduce the protective action of 1-alkyl derivatives.	O'Sullivan and Wallis, 1963b
	Parent compound has only slight activity against PV 1. 5-methoxy derivative is 10x more active than 1-phenyl HBB and 100x more active than HBB.		O'Sullivan and Wallis, 1963a Akihama et al., (1968)

Structural Change	Structural Formula	Example
Replacement of hydroxybenzyl group by guanidino group		2-N^3-phenylguanidino-benzimidazole
Substitution of H on α-carbon		2-(α-methyl-α-hydroxybenzyl)- 2-(α-ethyl-α-hydroxybenzyl)- benzimidazole
Introduction of electron withdrawing grouping at carbon-5 in benzimidazole moiety Introduction of electron donating groups at carbon-4 or 5 of benzimidazole moiety		5-trifluoromethyl-5-phenyl- 5-nitro-2-(α-hydroxybenzyl)- benzimidazole 5-methyl-2-(α-hydroxybenzyl)- benzimidazole
Replacement of phenyl group of hydroxybenzyl moiety with other cyclic groups		2-thienyl- p-chlorophenyl- β-benzylthio-2-(α-hydroxy) benzimidazole
Replacement of phenyl group of hydroxybenzyl moiety with deactivated aromatic ring or non-aromatic ring		m-nitrophenyl- cyclohexyl-2-(α-hydroxy) benzimidazole
Substitution of hydroxyl hydrogen		2-(α-methoxybenzyl)- 2-(α-acetoxybenzyl) benzimidazole
Replacement of hydroxybenzyl moiety by alkyl or aryl group.		2-(2-thienyl)- 2-(2-furyl)- 2-(phenylbenzimidazole)

	Activity	Conclusion	References
	This derivative most effective against poliovirus. More active than HBB but less selective (i.e. more toxic)		Sato et al., 1966
	α-methyl deriv. more active v. echo 6, but less selective than HBB. α-methyl less active than HBB against poliovirus. Three or four carbon aliphatic substituents have decreased activity	Suggests that decrease of the carbinol function may be of importance for activity. Unfavourable steric effects of large substituents.	Eggers and Tamm, 1961a O'Sullivan and Wallis 1963a
	5-trifluoromethyl and corresponding α-methyl substituted compound have highest inhibitory activity among group, but are more toxic. 5-phenyl moderate increase in activity but marked toxicity inc. 5-nitro reduced activity but inc. toxicity. 5-methyl as active as HBB but less selective, little protective effect against poliovirus at max. tolerated concentration.		Eggers and Tamm, 1961a Eggers and Tamm, 1961a O'Sullivan and Wallis, 1963a
	Little effect on activity but toxicity of p-chlorophenyl and β-benzyl thio derivatives much increased		
	Activity destroyed	Importance of aromaticity of the 2-substituent relative to the steric or geometric effect	Eggers and Tamm, 1961a
	Both as active as HBB against echo 6. 2-α-methoxy derivative also as active against polioviruses	No intramolecular hydrogen bonding involving the hydroxyl is possible. Such bonds not essential for activity in benzimidazole series	Tamm and Eggers, 1963a O'Sullivan et al., 1965
	No virus inhibitory activity	Aralkyl nature of the 2-substituent essential for activity in the benzimidazole series	Tamm et al., 1969

Tamm and Caliguiri conclude that the mechanism of inhibitory activity of HBB is dependent upon the structural characteristics of the molecule per se. As previously mentioned the authors' evidence suggests that one (or both) of the nitrogen atoms, rather than the hydroxyl group of HBB, are essential for binding HBB to its target. They state that the overall geometry of the entire HBB molecule appears to be of importance for activity and that the electron withdrawing capacity of the entire molecule is also reasonably essential. The spatial relationship of the planar phenyl moiety to the planar benzimidazole moiety is apparently important.

Comparing HBB with guanidine (Fig.1.6) the authors note that although these two compounds differ markedly in molecular size, aromaticity, steric points, number of nitrogen atoms and basicity they still have similar actions. Hence one would expect them to share a common feature involved in the virus-inhibiting activity (Tamm and Eggers, 1963a). The authors suggest that the $\rangle N\text{-}\overset{|}{C}=N-$ sequence may be such a feature. However, this sequence is not sufficient for activity in HBB since 2-(α-hydroxybenzyl)imidazole and 2-hydroxymethylbenzimidazole are both completely inactive. Tamm and Caliguiri conclude that the overall configuration of HBB appears to be of critical importance to its activity.

A different aspect of structure-activity relationships, namely the importance of the lipophilic characters of 1-substituted derivatives of HBB in relation to antiviral activity has been studied (O'Sullivan and Ludlow, 1972).

Mode of Action

HBB does not act by inhibiting some vital metabolic activity in host cells. At virus inhibitory concentrations it has no effect on metabolic activity, morphological appearance or division of uninfected cells (Eggers and Tamm, 1962, 1963). Gwaltney (1968) found a linear increase in the proportion of rhinovirus strains inhibited by increasing concentrations of HBB. At high concentrations (383 and 574 μM, Gwaltney, 1968 or 493 μM Kadin et al., 1964) morphological changes in the cells were observed. At such concentrations the virus strains could be inhibited for two possible reasons. The inhibition could be non-specific, just as a secondary effect on cell toxicity, or the inhibition could be virus-specific, where the cytotoxic effects produced by the drug are of no consequence to virus replication. The correlation between proportion of virus strains inhibited and compound concentration suggested that inhibition at higher concentrations was due in part to some direct effect upon the virus. However, it did also suggest that no new mode of action such as cell poisoning had become predominant. This was supported by the fact that in the presence of cytotoxic concentrations of D-HBB-HCl, WI-38 cells still supported the growth of a number of rhinovirus strains.

HBB does not affect virus adsorption, penetration and uncoating (Eggers and Tamm, 1962; Eggers et al., 1963; Eggers et al., 1965).

The antiviral activity of HBB has been attributed to interference with the replication of virus RNA (Eggers and Tamm, 1962, 1963). Baltimore and co-workers (1963) reported that HBB interferes with the synthesis of poliovirus-induced RNA polymerase activity. RNA chains can be completed in the in vitro system but few or no new chains can be initiated. Conclusions from these findings were therefore that HBB might interfere with the messenger function of virus RNA in the synthesis of virus RNA polymerase (Baltimore et al., 1963). However, from more recent findings (Caliguiri and Tamm, 1968 a,b) this conclusion appears to be invalid.

Caliguiri and Tamm (1968 a,b) provide evidence to support the view that HBB, like guanidine, blocks the initiation of new chains of virus RNA. HBB has no detectable effect on the messenger function of virus RNA. Their argument for this view was as follows.

Results obtained with guanidine indicate that the inhibitor has an effect on the initiation of virus RNA chains. If this is true virus RNA polymerase in inefected cells would be expected to become deficient in or devoid of RNA template soon after treatment with inhibitor is begun. Since the enzyme assayed *in vitro* is enzyme-template aggregate, the failure of reassociation with template RNA molecules in drug-treated cells could manifest itself as decreased polymerase activity of the enzyme assayed *in vitro*. The fact that HBB and guanidine have no effect on RNA synthesis when added to the *in vitro* assay system for virus RNA polymerase (Baltimore *et al.*, 1963) may be taken as evidence that the inhibitors have no effect on the growth of previously initiated RNA chains. Very little, if any, chain initiation is likely to take place in aggregate enzyme preparation assayed *in vitro*.

The inhibition of the formation of virus proteins, virus particles and virus-induced cytopathic effect in the presence of HBB are secondary to the primary effect of virus RNA synthesis (Eggers and Tamm, 1962; Halperen *et al.*, 1964 a,b; Bablanian *et al.*, 1966; Skinner *et al.*, 1968). HBB has no detectable effect on the messenger function of virus RNA in the synthesis of virus capsid proteins. Although it seems likely that HBB has no effect on the assembly of complete virus particles no findings have been obtained to provide conclusive evidence for this (Caliguiri and Tamm, 1968b).

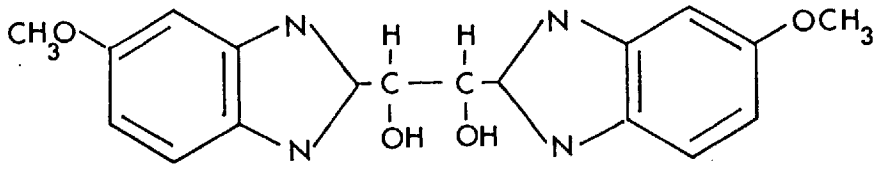
It is important to note that in the studies of benzimidazole derivatives so far carried out, no evidence of a connection between antiviral activity and the function of vitamin B_{12} in nucleic acid biosynthesis has been found.

Bisbenzimidazoles

The most promising rhinovirus inhibitory compounds yet to be found among the benzimidazoles are certain bisbenzimidazoles.

The antipoliovirus activity of the bisbenzimidazole, 1,2-bis(2-benzimidazolyl)-1,2ethanediol, was first reported by O'Sullivan and Wallis (1963a). The synthesis and antipoliovirus activity of 3 derivatives of this compound including 1,2-bis(5-methoxy-2-benzimidazolyl)-1,2-ethanediol (Fig.1.6) was later reported by Akihama et al., 1968). This compound was found to have a minimal inhibitory concentration of 0.2μM against poliovirus type 1 (Mahoney strain) by the tube dilution method. This activity is about 1000 times greater than that of HBB or guanidine.

Fig.1.6 Structural formula of
1,2-bis(5-methoxy-2-benzimidazolyl)-1,2-ethanediol



The antiviral effect of this 5-methoxy derivative, designated Abbott 36683, was further tested against 55 strains of rhinovirus, 3 poliovirus types and strains of types A and B Coxsackie virus (Schleicher et al., 1972). It was found to be active against all viruses tested and hence has a spectrum of picornavirus inhibitory activity wider than any other benzimidazole tested. Moreover the compound was found to possess activity both prophylactically and therapeutically against rhinoviruses

in that infected cultures could be cleared of virus-induced cytopathic effects up to 90 hours after infection.

A series of bisbenzimidazoles and related monobenzimidazoles were tested for antirhinovirus activity and structure activity relationships were determined (Roderick et al., 1972) but none was found to be more active than Abbott 36683. Roderick and co-workers (1972) found that the following structural features of the bisbenzimidazoles were essential for activity: (1) no substituent in the 1 position of the benzimidazole (2) a 5-methoxy or 5-ethoxy substituent, and (3) a 2-carbon chain, unsubstituted or substituted by hydroxyl, connecting the 2 benzimidazoles.

As yet no detailed studies to determine the mode of action of this benzimidazole have been carried out. However, the antiviral activity of Abbott 36683 has been found to be due neither to virus inactivation nor to inhibition of virus adsorption to or penetration into the cell (Schleicher et al., 1972).

Limited in vivo studies with Abbott 36683 have been made. It was shown that antiviral levels of this compound can be attained in mouse sera with an oral dose as small as 10 mg/kg (Shipkowitz et al., 1972). Virus shedding in chimpanzees infected with rhinovirus strains was prevented with a high dose of compound (100 mg/kg for 4 days) but adverse side effects developed. A single dose of 100 mg/kg afforded no protection and an intermediate dose produced only partial protection. Systemic use of this compound as a treatment for colds does not therefore look very promising.

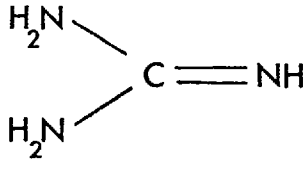
1.7.3 GUANIDINE

A detailed review of the picornavirus inhibitory compound, guanidine, has been made by Tamm and Caliguiri (1972) and it is felt unnecessary to duplicate their work in this survey.

However, it should be stated that this compound, like HBB, has been of major importance in the study and elucidation of virus-induced inhibition of cell metabolism, translation and transcription of virus RNA and assembly of virus particles.

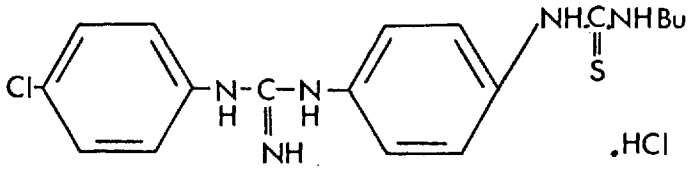
Of the picornaviruses the human enteroviruses form the largest group of guanidine sensitive viruses. Only a few rhinoviruses have so far been examined for sensitivity to this compound and have been shown to have varying sensitivities. Additionally guanidine is not useful in the chemotherapy of virus infections in animals and hence remains only of academic significance.

Fig.1.7 Structural formula of guanidine



High rhinovirus inhibitory activity *in vitro* has recently been reported for a substituted guanidine (Bucknall *et al.*, 1973). This compound, 1-*p*-chlorophenyl-3-(*m*-isobutylthioureido) phenyl guanidino hydrochloride, designated ICI 65,709,(Fig.1.8) was tested against 25 rhinovirus serotypes and a wide range of other (DNA and RNA) viruses, by the tube culture technique.

Fig.1.8 Structural formula of 1-*p*-chlorophenyl-3-(*m*-isobutylthioureido) phenyl guanidino hydrochloride (ICI 65,709)



Activity against all of the 25 rhinovirus serotypes was reported, the compound inhibiting virus-induced cytopathic effects by 50% at concentrations ranging from 0.05 to 0.4 μg/ml. Also the therapeutic ratio of the compound (the concentration producing 50% cytotoxic effects divided by the concentration producing 50% inhibition of virus-induced cytopathic effects) was found to range from 15 to 120 for these rhinovirus serotypes. The compound was also found to be active against certain echo-, coxsackie-, vaccinia, herpes, Semliki Forest and Sindbis viruses. It was not active, however, against equine rhino, pseudorabies, influenze A_0, A_1, A and A_2, parainfluenza, corona- 229E or respiratory syncytial viruses.

The cell line in which the compound was tested was found to be of importance. For example, rhinovirus type 2 was found to be sensitive to inhibition by the compound when grown in cultures of human embryo lung cells, but in cultures of KB, HeLa or primary monkey kidney cells this serotype was insensitive to inhibition by the compound.

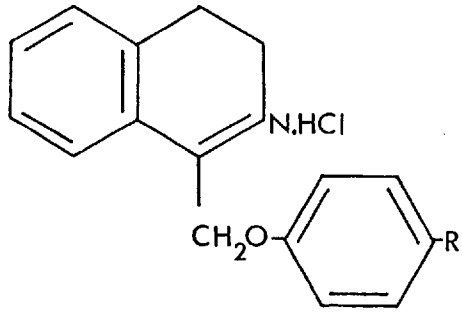
1.7.4 THE ISOQUINOLINES

A third group of compounds to receive attention as possible antirhinovirus agents is the synthetic isoquinolines, first investigated by Larin and co-workers (1967) and Brammer and co-workers (1968). The rationale for this series of compounds was to synthesise a compound with anti-neuraminidase activity. The enzyme neuraminidase is possessed by a number of different viruses, including influenza (Howe et al., 1960), parainfluenza (Darrell and Howe, 1964), Newcastle disease (Rott, 1963) and mumps (Tamm and Horsfall, 1962) viruses. Neuraminidase is present on the surface of particles of such viruses and evidence suggests that it facilitates the release of the virion from infected cells (Ackerman and Maassab, 1954; Seto and Rott, 1966; Kilbourne et al., 1968; Webster and Laver, 1967). Inhibition of neuraminidase prevents the release of the virion and it was therefore thought that a compound having affinity for the neuraminidase might also exhibit activity against viruses possessing neuraminidase.

The isoquinolines were developed after the discovery of activity against both bacterial (Vibrio cholerae) and viral (influenza) neuraminidase within this series of compounds (Larin et al., 1967; Brammer et al., 1968). The compounds to be found with highest activity were memotine, 1-(p-methoxyphenoxymethyl) -3,4-dihydroisoquinoline hydrochloride, and famotine, 1-(p-chlorophenoxymethyl) -3,4-dihydroisoquinoline hydrochloride, designated UK 2371 and UK 2054 respectively: their structural formulae are given in Fig.1.9.

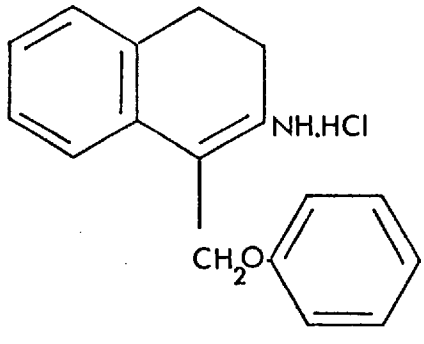
Two methods were used to determine antiviral activity (Larin et al., 1967; Brammer et al., 1968). In the first method the drug was incubated with the virus before titration of the remaining virus in a susceptible tissue culture or embryonated egg system. In the second method activity was demonstrated by methods based on inhibition of specific viral cytopathic effect and plaque inhibition when the compound was added at the time of virus inoculation. A summary of the results obtained by both sets of workers is shown in Table 1.7.

Fig.1.9 . Structural formulae of isoquinolines



dihydroisoquinolines

1-(p-methoxyphenoxymethyl) -3,4-dihydroisoquinolinehydrochloride (memotine)	UK 2371 (memotine)	R = OCH_3
1-(p-chlorophenoxymethyl)-3,4-dihydroisoquinolinehydrochloride (famotine)	UK 2054 (famotine)	R = Cl



tetrahydroisoquinoline

1-(phenoxymethyl)1,2,3,4-tetrahydroisoquinoline	UK 2792

Table 1.7 Antiviral spectrum of isoquinoline derivatives UK 2054, UK 2792 and UK 2371. (Summary of results obtained by Brammer et al., 1968 and Larin et al., 1967).

Method used to demonstrate anti-viral activity	UK 2054	UK 2792 *	UK 2371 ≠
Loss of infectivity following a preincubation period of virus with compound	Influenza A, B & C Fowl Plague Parainfluenza 1, 2 & 3 Newcastle disease Measles Herpes Rubella	Influenza A_2 & B Fowl Plague Parainfluenza 1 Newcastle disease	Influenza A,B & C Parainfluenza 1,2 & 3 Fowl Plague Newcastle disease Rubella
Inhibition of virus replication when compound added at time of virus inoculation	Echovirus 2,5,6, 11,12,13 Rhinovirus 1A & 2 Respiratory syncytial Reovirus 2 Rubella	Echovirus 6,7,8, 9,11,12 Coxsackie viruses A9, A21, B1, B4, B5 Fowl Plague	Echovirus 6,7,11 Reovirus 2 Rubella

* Tested by Brammer et al. only ≠ Tested by Larin et al. only

Larin and co-workers (1967) tested a wide range of picornaviruses against UK 2054 and UK 2371 and found the drugs to have no effect on poliovirus types 1 and 2 and many coxsackie and echovirus serotypes under either of the testing conditions. Those echovirus serotypes that were sensitive were found to vary greatly in their degree of sensitivity to the drugs.

Results obtained by Brammer and co-workers (1968) show that the activity of the dihydroisoquinolines as represented by UK 2054 is mainly a direct virus inactivating effect, shown especially by its effect on myxoviruses. The activity of tetrahydro derivatives like UK 2792, 1, 2, 3, 4 tetrahydro isoquinoline, is chiefly associated with virus cell relationships and unlike the dihydro derivatives these compounds do not inhibit neuraminidase.

Further studies on the antineuraminidase activity (Coombes et al., 1970) and in vitro and in vivo antiviral activity (Reed and Bynoe, 1970; Coombes et al., 1970) of these compounds have been published.

Mode of Action

At virus inhibitory concentrations of compounds UK 2054 and UK 2371 have been found to have no cytotoxic effects and no effect on the replication of insusceptible viruses (Larin et al., 1967). This shows that the virus inhibitory effect is specific and not due to any cytotoxic effects. In addition these compounds had no effect on influenza A_2 virus RNA or haemagglutinin production in BHK cells or on the influenza replicase enzyme (Ruck et al., 1969) isolated from the chorioallantoic membrane of influenza A_2 infected eggs (Coombes et al., 1970). UK 2371 has no direct action on viral haemagglutinin (Hobson, Flockton and Gregory, 1969).

From further experiments carried out by Coombes and co-workers (1970) on the antineuraminidase, virus inactivating and virus inhibitory effect

of these compounds in single and multicycle experiments, they concluded that the antineuraminidase activity observed at high concentrations (100 µg/ml) of UK 2054 was a secondary effect arising from extensive disruption of the structure of the virus particle. This activity they concluded was not associated *per se* with the antiviral effect of this compound, as had previously been suggested by Brammer and co-workers (1968) who reported that dihydroisoquinolines possessing both strong and weak antineuraminidase activity showed comparable virus inactivating activity.

However, it is true that the mechanism of action of dihydroisoquinolines has been presumed to be due at least in part to their antineuraminidase activity. This activity has of course been the basis of methods for antiviral screening (Edmond *et al.*, 1966; Wagner *et al.*, 1967; Brammer *et al.*, 1968; Haskell *et al.*, 1970). Such screens involved the estimation of neuraminidase activity as follows.

Viral (influenza) or bacterial (*Vibro cholerae*) neuraminidase is incubated with a suitable substrate such as N-acetylneuraminyl-lactose. The N-acetylneuraminic acid (NANA) released through enzyme action is then determined colormetrically according to the thiobarbituric acid assay procedure described by Warren (1959).

If a drug is to be tested for neuraminidase inhibitory activity it is added to the enzyme-substrate reaction mixture and is still present in the mixture on which an NANA determination is made.

Recently it was discovered that compounds UK 2054 and UK 2371 in fact do not inhibit the enzyme neuraminidase but inhibit only the development of colour in the thiobarbituric acid procedure (Shinkai and Nishimura, 1972). Hence results from screens using this procedure are false. UK 2054 and UK 2371 are the only isoquinolines so far to be tested for their ability to inhibit the colour development reaction and, although it seems likely that other so-called antineuraminidase compounds within this series also inhibit the reaction, this has not yet been proven.

Clinical Trials of Isoquinolines

In vivo studies of the antiviral effect of famotine and memotine have involved mainly influenza virus serotypes. A paper summarises and evaluates the results of clinical trials with famotine and memotine before 1969 (Williamson and Jackson, 1969). In man, challenge, prophylactic and therapeutic studies have been carried out.

Famotine and memotine were first evaluated in man in challenge studies. Reaction to challenge of memotine (Beare et al., 1968) and famotine (Meenan and Hillary, 1969 a,b) by influenza B was assessed on the parameters of viral isolation, antibody response and clinical reactions. In both studies, and in a later one with influenza B, the response to challenge was less in the drug treated group, although the difference was not always statistically significant. With influenza A_2 challenge studies only that in which strain A_2 Leningrad/4/65 was used did memotine show any significant effect (Beare et al., 1968; Reed et al., 1970). Other strains of influenza A_2 were not found to be affected in vivo although in vitro tests involving direct inactivation of the virus had shown them to be as sensitive at the Leningrad strain (Reed et al., 1970). However, some of the strains tested in vivo had previously been tested by other workers in ovo (Larin et al., 1967) and similar differences in sensitivity had been observed. Hence the nature of the host cell is important in determining the antiviral activity.

Prophylactic administration of famotine orally or topically reduces the incidence of clinical reaction to certain influenza A and B strains. The reduction is particularly evident with topical administration where decreased symptomatology is not associated with impaired antibody responses as is the case with oral administration. Memotine given orally reduces antibody responses and incidence of clinical reactions (Williamson and Jackson, 1969). In a more recent trial of famotine and memotine in mice (Coombes et al., 1970) the drugs were found to be

effective in prolonging the mean survival time of mice infected with influenza A/PR8, although a significant increase in mean survival time was not reproducible. Both compounds were also effective in protecting such infected mice from lung consolidation.

Studies on the in vitro and in vivo antirhinovirus activity of famotine and memotine have been made. Reed and Bynoe (1970) found that famotine but not memotine decreased the yield of rhinovirus types 2, 4, 9 and 42 in HeLa cells but this activity was not evident in human embryo lung cells. In vivo challenge studies of memotine against parainfluenza type 2 (sensitive to the drug in vitro) and of famotine against thinovirus type 9 gave negative results, however. In another study famotine was found to be ineffective in altering the course of a rhinovirus 1B infection in a group of volunteers (Meenan and Hillary, 1969)

No more recent trials on the efficacy of the isoquinolines, famotine and memotine in human respiratory infection have been carried out. It would appear that existing reports of trials have not been encouraging enough to warrant further investigation of these particular drugs.

1.7.5 THIOUREAS

The thioureas is another series of compounds containing the -NH-CS-NH- group found in the thiosemicarbazones and to which some workers ascribe the antiviral activity of the thiosemicarbazones (Thompson, 1964). On screening a series of N-2-hydroxyphenylthiourea (I) and N-2-hydroxyphenylurea (II) compounds (Fig.1.10), N-methyl-N-(2-hydroxyphenyl)-N-benzylthiourea (III) was found to reduce virus-induced cytopathic effects and virus yields in cells infected with poliovirus types 1, 2 and 3 (Shindarov et al., 1970 a). At a concentration of 25 µg/ml (less than the maximum subtoxic concentration) virus yields were reduced by 1.0 to 1.5 log units.

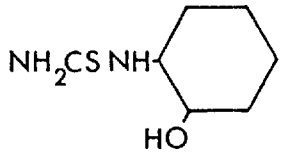
The antiviral activity of 12 derivatives of N-2-hydroxyphenylthiourea against other picornaviruses was later studied by gradient plate plaque inhibition tests and yield reduction studies (Shindarov et al., 1971). Again the reduction in yield at the maximum subtoxic concentration (generally less than 50 µg/ml) was less than 2 log units.

Shindarov and co-workers (1970b, 1971) found that the inhibitory effect of the compounds in experiments on cultures in liquid medium was more pronounced than in plaque reduction tests. This they thought was probably due to the quicker diffusion of the compounds into the cells.

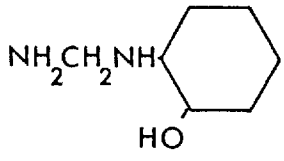
The activity of the N-2-hydroxyphenylthiourea derivatives and N-2-hydroxyphenylurea derivatives prompted the study of N-phenyl-N'-aryl- or alkylthioureas (Galabov et al., 1972a,b). In a screen of 54 derivatives against poliovirus by the gradient plate plaque inhibition test 12 were found to have high or significant activity and this was confirmed in yield reduction studies. The authors noted a correlation between activity and toxicity of the compounds in that the more toxic compounds were less active.

Eleven of the above 12 compounds found to have antipoliovirus activity were tested against other picornaviruses (Coxsackie B1, ECHO 19

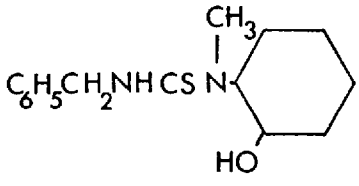
Fig.1.10 Structural Formulae of thioureas and related compounds



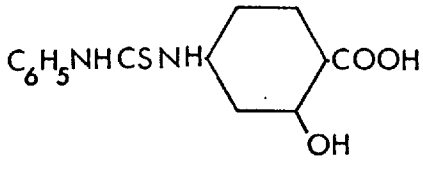
I N-2 hydroxyphenylthiourea



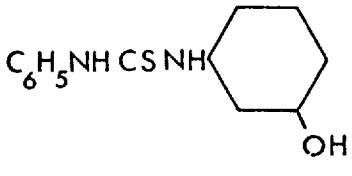
II N-2 hydroxyphenylurea



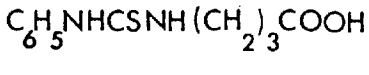
III N-methyl-N(2-hydroxyphenyl)-N′-benzylthiourea



IV N-phenyl-N′-4-carboxy-5-hydroxy-phenylthiourea



V N-phenyl-N′-3-hydroxyphenyl-thiourea



VI γ - phenylthioureidobutyric acid

and foot-and-mouth disease virus). Most viruses were inhibited to a similar extent by the most active compounds, N-phenyl-N'-4-carboxy-5-hydroxyphenylthiourea (IV), N-phenyl-N'-3-hydroxyphenylthiourea and 8-phenylthioureidobutyric acid (VI), but the effect of less active compounds was differential.

Structure Activity Relationships

Structure activity relationships among the N-2-hydroxyphenylthioureas (Shindarov et al., 1971) and the N-phenyl-N'-aryl or alkylthioureas (Galabov et al., 1972a) were studied. Shindarov and co-workers (1971) concluded that the simultaneous presence of an aromatic ring and a methyl or propyl radical at the N atom and of a benzyl radical at the N' atom are essential to activity in the N-2-hydroxyphenylthioureas (as in III). Respective urea derivatives were also active but less so.

In the N-phenyl-N'-aryl- or alkylthioureas (Galabov et al., 1972a) derivatives with the hydroxyphenyl radical at the N' atom have a strongly pronounced antiviral effect when the hydroxyl group is in the meta-position to the N-phenylthiourea residue as well as in the combination with carboxyl in the para-position (as in IV & V). The effect of active derivatives of N-phenyl-N'-aryl or alkylthiourea is probably determined by the RNH-CS-NH moiety (refs. Shindarov et al., 1971; Thompson 1964) and the presence of the hydroxyl group in the meta-position in the benzene ring at the N'-atom. Antiviral activity requires the hydroxyl group to be unsubstituted. The spatial location of the hydroxyl group in the molecule is of major importance, and Galabov and co-workers (1972a) show that in the highly active compounds the hydroxyl group is almost equally removed from the NH-CS-NH- moiety.

Mode of Action

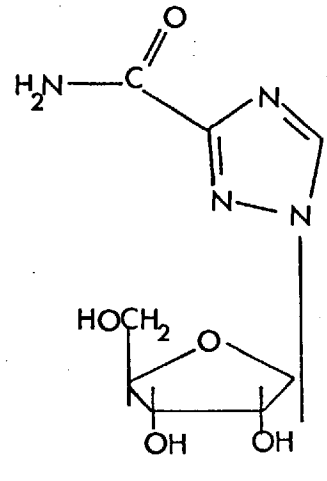
Limited studies on the mode of action of thioureas have been made. Shindarov and co-workers (1970 a,b) found N-methyl-N-(2-hydroxyphenyl) N'-benzylthiourea to have no virucidal effect nor any effect on adsorption

or penetration of poliovirus to HeLa, human embryonic kidney or KB cells. The process sensitive to inhibition in the multiplication cycle begins about 2 hours before the end of the latent phase and continues during the exponential phase. Having in mind the data regarding the stages in the synthesis of virus-induced proteins and replication of viral RNA these workers suggested that the compound most probably inhibits the synthesis of early as well as late viral proteins.

1.7.6 VIRAZOLE

Virazole, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Fig.1.11) was first synthesised by Witkowski and co-workers (1972) and found by them to possess reproduble and significant activity against a broad spectrum of DNA and RNA viruses, including rhinovirus, measles, herpes simplex virus and vaccinia virus, in tissue culture. The drug was further tested in vitro against herpes simplex types 1 and 2, pseudorabies, murine cytomegalo, vaccinia, myxoma, adeno, parainfluenza types 1 and 3, influenza types A_2 and B, rhino types 1A, 1B and 56, Coxsackie B_1, polio type 2, vesicular stomatitis and Semliki Forest viruses, (Sidwell et al., 1972). Virazole has a virus rating of 0.5 or greater against all the tested viruses except pseudorabies, Coxsackie and poliovirus. When drugs known to be active against the above viruses were tested in parallel with Virazole, the latter was found to have the greater activity in most cases (Sidwell et al., 1972). A more detailed quantitative study of the in vitro activity of Virazole was made by Huffman and co-workers (1973). In addition to the viruses already mentioned infectious bovine rhinotracheitis, rhino types 2 and 8, subacute sclerosing panencephalitis, and Newcastle disease viruses were found to be sensitive. Again Virazole was found to have comparable or greater activity against these viruses than known active compounds. In the case of rhinovirus type Virazole had a virus rating (0.7) equal to that for 2-methyl-4[(5-methyl-5H-as-triazino (5,6-b)indol -2-yl)amino]-2-butanol (SK&F30097).

Fig.1.11 Structural Formula of Virazole 1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide



The activity of Virazole was discernable in a variety of cell systems and parameters for measuring antiviral activity but it was noteworthy that the compound was less active against DNA viruses when grown in VERO cells rather than in chick embryo, Rk-13, KB, or HeLa cells. This then emphasises the importance of determining the activity of an antiviral compound in a number of cell systems.

The *in vivo* activity of Virazole against a number of viruses has been studied. Applied topically it has been found to inhibit herpetic keratitis in rabbits and tail lesions induced by herpes, vaccinia, and vesicular stomatitis viruses in mice (Sidwell *et al.*, 1973). Influenza A_0, A_2 and B and parainfluenza virus infection in mice is inhibited by intraperitoneal administration and oral or aerosol treatment of parainfluenza virus infections is also effective (Sidwell *et al.*, 1972; 1973; Khare *et al.*, 1973). No study of *in vivo* testing against rhinoviruses has been reported.

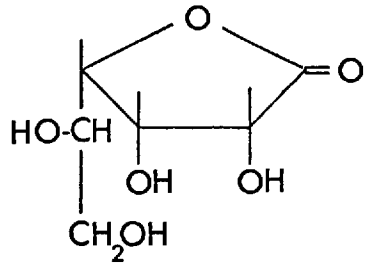
The mode of antiviral action of Virazole has not been fully explored. However, experiments have indicated that it is not virucidal and it is not an inducer of interferon (Sidwell *et al.*, 1972). The drug appears to act at an early stage in viral replication, although its effect on adsorption to or penetration into the cell has not been studied (Huffman *et al.*, 1973). The antiviral effect of the compound is reversible upon removal of the compound from the medium surrounding infected cells. The $CCED_{50}$ (50% cellular cytotoxic dose) of Virazole to chick embryo cells is approximately 1,000 µg/ml, although concentrations as low as 10 µg/ml have caused slight (15%) inhibition of total cell protein after 72-hour incubation (Huffman et al., 1973). This cytotoxicity would seem to be quite great considering for example that at 10 µg/ml the yield of infectious virus from type 1 herpes simplex infected KB cells was reduced by only 0.7 log unit (from $10^{4.7}$ $TCID_{50}$/0.2ml to $10^{4.0}$ $TCID_{50}$/0.2ml).

Virazole therefore appears to be one of the first synthetic, non-interferon inducing broad-spectrum antiviral agents. However, despite its broad spectrum its future as a clinically useful drug is not yet obvious due to its high toxicity.

1.7.7 ASCORBIC ACID

The efficacy of ascorbic acid (Fig.1.12) as a prophylactic and therapeutic agent against the common cold is controversial. However, it is widely recommended for this purpose by medical practitioners and many people use this treatment on themselves and believe in its effectiveness.

Fig.1.12 Structural formula of ascorbic acid



In vitro studies have shown no evidence that ascorbic acid protects cell cultures against several respiratory viruses (including rhinovirus strains) (Walker et al., 1967). However, many claims have been made that the ingestion of the vitamin in doses ranging from 30 mg to 1000 mg or more a day has a beneficial effect on the course of respiratory infection. Controlled trials have produced evidence both for and against the efficacy of ascorbic acid. Early trials based on daily supplements of 200 mg of ascorbic acid showed the vitamin to have little or no effect on the severity of colds (Cowan et al., 1942; Glazebrook and Thomson, 1942). However, in the trial by Glazebrook and Thomson the vitamin did have a beneficial effect on some respiratory tract infections (probably bacterial). In a later trial volunteers were inoculated with viruses known to cause colds and were given 3g of ascorbic acid or placebo tablets daily for three days (Walker et al., 1967). The number, duration and severity of the resulting colds were the same in the treated and control groups, thus leading the authors to conclude that the administration of ascorbic

acid is of no value in the prevention and treatment of colds produced by five known viruses. A recent randomized trial in England also found doses as high as 3g per day to be ineffective in treating colds (General Practitioner Trial, 1968).

Many trials, however, show ascorbic acid to have a beneficial effect in respiratory illness. Results of a trial in 1961 involving 279 skiers showed a reduction of 61% in the number of days illness from upper respiratory tract infections and a reduction of 65% in the incidence of individual symptoms, in groups treated with 1g of ascorbic acid daily compared with those groups given placebo tablets (Ritzel, 1961). However, although this difference was significant the results were taken from only one week's observations of the volunteers. In two later trials subjects were observed for much longer. In a blind trial of 90 volunteers (half given 1g of ascorbic acid daily and half given placebo tablets) records were made for 15 weeks (Charleston and Clegg, 1972). The incidence of colds was reduced by 49% in the supplemented group compared with the control group, this difference being significant at the 0.002 level. In another trial involving 1000 volunteers (of which 818 subjects remained in the trial for at least 2 months), the group being treated with 1g ascorbic acid showed less illness in terms of the average number of colds and days of sickness (Anderson et al., 1972). The difference, however, was not statistically significant. In contrast there was a significant difference in the number of subjects who remained well throughout the period studied: the treated group had 30% fewer days off than the control group (significant at the 0.001 level). This result is important both theoretically and practically. In a later trial made by Wilson and Loh (1973) the authors concluded, among other things, that ascorbic acid at a daily dose of 500 mg reduces the severity and total intensity of colds in girls, but does not benefit cold symptoms in boys. Criticism of this conclusion was made by other authors (Kinlen and Peto, 1973) who say that none of their tests were significant at the 1% level

and of those significant at the 10% level 4 were in favour of ascorbic acid and 5 against. In their opinion the data of Wilson and Loh (1973) can best be explained if ascorbic acid is entirely without effect on the frequency and severity of colds. It therefore appears that still more trials are required before any conclusion as to the future of ascorbic acid as a prophylactic or therapeutic agent in virus respiratory disease can be made.

The possible effect of ascorbic acid in the production of tissue resistance has been investigated. Ascorbic acid concentrations diminish in the leucocytes and increase in the plasma during the early days of a cold and return to normal 10 days after the onset of symptoms (Wilson and Loh, 1969). This has been confirmed by other workers (Hume and Weyers, 1973). The leucocyte concentrations of ascorbic acid can be raised by taking doses of ascorbic acid from 0.2 to 10g daily. The change in leucocyte and plasma concentration of ascorbic acid during a cold enables common-cold symptomatology to be correlated with alterations in ascorbic acid metabolism.

Pauling has long been an advocate of the use of ascorbic acid to fight the common cold. He presents his views in the paper-back book 'Vitamin C and the Common Cold' (Pauling, 1970). He believes that although the minimum daily requirements of ascorbic acid, perhaps 50 mg a day, are sufficient to prevent gross disease such as scurvy, a higher intake of up to 50 or 100 times more is the optimal daily intake of this vitamin for man, and will increase health further. This, he believes, will decrease the incidence and the severity of symptoms of common upper respiratory tract infections. However, if ascorbic acid is to be taken routinely at the high doses that Pauling recommends, the possible harmful effects of such doses must first be thoroughly investigated.

An early study of ascorbic acid found no harmful effects when the vitamin was taken for 3 months at a daily dose of 1g (Lowry et al., 1954)

and ascorbic acid has been prescribed in large doses without observing any harmful side effects (Poser, 1972). Some workers, in contrast, have observed that ascorbic acid at 4 to 9g daily increases the excretion of urinary oxalate (Lambden and Chrystowski, 1954) and it is thought to be a causative factor in the production of oxalic acid stones (Smith et al., 1972). Finally, mild gastrointestinal disturbances have been observed with large doses (Hume and Weyers, 1973) and a tendency to develop scurvy on coming off prolonged high dosages of ascorbic acid has been reported (Rhead and Schrauzer, 1971). This is probably a result of enhanced ability to metabolize ascorbic acid.

1.7.8 OTHER RHINOVIRUS INHIBITORY COMPOUNDS

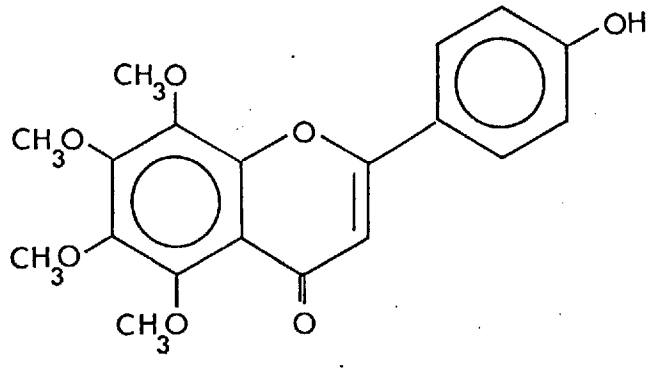
Compounds which have received minor attention as rhinovirus inhibitory compounds are isoprinosine and flavinoids.

Isoprinosine is the paracetamidobenzoic acid salt of inosine dimethylaminoisopropanol in a 1:3 molar ratio. It has been reported to inhibit the multiplication of both RNA and DNA viruses including herpesvirus hominis, vaccinia, poliomyelitis (type 3), Echo (type 11), influenza (Muldoon et al., 1972; Chang and Weinstein, 1973) and rhinovirus types 2,21 and 44 to be resistant to isoprinosine in vitro.

A trial of isoprinosine against simultaneous rhinovirus type 9 and type 31 infection of volunteers was carried out in view of the fact that the then existing reports of the antiviral activity of isoprinosine conflicted (Soto et al., 1973). Results however showed no evidence of useful rhinovirus inhibitory activity. Results of in vivo trials of the compound against influenza virus have shown demonstrable activity in animals (Muldoon et al., 1972) but not in humans (Longley et al., 1973).

Antirhinovirus activity has also been claimed for 4'-hydroxy-5,6,7,8-tetramethoxyflavone (Fig.1.13). This compound is found to have activity against types 13 and 56 but not against type 1A rhinovirus (Burnham et al., 1972). The criterion used for the evaluation of activity was the inhibition of virus-induced cytopathic effect and the virus rating system was used to assess activity. The drug had virus ratings of 0.1-0.2, 0.5 and 0.5-0.7 against types 1A, 56 and 13 respectively (for comparative purposes SK&F 30097 also had a virus rating of 0.5-0.7 against rhinovirus 13). However, the toxicity of the compound was high. In virus yield experiments the compound reduced virus yield by 0 to 10^5 $TCID_{50}$ at concentrations ranging from 10 - 1000µg/ml (a more or less linear relationship) and the drug was found to be cytotoxic, as deduced from morphological appearance of cells, from 32µg/ml. The compound is therefore far less selective than SK&F 30097.

Fig.1.13 Structural formula of 4'-hydroxy-5,6,7,8-tetramethoxyflavone



It is probably of interest to note here that the antiviral action of hydrogen peroxide against rhinoviruses has also been studied (Mentel and Schmidt, 1973). These workers thought that in view of the good compatibility of this substance with the nasal mucosa its in vitro rhinovirus inhibitory activity should be tested. They found that three strains of rhinovirus (types 1A, 1B and 7) could be inactivated in 6 to 8 minutes by a 3% hydrogen peroxide solution. The time for inactivation increased with decreasing peroxide concentration. On the basis of these results Mentel and Schmidt suggest that the virus inhibitory activity of hydrogen peroxide should be checked in volunteers.

1.8 AIM OF WORK

From the study of the literature it has become apparent that a comparison of the results obtained by different workers on the study of rhinovirus inhibitory activity is extremely difficult. This is due to the fact that different workers have used different techniques for the detection and further study of virus inhibitory compounds.

The aim of this work is therefore to compare some of the techniques which have been used for the detection and further study of rhinovirus inhibitory compounds. Particularly this comparison will be made with respect to the sensitivity, reliability, reproducibility and ease of use of the techniques, both as methods for detection and quantitation of virus inhibitory activity. The relative activity of 3 drugs compared with a standard drug will be determined using different systems and the results will be compared. Additionally the relative sensitivities of a number of rhinovirus serotypes compared with a standard rhinovirus will be determined by the same techniques. This is of great practical importance, as little work has previously been carried out to determine whether different rhinovirus serotypes display varying sensitivities to inhibitory compounds. If it is shown that rhinoviruses do exhibit differing sensitivities to the same drug, then it is obviously of importance to determine why such differences exist, and this will also be studied.

It is considered that the effect of variations in experimental conditions, such as changes in the multiplicity of infection used and incubation time of cultures, on the results obtained should be studied, if one wishes to find a technique which will give reproducible results in successive experiments, where such variations may exist. A detailed study of the effect of variations in experimental conditions

has long been necessary, since the results obtained may well give indications as to why discrepancies exist between various workers' results.

In studying the effect of variations in experimental conditions in each of the methods used it is hoped to be able to assess which technique is least affected by such variations, and if possible to find a system in which the value of a drug's activity will be virtually invariant. The basic object of this standardisation is then to be able to measure the absolute activity of a number of drugs against some rhinovirus serotypes.

Additionally experiments are to be carried out to help elucidate the mechanism of action of three rhinovirus inhibitory compounds.

2.0 MATERIALS AND METHODS

The experimental techniques used in this study are described in detail in this section. However, it will be of help to outline here the general plan for experimentation.

The aim of the study was first to compare techniques used for the detection of rhinovirus inhibitory activity. Rhinovirus serotypes and poliovirus type 1 were used as the test viruses, and a large number of compounds, mainly previously untested compounds but including 3 previously tested rhinovirus inhibitory compounds, were tested. Two techniques were used to detect rhinovirus inhibitory activity, the inhibition of virus-induced cytopathic effects in tube cultures and the inhibition of plaque formation.

Tests were then taken further to quantitate virus inhibitory activity, using again tests based on the inhibition of CPE in tube cultures and the inhibition of macroplaques, and also the inhibition of virus yield. Three rhinovirus inhibitory compounds were used in this study, and also a compound found to be active in the general screen for virus inhibitory activity. Techniques for quantitating the activity of these compounds were compared, using again a number of rhinovirus serotypes and poliovirus type 1.

Finally mode of action studies were carried out with the 4 active compounds against 2 rhinovirus serotypes and poliovirus type 1.

2.1 CELLS

2.1.1 Cell lines used

Three continuous and 1 semi-continuous cell lines were used in this study. They were the M-HeLa, HeLa OH3, Vero and Wistar - 38 cell lines.

M-HeLa cells are a rhinovirus sensitive strain of the HeLa cell line, first isolated by Gey et al., (1952) from a carcinoma of the cervix. The rhinovirus sensitivity of the M-HeLa strain was first described by Fiala and Kenny (1966).

HeLa OH3 cells are also a rhinovirus sensitive strain of the HeLa cell line. They are a strain of the HeLa OHIO cell strain described by Conant and Hamparian (1968). The cells were originally obtained from the Common Cold Research Centre, Salisbury, and had been kept in liquid nitrogen storage for 3 years before use.

Vero cells are a continuous epithelial cell line derived from the kidney of a normal adult African green monkey (Cercopithecus aethiops) (Yasumura and Kawakita, 1963). The line used in this study has been propagated in this laboratory for many years and was originally obtained from Flow Laboratories, Irvine, Scotland, who in turn obtained it at a passage number of less than 25 from the American Type Culture Collection.

The Wistar-38 (WI-38) cell line was originally derived from normal embryonic (3 month gestation) lung tissue of a Caucasian female (Hayflick, 1965). The cell line is semi-continuous, having a finite lifetime of 50 population doublings. It has been shown to have one of the broadest human viral spectra of any cell line that has been tested and is especially useful for the isolation of rhinoviruses. The cell line used in this study was kindly supplied by the tissue culture department of Wellcome Research Laboratories, Beckenham, and was priginally obtained from the Medical Research Council, Hampstead Laboratories, London, N.W.3.

2.1.2 Cultivation

All cell lines were propagated in this laboratory. The continuous cell lines were grown as monolayers in 12oz medical flats and WI-38 cells were grown as monolayers in 4oz medical flats, all cells being incubated at 37°. The growth medium for normal propagation consisted of Eagle's medium (Wellcome Reagents, Beckenham, England), 'basal' (EBM) for WI-38 cells or 'minimal essential' (MEM) for other cell lines, and added serum, (10% adult bovine serum (Wellcome Reagents) for M-HeLa and OH3 cells, or 5% foetal calf serum (Flow Laboratories) for WI-38 and Vero cells). For all the cell lines the growth medium also contained 13 mM sodium bicarbonate (Wellcome Reagents) as a buffer and 75 µg/ml penicillin and 125 µg/ml streptomycin sulphate, ('Crystamycin', Glaxo Laboratories, Greenford, Middlesex) to prevent bacterial growth in the cultures.

Cells were removed from the culture vessel surface with 0.02% (w/v) versene in phosphate buffered saline (for HeLa cell lines) or 0.2% (w/v) trypsin in the same solution (for Vero or WI-38 cells). The cell suspension was then spun down in universal bottles on an M.S.E. 'Multex' centrifuge at 2000 r.p.m. for 2 minutes. The supernatant was discarded and the cell pellet was resuspended in growth medium. For routine culturing of cells the continuous cell lines were subcultured twice weekly at a split ratio of 1:6. WI-38 cells were subcultured weekly at a split ratio of 1:2.

Counting of Cells

For purposes other than routine subculturing cells were required to be seeded at a set concentration and were therefore counted. Pellets of cells were suspended in 10 ml of growth medium and 0.1 ml of this was then mixed with 0.1 ml of 0.5% aqueous trypan blue (British Drug Houses, Poole, England). The stained cells were then counted in a Neubauer counting chamber (Hawksley and Sons Ltd., Lancing, England).

Procedure for Cultivation of Cells in other Culture Vessels

Where cell cultures were required for purposes other than routine subculture, cultures were seeded into the appropriate vessel so that they should be at the required state of confluence after incubation at 37° for 1 day (all continuous cell lines) or 2 days (WI-38 cells). For one-step growth cycle experiments confluent cell monolayers were used, but for plaquing, production of rhinovirus stocks and for tube tests 80 - 90% confluent monolayers were required. The following types of culture vessel were used: 60 x 15mm and 100 x 20mm plastic tissue culture dishes (Falcon Plastics, Oxnard, U.S.A.), 25 cm^2 (30 ml) plastic tissue culture flasks (Falcon Plastics), 151 x 15.5mm bacteriological grade soda glass test tubes (Anderman & Co., London , S.E.1) and 200 ml glass centrifuge buckets (Pyrex).

Cells were seeded in growth medium as follows:-

Culture Vessel	No. of cells
Tubes	1×10^5
25 cm^2 flasks	2×10^6
60 x 13 mm dishes	2×10^6
100 x 20 mm dishes	6×10^6
200 ml buckets	6×10^6
4 oz medical flats	6×10^6

Cultures were incubated as described. Dish cultures were incubated in a 'Hotpack' humidified CO_2 incubator (Hotpack, Waterloo, Ontario, Canada) where the atmosphere was maintained at 5% CO_2 in air.

2.2 VIRUSES

2.2.1 Virus Types Used

Poliovirus

Poliovirus type 1 was a laboratory strain obtained from the Virology Department, Wellcome Research Laboratories, Beckenham, Kent.

Rhinoviruses

The rhinovirus serotypes used are listed in Table 2.1

Table 2.1 Rhinovirus serotypes used in study

Rhinovirus Type	Strain	Source
1B	633/16	b
2	HGP	a
3	D-3	b
4	16/60	a
5		b
6	Thompson	a
7	CV11	c
8	CV12	c
9	H-2	b
10	CV14	c
11	CV15	c
12	CV16	c
13	353	c
14	1059	c
15		c
16	11757	b
17	33342	c
18	CV17	c
19	CV18	c

Table 2.1 continued

Rhinovirus Type	Strain	Source
21	CV21	c
22	CV22	c
27	CV28	c
28	H3E	c
29	J-3	b
30	106F	c
31	J-1	b
33	1200	c
36	342H	c
38	201-3C	c
39	209	c
40	1794	c
41	56110	c
43	58750	c
53	FOI 3928	c
54	FOI-3774	c

a. given by Dr. D.A.J. Tyrrell, Clinical Research Centre, Watford Road, Harrow, Middlesex

b. given by Dr. C.R. Spilling, Glaxo Laboratories, Greenford, Middlesex

c. given by Dr. S.E. Reed, Common Cold Research Unit, Harvard Hospital, Salisbury, Wiltshire.

2.2.2 Preparation of Virus Stocks

All rhinovirus stocks were prepared in roller cultures of M-HeLa cells in 200 ml Pyrex glass centrifuge buckets. Poliovirus stocks were prepared in stationary cultures of Vero cells in 4 oz medical flats.

Culture vessels were seeded with cells as described and incubated overnight at 37°. Cell monolayers were then infected with 5 ml of virus inoculum (containing about 2.5×10^6 $TCID_{50}$) to give a multiplicity of infection (MOI) of about 0.5 $TCID_{50}$ per cell. Virus was allowed to adsorb for 1 hour at 34° (37° for poliovirus) and the inoculum was then removed. Maintenance medium (Eagle's MEM, sodium bicarbonate and Crystamycin at previously described concentrations, plus 2% serum) was then added to the bottles (30 ml per bottle). Cultures for rhinovirus stocks were then incubated at 34° overnight in a roller drum apparatus (Matburn) at 10 revolutions per hour. Cultures for poliovirus stocks were incubated stationary overnight at 37°. After overnight incubation microscopic observation of the cultures usually showed all the cells to be rounded and thus infected. Many of the cells were detached from the glass but the remaining ones were removed by shaking. Cultures were then frozen and thawed 3 times and the resulting virus stock was distributed in 1.2 ml aliquots into bijou bottles and stored at -70°.

2.3.1 Compounds

Four compounds were used for the main part of this study. These were 1,2-bis(5-methoxy-2-benzimidazolyl)-1,2-ethanediol (Abbott 36683),* Wellcome 71-123 , 2-methyl-4[(5-methyl-5H-as-triazino (5,6-b)indol-3-yl)amino]-2-butanol (SK&F 30097)≠ and 4-[(8-amino-7-chloro-5-methyl-5H-as-triazino(5,6-b)indol-3-yl)amino]-2 methyl -2-butanol (SK&F 40491)≠ The formulae of these compounds have been given (Figs. 1.3 and 1.6)

* Kindly supplied by Abbott Laboratories, Ltd.
≠ Kindly supplied by Smith, Kline and French Laboratories, Ltd.

2.3.2 Preparation of Solutions

Wellcome 71-123 was readily soluble in water at the concentrations required (up to 200 μM). Solutions of the other 3 compounds were prepared as follows. The required weight of compound was dissolved in 3 or 4 drops of dimethylformamide (DMF) from a Pasteur pipette. This solution was then pipetted into a carefully measured volume of water (usually 100 or 200 ml) which had been heated to about 90°. All compounds could be dissolved up to a concentration of 200 μM by this method. However, solutions of SK&F 30097 and 40491 of greater than 20 μM were always stored at 37° after preparation, since at room temperature such concentrations were found to come out of solution.

Solutions were generally kept for less than 1 month. Experiments were performed to test their stability and retention of antiviral activity over this period.

2.4 TUBE TESTS

2.4.1 Infectivity Titrations

Certain rhinoviruses will not produce macroplaques easily. For the titration of samples of such serotypes the end-point dilution technique was used. It was also used in certain cases as a comparison with macroplaque assays.

For tube titrations M-HeLa or WI-38 cells were used. Tubes were seeded with 1 x 10^5 cells and the cells were incubated as described (2.1.2). Serial tenfold dilutions of virus samples in maintenance medium were then prepared. The 80 to 90% confluent monolayers were then infected with a 0.2 ml inoculum of the appropriate virus dilutions, 3 tubes per dilution. To titrate a stock initially all dilutions from neat to 10^{-6} would be used but a smaller range of dilutions was used if the approximate titre of the stock was known. Virus was allowed to adsorb for 2 hours at 34° and the inoculum was then removed and replaced by 1 ml of maintenance medium. Cultures were then incubated at 34° on a roller drum apparatus at 10 revolutions per hour. Cultures were examined microscopically for CPE twice daily for 4 days and the extent of involvement by CPE was recorded as a percentage of the total cell sheet affected (less than 25%, 25 to 50%, 50 to 75% or greater than 75%).

The titre of the stock was then calculated using the Kärber equation.

$$\log_{10} \text{TCID}_{50} = m - \triangle (\Sigma p - \tfrac{1}{2})$$

The TCID_{50} is the 50% tissue culture infective dose, i.e, that dose which will infect 50% of all cultures. The equation gives the $\log_{10}$ TCID_{50}, which is the log of the dilution of the stock which will infect 50% of all cultures.

In the equation

m = the log dilution corresponding to the largest proportion (i.e. the lowest dilution from which p is calculated).

p = The proportion of cultures infected (i.e. where there is visible CPE).

Σp = the sum of the proportions for the dilutions from m to the highest dilution at which some CPE was produced.

$\triangle$ = the constant dilution interval; for serial tenfold dilutions $\triangle = 1$, hundredfold dilutions $\triangle = 2$.

2.4.2 Detection of Antiviral Activity

Antiviral activity may be detected by the inhibition of virus-induced cytopathic effects in cell cultures. Tube tests were used as one method to detect antiviral activity, in order to determine the spectrum of rhinovirus inhibitory activity of compounds Abbott 36683, SK&F 40491 and SK&F 30097. The rhinovirus inhibitory activity of Wellcome 71.123 was also tested by this method.

Tube cultures of M-HeLa and WI-38 cells were used for this study, since it was hoped to compare results obtained with each of the cell lines. Tube cultures were seeded, incubated and infected as described for infectivity titrations (2.4.1). Each serotype was tested at at least 2 input inocula, 10 and 100 $TCID_{50}$ per culture. Approximately half the serotypes used were also tested at 1 $TCID_{50}$ per culture.

After virus adsorption the inoculum was removed and replaced by 1 ml of normal maintenance medium or maintenance medium containing drug (Abbott 36683 at 2 and 4 μM, SK&F 49491 and 30097 at 20 and 40 μM and Wellcome 71.123 at 80 μM). Uninfected control cultures were given the same range of media and all tests were in triplicate. Cultures were rolled at 34° and the monolayers were examined microscopically

for virus-induced cytopathic effects (CPE) or drug induced cytotoxic effects (CTE) twice daily for 4 days.

Virus-induced cytopathic effects were scored on the following basis:

0 no CPE

1 less than 25% CPE

2 greater than 25% but less than 50% CPE

3 greater than 50% but less than 75% CPE

4 greater than 75% CPE

Toxic effects of the compounds were also scored on this basis.

2.4.3 Quantitation of Antiviral Activity

The method used to quantitate activity in tube tests involves only the technique already described in the detection of antiviral activity (2.4.2). For this, tube cultures were seeded, incubated and infected as described. There were 2 ways in which this test was used to quantitate activity.

In the first case, one virus dose and a range of drug concentrations (doubling concentrations) were used. When observing cultures the extent of CPE was recorded as an approximate percentage of the total cell sheet involved, and from these data a graph of percentage inhibition of CPE against drug concentration (when CPE in the control cultures had reached a standard percentage, 100%) was plotted. Such dose-response lines were then plotted for different virus serotypes and different drugs.

Alternatively, 2 or 3 virus doses (serial tenfold dilutions) and 1 drug concentration were used. The drug concentration chosen was such that CPE was not entirely inhibited but delayed for a number of days. When observing cultures the extent of CPE was recorded on the 0 to 4 scoring

basis. It was found that the time at which CPE first appeared (i.e. a score of 1) could be more accurately determined than the time taken to reach 50%, 75% or 100% involvement of CPE, since on observing cultures the difference between no CPE at all and the first appearance of CPE (up to 25%) is far clearer than any of the other dividing lines. It was therefore decided that for each culture the time taken for CPE to appear should be determined to the nearest ¼ day. Obviously one cannot observe cultures continuously and therefore in some cases the cultures may have reached 50 or 75% CPE between observations. In such cases the following rule was followed. For cultures showing 25 to 50% CPE at a particular time of observation, 6 hours was taken off that time and the difference given as the time taken for CPE to appear. For cultures showing 50 to 75% CPE 12 hours was taken off that time and the difference given as the time taken for CPE to appear. Having determined the time taken for CPE to appear in each culture the data were treated as follows: the time taken for CPE to appear in control cultures was subtracted from the time taken for CPE to appear in test (drug treated) cultures. The resulting 'delay in time taken for CPE to appear', T, was averaged for triplicate cultures and was plotted against the virus input inoculum (on a log scale). Additionally $^{1}/_{T}$ was plotted against the virus input inoculum (a method used by O'Sullivan and Wallis, (1963a)). However, this method was found to put too much weight on small values of T. For example, values of T of 0.33 and 0.5 days give reciprocals of 3 and 2 days^{-1} respectively, whereas values of 2 and 3 days give reciprocals of 0.5 and 0.33 respectively. It was therefore thought better to plot T against the virus input inoculum.

2.5 Macroplaque Technique

2.5.1 Infectivity Titrations

Rhinoviruses

The macroplaque assay technique employed for rhinoviruses was similar to that first described by Fiala and Kenny (1966). Initial attempts to produce rhinovirus plaques using this technique exactly were not successful and the method described here is the one eventually found to produce plaques consistently.

Plaque assays were carried out in M-HeLa cells. Tissue culture dishes (60 x 13 mm) were seeded and cultures were incubated as previously described (2.1.2).

Serial tenfold dilutions of virus stocks were prepared in maintenance medium. The monolayers were then infected with 1 ml volumes of the appropriate dilutions of virus to be titrated. Since no CO_2 incubator at 34° was available virus was allowed to adsorb for 2 hours at 36° in the 'Hotpack' CO_2 incubator. Monolayers were then aspirated and overlaid with medium at $41^{\circ}C$.

Overlay medium was prepared by combining 1 volume containing twice the normal concentrations of Eagle's MEM, sodium bicarbonate and Crystamycin, 8% tryptose phosphate broth (Wellcome Reagents, Ltd.), 4% adult bovine serum and 20 mM magnesium chloride (Ultrar, Hopkins and Williams, Chadwell Heath, Essex) with 1 volume of 1.0% agarose Indubiose A 37, l'Industrie Biologique Francaise, S.A.) at 41°. The overlay was allowed to set at room temperature and the culture then incubated at 36° in the CO_2 incubator for 2½ to 5 days to allow plaques to develop. The time of incubation required for different virus types to produce plaques is given in Table 2.2. Cell monolayers were then fixed in 10% formalin in PBS A (Wellcome Reagents, Ltd.) for 1 to 2 hours. The solid overlay was then removed by shaking and the

cell monolayers were then stained with 1.0% methyl violet in 20% methanol. Only healthy uninfected cells remain attached to the surface of the tissue culture dish and plaques are therefore seen as circular areas in the monolayer where cells are absent.

Table 2.2 Incubation time for cultures for production of rhinovirus plaques

Rhinovirus Serotype	Hours Incubation
1B	72
2	72
12	72
14	66 - 72
17	66 - 72
19	108
22	90 - 96
30	96
53	108

Poliovirus

Poliovirus plaques were obtained in either M-HeLa or Vero cells with the technique described above, except that virus was allowed to adsorb for 1 hour only and the overlaid plates were incubated for 2 days only. Additionally for Vero cells the overlay medium was different, being prepared by combining 1 volume containing twice the normal concentrations of Eagle's MEM, sodium bicarbonate and Crystamycin and 4% foetal calf serum, with 1 volume of 1.0% agarose at 41°.

2.5.2 Detection of Antiviral Activity

Disc Plate Plaque Inhibition Method

It was found possible to plaque a number of rhinovirus serotypes consistently, although some with greater ease than others. The plaque inhibition test was therefore used as a method for detecting rhinovirus inhibitory activity.

The procedure for plaquing was carried out exactly as described in infectivity titrations (2.5.1). However, a standard virus inoculum was required for this test, i.e. an inoculum sufficient to give about 200 plaques per plate (60 mm diameter). After infecting and overlaying the monolayers and allowing the overlay to set, filter paper discs containing the test compound (50 µg) were placed on the overlay (1 or 2 discs per plate).

Discs were prepared from filter paper (Whatman 3MM) with a metal paper punch (6 mm diameter). They were sterilized by wrapping in aluminium foil (about 10 discs per pack) and heating at 150° for 2 hours. All test compounds were dissolved in an appropriate solvent (water, absolute ethanol or DMF) usually at a concentration of 5 mg/ml. Aqueous and DMF solutions were sterilized by heating at 100° for 10 minutes. By just touching the disc on to the surface of the liquid and allowing the liquid to rise into the disc, it was found that each disc would take up 0.01 ml of liquid (containing 50 µg of compound).

After placing the discs on the overlay, cultures were incubated for 2 to 4 days at 36° to allow plaques to develop. For plaque inhibition an even spread of fairly small plaques (up to 1.5 mm) over the plate is desirable. To obtain such plaques different incubation times were required for different serotypes. Prolonged incubation of cultures was found to lead to increased plaque sizes and eventually to confluent plaque formation.

The plates were fixed and stained 2 to 4 days after infection and examined for any zones free from plaques. An active compound produced a circular plaque-free zone, or a zone containing smaller plaques, in the area surrounding the position of the filter paper disc. In this area the concentration of the drug in the overlay and in the cells is sufficient to inhibit multiplication of the virus, but further away from the disc the concentration of drug decreases and an increase in plaque size and number is observed until the density of plaques is the same as that in the control.

The plaque inhibition method does not require untreated virus controls nor drug treated cell controls (to test for compound cytotoxicity). Firstly, a large proportion of inactive compounds will be tested and these will act as virus controls. Secondly the test itself acts as a control for cytotoxicity since any cytotoxicity is observed as an area of cell degeneration surrounding the discs position.

The disc plate plaque inhibition test was used to detect antiviral activity of a large number of compounds against poliovirus type 1 and rhinovirus types 1B and 2. All these compounds were substituted adenine derivatives of a kind not previously tested for poliovirus or rhinovirus inhibitory activity.

The plaque inhibition test was also used for determining the activity of compounds Abbott 36683, SK&F 40491, SK&F 30097 and Wellcome 71-123 (a compound found active against rhinovirus types 1B and 2 in the above tests), against 8 plaque-forming rhinoviruses (types 1B, 2, 12, 14, 17, 19, 22, and 30). It was hoped also that the diameters of the zones of inhibition obtained in this test would give an indication of the relative potencies of the 4 compounds.

2.5.3 Quantitation of Antiviral Activity

(a) Disc Plate Plaque Inhibition Technique

The disc plate plaque inhibition method (described above) can be used to quantitate antiviral activity roughly, by comparing the diameters of the zones of inhibition produced by different compounds. The diameter of the zone of inhibition would therefore be roughly proportional to the activity of the compound, although the molecular weight and solubility of the compound must be taken into account, since these would affect its diffusion through the agarose overlay.

(b) Plaque Reduction Technique

The plaque reduction technique for quantitation of antiviral activity involves the technique for plaque production already described. Monolayers of M-HeLa cells (80 to 90% confluent) in 60 mm plastic tissue culture dishes were infected with a virus inoculum sufficient to give a large but countable number of plaques (100 to 150). After virus adsorption the monolayers are drained and overlaid with overlay medium, either normal or containing compound in a number of doubling concentrations (each test being performed in triplicate). Overlay medium containing compound was prepared by combining 1 volume containing 4 times the normal concentration of overlay medium constituents with 1 volume containing 4 times the highest final concentration of compound required. Doubling dilutions (usually 4 successive dilutions) of this 2 times compound plus overlay medium mixture were then made in 2 times overlay medium. For the final overlay medium 1 volume of 2 times overlay medium plus compound was combined with 1 volume of 1.0% agarose at 41°.

After incubation of the cultures they were fixed and stained as described and the number of plaques on each plate counted.

Dose-response lines of plaque counts against the log of the concentration of the compound were then plotted from the results.

The activity of different compounds against 1 virus or of 1 compound against different viruses may then be compared by observing the dose-response lines produced.

(c) Gradient Plate Plaque Reduction Technique

The gradient plate plaque reduction technique, first described by Kucera and Herrmann (1966) was also used in this study as an additional method for quantitating antiviral activity. Monolayers of M-HeLa cells (80 to 90% confluent) in 60 mm plates were infected with a virus inoculum sufficient to give about 200 plaques (as for disc plate plaque inhibition method). After adsorption and removal of virus inoculum the cells were overlaid with 2 layers of agarose containing medium. The first 5 ml of overlay was added and allowed to set with the plates inclined at an angle so that the overlay just covered the uppermost area of the monolayer. The second 5 ml of overlay (containing drug at the required concentration) was then placed over the first and allowed to set with the plates returned to the horizontal. The concentration of compound in the second overlay was a concentration which inhibited plaque formation completely.

By this technique a continuously varying concentration of compound is obtained in the overlay. After incubating, fixing and staining the cell cultures the monolayers are examined to find the position at which plaque formation is completely inhibited. By measuring the distance between this position and the point of minimum or maximum compound concentration, the plaque inhibitory concentration can be calculated (see Fig.1.2 in the Introduction).

The activity may therefore be quantitated by finding the plaque inhibitory concentration (C_i) for different compounds. Alternatively, the sensitivity of different virus serotypes can be found by finding C_i for different serotypes.

2.6 YIELD STUDIES

2.6.1 Establishment of Virus Growth Cycles

For growth cycle studies confluent monolayers of M-HeLa cells were used in every case. For single cycle conditions it is necessary to infect cultures with a high multiplicity of virus particles so that any new virus released during the growth cycle will not be able to reinfect further cells.

Confluent monolayers of M-HeLa cells in 30 ml tissue culture flasks were seeded and incubated as described (2.1.2), and infected with a virus inoculum (1 ml) sufficient to give a multiplicity of infection of 2 pfu per cell. After virus adsorption, the inoculum was removed, the cell monolayers were washed in 5 ml of PBS A and each monolayer was then given 10 ml of maintenance medium. Cultures were then incubated at 34°. In order to follow one virus growth cycle duplicate cultures were taken at hourly intervals and frozen at -70°. Samples were harvested by freezing and thawing 3 times and titrated by plaque assay.

In certain experiments the course of the growth cycle in the presence and absence of an inhibitory compound was followed. In this case cultures were set up as described above and after adsorption of virus half the cultures received normal maintenance medium while the other half received maintenance medium containing the active compound. Duplicate test and control cultures were then taken at hourly intervals and the growth cycle of the virus in the presence and absence of compound was then determined.

2.6.2 Quantitation of Antiviral Activity

Yield Reduction Assay

Yield reduction assays were used in addition to tube tests and plaque reduction assays as a method for quantitating antiviral activity.

Confluent monolayers of M-HeLa cells in 30 ml tissue culture flasks were infected with virus at a multiplicity of infection of 2 pfu

per cell. After virus adsorption the inoculum was replaced by normal maintenance medium or maintenance medium containing drug at a range of concentrations (usually doubling concentrations). Cultures were then incubated at 34° for 16 hours and virus samples were then harvested by freezing and thawing 3 times and titrated by plaque assay.

Dose-response lines of the log of the virus yield or the percentage of the control virus yield against the log of the concentration of the drug were then plotted.

2.7 MODE OF ACTION STUDIES

The techniques already described were used to carry out mode of action studies with Abbott 36683, SK&F 30097 and Wellcome 71.123. The individual procedures used will be described with the results in Section 3.3.

3.0 RESULTS

3.1 DETECTION OF ANTIVIRAL ACTIVITY

3.1.1 Detection of Compounds active against Rhinovirus and Poliovirus

The plaque inhibition test was used as a method to determine the activity of a number of adenine derivatives against poliovirus type 1, and rhinovirus types 1B and 2. The compounds tested were all of a series not previously tested for rhinovirus inhibitory activity, but thought possibly to possess activity because of structural resemblances with known rhinovirus inhibitory compounds.

The activity of the compounds was scored on the following basis. Compounds were denoted inactive (-) when they gave no zone of protection in the test, i.e. not even a zone in which plaque sizes were observably reduced. A score of doubtful activity (±) was recorded when the compound produced a zone in which the plaque sizes were observably reduced when this zone was less than 9 mm in diameter. To be recorded as active (+) a compound must give a zone of protection (complete plaque suppression or reduced plaque size) of at least 10 mm diameter.

Of the compounds screened, 121 were found to be inactive, 6 were found to have doubtful activity and 11 were active against poliovirus type 1. Fewer compounds were found to be active against rhinovirus types 1B and 2.

The diameters of the zones of plaque suppression, either complete (plaque free zone) or incomplete (small plaque zone) for the compounds showing activity against poliovirus or rhinovirus are given in Table 3.1.

It is obvious from this that the compounds are not equally active against all the virus types tested. Especially of note is the fact that 71 - 329 is completely inactive towards poliovirus but active towards rhinovirus.

Observations of test plates also demonstrated the importance of having virus control plates, since on occasions cell sheets settle with thick areas at the centre. Plaques formed in this area are smaller

than those formed at the periphery of the plate and the appearance of the plate is thus very similar to that obtained on a test plate with an active compound. However, this problem can be overcome if large tissue culture plates are used, since then the compound-containing discs (usually 4 discs per large plate are used) need not be placed centrally.

Table 3.1 Activity of Compounds tested against Poliovirus type 1, and Rhinovirus types 1B and 2. Diameter of Zones of Protection Produced by Compounds showing some activity. (Number outside brackets indicates the diameter of the plaque free zone and number inside brackets indicates the diameter of the zone with reduced plaque size).

Compound	Diameter of Zone of Protection (mm)			Diameter of Toxic Zone
	Polio type 1	Rhino type 1B	Rhino type 2	
70-219	-	20	20(40)	15
70-223	30(32)	(10)	(10)	7
70-225	(9)	-	-	-
70-227	(9)	-	-	-
70-332	-	(20)	-	-
71-123	19(25)	20	20	-
71-124	10(12)	10(14)	10(16)	-
71-171	(12)	(11)	10(23)	-
71-172	(6)	-	-	-
71-225	(11)	-	-	-
71-228	(8)	-	-	-
71-238	3(11)	-	-	-
71-261	(10)	-	-	-
71-301	(5)	-	-	-
71-320	(10)	-	-	-
71-322	15(20)	-	-	-
71-329	-	5	10	-
71-335	(10)	-	-	-
71-336	13(20)	12(20)	20(45)	11
72-108	15(20)	-	-	-

3.1.2 Determination of the Spectrum of Rhinovirus Inhibitory Activity of Abbott 36683, SK&F 40491, SK&F 30097 and Wellcome 71-123.

3.1.2(a) Tube Culture Technique

Abbott 36683 (2μM and 4μM), SK&F 40491 and SK&F 30097 (20μM and 40μM) were each tested for activity against 33 rhinovirus serotypes, using the inhibition of virus induced CPE as the criterion for compound activity as described in the methods (2.4.2). Wellcome 71-123 was tested against 9 rhinovirus serotypes by this method.

Figs. 3.1a, b, c and d demonstrate the method of scoring CPE. The appearance of cultures of M-HeLa cells infected with different input inocula of rhinovirus type 2 after 36 hours incubation at 34° is shown. The cultures were seeded on to glass coverslips in Leighton tubes and after overnight incubation there were about 10^{5} cells per coverslip. Cultures were then infected with 10, 100, or 1000 $TCID_{50}$ of virus inoculum or left uninfected. Cultures were incubated for 36 hours at 34° and were then fixed in acetone for 2 minutes and stained with Giemsa. On the 0 to 4 scoring basis for CPE the cultures shown in Figs. 3.1a, b, c, and d would be scored as 0, 1, 3 and 4 respectively.

Table 3.2 shows the spectrum of virus inhibitory activity of Abbott 36683, SK&F 40491 and SK&F 30097. Activity was scored on the following basis: '+' denotes a delay in the appearance of CPE for 2 days or over, '$\pm$' denotes a delay of 1 to 2 days and '-' denotes a delay of less than 1 day. The number in brackets is the size of the input inoculum of virus ($TCID_{50}$ per tube) against which the compound is tested. Results for the compound Wellcome 71-123 have not been tabulated. The compound was found to have a '-' rating against each of the virus serotypes tested (types 1B, 5, 9,12, 14, 17, 19, 22 & 30).

The results show that both Abbott 36683 and SK&F 40491 were active against all rhinovirus serotypes tested at both concentrations of compound and at all virus input inocula tested.

Fig. 3.1. a, b, c and d. Monolayers of M-HeLa cells on glass coverslips infected with different inocula of rhinovirus type 2, 10, 100 or 1000 $TCID_{50}$ per culture or no virus. Cultures were incubated for 36 hours at 34° and were then fixed in acetone and stained with Giemsa. All plates are at a magnification of x300.

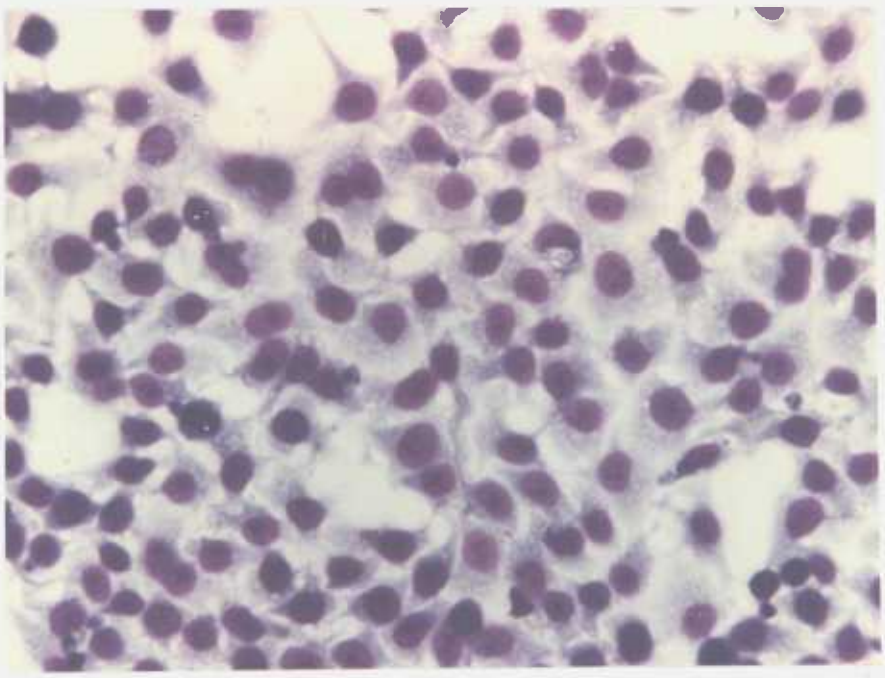


Fig. 3.1a. Control- uninfected.

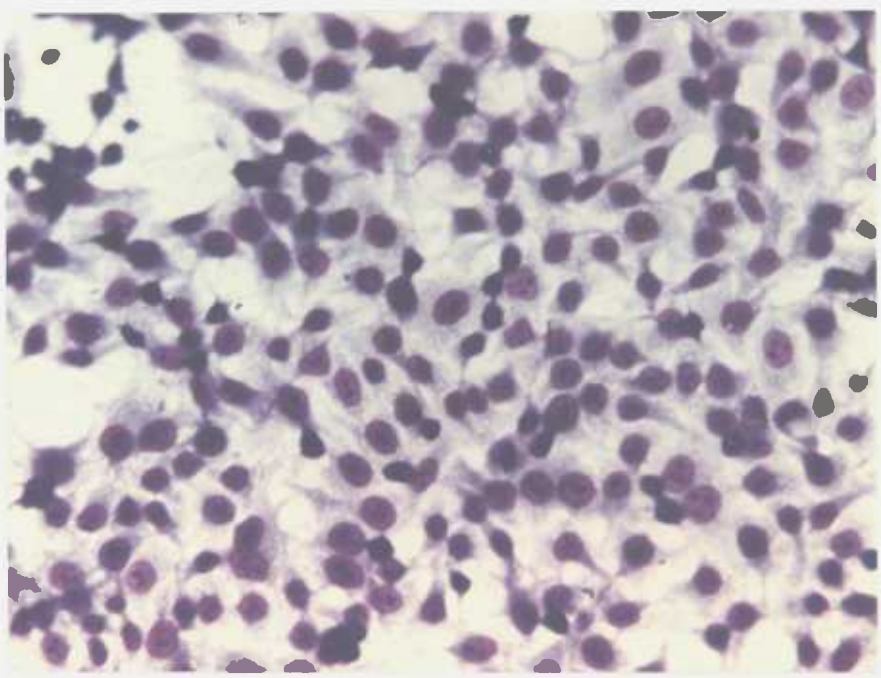


Fig. 3.1b. 10 $TCID_{50}$ of virus per culture.

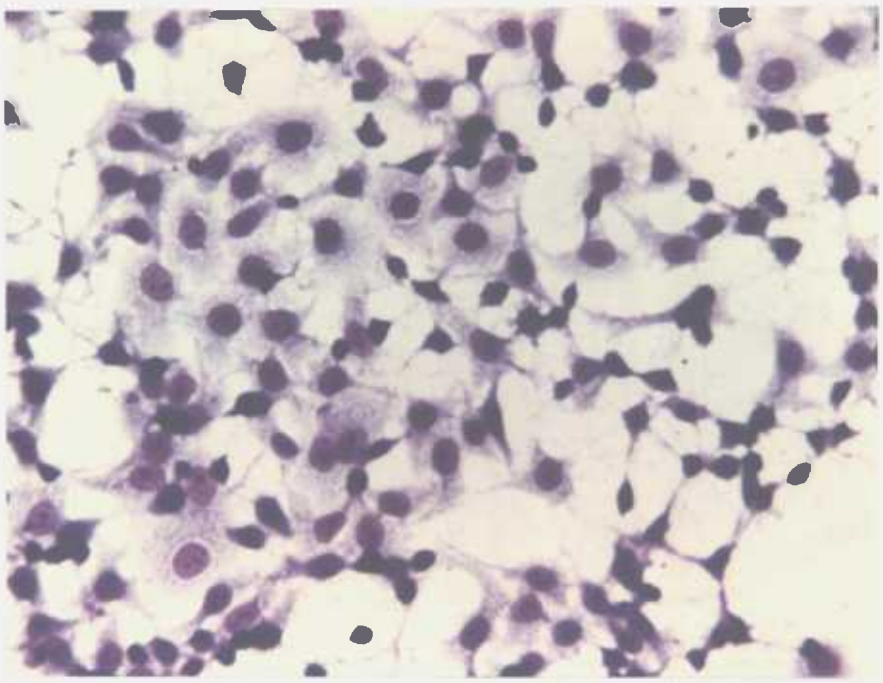


Fig. 3.1c. 100 $TCID_{50}$ of virus per culture.

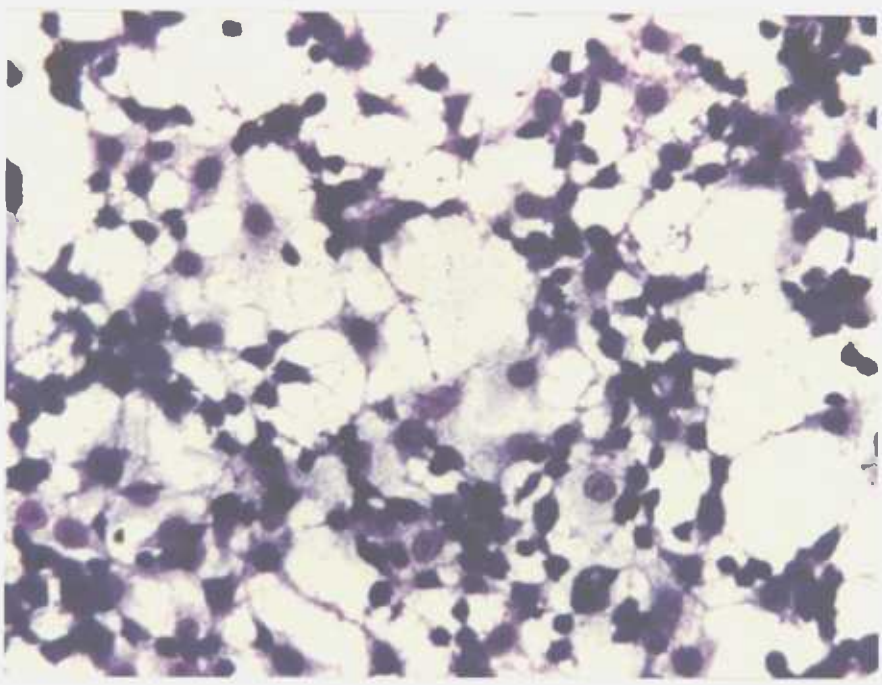


Fig. 3.1d. 1000 $TCID_{50}$ of virus per culture.

TABLE 3,2 Spectrum of rhinovirus activity of compounds Abbott 36683, SK+F 40491 and SK+F 30097 as found by inhibition of CPE in tube cultures

Rhino-virus type	Activity of compound					
	Abbott 36683		SK+F 40491		SK+F 30097	
	2 µM	4 µM	20 µM	40 µM	20 µM	40 µM
1B	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(10;100)	+(1) ±(10) -(1(
2	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(10;100)	-(1;10;100
3	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(10;100)	+(10) -(100)
4	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(1;10;100)	+(1) -(10;100)
5	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(10;100)	+(10) -(100)
6	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(1;10;100)	-(1;10;100
7	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(1;10;100)	+(1) -(10;100)
8	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(1;10;100)	+(1) -(10;100)
9	+(10;100)	+(10;100)	+(10;100)	+(10;100)	+(1) -(10;100)	+(1) ±(10) -(100)
10	+(10;100)	+(10;100)	+(10;100)	+(10;100)	+(1) ±(10) -(100)	+(1) ±(10) -(100)
11	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(1;10;100)	-(1;10;100
12	+(10;100)	+(10;100)	+(10;100)	+(10;100)	+(1;10) ±(100)	+(1;10;100
13	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(1;10;100)	±(1) -(10;100)
14	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(10;100)	-(10;100)
16	+(10;100)	+(10;100)	±(10) -(100)	+(10) ±(100)	-(10;100)	-(10;100)

Rhino-virus type	Activity of compound					
	Abbott 36683		SK+F 40491		SK+F 30097	
	2 µM	4 µM	20 µM	40 µM	20 µM	40 µM
17	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(10;100)	-(10;100)
18	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(10;100)	±(10) -(100)
19	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(1;10;100)	+(1;10) ±(100)
21	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(10;100)	-(10;100)
22	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(10;100)	-(10;100)
27	+(10;100)	+(10;100)	+(10;100)	+(10;100)	±(10) -(100)	+(10) ±(100)
28	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(1;10;100)	+(1;10) -(100)
29	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(1;10;100)	+(1) -(10;100)
30	+(10;100)	+(10;100)	+(10;100)	+(1;10;100)	-(1;10;100)	±(1) -(10;100)
31	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(10;100)	-(10;100)
33	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(10;100)	±(10) -(100)
36	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(10;100)	±(10) -(100)
38	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(10;100)	-(10;100)
39	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(10;100)	±(10) -(100)
40	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(1;10;100)	+(1) ±(10) -(100)
41	+(10;100)	+(10;100)	+(1;10;100)	+(10;100)	-(10;100)	±(10) -(100)
43	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(10;100)	±(10) -(100)

Rhino-virus type	Activity of compound					
	Abbott 36683		SK+F 40491		SK+F 30097	
	2 µM	4 µM	20 µM	40 µM	20 µM	40 µM
53	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(1;10;100)	+(1) ±(10 -(100)
54	+(10;100)	+(10;100)	+(10;100)	+(10;100)	-(1;10;100)	+(1) ±(10) -(100)

\+ delays appearance of CPE for 2 days

± delays appearance of CPE for 1 - 2 days

\- delays appearance of CPE less than 1 day

Numbers in brackets indicate input inoculum of virus at $TCID_{50}$ per tube

(Approximate multiplicity of infection is this number divided by 10^5)

However, SK&F 30097 at a concentration of 20μM generally exhibited no antiviral activity, and at 40μM activity was variable against different serotypes and against different virus input inocula. In only one instance (i.e. against rhinovirus type 12) did the compound have a '+' rating against an input inoculum of 100 $TCID_{50}$ per culture.

All 4 compounds were non-cytotoxic at the concentrations used.

The rhinovirus inhibitory spectrum of SK&F 30097 is more clearly understood from Table 3.3, where the rhinovirus serotypes tested have been put into +, ± or - rating groups for each of the 3 virus input inocula used.

As can be seen, not all serotypes were tested at an input inoculum of 1 $TCID_{50}$ per culture, but such serotypes can be expected to be at least as sensitive at this input as at an input of 10 $TCID_{50}$ per culture. Rhinovirus types 12, 19 and 27 were most sensitive to SK&F 30097. The compound was found to have a '-' activity rating for most serotypes when tested at 100 $TCID_{50}$ per culture. At an input inoculum of 10 $TCID_{50}$ per culture a far greater number of serotypes appeared sensitive to the compound. Finally at an input inoculum of 1 $TCID_{50}$ per culture SK&F 30097 had a '-' rating against only 3 of the 18 serotypes tested. This therefore shows very clearly the effect of virus inoculum size on the detection of virus inhibitory activity in tube culture tests. If a large virus inoculum is employed an active compound may evade detection.

Table 3.3

Spectrum of Rhinovirus Inhibitory Activity of SK&F 30097 (40μM) as determined by the inhibition of virus induced cytopathic effects in tube cultures. Serotypes listed as showing '+', '±' or '-' activity at 3 sizes of virus input inoculum

Activity	Input Inoculum of Virus ($TCID_{50}$ per culture)		
	1.0	10.0	100
+	1B, 9, 10, 12, 19, 28, 29, 40, 53,	12, 19, 27, 28,	12,
±	4, 7, 8, 13 30, 22,	1B, 3, 5, 9, 10, 18, 33, 36, 39, 40, 41, 43, 53,	19, 27
-	2, 6, 11,	2, 4, 6, 7, 8, 11, 13, 14, 16, 17, 21, 22, 29, 30, 31, 38, 54,	1B, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 14, 16, 17, 18, 21, 22, 29, 30, 31, 33, 36, 38, 39, 40, 41, 43, 53, 54,

3.1.2(b) Plaque Inhibition Technique

The spectrum of virus inhibitory activity of Abbott 36683, SK&F 30097 and 40491 and Wellcome 71-123 was also investigated by the plaque inhibition test. Each compound was tested against 8 rhinovirus serotypes (types 1B, 2, 12, 14, 17, 19, 22 and 30).

The amount of compound administered on the filter paper disc was not the same for all compounds, since Abbott 36683 and SK&F 40491 are far more active than the other compounds. Hence they produced zones of inhibition which covered the entire plate (50 mm diamter) when tested at the usual amount (50µg per disc). For this reason SK&F 30097, Wellcome 71-123 and 71-329 were tested in equimolar amounts, 0.2µMole, and SK&F 40491 and Abbott 36683 were tested at 0.15 and 0.075, and 0.018 and 0.009 µMoles respectively. Some compounds were tested on 80mm diameter plates.

The average diameter of the zone of inhibition for each compound against each virus is given in Table 3.4.

The results indicate that all the compounds tested are active against all the serotypes used. Abbott 36683 is seen to be the most active compound, with SK&F 40491, SK&F 30097, Wellcome 71-123 and Wellcome 71-329 having successively smaller activity.

It is of interest to note that serotypes 14 and 17 appear to be the least sensitive to inhibition by all the compounds. These serotypes were shown to be insensitive to inhibition by 40µM SK&F 30097 in the tube test technique. Additionally, types 12 and 19 appear to be most sensitive to inhibition in the plaque inhibition test, in agreement with results obtained in the tube tests, using SK&F 30097. The plaque inhibition test would therefore seem to be a more sensitive technique for determining antiviral activity. However, the main drawback of this technique is that plaques are not easily obtainable with all rhinovirus serotypes.

All of the compounds tested, except Wellcome 71-123, produce zones of inhibition which vary from serotype to serotype. The reason for this difference with Wellcome 71-123 is not known, but it may possibly be due to the very high molecular weight of this compound which possibly makes its diffusion through the agarose overlay slower in comparison with the other compounds.

Table 3.4

Rhinovirus Inhibitory Activity of Abbott 36683, SK&F 40491, SK&F 30097, Wellcome 71-123 and Wellcome 71-329, as determined by the Plaque Inhibition Method.

(Zone diameter, mm, given for various amounts of compound on discs)

Rhino-virus Type	Abbott 36683		SK&F 40491		SK&F 30097		Wellcome 71-123	Wellcome 71-329
	0.018* 6.25 µg	0.009* 3.125 µg	0.15* 50 µg	0.075* 25 µg	0.2* 57.2 µg	0.1* 28.6 µg	0.2* 93.0µg	0.2* 52.3µg
1B	50	52.5	50	45.5	31.0		20.0	18.5
2	50	49.5	50	42.5	23.5		20.0	
12	50	43.5	50	42.5	26.0	17.5	20.0	
14	41.5		45.5		19.0		20.0	
17	43.5		49.5		24.0		20.0	9.5
19	50	48.5	50	44.5		22.5	20.0	
22	50	48.5	50	44.5		29.5	20.0	
30	50	45.5	50	43.5		21.5	20.0	14.5

* amount of compound on disc in micromoles

From the observed diameters of the zones of inhibition the rhinovirus serotypes were placed in order of increasing sensitivity to each of the compounds except Wellcome 71-123 (Table 3-5). It can be seen from this table that the serotypes follow roughly the same order of sensitivity to each compound, types 14 and 17 showing least sensitivity and types 22, 19 and 1B showing generally greatest sensitivity.

Table 3.5

Rhinovirus serotypes placed in order of increasing sensitivity to compounds Abbott 36683, SK&F 40491, SK&F 30097 and Wellcome 71-329

Abbott 36683	SK&F 40491	SK&F 30097	Wellcome 71-329
14	14	14	17
17	17	2	30
12	2=12	17	1B
30	30	12	
19=22	19=22	1B	
2	1B	30	
1B		19	
		22	

3.2 QUANTITATION OF ANTIVIRAL ACTIVITY

3.2.1 Tube Culture Technique

3.2.1(a) Use of Tube Tests to Quantitate the Rhinovirus Inhibitory Activity of Abbott 36683, SK&F 40491 and SK&F 30097

As described in materials and methods tube tests were used to attempt to quantitate the antiviral activity of Abbott 36683, and SK&F 40491 and 30097, particularly with an aim to determine whether rhinovirus serotypes displayed varying sensitivities to these compounds.

For this study the concentration of each of the compounds required was that which would delay CPE for no longer than 2 days (at the lowest virus inoculum used).

In order to determine a working concentration for each of the compounds a titration was set up with rhinovirus type 12, which appeared to be the serotype most sensitive to SK&F 30097 in the determination of the antiviral spectrum of the latter (section 3.1.2). Hence the maximum delay would be with rhinovirus type 12. Tube cultures of M-HeLa cells infected with 10 or 100 $TCID_{50}$ of rhinovirus type 12 were treated with normal medium or medium containing one of the compounds at one of 4 concentrations (Abbott 36683, 0.25, 0.5, 1.0 or 2.0 μM; SK&F 30097, 40, 80, 120 and 160 μM; and SK&F 40491, 2.5, 5.0, 10 or 20 μM). Uninfected control cultures were also similarly treated. Cultures were incubated on a roller drum apparatus at 36° and observed twice daily for virus-induced cytopathic or compound-induced cytotoxic effects, which were recorded on the 0 to 4 scoring basis. From the data obtained the concentration of each compound required to delay the appearance of CPE for no longer than 2 days was determined. This concentration was found to be 0.5, 5.0 and 80 μM for Abbott 36683, SK&F 40491 and SK&F 30097 respectively.

The activity of the 3 compounds against 14 different rhinovirus serotypes was then determined. Tube cultures were infected with 10,

100 or 1000 $TCID_{50}$ of virus inoculum and then treated with normal medium or medium containing one of the 3 compounds at the determined concentration. Cultures were incubated as usual and observed twice daily in order to record CPE. For each virus input inoculum and for each compound the delay in time taken for CPE to appear (compared with the control) was calculated. This time was then plotted against the log of the virus input inoculum (Fig. 3.2). It was thought better to plot this than to plot the reciprocal of the delay in time since the latter gives too much weight to short time intervals, i.e. those which cannot be so accurately assessed in the test.

The reproducibility of this test system was determined as follows. From the set of lines obtained for each compound 5 viruses were chosen whose sensitivity appeared to be very similar, i.e. not necessarily the same serotypes for all 3 compounds. The tests were repeated for all 3 compounds against the chosen serotypes.

The series of plots in Fig.3.2 shows that the working concentrations chosen for each compound have approximately the same rhinovirus inhibitory activity since the range of delay times for each compound is similar. That is, in terms of activity 0.5μM Abbott = 5.0μM SK&F 40491 = 80μM SK&F 30097.

From the series of plots for each compound a rough estimation of the sensitivities of each serotype to each compound can be made, since the greater the delay in the appearance of CPE the more sensitive the virus. The serotypes are listed below in order of increasing sensitivity (Table 3.6).

The order is fairly difficult to determine from the plotted results since not all the lines are parallel and where lines cross a ranking of sensitivity becomes difficult. This is especially true for SK&F 40491 where many of the lines are seen to cross one another. Serotypes 6, 10 and 13 are particularly difficult to place in order of sensitivity for this reason.

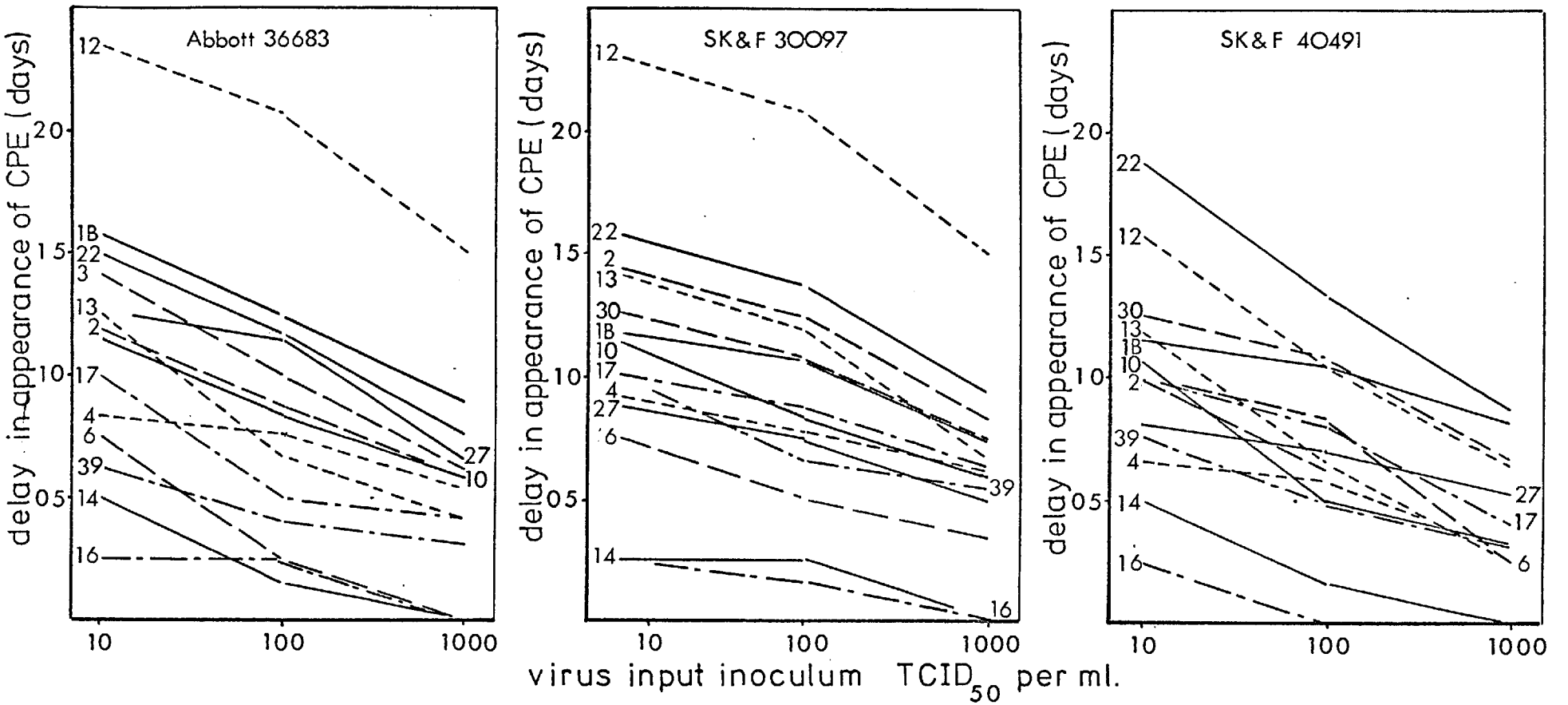


Fig. 3.2. Activity of Abbott 36683 (0.5μM), SK+F 30097 (80μM), and SK+F 40491 (5.0μM) against 14 rhinovirus serotypes as determined by the tube culture technique. The individual plots show the delay in the appearance of virus induced cytopathic effects in compound treated cultures compared with untreated cultures for virus input inocula of 10, 100, and 1000 $TCID_{50}$ per ml.

The repeat experiments carried out for each of the compounds showed varying agreement with the results. For Abbott 36683, which was retested against rhinovirus types 22, 27, 30, 2 and 10, results obtained gave a sensitivity ranking of 22 > 27 > 30 > 10 > 2, which is almost the same as in Table 3.5. Similarly for SK&F 30097, which was retested against types 2, 13, 30, 1B and 10, results were comparable, giving a sensitivity ranking of 2 > 13 > 30 = 1B > 10.

Table 3.6

Fourteen rhinovirus serotypes listed in order of increasing sensitivity to Abbott 36683 (0.5μM), SK&F 30097 (80μM) and SK&F 40491 (5.0μM), as determined by the inhibition of virus induced cytopathic effects in tube cultures

Abbott 36683	SK&F 30097	SK&F 40491
14=16	16	16
6	14	14
39	6	4=39
17	27=39	10
4	4	2
13	10	27
10	17	6
2	1B	13
30	30	17
27	13	1B
22	2	30
1B	22	12
12	12	22

However, for compound SK&F 40491, where types 2, 27, 6, 17 and 1B were retested, the results obtained gave lines which were no more parallel than those already obtained, again making ranking of sensitivity difficult. However, the latter was as follows; 1B>30=13>2>10, compared with the previous results of 30>1B>13>2>10.

It can be seen that while some differences do occur between the compounds in the ranking of virus sensitivity the general trend for virus sensitivity is the same for each compound. Serotypes 1B, 12 and 30 consistently appear to be most sensitive and types 4, 14, 16 and 39 most insensitive.

The most marked difference in compound sensitivity ranking of the serotypes is for compound SK&F 40491, where types 2, 6, 10, 12, 13 and 22 are very differently placed compared with their positions of sensitivity ranking against Abbott 36683 and SK&F 30097. Type 22 is found to be most sensitive to SK&F 40491 and is far more sensitive to this compound than to the others at the concentrations used. Type 12, which is found to be most sensitive to Abbott 36683 and SK&F 30097, is in fact far less sensitive to SK&F 40491. However, since the positioning of types 6, 10 and 13 in the table was initially in doubt, it is thought that the difference in their sensitivity ranking against SK&F 40491 compared with that against the other two compounds possibly demonstrates a failure of this test method to define correctly the differences in sensitivities of serotypes to SK&F 40491.

The activities of Abbott 36683, SK&F 40491 and SK&F 30097 were also measured by the tube technique using WI-38 cell cultures, in order to compare results with those obtained with M-HeLa cells. Tube cultures of WI-38 cells were seeded and incubated as described (section 2.1.2) and infected as for M-HeLa cells. In order to make a direct comparison of the 2 cell lines tests were set up with WI-38 and M-HeLa cells concurrently. The activities of each of the 3 compounds were measured

against 3 rhinovirus serotypes. Originally it had been hoped to test a far greater number of serotypes, but it was found that WI-38 cells were difficult to culture since they could not be obtained at a low passage number.

Control and test cultures were observed and the extent of CPE noted as previously described. Results were also analysed as for previous tests, and Fig.3.3a shows plots of the delay in the appearance of CPE against the size of the virus input inoculum, for the serotypes and compounds tested in both WI-38 and M-HeLa cells.

The delay in the appearance of CPE in WI-38 cells (shown by the plots with open symbols) is in all cases greater than the corresponding delay in M-HeLa cells. Also the 3 serotypes exhibit the same comparative sensitivity to each of the compounds in both cell lines. One exception to this is with SK&F 30097. In WI-38 cells type 17 appears more sensitive to this compound than type 2, whereas in M-HeLa cells type 2 is more sensitive than type 17. Also the plots for SK&F 40491 show that the difference in sensitivity between types 2 and 17 is apparently greater in WI-38 cells than in M-HeLa cells. This can probably be attributed to the fact that the delay in appearance of CPE is generally longer in WI-38 than in M-HeLa cells.

It was thought important to determine whether each of the compounds at the concentrations used had the same comparative activity in both cell lines. The delay in the appearance of CPE was therefore plotted against virus input inoculum for all 3 compounds against each virus serotype individually. This is shown in Fig.3.3b; open symbols show the results for WI-38 cells and closed symbols show the results for M-HeLa cells. For rhinovirus types 2 and 17 Abbott 36683 (0.5μM) is shown to be more active than SK&F 40491 (5.0μM), and the latter is more active than SK&F 30097 (80μM). However, for rhinovirus type 22 results in the 2 cell lines are not consistent. In WI-38 cells the order of decreasing activity is Abbott 36683 > SK&F 40491 > SK&F 30097, whereas in M-Hela cells the order is SK&F 30097 > SK&F 40491 > Abbott 36683.

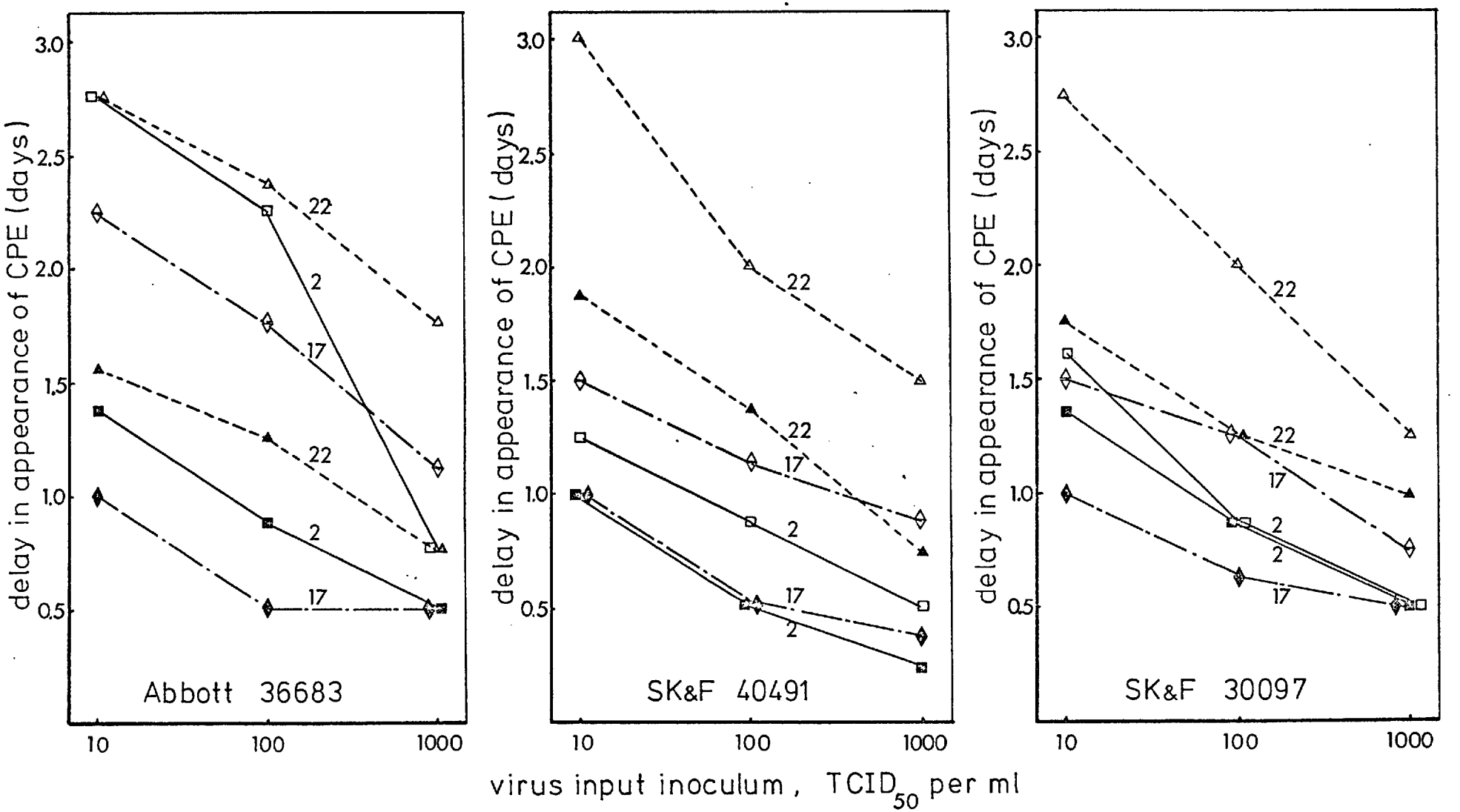


Fig. 3.3a. Activity of Abbott 36683 (0.5μM), SK+F 40491 (5.0μM) and SK+F 30097 (80μM) against rhinovirus types 2 (□——□), 17 (◇—·—◇) and 22 (△----△) in M-HeLa cells (closed symbols) and WI-38 cells (open symbols). The individual plots show the delay in the appearance of virus induced cytopathic effects in compound treated cultures compared with untreated control cultures for virus input inocula of 10, 100 and 100 $TCID_{50}$ per ml.

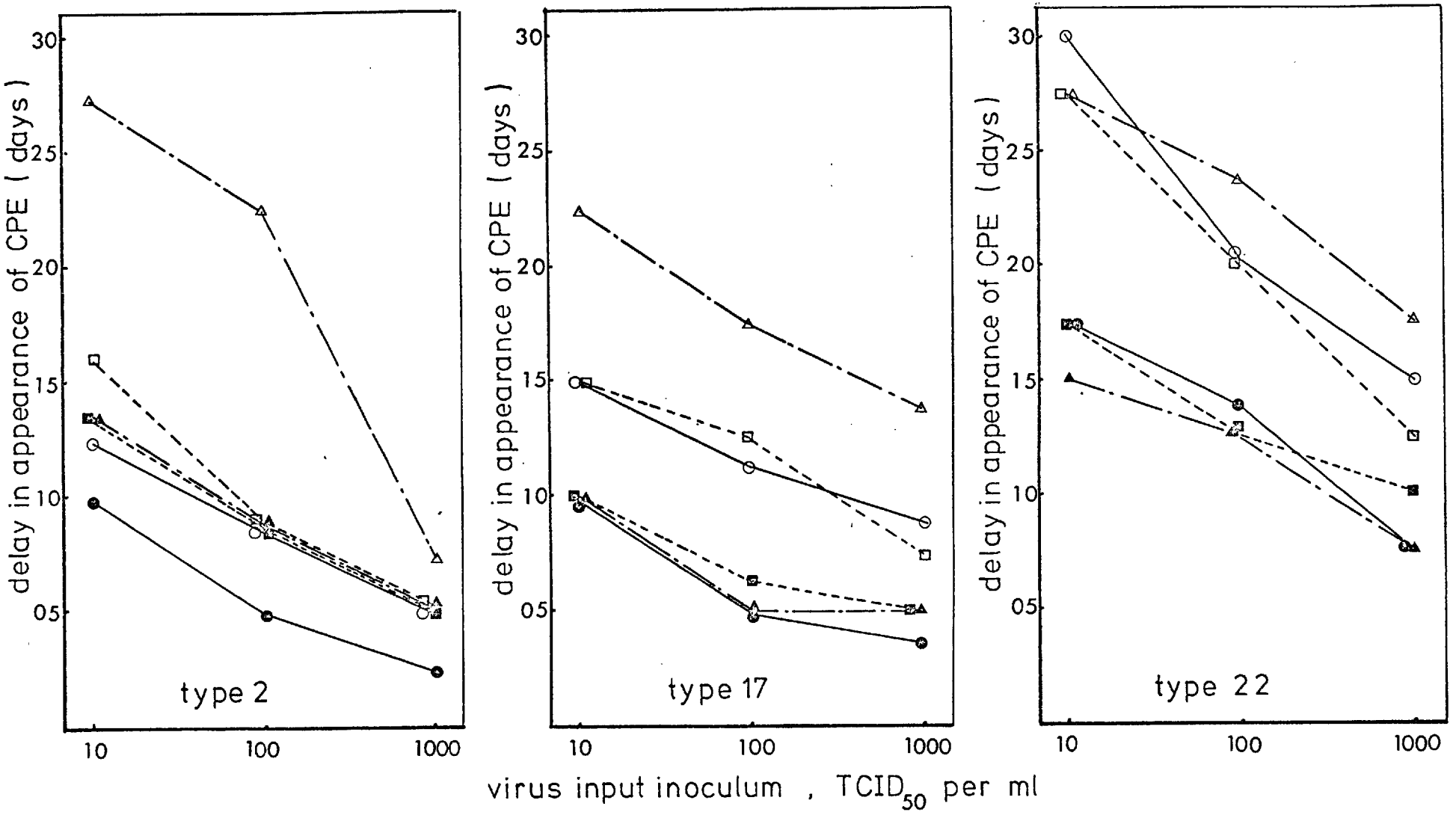


Fig. 3.3b. Activity of Abbott 36683 (0.5μM) (△—·—△), SK+F 40491 (5.0μM) (○——○) and SK+F 30097 (80μM) (□----□) against rhinovirus types 2, 17 and 22 in M-HeLa cells (closed symbols) or WI-38 cells (open symbols). The individual plots show the delay in the appearance of virus induced cytopathic effects in compound treated cultures compared with untreated cultures for virus input inocula of 10, 100 and 1000 $TCID_{50}$ per ml.

3.2.1(b) Effect of multiplicity of infection on the apparent measured activity of a compound

It has been shown in the previous section (3.2.1.a) that the activity of a compound (if measured as the delay in the appearance of cytopathic effects in tissue culture) will depend on the size of the infecting inoculum of virus. Hence the delay in the appearance of CPE produced by a compound against a small virus inoculum will be longer than that produced by the same concentration of drug against a large virus inoculum.

If one wishes to determine the minimal inhibitory concentration of a compound using the tube test technique, it is obvious that this concentration also will depend on the size of the infecting inoculum Hence it was proposed to determine what relationship existed between the minimal inhibitory concentration and the size of the infecting inoculum in tube tests.

This was done using Abbott 36683 and poliovirus type 1. Tube cultures of M-HeLa cells were infected with poliovirus at multiplicities of infection ranging from 0.006 to 1000 pfu per cell. After infection cultures were treated with maintenance medium or medium containing compound at a range of concentrations from 0.105μM to 800μM. Uninfected control cultures were also treated with the same compound-containing medium. Cultures were incubated on a roller drum apparatus at 36° and observed twice daily for cytopathic and cytotoxic effects which were scored on a 0 to 4 basis. The minimal inhibitory concentration, defined as the lowest concentration of compound which would delay the appearance of CPE for 1 day, was then determined for each multiplicity of infection. The log of the minimal inhibitory concentration was then plotted against the log of the multiplicity of infection (Fig.3.4).

It was found that CPE in cultures infected with an inoculum of up to 10 pfu per cell could be delayed for 1 day by a non-cytotoxic

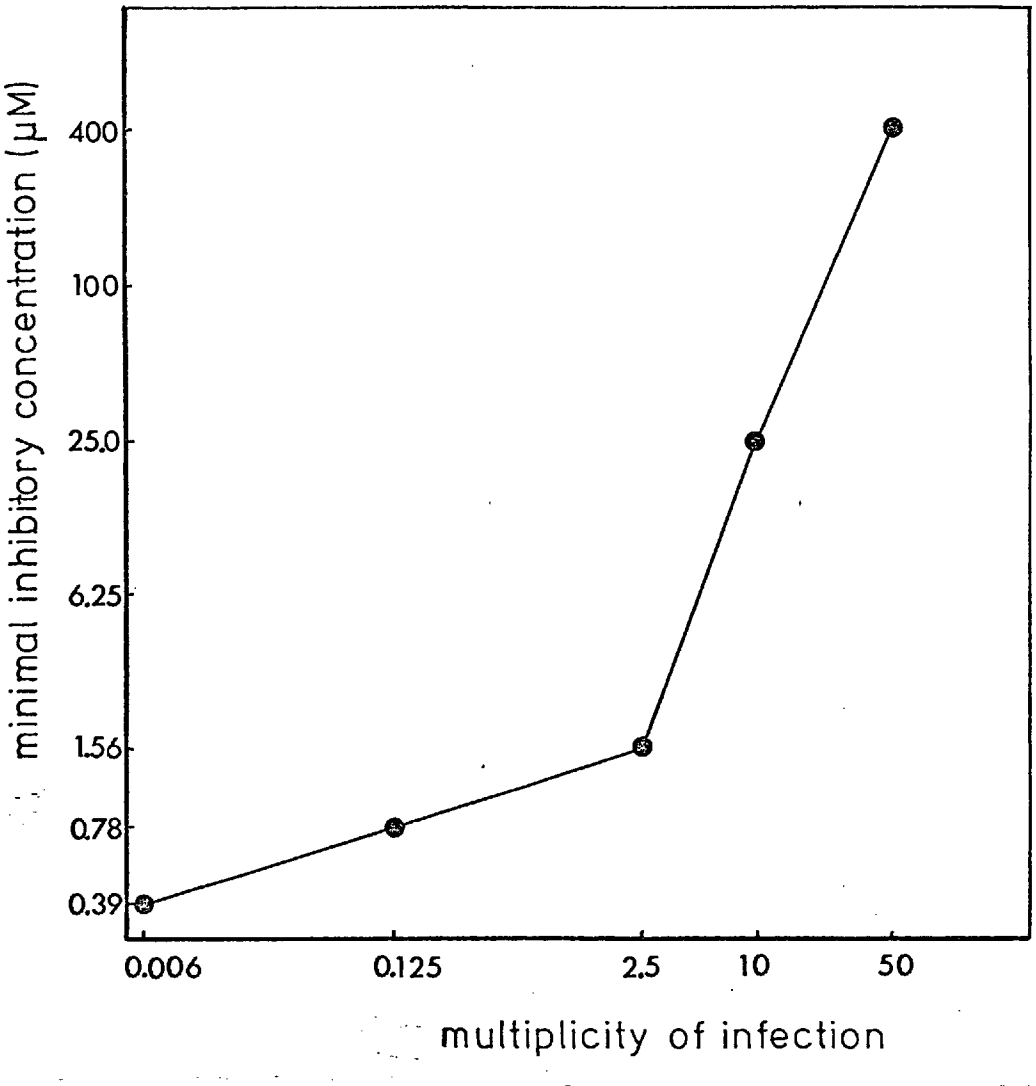


Fig. 3.4. Effect of changes in the multiplicity of infection on the minimal inhibitory concentration of Abbott 36683 against poliovirus type 1 as determined by the inhibition of virus induced cytopathic effects in tube cultures of M-HeLa cells. The log of the minimal inhibitory concentration, defined as the lowest concentration of compound which will delay the appearance of CPE for 1 day, is plotted against the log of the multiplicity of infection.

concentration of Abbott 36683. However, at a multiplicity of infection of 50 pfu per cell a 400μM concentration of compound was required to delay CPE for 1 day and this concentration was found to be cytotoxic after 36 hours incubation with the cells. At 250 and 1000 pfu per cell CPE was not delayed by a 800μM concentration of compound and above this concentration the compound was cytotoxic after 24 hours incubation with cell cultures.

It can be seen then that the log of the minimal inhibitory concentration increases linearly with the log of the multiplicity of infection, at low multiplicities of infection (in this experiment, up to 2.5 pfu per cell). With larger multiplicities of infection the minimal inhibitory concentration increases more rapidly and in this system, at multiplicities of infection of greater than 10 the minimal inhibitory concentration is toxic.

3.2.2 Plaque Reduction Technique

3.2.2(a) Effect of various parameters on Plaque Reduction Results

It becomes apparent from carrying out plaque reduction experiments that results were not always consistent from one experiment to another. This was thought to be due perhaps to changes in experimental conditions, such as the length of time of incubation of the plaque plates or the size of the infecting inoculum. The effect of these 2 parameters on results obtained in the plaque reduction technique was therefore studied. Additionally the difference in results obtained from the plaque reduction technique when using different cell lines was studied.

Incubation Time

A study of the effect of varying the incubation time of cultures in plaque reduction tests was carried out using the compound Abbott 36683 to challenge poliovirus type 1 in Vero cells.

Monolayers of Vero cells (80 to 90% confluent) in 10cm diameter tissue culture dishes were infected with approximately 150 pfu of poliovirus type 1 in 5ml of maintenance medium. After virus adsorption the monolayers were drained and overlaid with normal overlay or overlay containing the compound at one of 4 concentrations, 0.025, 0.05, 0.10 or 0.20 μM. Cultures were then incubated at 36° and batches of plates (containing triplicate plates for control and each compound concentration) were fixed and stained at 3 different times after infection (27, 43, and 49 hours).

Plaque Counts

The number of plaques on each plate was counted and recorded. Average plaque counts per plate were plotted against the concentration of compound (on a $\log_2$ scale) for each incubation time (Fig.3.5). It was also of interest to plot the average plaque counts per plate against the incubation time of the cultures for each concentration of compound used (Fig.3.6).

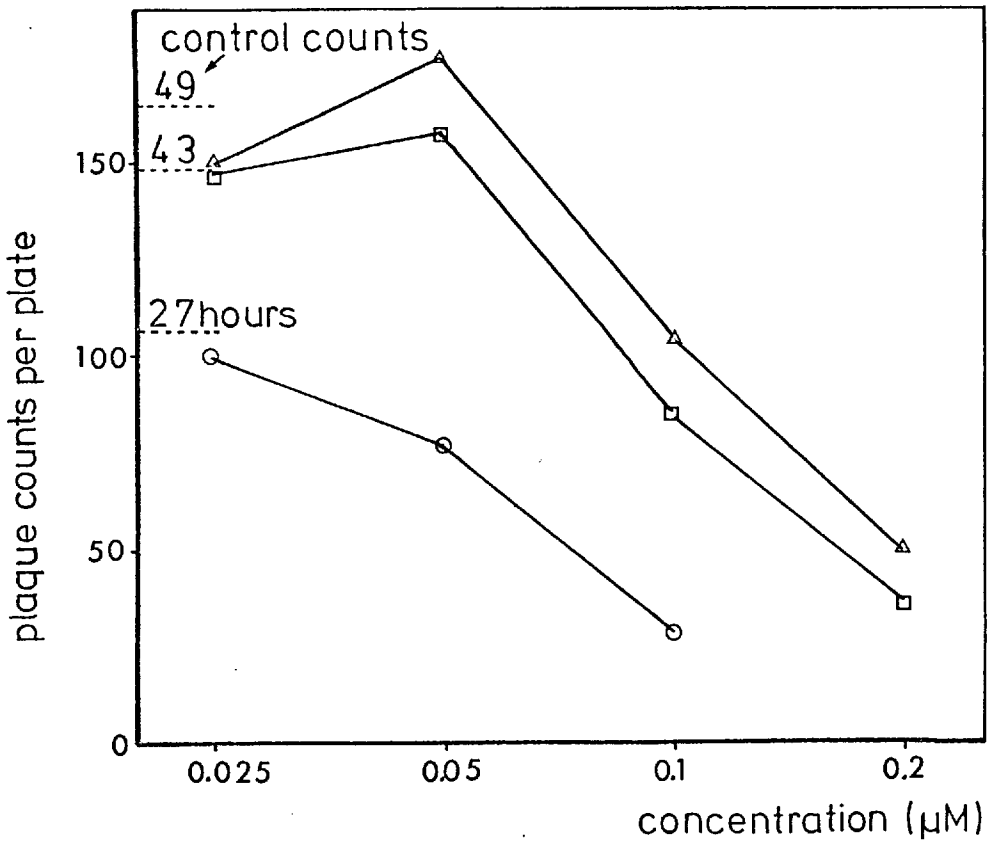


Fig. 3.5. Plaque reduction of poliovirus type 1 with Abbott 36683 in Vero cell cultures. Plaque counts per plate plotted against the compound concentration (log scale) at 3 different incubation times.

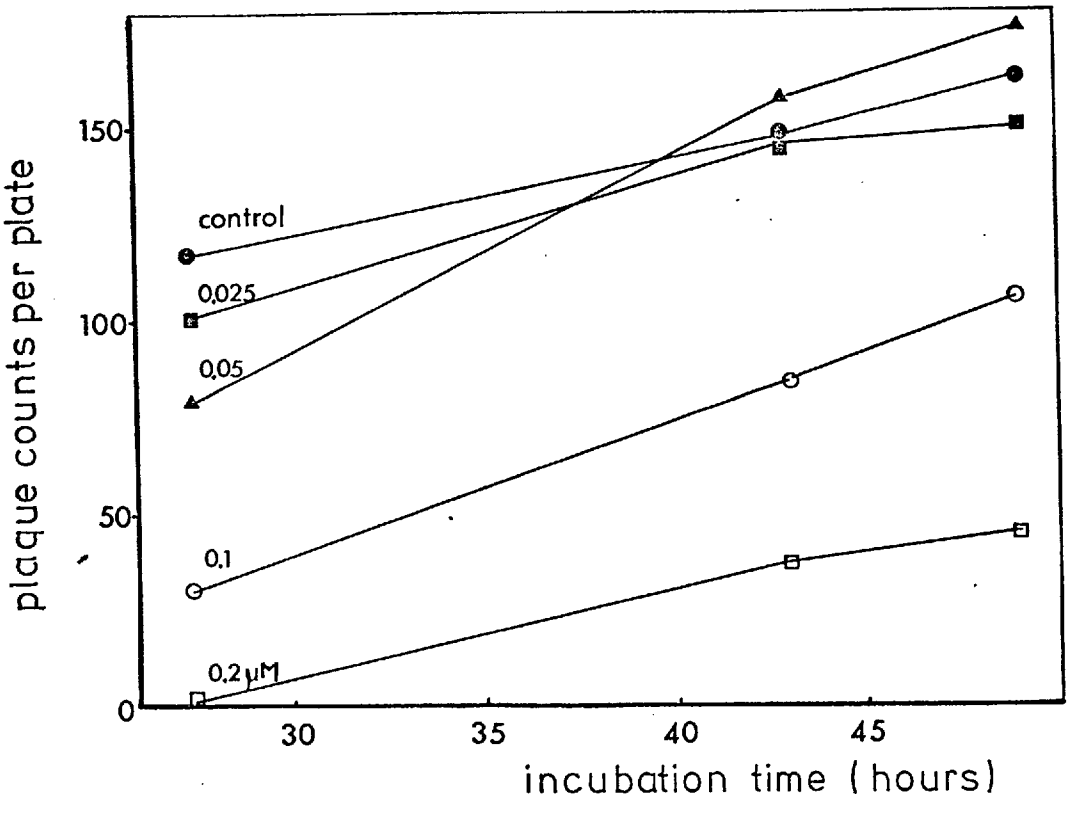


Fig. 3.6. Plaque reduction of poliovirus type 1 with Abbott 36683 in Vero cell cultures. Plaque counts per plate plotted against the incubation time, for control cultures and treated cultures at 4 different compound concentrations.

Figs. 3.5 and 3.6 show the importance of standardizing the incubation time for cultures in the plaque reduction test.

In Fig.3.5, the dose-response lines obtained when the cultures have been incubated for only 27 hours indicate that the compound appears to have a greater activity at this time than when the cultures have been incubated for 43 or 49 hours. For example, the concentration of compound required to decrease plaque counts to 50 per cent of the control plaque count are 0.071, 0.118 and 0.135 μM at 27, 43 and 49 hours respectively. Additionally after 27 hours incubation a 0.2μM concentration of compound in the overlay will cause complete inhibition of plaques. However after 43 or 49 hours incubation complete plaque suppression is not obtained with a 0.2μM concentration of compound. Since the activity of one compound against one virus must be an invariant quantity, these results indicate a drawback in the plaque reduction technique, in that the incubation time of cultures must be constant in successive experiments.

It is of interest to note that after 43 and 49 hours incubation a 0.05μM concentration of compound enhances plaque formation (i.e. increases the plaque count over the control plaque count). This is also apparent from Fig.3.6, which shows the increase on plaque count with time at various compound concentrations. At all compound concentrations except 0.05μM the plaque count increases with time approximately at the same rate as the control cultures in the absence of compound.

Plaque Size

It was thought that it might be of interest to compare the effect of Abbott 36683 on plaque number with its effect on plaque size. For each concentration of Abbott 36683 used and control cultures, except the 0.2μM cultures, 90 plaques were measured (to the nearest mm). On the 0.2μM test plates only 82 plaques in total were present and these were all measured.

From the data obtained graphs of average plaque diameter against compound concentration (Fig.3.7) for each incubation time, and of plaque diameter against incubation time for each compound concentration used (Fig.3.8) were plotted. Also histograms were drawn to show the distribution of plaque sizes for each incubation time and compound concentration (Fig.3.9).

It is apparent from Fig.3.7 that there is a great difference in the dose-response lines obtained at the 3 incubation times. Particularly, the dose-response lines at 43 and 49 hours show a greater difference than the 43 and 49 hours plaque count dose-response lines (Fig.3.5).

Also Fig.3.8 shows that in control cultures there is a linear increase in plaque size between 27 and 49 hours. However, for low concentrations of Abbott 36683 the rate of increase in plaque size is slower than in control cultures between 27 and 43 hours, but is greater than in control cultures between 43 and 49 hours.

It is of interest to note that a 0.05μM concentration of compound does not enhance plaque size although it increases plaque count.

Fig.3.9 showing the distribution of plaque sizes in the cultures for various compound concentrations and incubation times used, shows very clearly the effect of compound concentration and incubation time on plaque size distribution. At 27 hours nearly all plaques are no greater than 2mm in diameter. However, at 43 and 49 hours plaque sizes reach 6mm in diameter in control cultures. At 27 hours all concentrations of compound used have a marked effect on plaque size distribution, but at 43 and 49 hours a concentration of at least 0.05μM is required to alter the distribution of plaque sizes.

Fig.3.10 shows photographs of portions of plates from which the distribution of plaque sizes shown in Fig.3.9 were determined. It can be seen that poliovirus produces large clear plaques in Vero cell

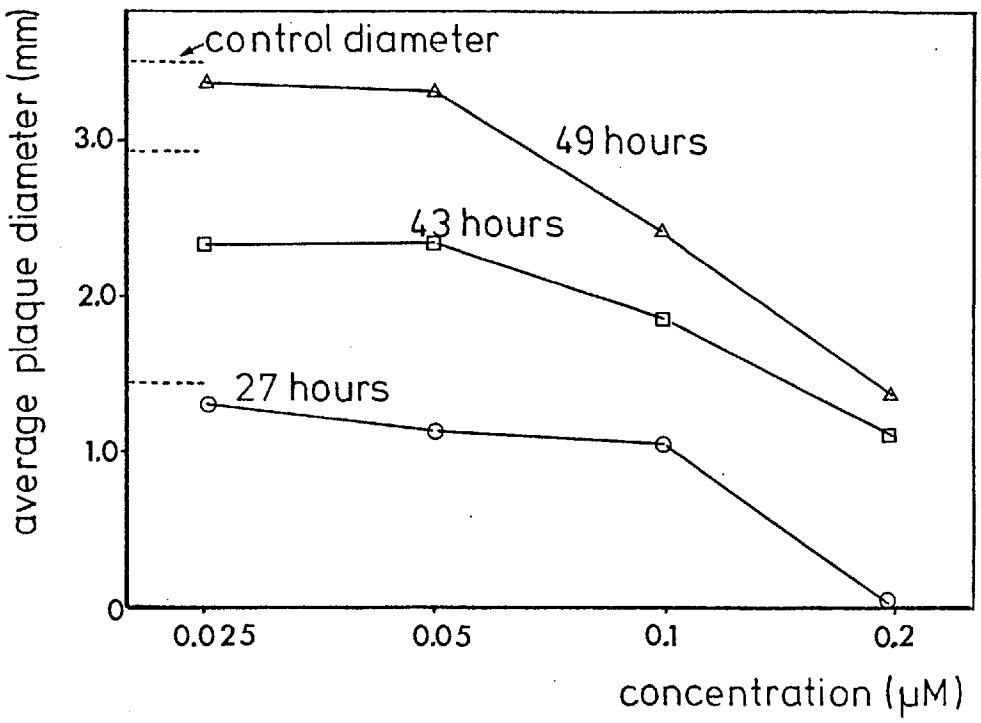


Fig. 3.7. Plaque reduction of poliovirus type 1 with Abbott 36683 in Vero cell cultures. Average plaque diameter plotted against the compound concentration (log scale) at 3 different incubation times.

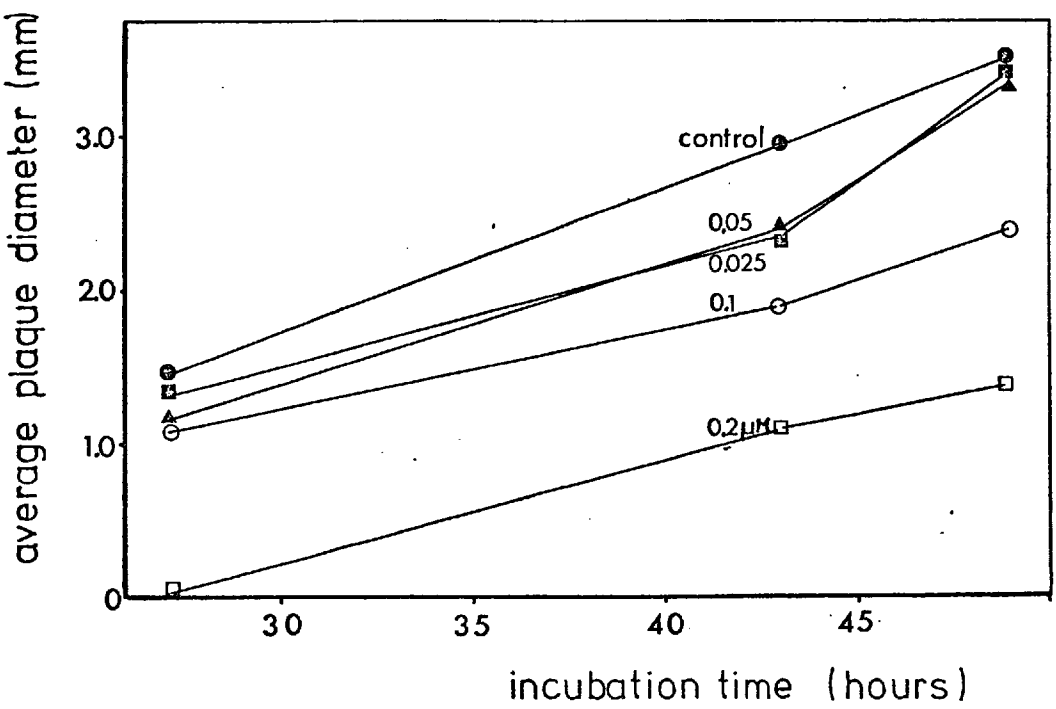


Fig. 3.8. Plaque reduction of poliovirus type 1 with Abbott 36683 in Vero cell cultures. Average plaque diameter plotted against the incubation time for control cultures and treated cultures at 4 different compound concentrations.

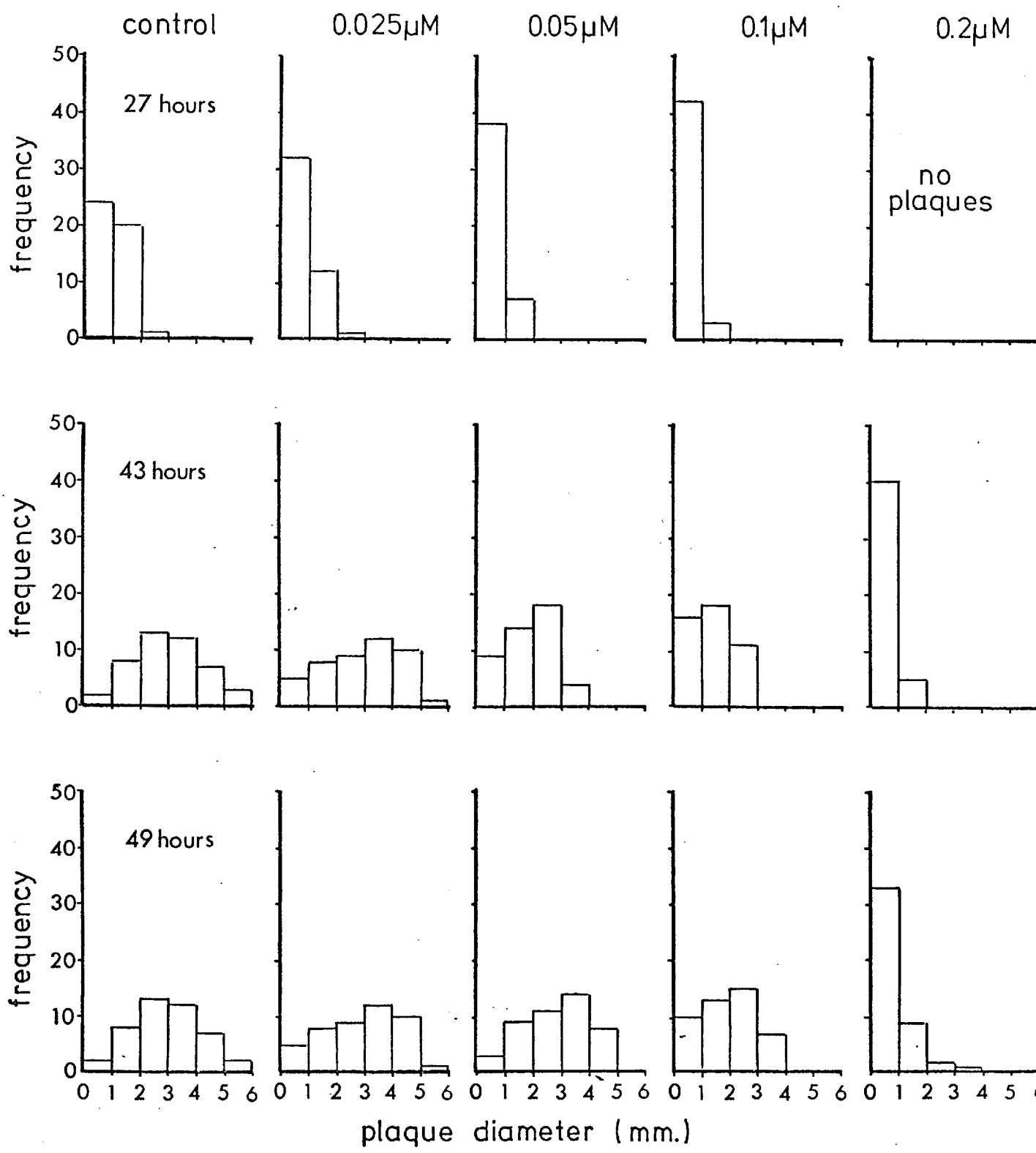


Fig. 3.9. Distribution of poliovirus type 1 plaque sizes in Vero cell cultures, control or treated with Abbott 36683 at 4 concentrations (0.025, 0.05, 0.1 or 0.2μM) after 3 different incubation times.

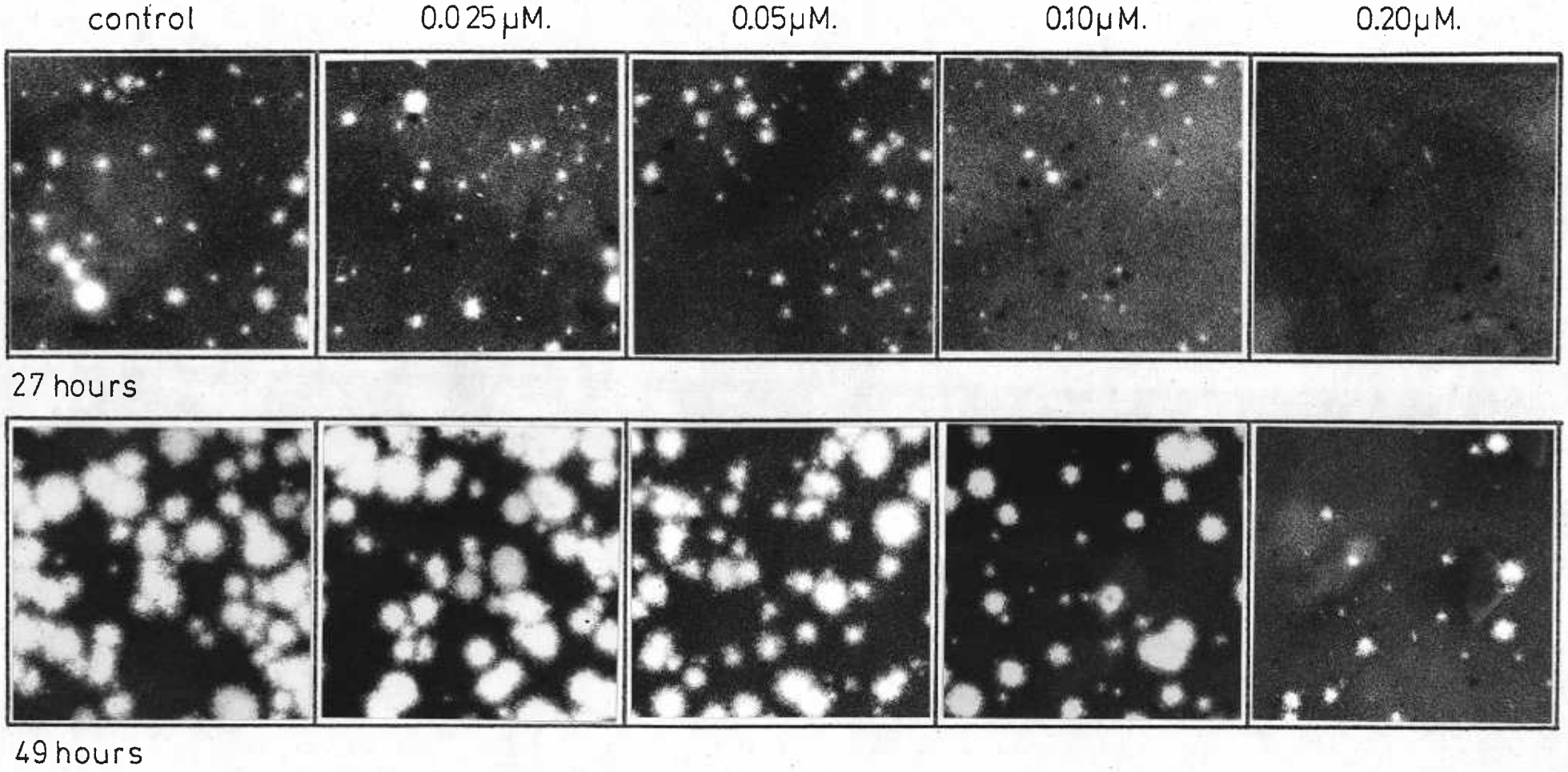


Fig.3.10. Plaque Reduction of Poliovirus type 1 with Abbott 36683 in Vero cells, after two different periods of incubation.

monolayers and the reduction in the size and number of these plaques by increasing concentrations of Abbott 36683 is apparent.

Size of Virus Input Inoculum

It was thought important to determine whether or not the size of the virus input inoculum has any effect on the measured activity of a compound, since a large part of the work in this thesis is to compare the sensitivity of different rhinovirus serotypes to certain compounds by the plaque inhibition method. Hence, determining whether the size of the virus input inoculum has any effect on the measured activity of a compound would dictate whether it is necessary to use virus input inocula of the same size for all the different rhinovirus serotypes used in the plaque inhibition experiments.

An experiment similar to the one just described was carried out, where infected cultures were treated with different concentrations of Abbott 36683 and the plaques which formed after various incubation times were counted. However, the virus inoculum used here was approximately 250 pfu per culture instead of 150 pfu per culture. Cultures were incubated for 29, 45 and 53 hours, plaques were visualised in the normal way and then the decrease in plaque count at each concentration as a percentage of the average control plaque count was plotted against the log of the concentration of the compound (Fig.3.11) for these data and for the data from the previous experiment. The results show that there is a great difference between the 2 sets of dose-response lines obtained for 2 virus input inocula. Again, however, it is seen that Abbott 36683 appears to be most active when the cultures have been incubated for only 29 hours. The 2 sets of dose-response lines would therefore suggest that the compound, in the concentration range 0.025 to 0.2μM is more active against an input inoculum of 150 pfu per culture than against an input inoculum of 250 pfu per culture. This is obviously unlikely.

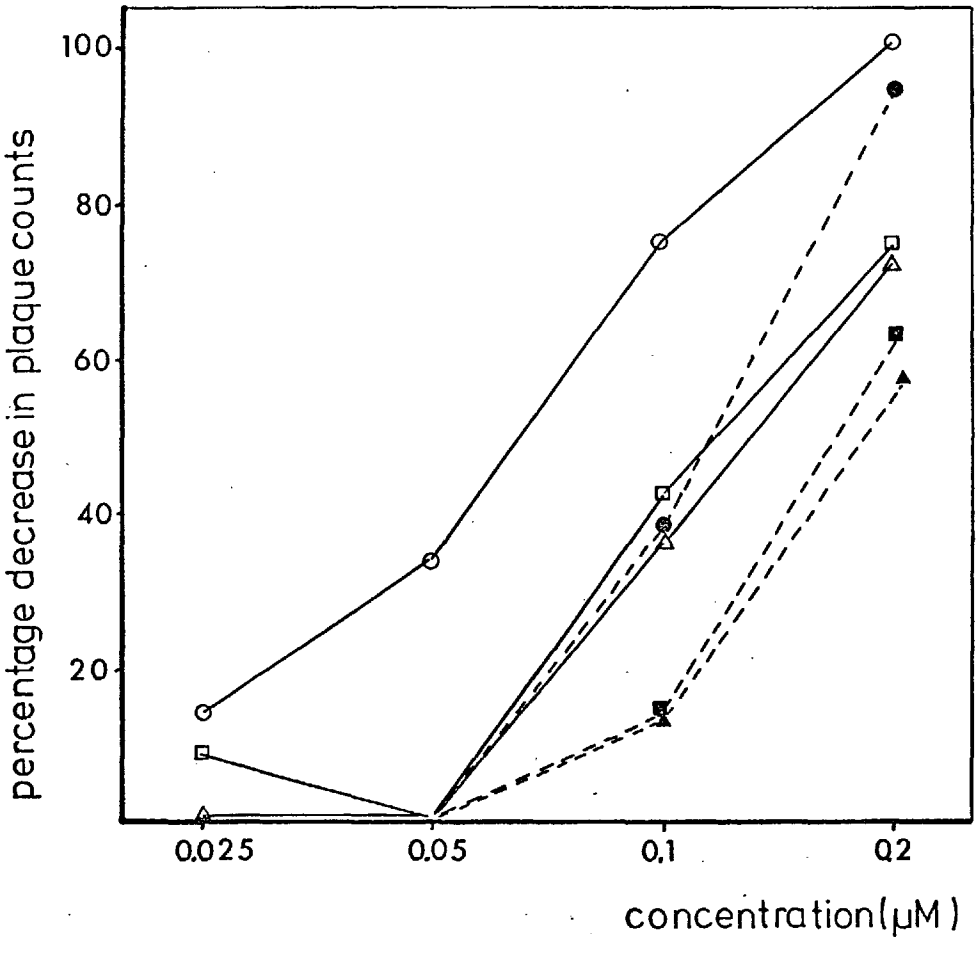


Fig. 3.11. Effect of virus inoculum size on activity of Abbott 36683 against poliovirus type 1 as measured by the plaque reduction method. Plaque reduction dose-response lines for Abbott 36683 against poliovirus type 1 in Vero cells.

——— cultures infected with approximately 150 pfu of virus.

------cultures infected with approximately 250 pfu of virus.

○——○27 hours ●---●29 hours

□——□43 hours ■---■45 hours

△——△49 hours ▲---▲53 hours

Moreover, from Table 3.7, which shows the numerical and percentage decrease in plaque counts for the 2 experiments, it can be seen that although the percentage decrease in counts in the cultures receiving the smaller virus inoculum is at all times greater than the percentage decrease in counts in the equivalent cultures receiving the larger inoculum, the numerical decrease in plaque counts is in many cases smaller.

This therefore makes the situation rather confused. It must therefore be a rule that to compare the sensitivity of serotypes to one compound or the activity of different compounds to one virus serotype, similar virus input inocula must be used.

Table 3.7

Average numerical and percentage decrease in plaque counts in cultures treated with Abbott 36683, compared with control cultures. Average decrease at three different incubation times and at two different sizes of virus input inoculum

(i) Input Inoculum, 270 pfu per culture

Concentration of compound (μM)	Numerical Decrease in Plaque counts			% Decrease in Plaque Counts		
	29	45	53	29	45	53
0.2	181	160	148	93.8	61.5	55.0
0.1	73	36	36	37.8	13.8	13.4
0.05	+ 8	+ 2	+ 14	0	0	0

(ii) Input Inoculum, 150 pfu per culture

	Numerical Decrease in Plaque counts			% Decrease in Plaque Counts		
	27	43	49	27	43	49
0.2	116	119	111	100	75.0	72.5
0.1	87	59	63	75	42.5	36.0
0.05	39	+16	+10	33.6	0	0

Comparison of activity of Abbott 36683 in two different Cell Lines

It was felt important to determine whether the activity of Abbott 36683 was a constant in 2 different cell lines.

A plaque reduction experiment was carried out again with poliovirus type 1 but in cultures of HeLa OH3 cells instead of Vero cells. The monolayers received either 150 or 25 pfu of virus inoculum and after adsorption the monolayers were given normal overlay medium containing compound at 0.025, 0.05, 0.1 and 0.2 μM. The cell cultures were fixed after 50 hours incubation at 36°, after which time the plaque size in the control cultures was seen to be obviously far smaller than in Vero cells. Dose-response lines of average plaque count against the log of the compound concentration were plotted (Fig.3.12) and compared with those obtained with Vero cells and a similar inoculum of 150 pfu per culture. (Fig.3.11).

Fig.3.12 shows that Abbott 36683 is far more active against poliovirus type 1 when tested in Vero cells compared with OH3 cells. At a 0.2μM concentration of Abbott 36683 the percentage decrease in plaque count is 73.0 and 11.0 % for Vero and OH3 cells respectively. This is despite the fact that plaque sizes are far larger in Vero than in OH3 cells (and one might well expect the activity of the compound to be less in Vero than in OH3 cells for this reason).

3.2.2(b) Use of the Plaque Reduction Test to Quantitate Rhinovirus Inhibitory Activity

The plaque reduction technique was used in order to quantitate the rhinovirus inhibitory activity of the 4 compounds Abbott 36683, SK&F 40491, SK&F 30097 and Wellcome 71-123. The test was carried out as described (section 2.5.3.(b). A large number of rhinovirus serotypes were used in this test in order to determine whether they displayed varying sensitivity to each of the compounds. As will be evident this necessarily resulted in the accumulation of a large number of data. Nine

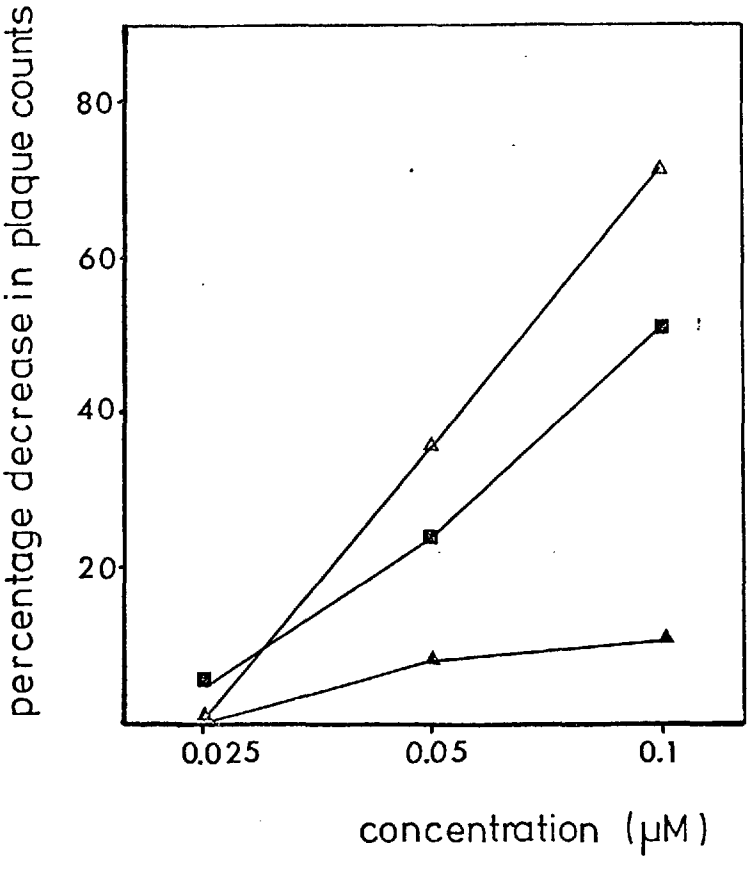


Fig. 3.12. Effect of cell line used and inoculum size on the activity of Abbott 36683 against poliovirus type 1 as measured by the plaque reduction method. Plaque reduction dose-response lines for Abbott 36683 against poliovirus type 1.

△——△ Vero cells, 150 pfu inoculum, 49 hours incubation.

▲——▲ OH3 cells, 150 pfu inoculum, 50 hours incubation.

■——■ OH3 cells, 25 pfu inoculum, 50 hours incubation.

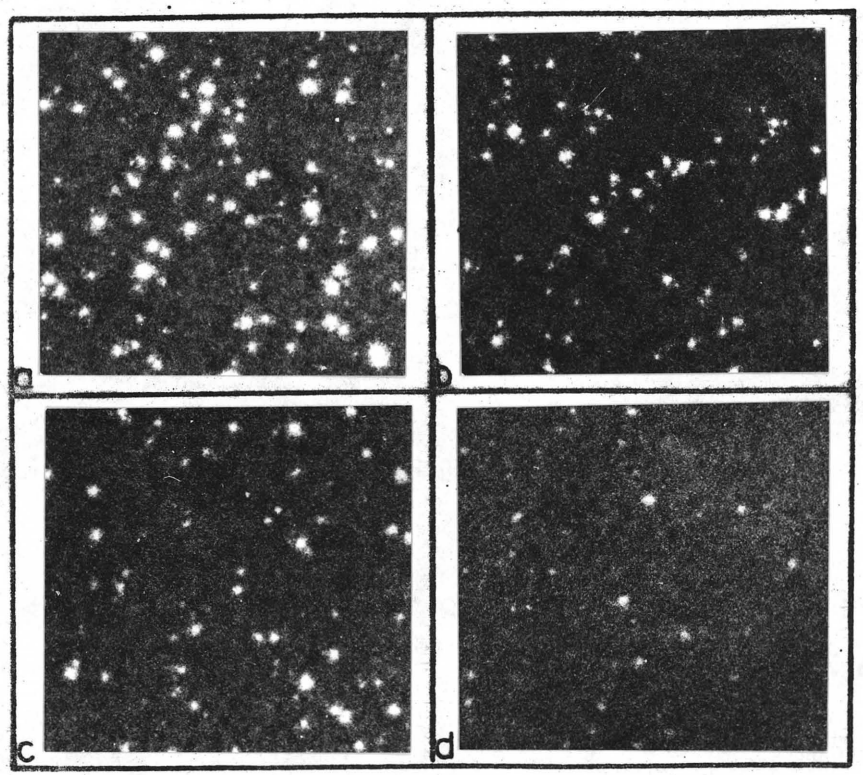
rhinoviruses were chosen for testing, 2 of which produced large plaques (types 14 and 17), 3 of which produced small plaques (types 53, 19 and 22) and 4 of which produced intermediate sized plaques (types 1B, 2, 12, and 30). Each of these viruses and poliovirus type 1 were tested against each of the compounds, with the exception of rhinovirus type 53, which was tested only against SK&F 40491, since plaquing attempts with this serotype were found to fail on many occasions.

It was first necessary to determine the concentration range over which the reduction in plaque number (calculated as a percentage of the number of control plaques) was dose-related. This was initially done with rhinovirus type 2 and the concentration ranges chosen from the results were: Abbott 36683, 0.05, 0.1, 0.2, and 0.4μM; SK&F 40491, 0.25, 0.5, 1.0, 2.0 and 4.0μM; SK&F 30097, 6.25, 12.5, 25.0 and 50.0μM; Wellcome 71-123, 3.125, 6.25, 12.5 and 25.0μM.

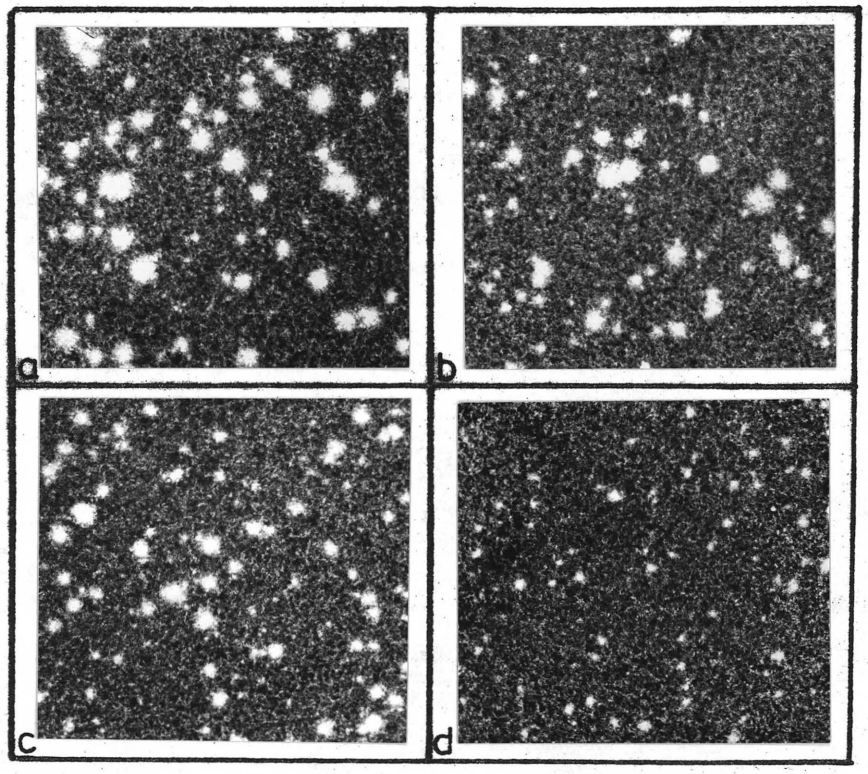
All 10 serotypes were tested against these concentrations. Figs. 3.13 to 3.15 show portions of the plates for 6 typical plaque reduction experiments. The decrease in plaque size and number with increasing compound concentration can be observed in each case. There is a variation in control plaque sizes from experiment to experiment, as can be seen from the plates for rhinovirus type 1B (Figs. 3.13 to 3.15).

In all experiments the number of plaques on each plate was counted. Initially the dose-response lines of percentage control count against the log of the concentration of the compound for each virus individually against all 4 compounds were plotted (Figs. 3.16 to 3.24). Then the dose-response lines for all viruses against each of the drugs individually were plotted (Figs. 3.25 to 3.28).

Figs. 3.16 to 3.24 show the difference in activity of the 4 compounds to each virus serotype. In all cases Abbott 36683 is the most active compound and SK&F 40491 the next most active. Generally SK&F 30097 is shown to be more active than Wellcome 71-123 although

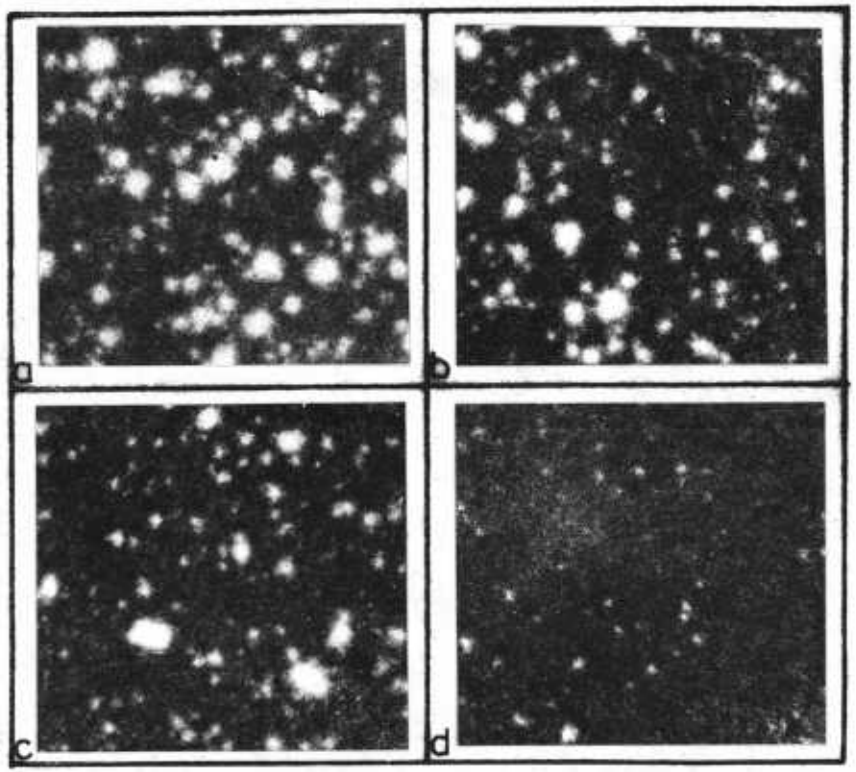


Rhinovirus type 1B

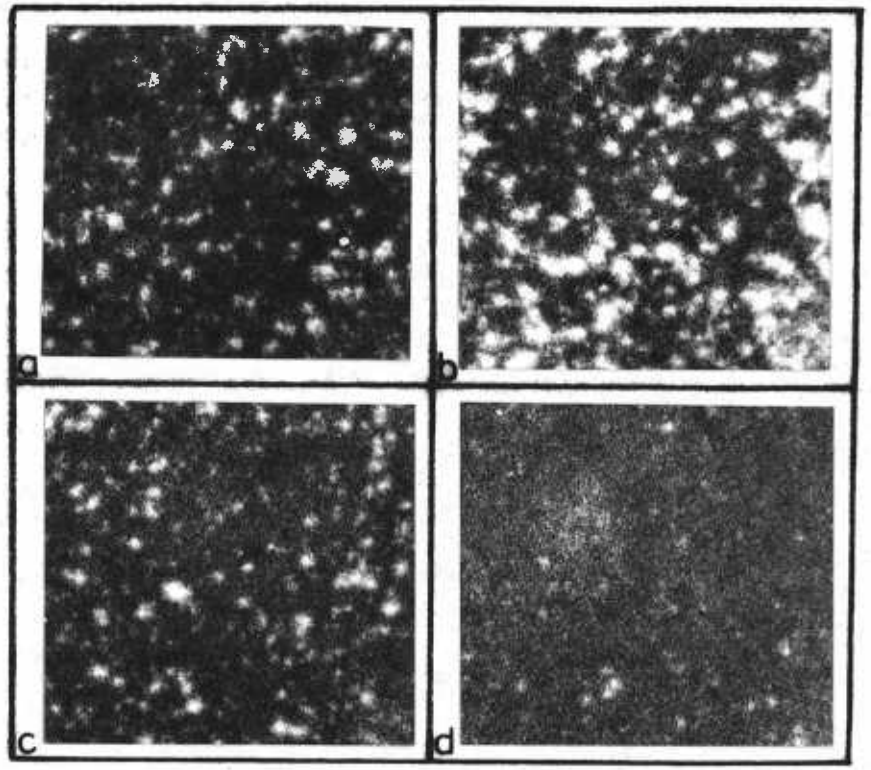


Rhinovirus type 2

Fig. 3.13. Plaque reduction of rhinovirus types 1B and 2 with SK&F 40491
a) control, b) 0.5 µM, c) 1.0 µM, d) 2.0 µM

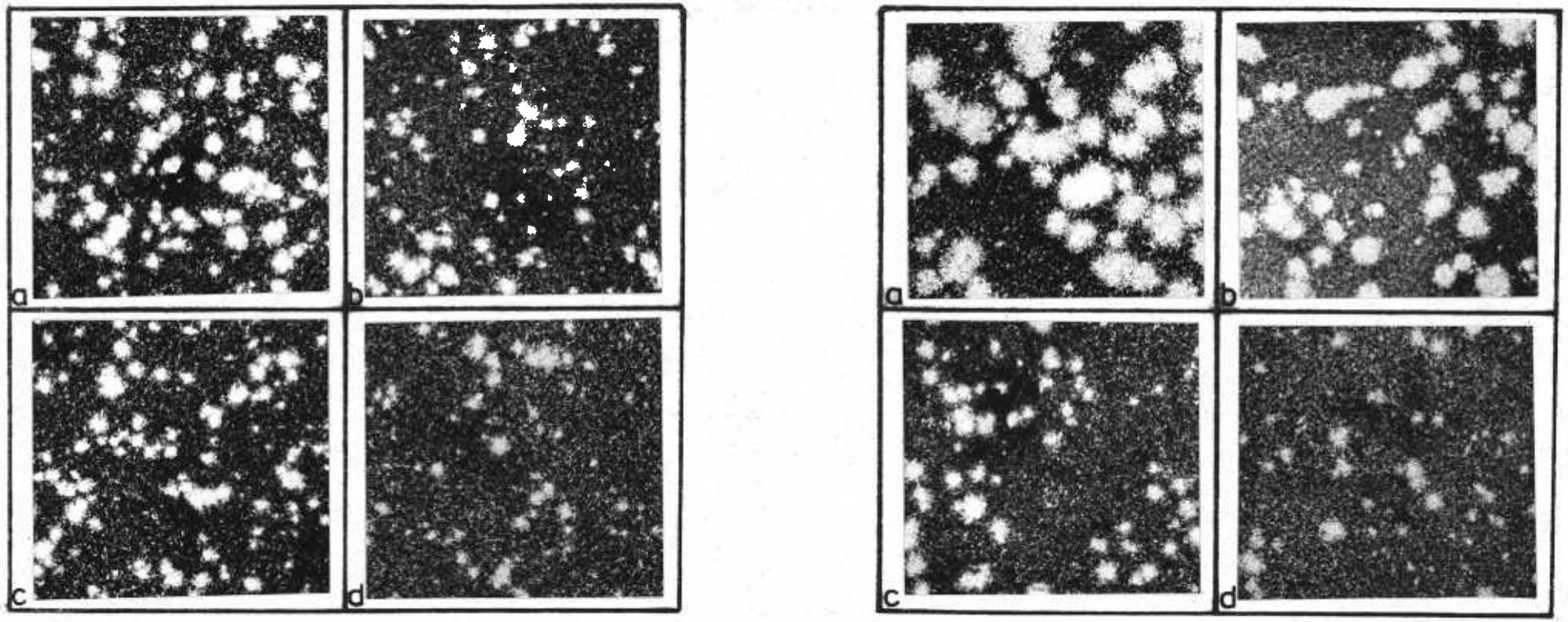


Rhinovirus type 1B



Rhinovirus type 12

Fig.3.14. Plaque reduction of rhinovirus types 1B and 12 with SK&F 30097
a) control, b) 6.25μM, c) 12.5μM, d) 25.0μM



Rhinovirus type 1B

Rhinovirus type 17

Fig.3.15. Plaque reduction of rhinovirus types 1B and 17 with Wellcome 71-123
a) control, b) 6.25μM, c) 12.5μM, d) 25.0μM.

Figs. 3.16 to 3.24. Plaque reduction dose-response lines for Abbott 36683 (○——○), SK+F 40491 (□——□), SK+F 30097 (△——△) and Wellcome 71-123 (▲——▲) against rhinovirus types 1B, 2, 12, 14, 17, 19, 22 and 30 and poliovirus type 1.
Percentage of the control plaque counts per plate in treated cultures plotted against the $\log_{10}$ of the concentration of the compound in the overlay medium.

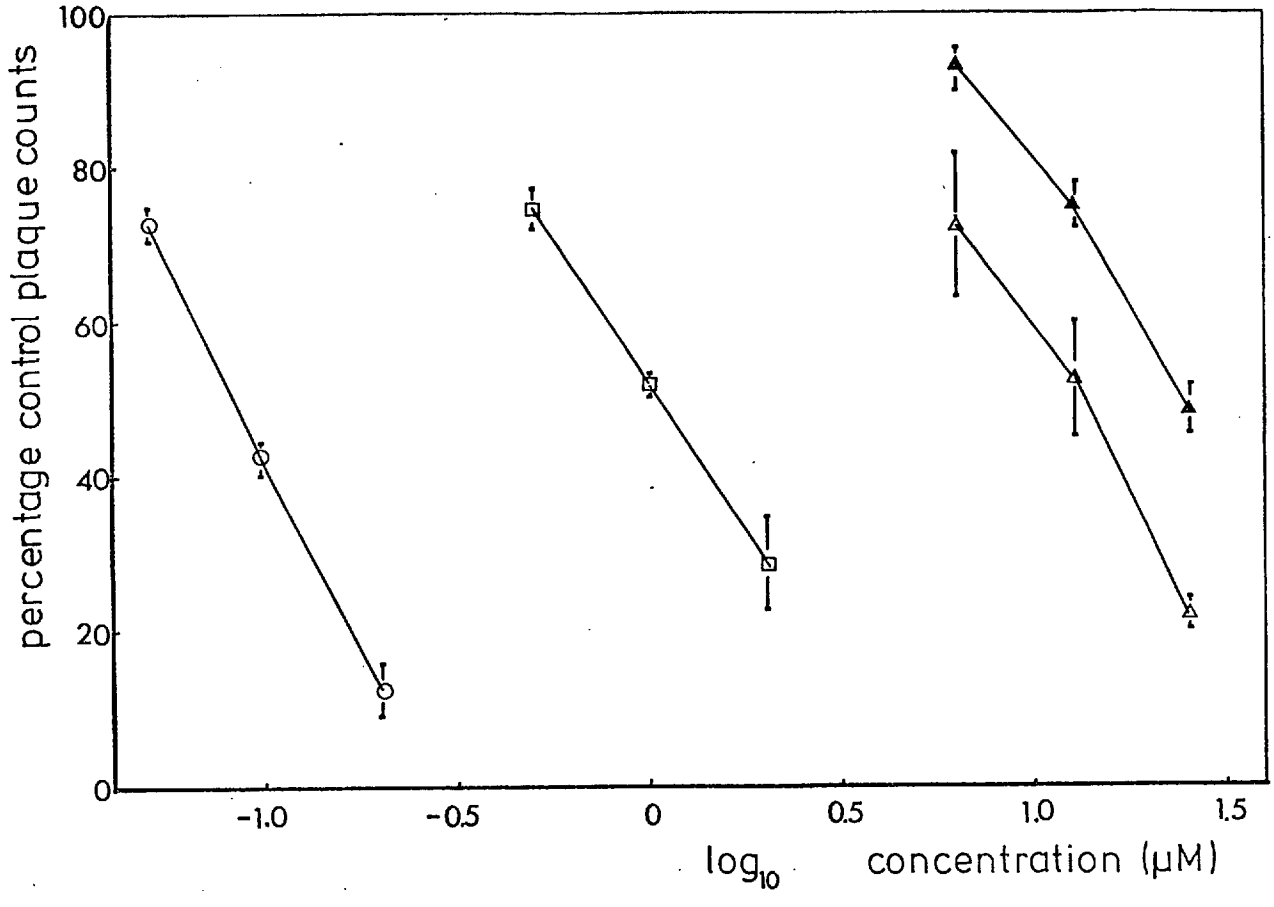


Fig. 3.16. Rhinovirus type 1B.

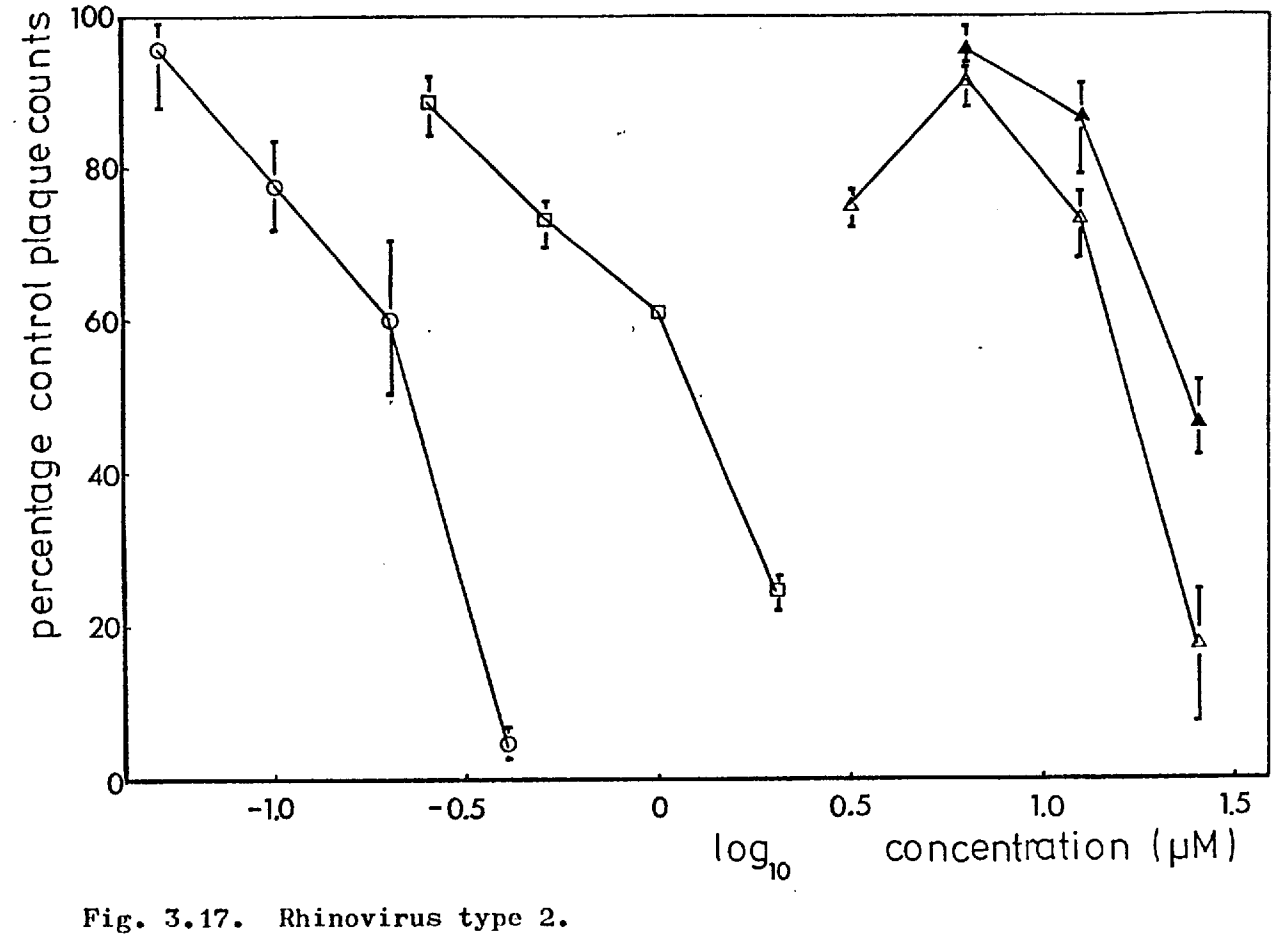


Fig. 3.17. Rhinovirus type 2.

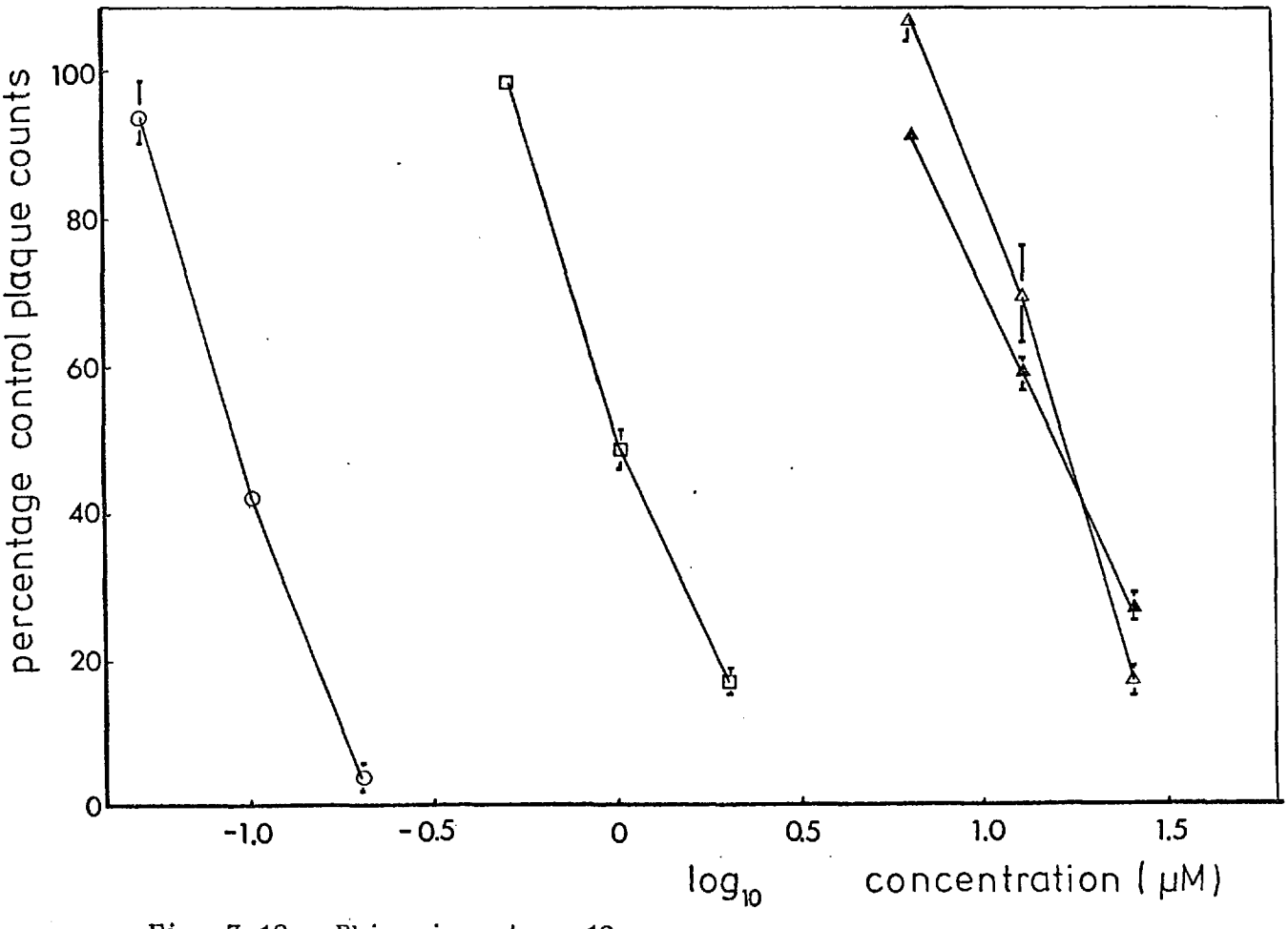


Fig. 3.18. Rhinovirus type 12.

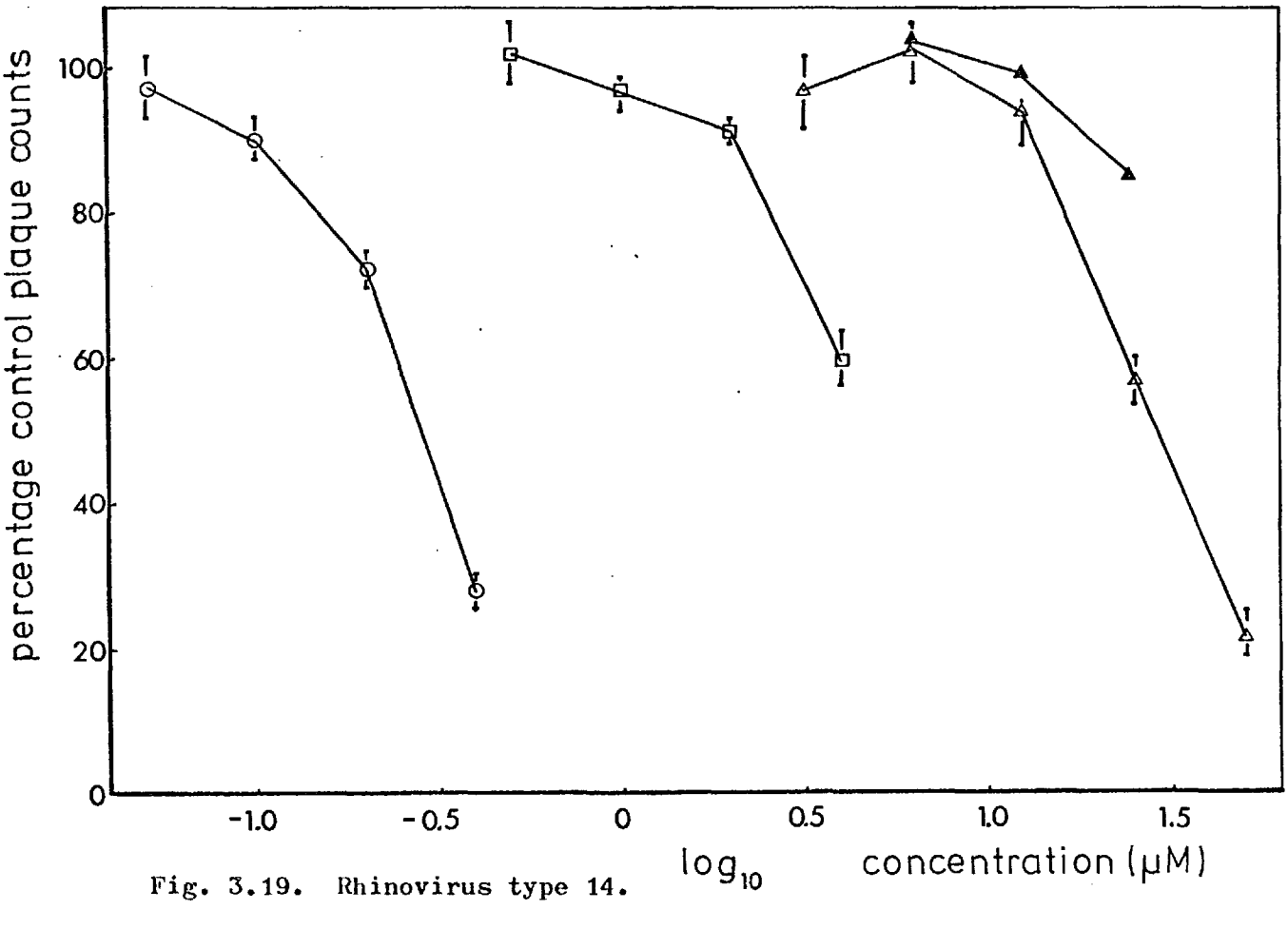


Fig. 3.19. Rhinovirus type 14.

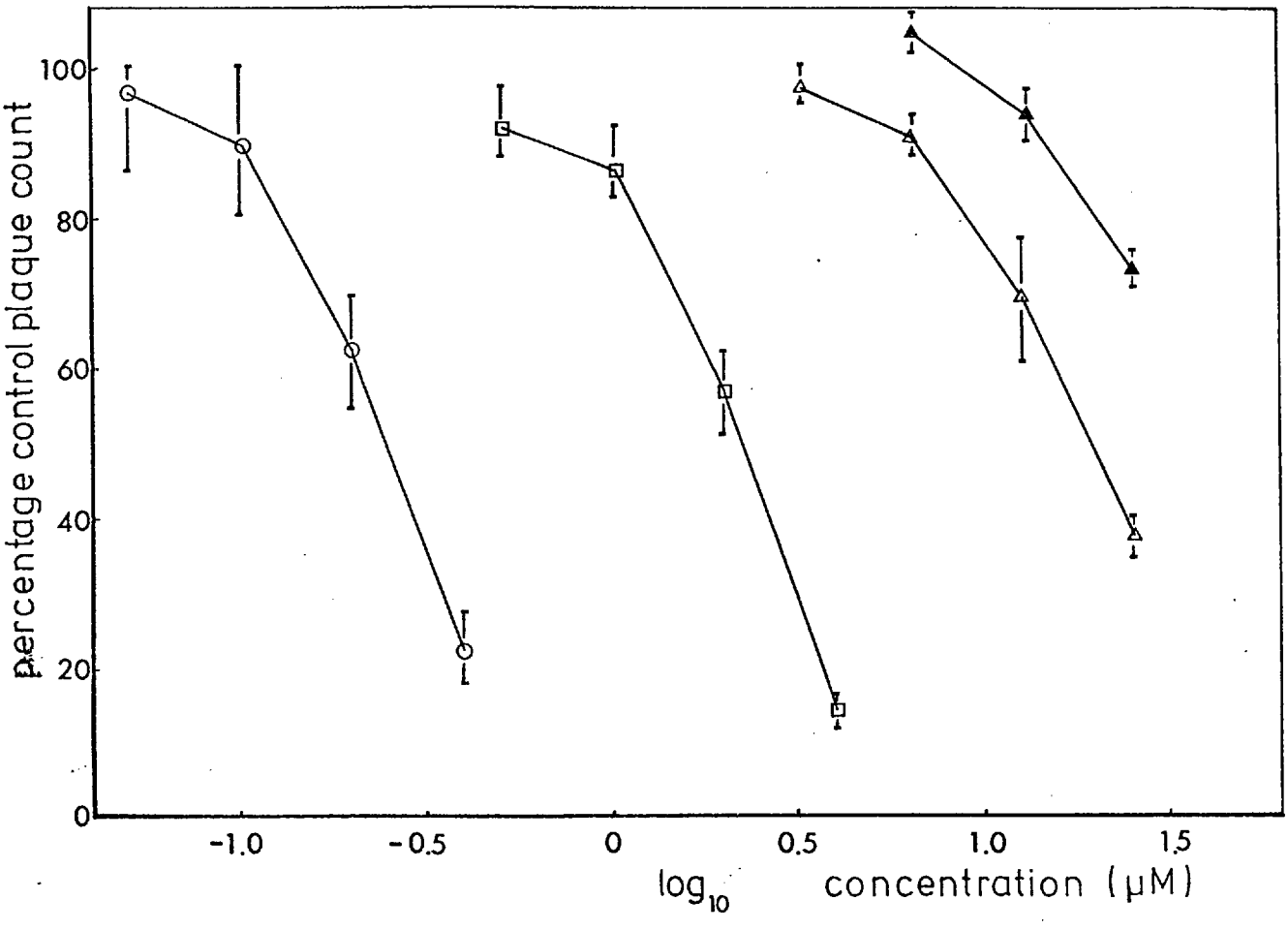


Fig. 3.20. Rhinovirus type 17.

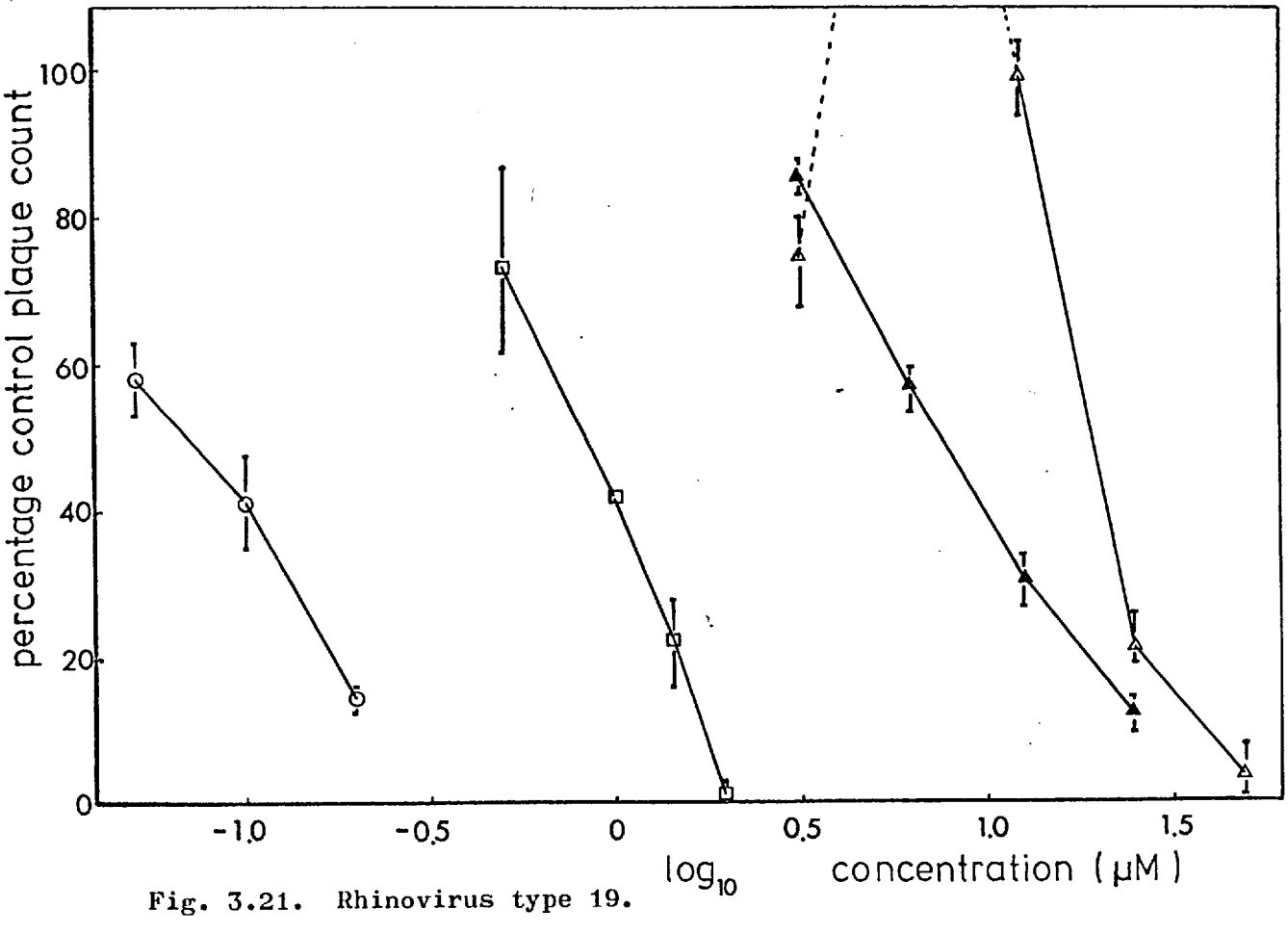


Fig. 3.21. Rhinovirus type 19.

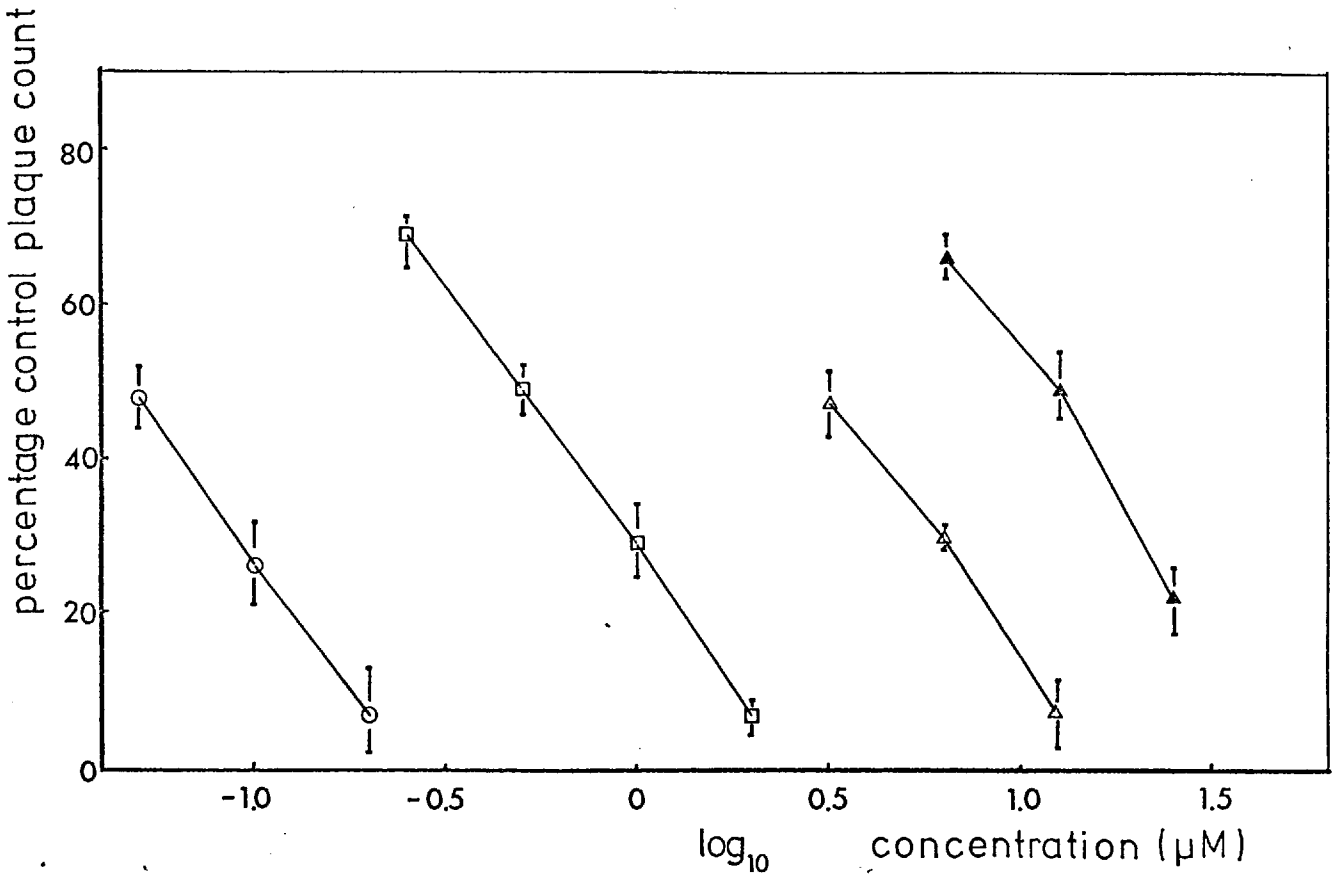


Fig. 3.22. Rhinovirus type 22.

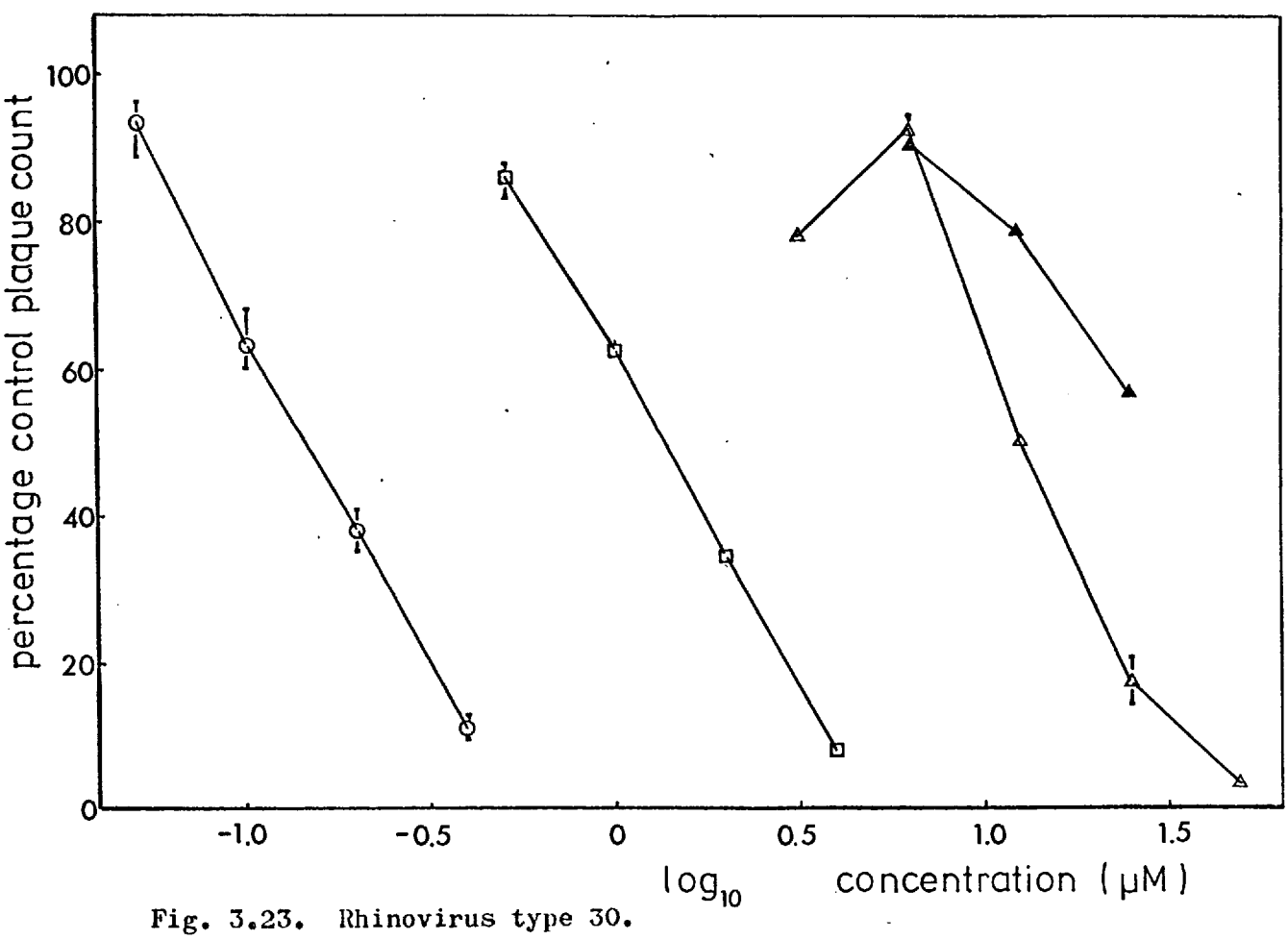


Fig. 3.23. Rhinovirus type 30.

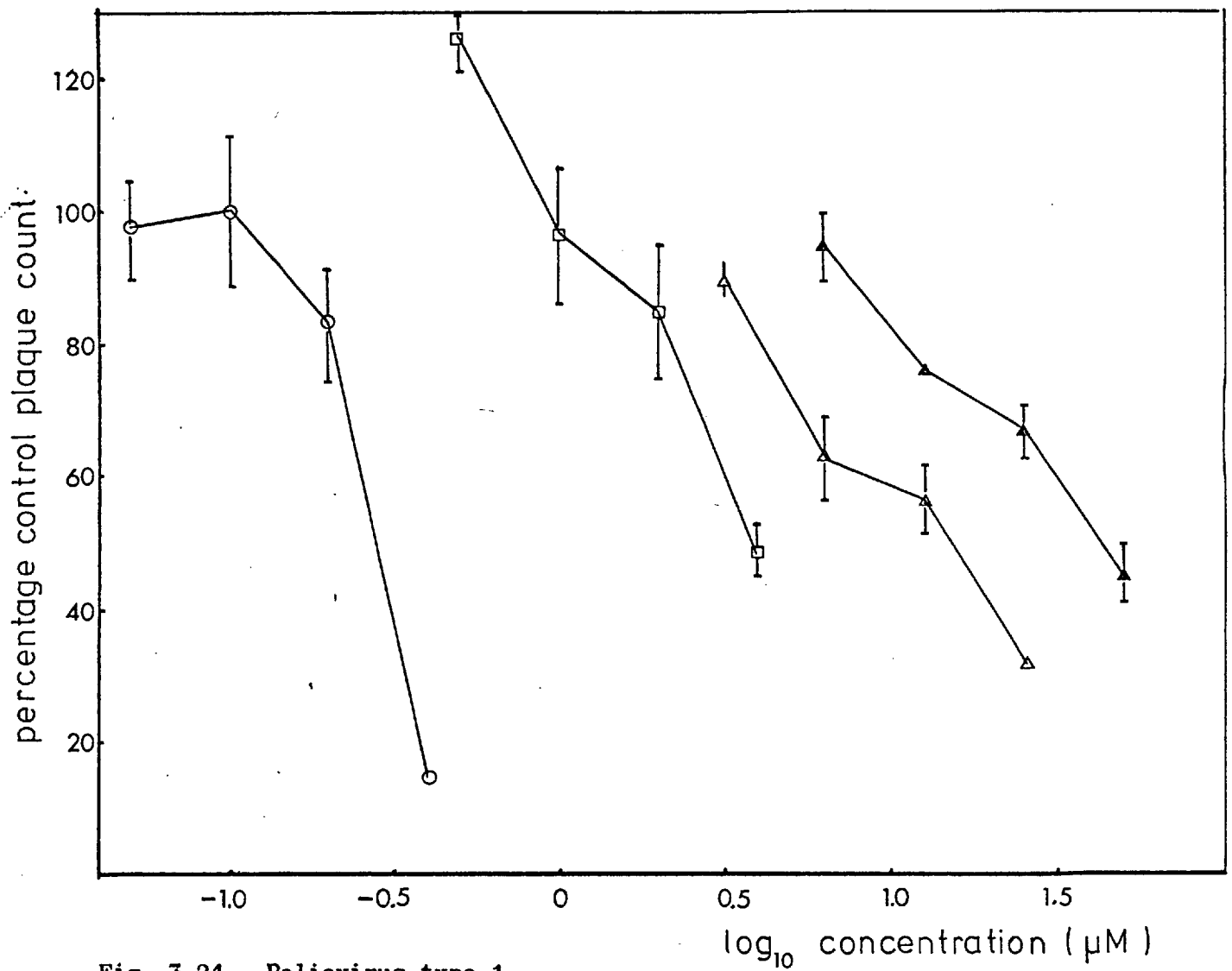


Fig. 3.24. Poliovirus type 1.

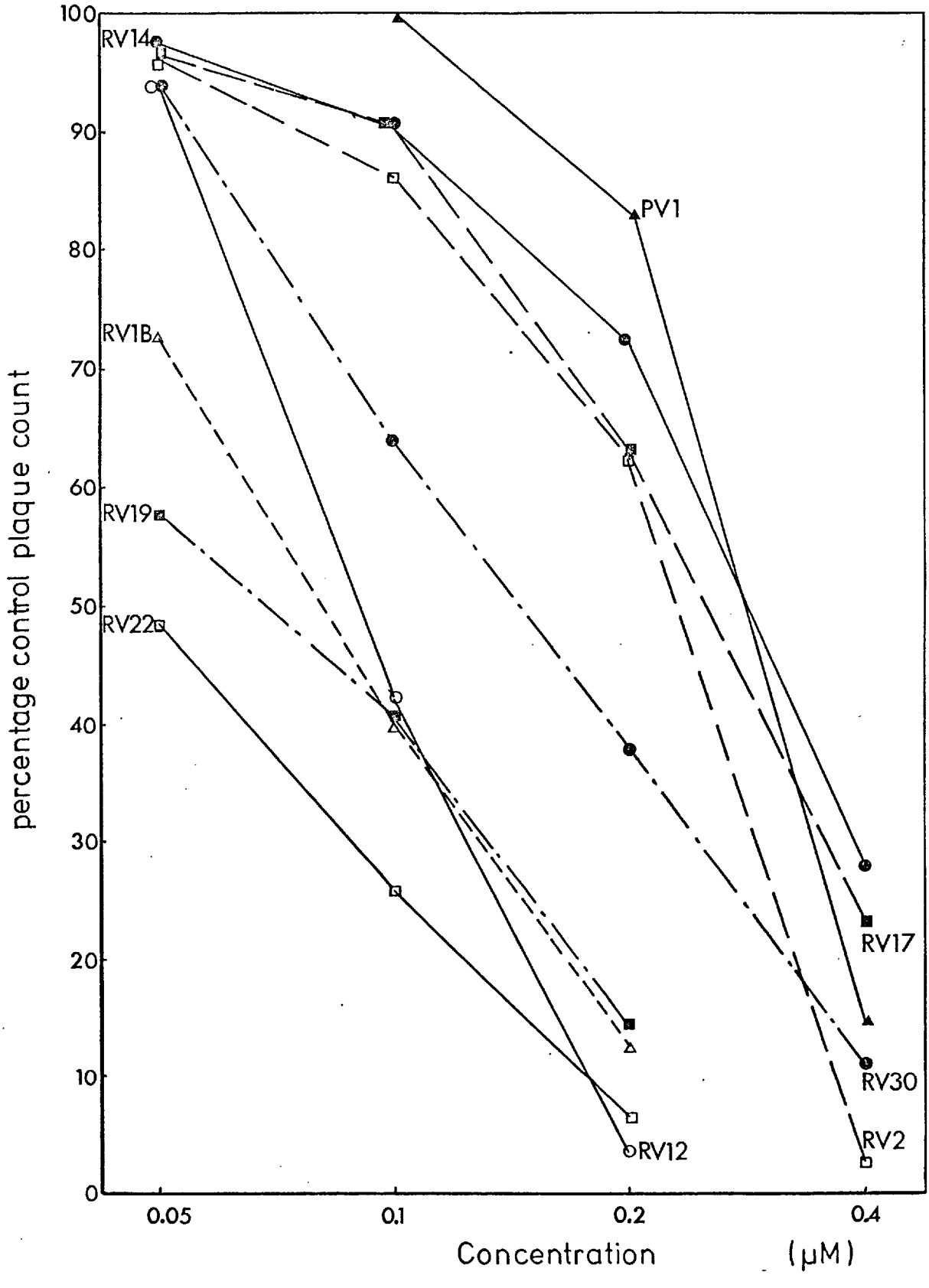


Fig. 3.25. Plaque reduction dose-response lines for Abbott 36683 against 8 rhinovirus serotypes and poliovirus type 1. Percentage of the control plaque counts per plate in treated cultures plotted against the concentration of the compound in the overlay medium ($\log_2$ scale).

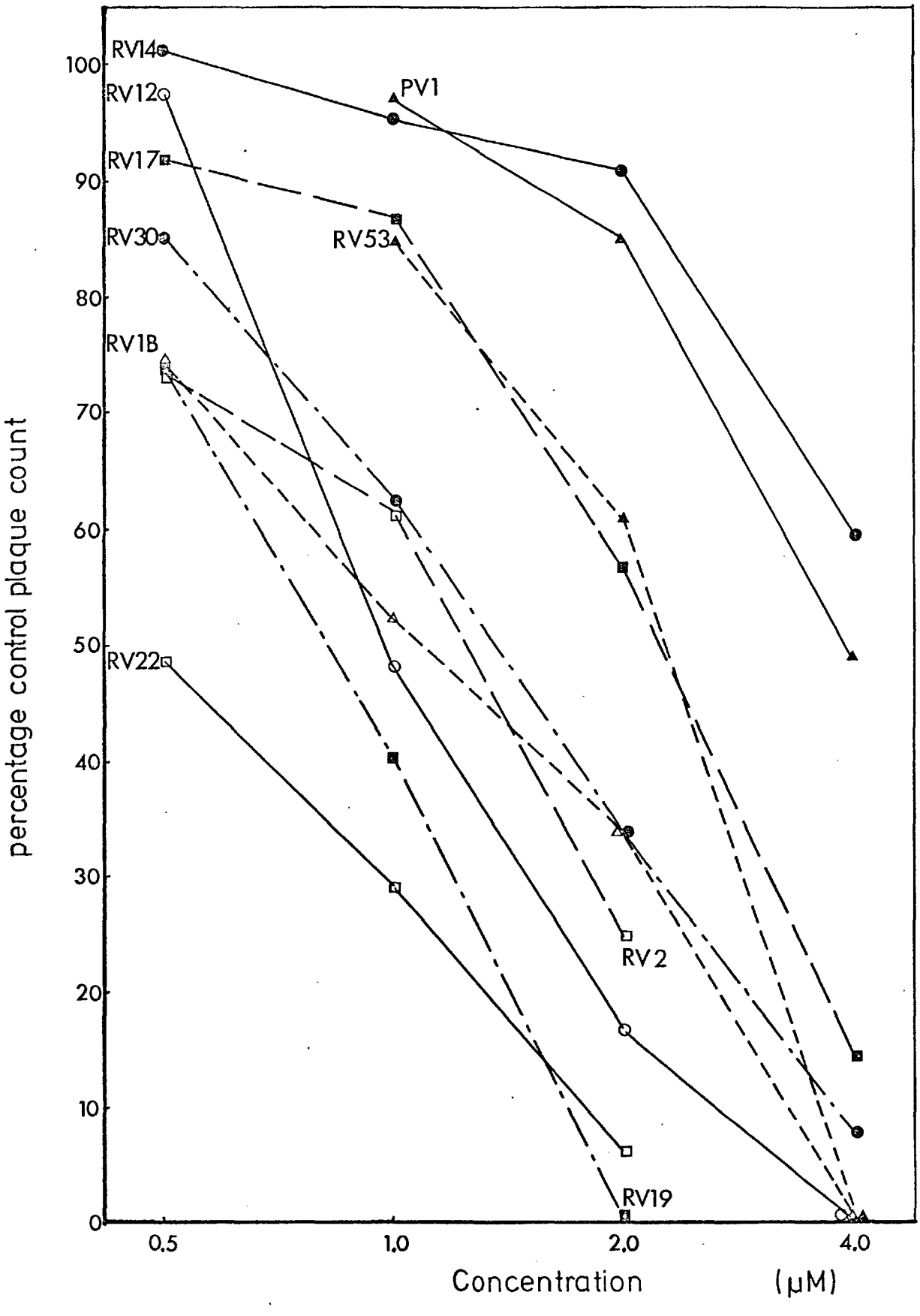


Fig. 3.26. Plaque reduction dose-response lines for SK+F 40491 against 9 rhinovirus serotypes and poliovirus type 1. Percentage of the control plaque counts per plate in treated cultures plotted against the concentration of the compound in the overlay medium ($\log_2$ scale).

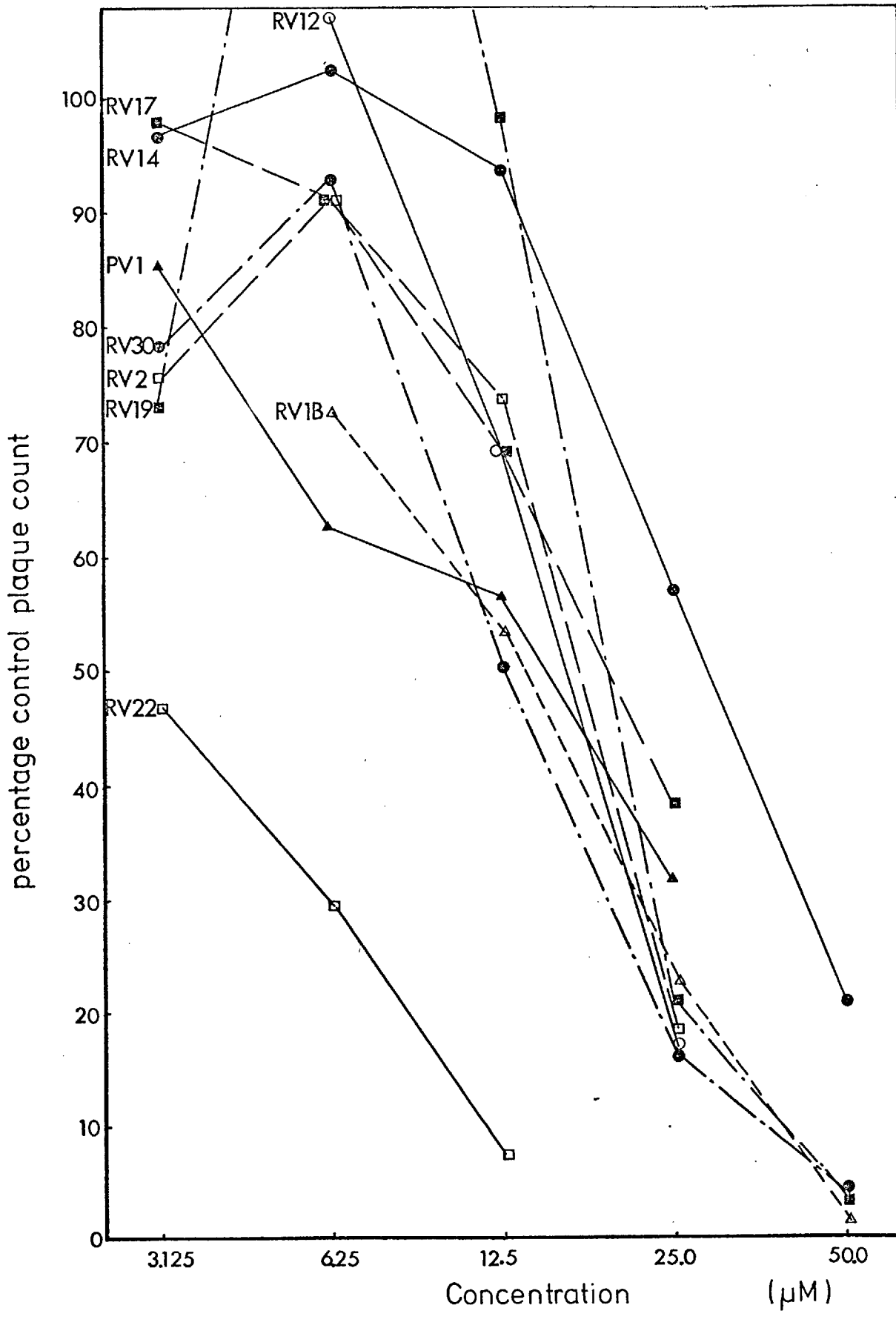


Fig. 3.27. Plaque reduction dose-response lines for SK+F 30097 against 8 rhinovirus serotypes and poliovirus type 1. Percentage of the control plaque counts per plate in treated cultures plotted against the concentration of the compound in the overlay medium ($\log_2$ scale).

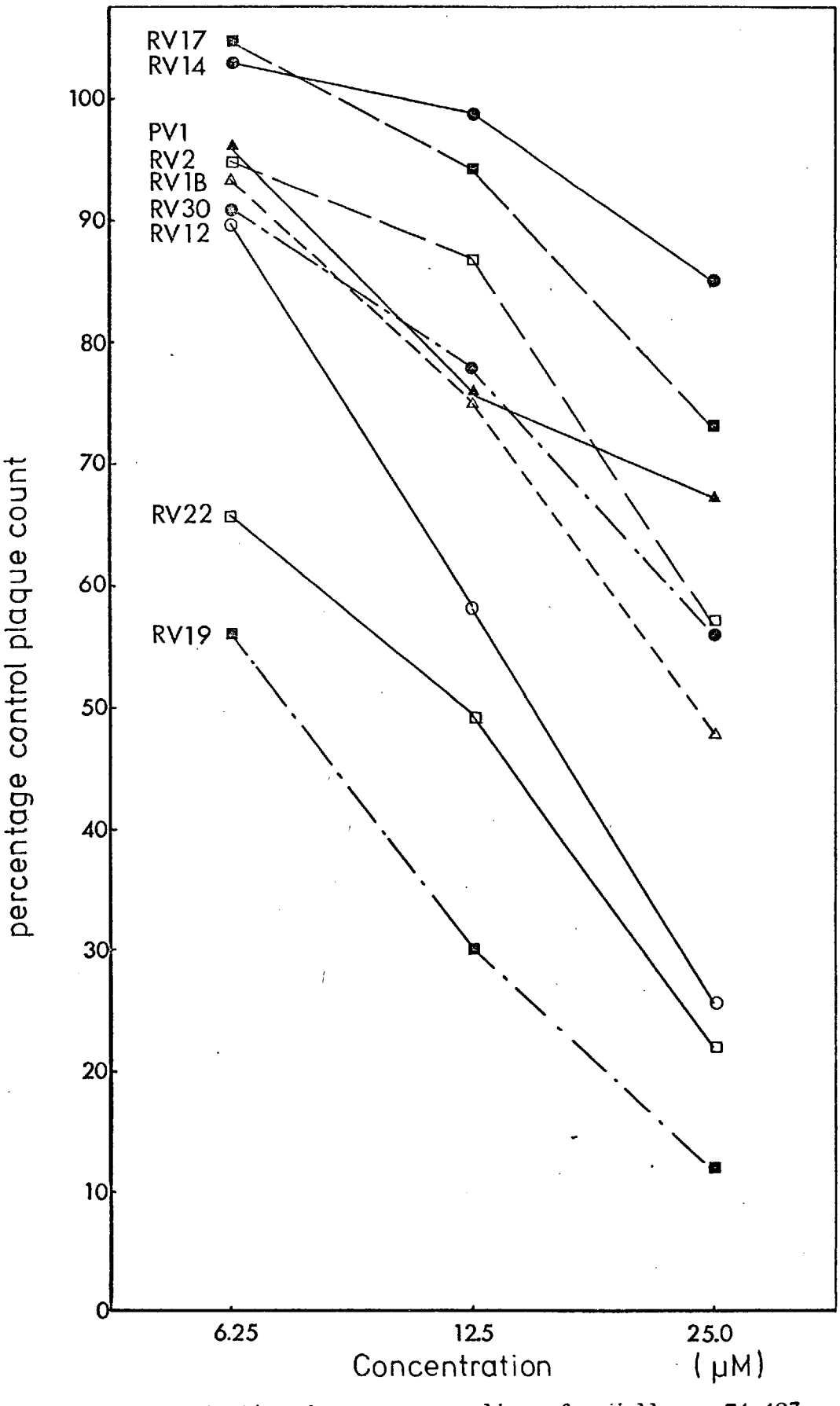


Fig. 3.28. Plaque reduction dose-response lines for Wellcome 71-123 against 8 rhinovirus serotypes and poliovirus type 1. Percentage of the control plaque counts per plate in treated cultures plotted against the concentration of the compound in the overlay medium ($\log_2$ scale).

against rhinovirus type 19, 71-123 appears more active than SK&F 30097. However, it must be noted here that a low concentration of SK&F 30097 (6.25μM) in fact enhances plaque formation by many serotypes, the effect being particularly pronounced with type 19. The plaque count for type 19 under an overlay containing 6.25μM SK&F 30097 was almost double that obtained under an overlay containing no compound. Hence the dose-response line produced (Fig.3.21) is rather unusual and one can see that if the 6.25μM point is omitted from this plot, SK&F 30097 would appear to have similar activity to Wellcome 71-123 against type 19. Additionally plaque counts for types 2, 12, 14, and 17 were greater at a compound concentration of 6.25μM than at 3.125μM or in control cultures.

The plaque enhancing effect was also apparent with serotypes 12, 19 and 53 under an overlay containing 0.25μM SK&F 40491 (not shown on graphs). However, most serotypes were slightly inhibited by this concentration of compound in the overlay.

From the results it was hoped to be able to obtain some numerical value for the relative activities of the 4 compounds against each virus serotype. The relative potency, R, of one compound to another may be determined from the dose-response lines of the 2 compounds (response plotted against the log of the concentration of the compound), provided that the lines are parallel. The relative potency is in fact a measure of the horizontal distance between the 2 lines and can be measured directly if the lines are definitely parallel, or can be calculated from the original data for the dose-response lines using the mathematical relation:

$$\log R = \log x_s - \log x_t - (\bar{y}_s - \bar{y}_t)\, b^{-1}$$

where x_s is the mean dose of the standard compound
x_t is the mean dose of the test compound
y_s is the mean response of the standard compound
y_t is the mean response of the test compound

and b is the combined slope of the 2 dose-response lines.

The relative potencies of the 4 compounds were determined, using Abbott 36683 as the standard compound, with the aid of a computer program devised for this purpose. For this, the dose of compound and the response (the individual values for percentage of control counts) is required. From the data given the program determines the mean dose, mean response and slope of each dose-response line. The deviations of each line from linearity are also determined and a test of significance is applied to these, showing whether the lines differ significantly from linearity. A chi-squared test is applied to the slopes as a whole to determine whether they differ significantly from one another. If the slopes do differ significantly, the relative potencies of the compounds cannot be determined, or at least the values calculated are not meaningful. In order to obtain good values for relative potency the slopes of the individual dose-response lines must not differ significantly.

Data from each of the sets of plots shown in Figs. 3.16 to 3.24 were computed in order to determine the relative potencies of the 4 compounds. In many cases not all the points on the graphs were used but only those which would give lines which did not differ significantly in slope. The relative potencies of SK&F 40491 and 30097 and Wellcome 71-123 compared with that of Abbott 36683 are given in Table 3.8.

The relative potency of SK&F 40491 measured against all 8 rhinovirus serotypes varies between 0.054 and 0.206, that is, Abbott 36683 is 4.85 to 18.5 times more active than SK&F 40491. Similarly Abbott 36683 is 55.5 to 200 times more active than SK&F 30097 and 91.0 to 333 times more active than Wellcome 71-123 as determined by the plaque reduction method.

Having determined the difference in the individual activities of the compounds it was hoped that some conclusions might be drawn from the dose-response lines shown in Figs. 3.25 to 3.28, as to the individual sensitivities of each virus to each particular compound.

Table 3.8 Relative potency of SK&F 40491, SK&F 30097 and Wellcome 71-123 compared with Abbott 36683 against 8 Rhinovirus Serotypes and Poliovirus type 1.

Serotype	Relative Potency against virus (and limits) compared with Abbott 36683 SK&F 40491	SK&F 30097	Wellcome 71-123
1B	0.079 (0.093-0.066)	0.007 (0.008-0.006)	0.004 (0.005-0.003)
2	0.167 (0.206-0.134)	0.013 (0.016-0.011)	0.009 (0.011-0.007)
12	0.087 (0.095-0.079)	0.006 (0.006-0.005)	0.007 (0.007-0.006)
14	0.063 (0.074-0.054)	0.010 (0.011-0.009)	
17	0.114 (0.140-0.094)	0.013 (0.016-0.011)	0.006 (0.008-0.005)
19	0.082 (0.126-0.053)	0.008 (0.012-0.005)	0.008 (0.012-0.006)
22	0.097 (0.113-0.083)	0.016 (0.018-0.013)	0.004 (0.005-0.004)
30	0.110 (0.119-0.102)	0.011 (0.012-0.010)	0.005 (0.006-0.005)
PV 1	0.062 (0.094-0.039)	0.028 (0.043-0.019)	0.010 (0.014-0.006)

In most cases the virus serotypes can be placed in a rough order of sensitivity to each of the compounds from observations of the dose-response lines. In some cases, especially for compound SK&F 30097, the dose-response lines for different rhinovirus serotypes cross one another, thus making a comparison of the sensitivity of the viruses very difficult.

However, an attempt has been made to place the viruses in order of increasing sensitivity to the 4 compounds and this is shown in Table 3.9.

Table 3.9

Rhinovirus serotypes and poliovirus type 1 listed in order of increasing sensitivity to Abbott 36683, SK&F 40491, SK&F 30097 and Wellcome 71-123, as determined by the plaque reduction method

Abbott 36683	SK&F 40491	SK&F 30097	Wellcome 71-123
PV1	14	14	14
14	PV1	17	17
17	17=53	2	2
2	30	12	PV1
30	2	PV1	30
1B	1B	1B	1B
19	12	30	12
12	19	22	22
22	22		19

It can be seen from the table that the virus serotypes show the same general trend in sensitivity to all 4 compounds. (It was found impossible to place rhinovirus type 19 in any position of sensitivity to SK&F 30097 due to the marked enhancement of plaque formation at a 6.25μM concentration of the compound.) Type 22 is most sensitive to

all compounds except Wellcome 71-123. Only type 19 is more sensitive to Wellcome 71-123 than type 22. Types 19 and 12 then generally are the next most sensitive serotypes. However, it can be seen that the sensitivity of poliovirus type 1 compared with the rhinovirus serotypes is not the same for each of the compounds, although the variation is no greater than that with rhinovirus type 30. The sensitivity of poliovirus type 1 to the compounds Abbott 36683 and SK&F 40491 is in fact similar to that of rhinovirus type 14. However, its sensitivity to Wellcome 71-123 and SK&F 30097 is closer to that of rhinovirus type 1B. It must be noted here that the dose-response lines for type 12 are in most cases steeper than those for other serotypes, and type 12 therefore appears more sensitive to the compounds in comparison with other serotypes at high concentrations of compound, whereas at low concentrations this type does not appear to be as sensitive as many serotypes (see plots for Abbott 36683, SK&F 40491 and SK&F 30097). Types 1B, 30 and 2 show more or less similar sensitivity to the 4 compounds, but none of the 3 types is consistently the most or least sensitive of the 3. Types 14 and 17 consistently appear to be the serotypes least sensitive to inhibition by all 4 compounds, type 14 being less sensitive than type 17. Types 53 and 17 display similar sensitivity to SK&F 40491.

It can be stated here that repeats of a great many of the above plaque reduction experiments gave results that did not alter the above conclusions on the sensitivity of different serotypes to inhibition by the 4 compounds.

From the results it was again hoped to obtain some numerical value for the relative sensitivities of different serotypes to each of the compounds. This is in fact just the same as determining the relative potency of one compound against the different serotypes. To determine the relative sensitivities of the serotypes to each compound the computer program was again used and rhinovirus type 1B was used as the virus standard.

It can be seen from Figs. 3.25 to 3.28 that the slopes of the complete dose-response lines of all the virus serotypes to any one compound differ significantly from one another in every case. It was therefore necessary to use only those points which gave dose-response lines of similar slope. In many cases this meant using only 2 points on the dose-response line. It must therefore be emphasized that the relative sensitivity values obtained from calculations from these results are obviously not absolute values. They were calculated to see if quantitative values of relative sensitivity could in fact be obtained and to see if such values gave a clearer indication of relative sensitivity than the less quantitative estimation from observations of the dose-response lines.

In some cases this is in fact not done. For example, rhinovirus type 2 is shown by calculation to be slightly less sensitive than type 17 to Abbott 36683, whereas general observation of the 2 complete dose-response lines shows that type 17 is less sensitive than type 2. This then is a great drawback in the calculation in that one needs to include only those portions of the dose-response lines which give parallel lines, and the method is therefore best not used when dose-response lines are not parallel.

In 4 cases (Abbott 36693 and SK&F 30097 against type 12 and SK&F 30097 and 40491 against type 19) it was not possible to find even 2 points on the dose-response lines which would give slopes not significantly different from the other dose-response lines in the group. Hence the relative sensitivity of these viruses to the particular compounds could not be determined by this mathematical method.

Table 3.10 gives the relative sensitivities of each of the viruses compared with rhinovirus type 1B to each of the compounds used.

Table 3.10

Relative Sensitivities of Eight Rhinovirus Serotypes and Poliovirus type 1 compared with Rhinovirus type 1B, to Abbott 36683, SK&F 40491, SK&F 30097 and Wellcome 71-123, as determined by the plaque reduction method

Virus Type	Relative Sensitivity to Compound (compared with type 1B)			
	Abbott 36683	SK&F 40491	SK&F 30097	Wellcome 71-123
2	0.238 (0.316-0.167)	1.018 (1.208-0.857)	0.799 (1.031-0.610)	0.727 (0.813-0.650)
12	NC	1.183 (1.456-0.969)	NC	1.345 (1.496-1.208)
14	0.196 (0.271-0.130)	0.190 (0.232-0.154)	0.470 (0.587-0.369)	0.429 (0.479-0.382)
17	0.258 (0.345-0.178)	0.416 (0.509-0.338)	0.723 (0.933-0.549)	0.525 (0.585-0.470)
19	0.955 (1.215-0.745)	NC	NC	3.019 (3.423-2.685)
22	1.424 (1.758-1.130)	1.888 (2.270-1.585)	3.033 (4.023-2.346)	1.831 (2.094-1.618)
30	0.481 (0.622-0.357)	0.799 (0.937-0.684)	1.103 (1.434-0.853)	0.818 (0.911-0.735)
53	NT	0.405 (0.495-0.329)	NT	NT
PV1	0.145 (0.226-0.080)	0.601 (0.870-0.412)	0.865 (1.106-0.669)	1.001 (1.192-0.832)

NC - relative sensitivity value not calculated due to non parallelism of lines.

NT - virus serotype not tested against compound

It can be seen that the relative sensitivity compared with type 1B is not the same against each of the compounds tested. However, only the relative sensitivity values for compound Abbott 36683 differ greatly from the others. Fig.3.25 shows that rhinovirus type 1B appears to be rather more sensitive to Abbott 36683 compared with the other virus serotypes, than to the other 3 compounds. This would therefore account for the fact that values for the relative sensitivity of the other virus serotypes are lower for Abbott 36683 than for the other 3 compounds. Hence it would perhaps be better to choose as the standard virus for determination of the relative sensitivity values, a serotype whose dose-response lines take approximately the same position in each of the 4 sets of plots. In fact, none of the serotypes are good choices. Rhinovirus type 22, for example, although keeping a very consistent position compared with the other serotypes for Abbott 36683, SK&F 40491 and SK&F 30097, is relatively less sensitive to Wellcome 71-123. Moreover it is possibly not a good idea to choose as a standard a serotype which is either most or least sensitive to the test compounds. This could be similarly argued for type 14, which exhibits least sensitivity to all 4 compounds.

It was therefore thought that to take another serotype as the standard would yield no more meaningful results.

The rhinoviruses tested have therefore shown the same general trend in sensitivity to each of the 4 compounds tested. It can be seen that the virus serotypes generally exhibiting greatest sensitivity to the drugs (types 19 and 22) are those which produce the smallest plaques and, conversely, those serotypes exhibiting least (types 14 and 17) are those which produce the largest plaques.

It was thought to be of interest to determine if the same relationship existed between the average plaque diameter and compound concentration as between the average plaque count and compound concentration in a plaque reduction experiment. This was studied in plaque reduction

experiments with SK&F 40491 and Abbott 36683 against 5 rhinovirus serotypes, types 1B, 2, 12, 17 and 22. Plaques were measured at 3 concentrations of each compound and on control plates. The size of 100 plaques on each plate was measured using a low power microscope (10x) containing a calibrated eyepiece graticule. The average plaque diameter was plotted against the compound concentrations (Figs. 3.29a and b).

Figs. 3.29a and b merely demonstrate the reduction in average plaque diameter (for 5 virus serotypes) produced by increasing concentrations of compound. Plots for Abbott 36683 show that there is in many cases a linear relationship between the reduction in plaque diameter and the log of the compound concentration. This is also observed with SK&F 40491 and the smaller plaque forming serotypes used.

One cannot in fact determine the relative sensitivities of the 5 serotypes from Figs. 3.29a and b since the control plaque diameters are different for each type.

In order to compare the sensitivities of the viruses the average plaque diameters must be plotted as a percentage of the average control plaque diameter. This is shown in Figs. 3.30a and b; these do not demonstrate the same trend in virus sensitivity as shown in the plaque count reduction dose-response lines. One would deduce from these 2 figures that rhinovirus type 17 is most sensitive and type 1B is least sensitive to SK&F 40491. Type 1B is also shown to be least sensitive to Abbott 36683 and type 12 is shown to be most sensitive to this compound.

However, a possible cause for this disagreement between the results was found. Serotypes which are found to be most sensitive to a compound by reduction in plaque number obviously have a larger proportion of plaques completely inhibited by a certain concentration of compound

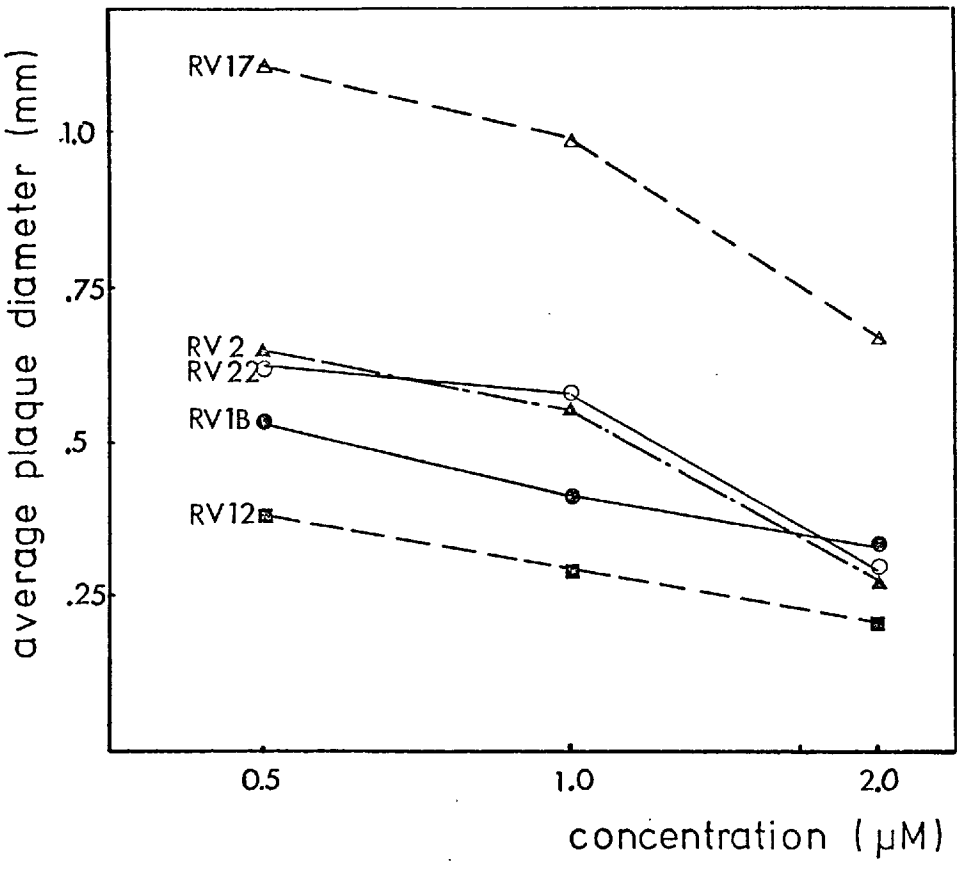


Fig. 3.29a. SK+F 40491.

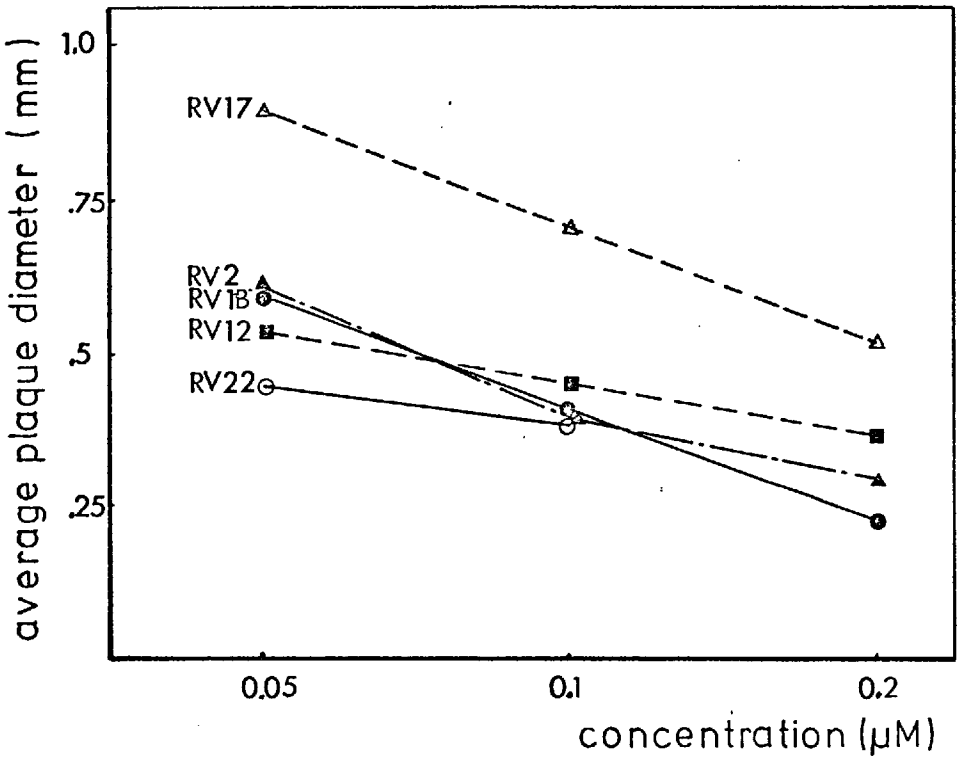


Fig. 3.29b. Abbott 36683.

Figs. 3.29a and b. Plaque reduction dose-response lines for SK+F 40491 and Abbott 36683 against rhinovirus types 1B, 2, 12, 17 and 22. Average plaque diameter plotted against the concentration of the compound in the overlay medium ($\log_2$ scale).

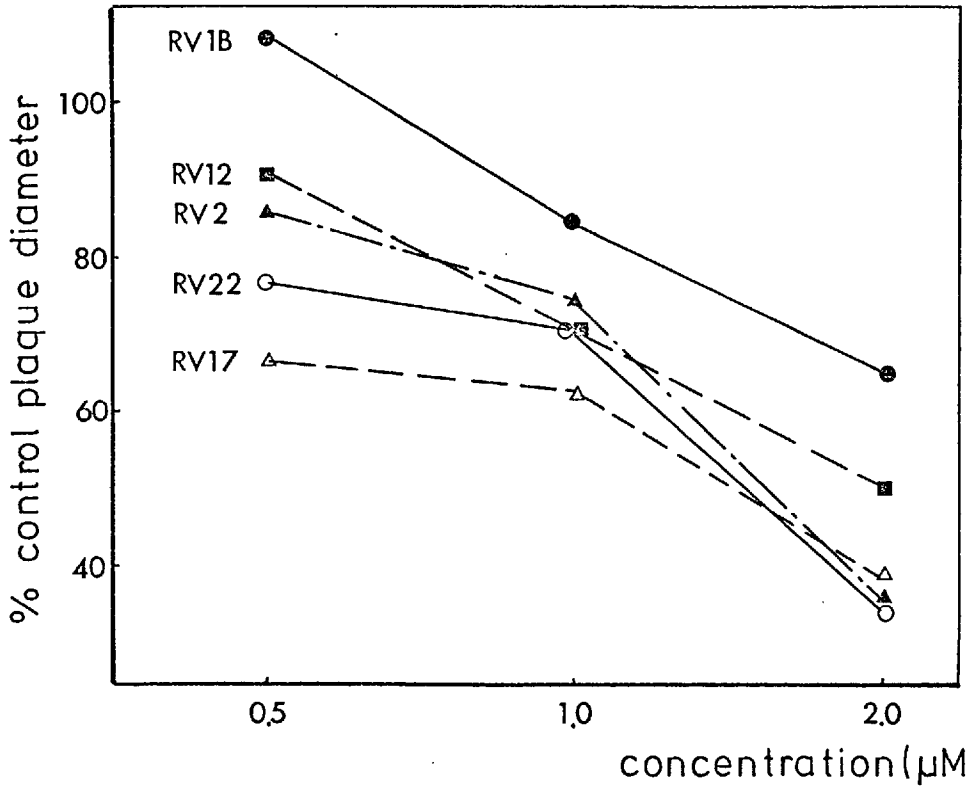


Fig. 3.30a. SK+F 40491.

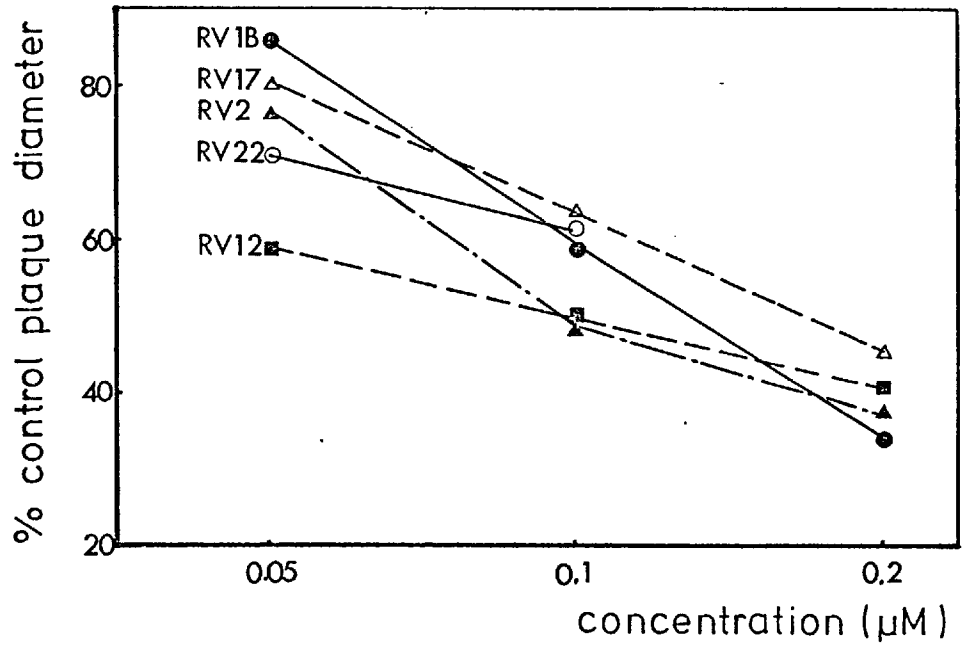


Fig. 3.30b. Abbott 36683.

Figs. 3.30a and b. Plaque reduction dose-response lines for SK+F 40491 and Abbott 36683 against rhinovirus types 1B, 2, 12, 17 and 22. Percentage of the control plaque diameters in the treated cultures plotted against the concentration of the compound in the overlay medium ($\log_2$ scale).

than the less sensitive serotypes. When measuring the diameter of plaques in compound treated cultures, one is measuring only the remaining plaques. Therefore it was decided that each plaque which was completely inhibited should be counted as a plaque of diameter 0 mm, and this will inevitably decrease the average plaque diameter in all of those cultures in which the plaque number is less than in the controls.

This was therefore tested. The reduction in plaque number at each concentration of compound was determined and for each absent plaque a value of 0 mm was given. The average plaque diameter was then recalculated as a percentage of the average control plaque diameter and plotted against the log of the concentration of each of the 2 compounds (Figs. 3.31a and b). The dose-response lines in Fig.3.31a cross in many places, therefore making it difficult to place the serotypes in order of increasing sensitivity to SK&F 30491. However, this was attempted. The orders of increasing virus sensitivity to the 2 compounds as determined from dose-response lines for plaque count reduction, plaque size reduction and plaque size reduction (where the plaques of 0 mm diameter were included) were compared (Table 3.11).

It can be seen that plaque size reduction dose-response lines for Abbott 36683 and SK&F 40491 demonstrate a similar relation between the individual sensitivities of 5 rhinovirus serotypes as is shown by the plaque count reduction dose-response lines, if plaques which are completely suppressed are counted as plaques with a diameter of 0 mm.

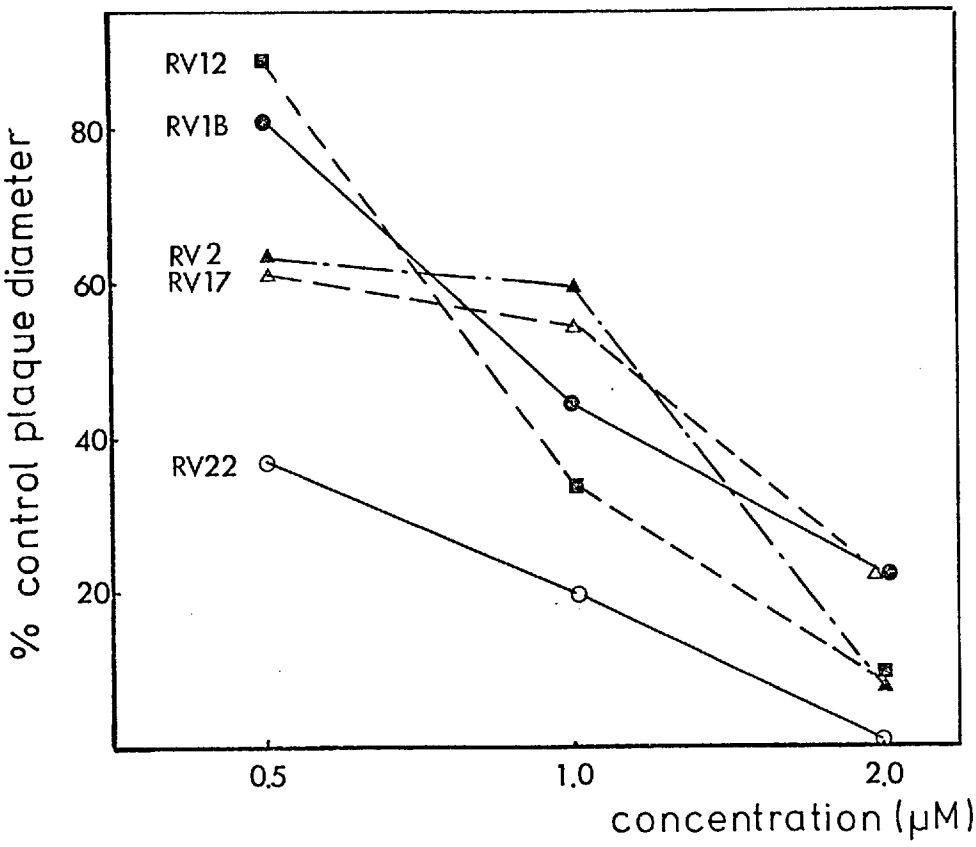


Fig. 3.31a. SK+F 40491.

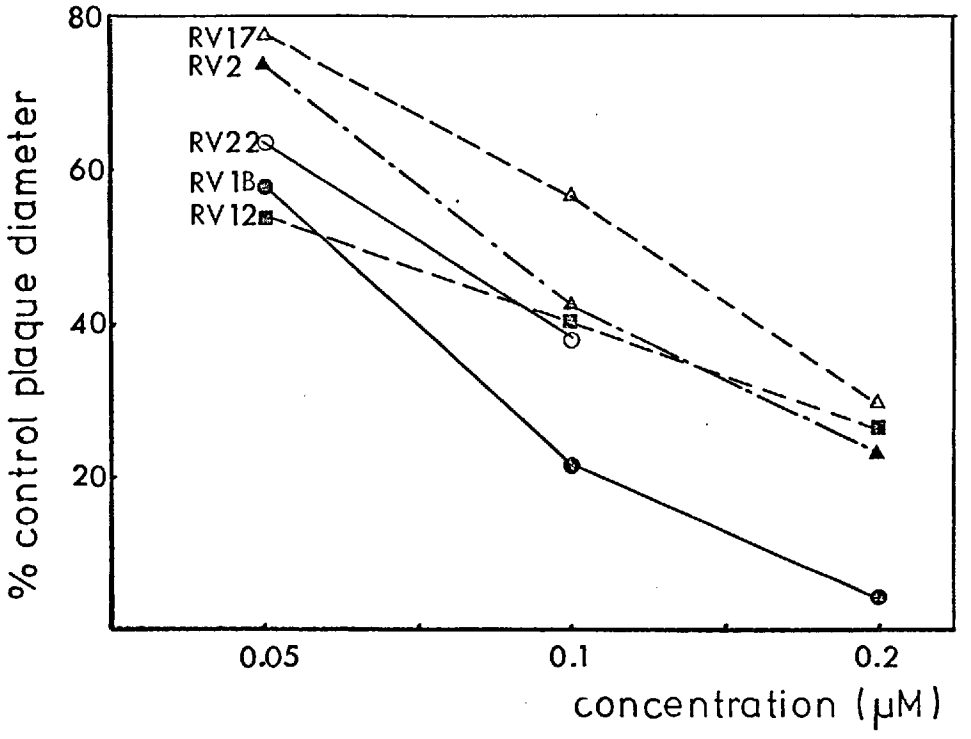


Fig. 3.31b. Abbott 36683.

Figs. 3.31a and b. Plaque reduction dose-response lines for SK+F 40491 and Abbott 36683 against rhinovirus types 1B, 2, 12, 17 and 22. Percentage of the control plaque diameter in treated cultures (giving a value of 0mm for all plaques completely suppressed by compound) plotted against the concentration of compound in the overlay (log scale).

Table 3.11

Rhinovirus types 1B, 2, 12, 17 and 22 placed in order of increasing sensitivity to SK&F 40491 and Abbott 36683, as determined from plaque count reduction dose-response lines (a) or plaque size reduction dose-response lines (b) and (c). (b) Plaques completely suppressed by the compounds are ignored. (c) Plaques completely suppressed by the compounds are counted as plaques of 0 mm diameter

SK&F 40491			Abbott 36683		
(a)	(b)	(c)	(a)	(b)	(c)
17	1B	17	17	1B	17
2	12	2	2	17	2
1B	2	1B	1B	22	22
12	22	12	12	2	12
22	17	22	22	12	1B

Gradient Plate Plaque Reduction Technique

The gradient plate plaque reduction technique was studied as an alternative method to the normal plaque reduction technique for the quantitation of rhinovirus inhibitory activity. The technique was carried out as described in section 2.5.3(c).

Few results were obtained by this technique since it was not found to work as easily or consistently as described by other workers and the normal plaque reduction technique was therefore used preferentially.

However, against rhinovirus type 1B the plaque inhibitory concentration, C_i, was found to be 115.0 and 37.0 μM for SK&F 30097 and Wellcome 71-123 respectively. These results therefore do not compare well with results obtained from plaque reduction dose-response lines, where SK&F 30097 was shown to be more active than Wellcome 71-123.

Generally the method was found to be more difficult to carry out than the straightforward plaque reduction technique, although it must be noted that fewer plates are required for each experiment with this technique than with normal plaque reduction. However, the main drawback of the technique was that straight and clear cut-off lines between plaque and no plaque zones were not observed. Plaques were found to decrease in size and number slowly over a wide portion of the plate and the dividing line between plaques and no plaques was curved (concave towards the end of the plate with maximum compound concentration) thus making an estimation of the distance d_1 difficult.

An attempt was made to overcome this drawback by using rectangular 25 cm^2 tissue culture flasks instead of the circular tissue culture dishes. This was found to present additional practical difficulties when overlaying infected monolayers. Monolayers in flasks could not be as thoroughly drained as those in plates and in order to add the second layer of medium the neck of the flask had to be raised, often resulting in the detachment of the first layer of medium from the surface of the cell monolayer.

The dividing line between plaque and no plaque zones was found to be as widespread although not as curved with rectangular flasks as with circular dishes.

3.2.3 Yield Reduction Technique

3.2.3(a) Use of Yield Reduction test to Quantitate Rhinovirus Inhibitory Activity

The yield reduction technique, as described in section (2.6.2) was used as another method for quantitating the rhinovirus inhibitory activity of Abbott 36683, SK&F 40491, SK&F 30097 and Wellcome 71-123.

The test was initially carried out with Abbott 36683 and poliovirus type 1 in M-HeLa cells. A wide concentration range (0.1 to 50μM) was employed in order to obtain a complete picture of the yield-reducing activity of this compound and to determine the concentration range over which the response in yield reduction was dose-related. The virus yields and extent of virus-induced CPE in infected control and treated cultures, was determined after 10 hours incubation. The dose-response lines (both $\log_{10}$ yield, pfu/ml, and percentage control yield against compound concentration) are given in Fig.3.32. It can be seen that even at a 0.1μM concentration of compound nearly 40% inhibition of virus yield is obtained. Nearly 100% inhibition of virus yield is obtained at a 3.125μM (log = 0.4) concentration of compound. The virus yield at 5.0μM is still above 10^4 pfu per ml. However, since this value then decreases little with increasing drug concentration, it may be assumed that this is the residual virus titre left from the original infecting inoculum.

From Fig.3.32 the concentration range over which the response in yield reduction to Abbott 36683 was dose-related was found to be 0.1 to 0.4μM.

It was then felt of importance to determine the relationship between the inhibition of virus yield and the inhibition of virus-induced CPE, i.e. to determine whether a certain percentage inhibition of virus yield produced the same percentage inhibition of virus-induced CPE. The cytopathic effects observed in infected control and treated cultures

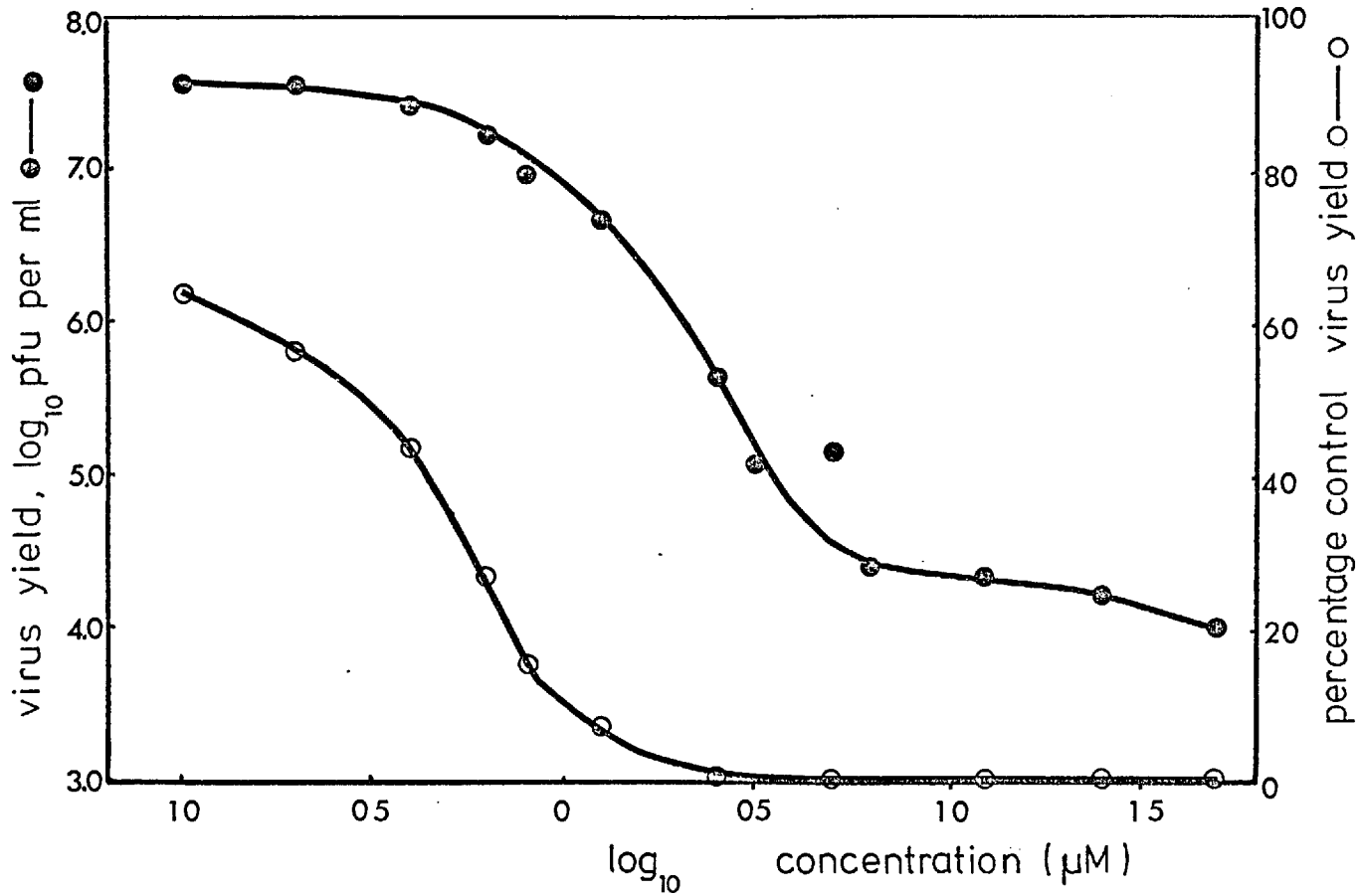


Fig. 3.32. Yield reduction dose-response lines for Abbott 36683 against poliovirus type 1 in M-HeLa cells. Virus yield, pfu per ml (●—●) and percentage control virus yield (O—O), plotted against the $\log_{10}$ of the concentration of the compound in the medium.

after the required incubation time were noted and then the extent of CPE in the treated cultures was calculated as a percentage of the CPE in the control cultures. Both the percentage of control CPE and the percentage of control virus titre were plotted against compound concentration. This was done for poliovirus type 1 and rhinovirus type 22 against Abbott 36683 (Fig.3.33).

The most obvious point arising from this is that a reduction in virus yield does not necessarily produce a reduction in virus-induced CPE. For example, maximum CPE is produced by less than maximum virus yield, as reported by Tamm et al.(1961). In the case of poliovirus type 1 44% of the normal virus yield produces maximum CPE. Additionally it should be noted that although the yield reduction dose-response lines are very similar for the 2 viruses, the CPE reduction dose-response lines would indicate that rhinovirus type 22 is in fact more sensitive than poliovirus type 1 to Abbott 36683, a greater percentage CPE reduction being produced in rhinovirus type 22 cultures than in poliovirus type 1 cultures by a concentration of compound producing the same percentage reduction in virus yield in both cultures. This would seem to be related to the fact that the yield in control cultures of rhinovirus type 22 is much less than that in control cultures of poliovirus type 1.

Yield reduction experiments were then carried out with 8 rhinovirus serotypes and poliovirus type 1, and each of the 4 compounds. Abbott 36683 was tested against rhinovirus types 1B, 2, 12, 17 and 22, SK&F 40491 against types 1B, 2, 12, 14, 17, 22 and 30, SK&F 30097 against types 1B, 2, 12, 14 and 17 and Wellcome 71-123 against 2, 14, 17 and 22. Abbott 36683 and SK&F 40491 were also tested against poliovirus type 1. The compounds were tested at the following concentrations: Abbott 36683, 0.1, 0.2, 0.4, 0.8 and 1.6μM; SK&F 40491 2.5, 5.0, 10, 20 and 40μM; SK&F 30097 10, 20, 40, 80 and 160μM; Wellcome 71-123 12.5, 25.0, 50 and 100μM.

Dose-response lines of percentage control titre against compound concentration (on a $\log_2$ scale) were plotted as for plaque reduction

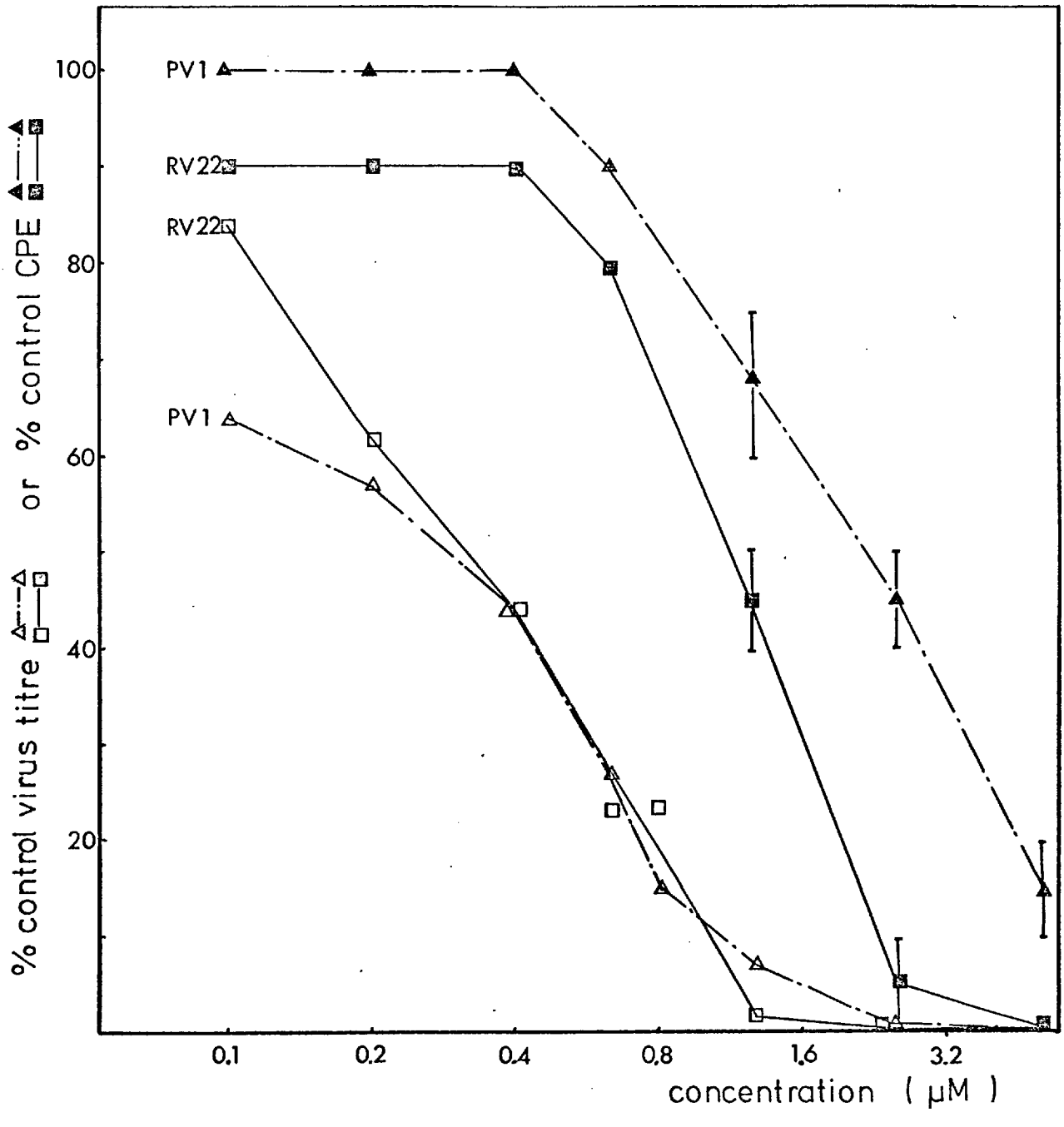


Fig. 3.33. Yield reduction and CPE reduction dose-response lines for Abbott 36683 against poliovirus type 1 (△—·—△) and rhinovirus type 22 (□——□) in M-HeLa cells. Percentage control virus titre (open symbols) or percentage control virus induced CPE (closed symbols) plotted against the concentration of the compound ($\log_2$ scale).

dose-response lines, i.e. for each virus individually against all 4 compounds (Figs. 3.34 to 3.40) and for each compound individually against all virus serotypes tested (Figs. 3.41 to 3.44).

It was decided not to carry out a statistical analysis of the results as was done for the plaque reduction results. Such analysis, it was found, gave misleading results in some cases, due to the fact that only those points on the dose-response lines which gave lines parallel to one another could be used for the analysis. This meant that points were chosen which were not typical of the dose-response line as a whole, thus leading to a misinterpretation of the results. It was therefore thought that it is in fact very difficult to make an accurate numerical assessment of relative potency or relative sensitivity.

It is apparent from Figs. 3.34 to 3.40 that Abbott 36683 is again shown to be the most active of the 4 compounds and SK&F 40491 the next most active. However, for 2 of the 4 serotypes tested against Wellcome 71-123 (types 14 and 17) this compound appeared to be more active than SK&F 30097. In the plaque reduction experiments against the same 2 serotypes Wellcome 71-123 had been shown to be less active than SK&F 30097 (Figs. 3.19 and 3.20).

Figs. 3.41 to 3.44 show the dose-response lines for various rhinovirus serotypes against Abbott 36683 (Fig. 3.41), SK&F 40491 (Fig.3.42), SK&F 30097 (Fig.3.43), and Wellcome 71-123 (Fig.3.44).

From observations of the plots the rhinovirus serotypes tested were placed in order of increasing sensitivity to each of the compounds. This is shown in Table 3.12.

Figs. 3.34 to 3.40. Yield reduction dose-response lines for Abbott 36683 (○——○), SK+F 40491 (□——□), SK+F 30097 (△——△) and Wellcome 71-123 (▲——▲) against rhinovirus serotypes 1B, 2, 12, 14, 17 and 22 and poliovirus type 1. Percentage of the control yield produced in treated cultures plotted against the $\log_{10}$ of the concentration of compound in the culture medium.

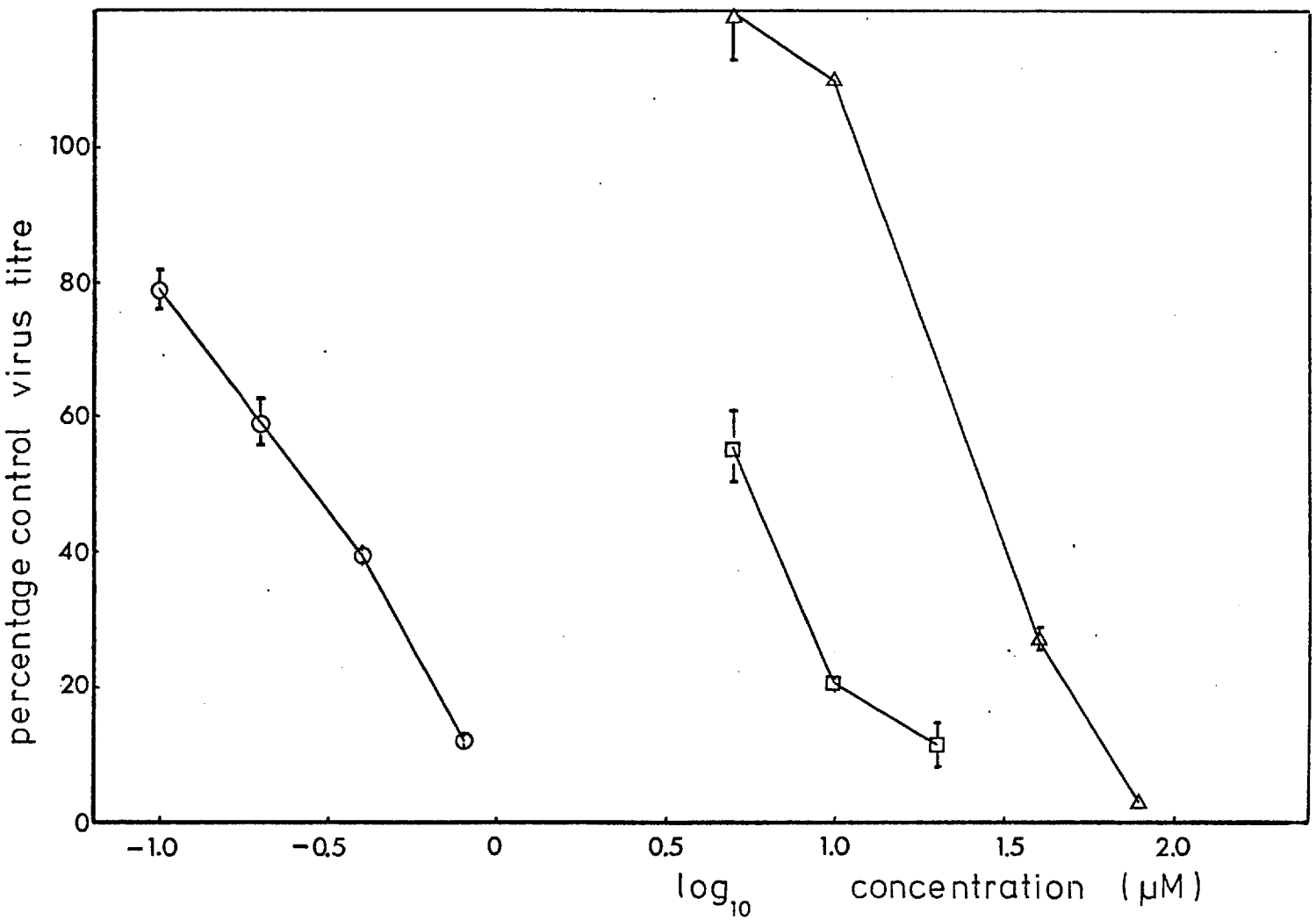


Fig. 3.34. Rhinovirus type 1B.

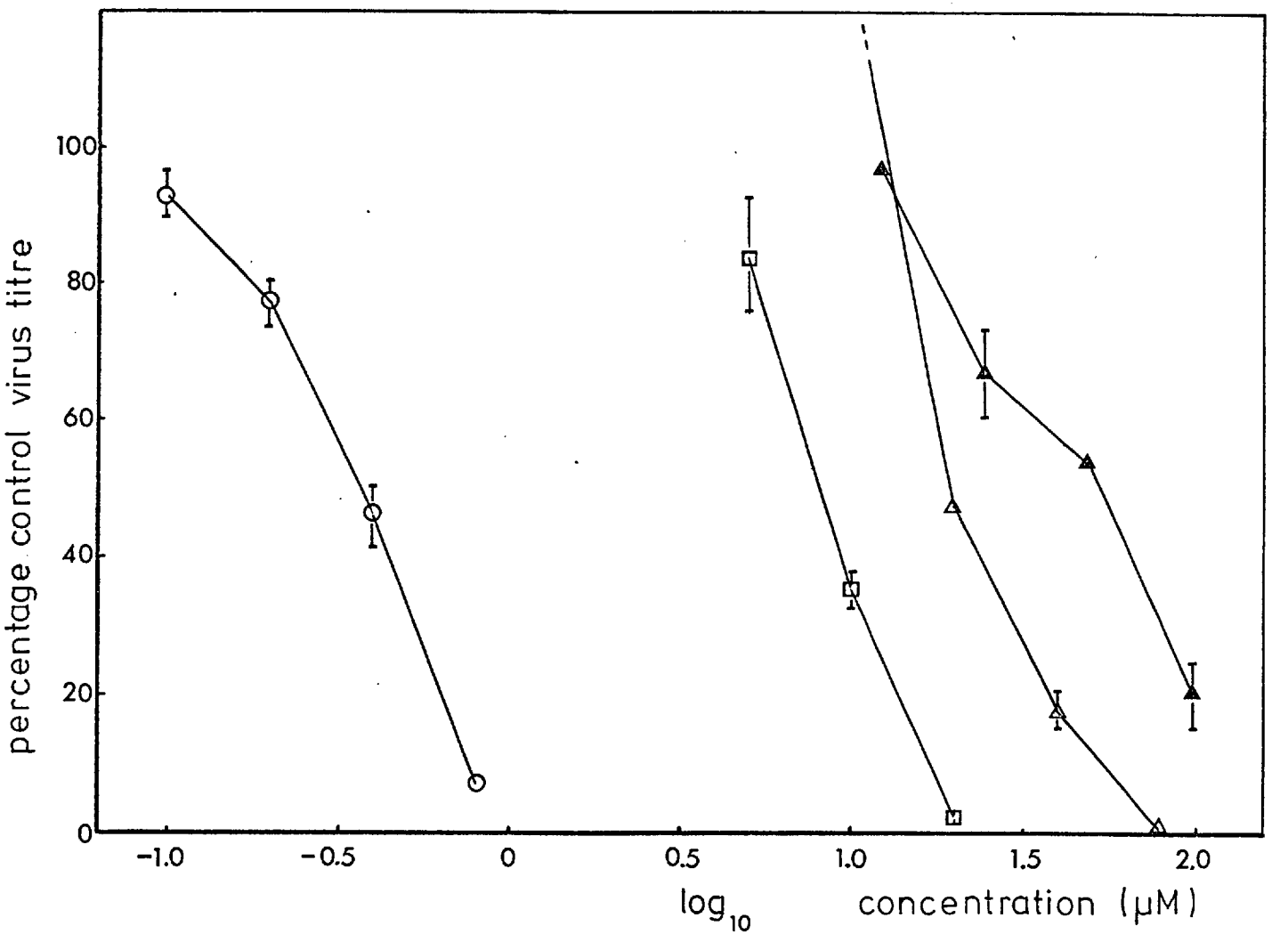


Fig. 3.35. Rhinovirus type 2.

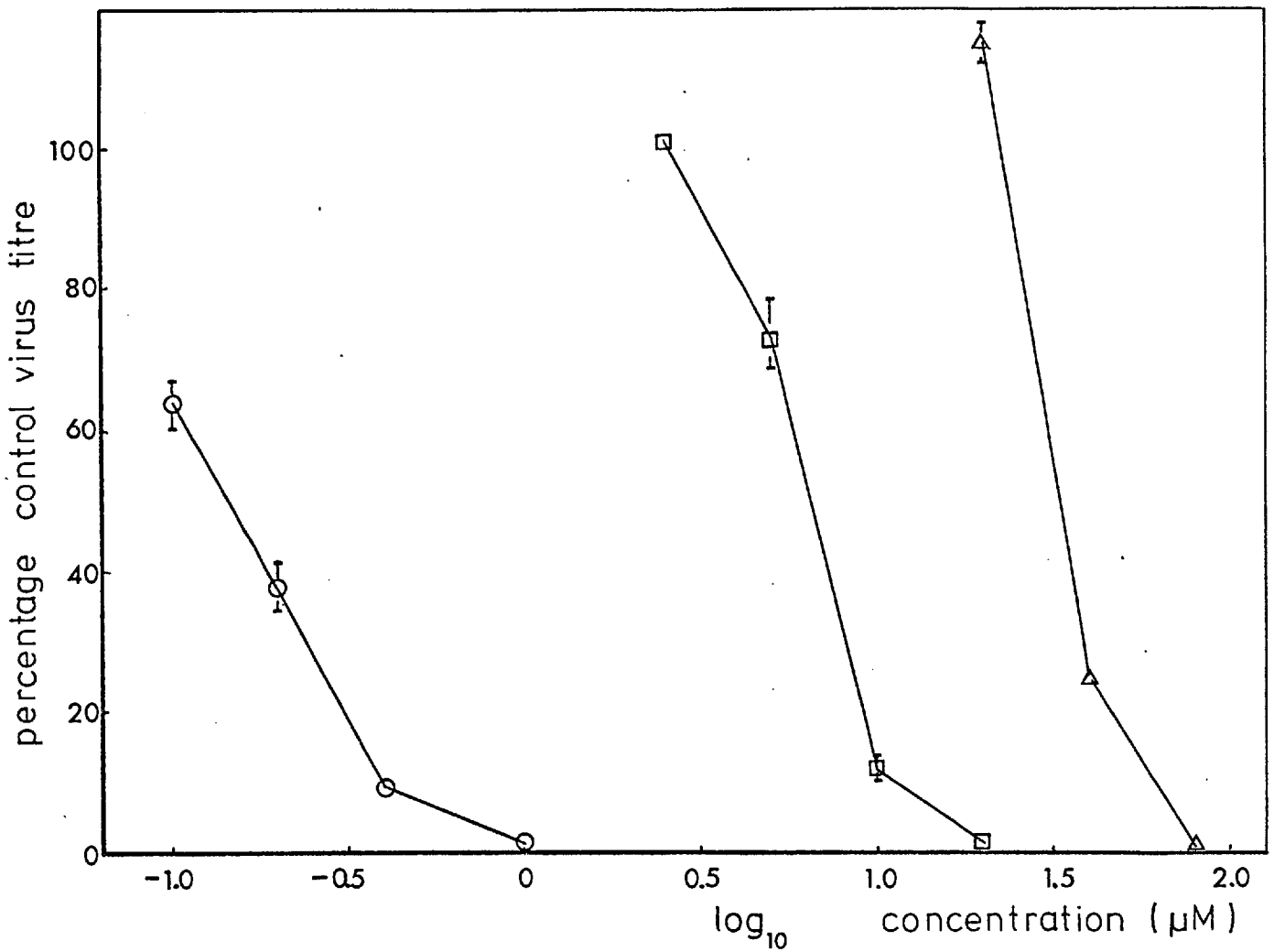


Fig. 3.36. Rhinovirus type 12.

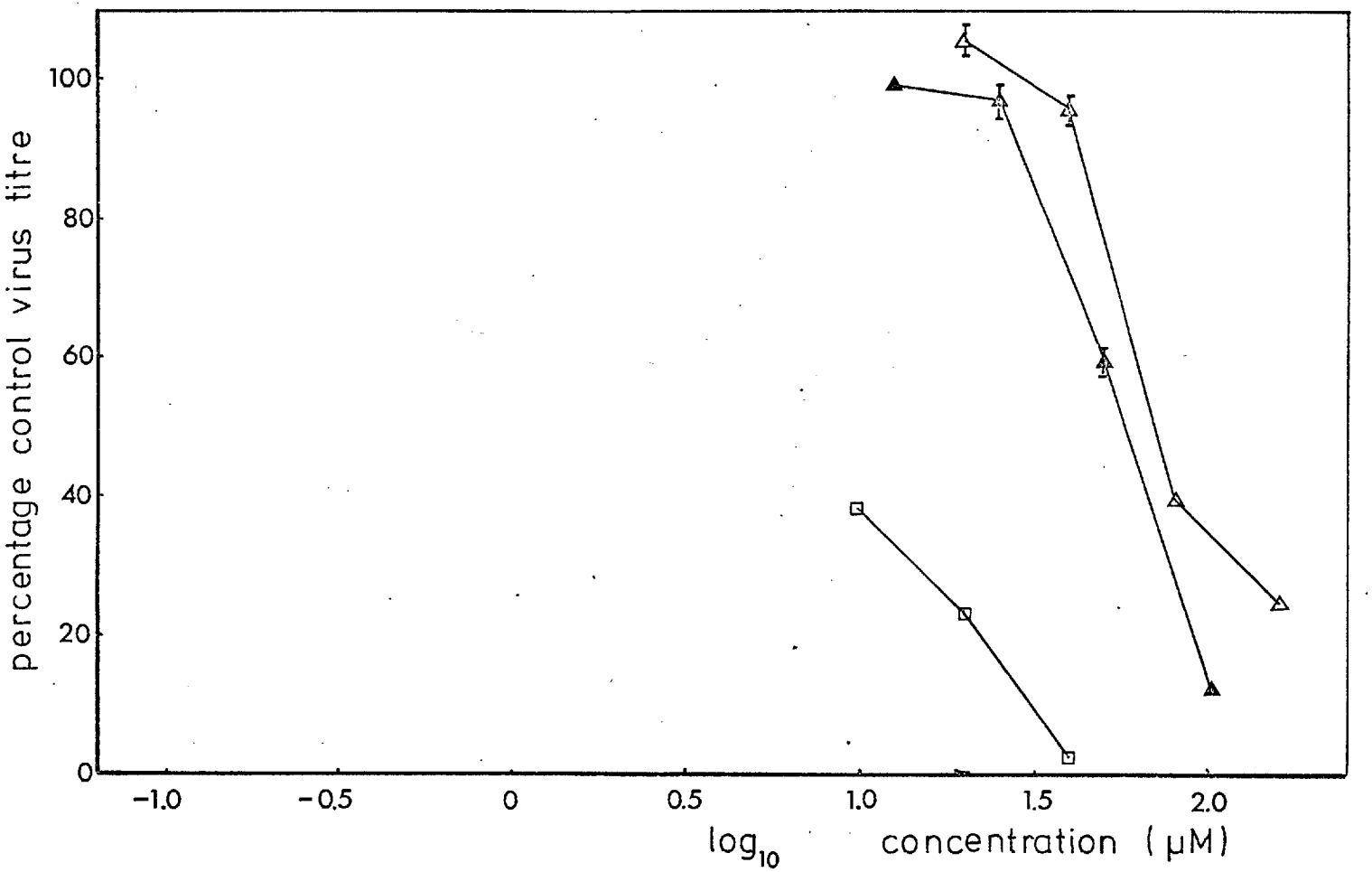


Fig. 3.37. Rhinovirus type 14.

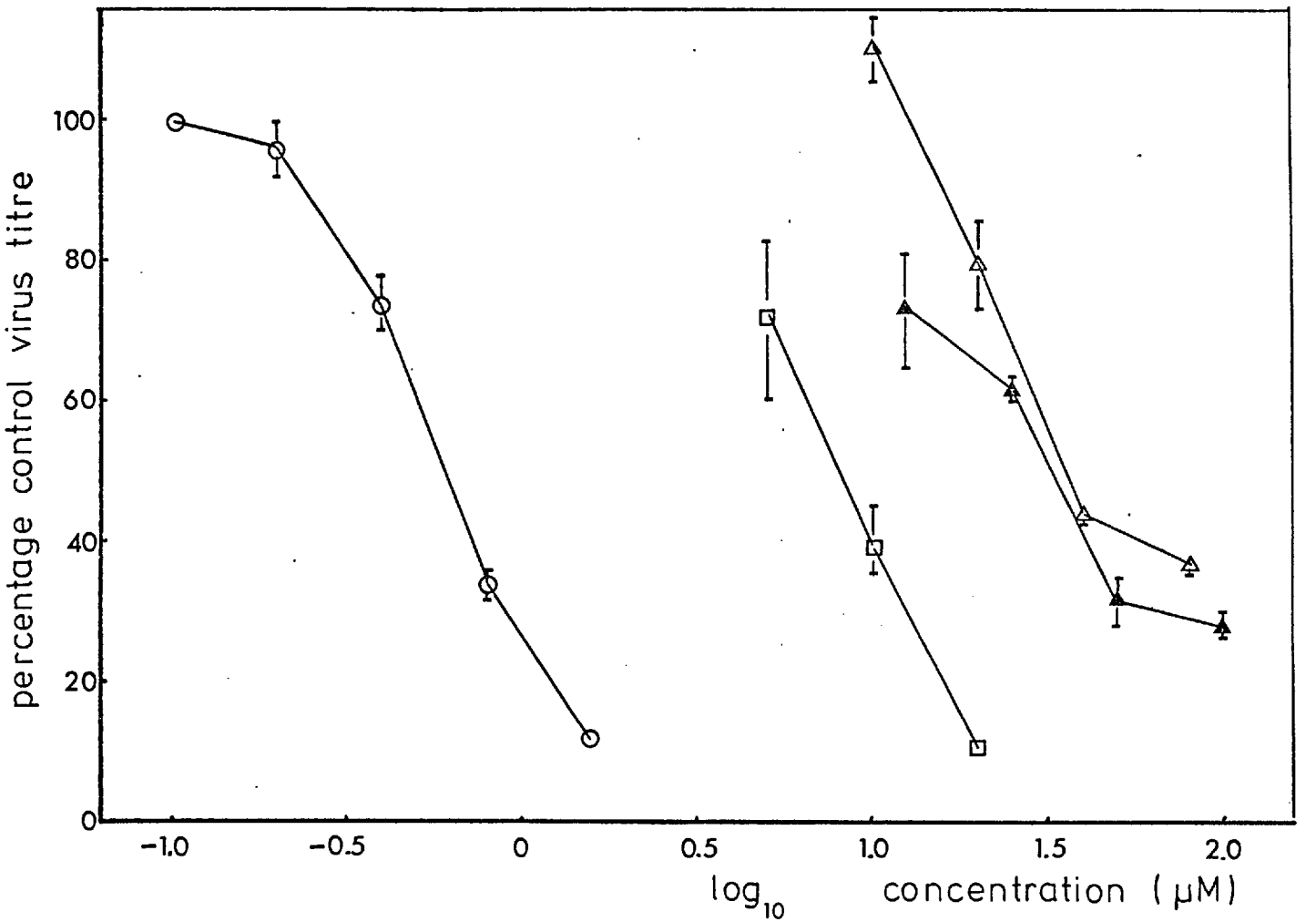


Fig. 3.38. Rhinovirus type 17.

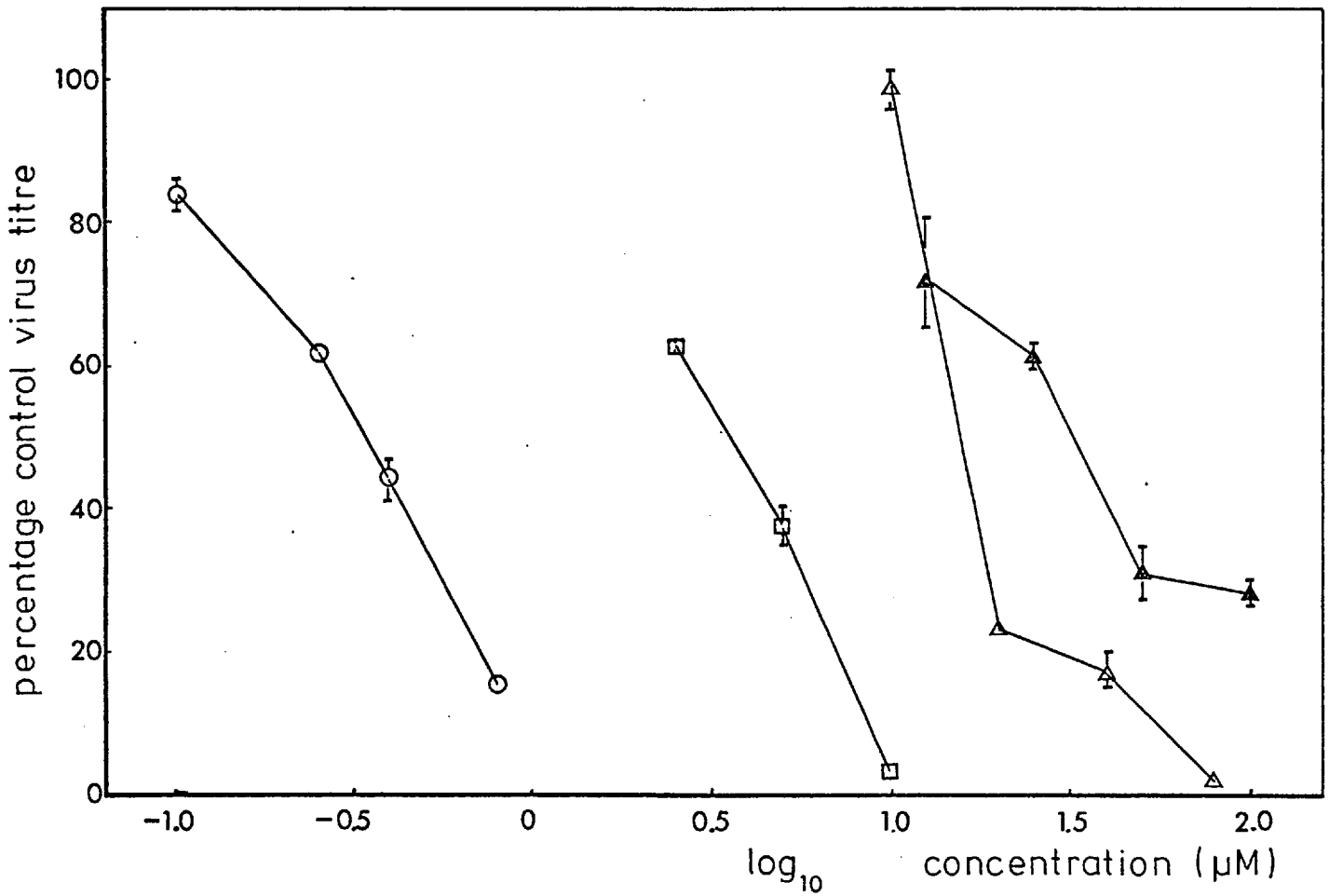


Fig. 3.39. Rhinovirus type 22.

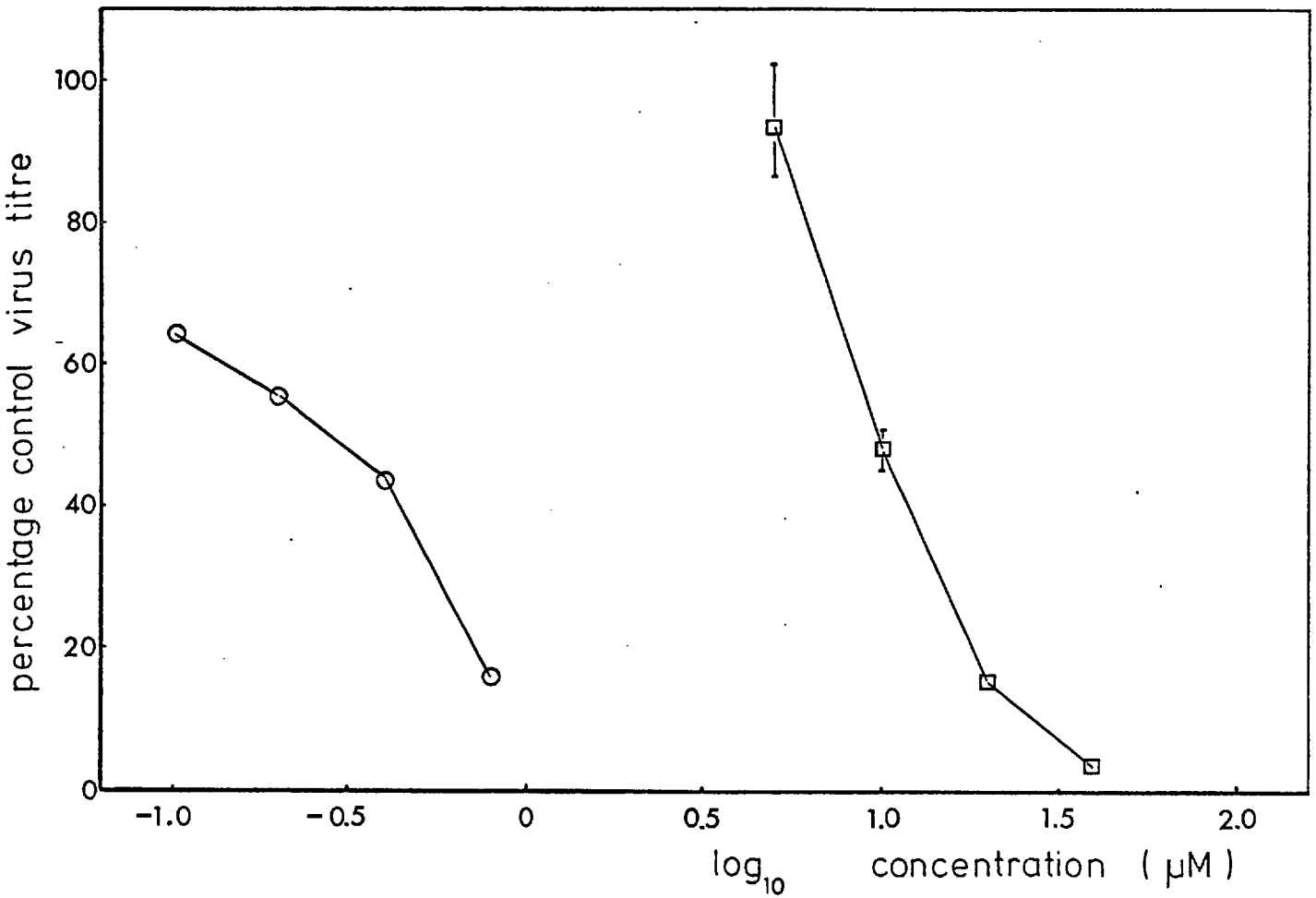


Fig. 3.40. Poliovirus type 1.

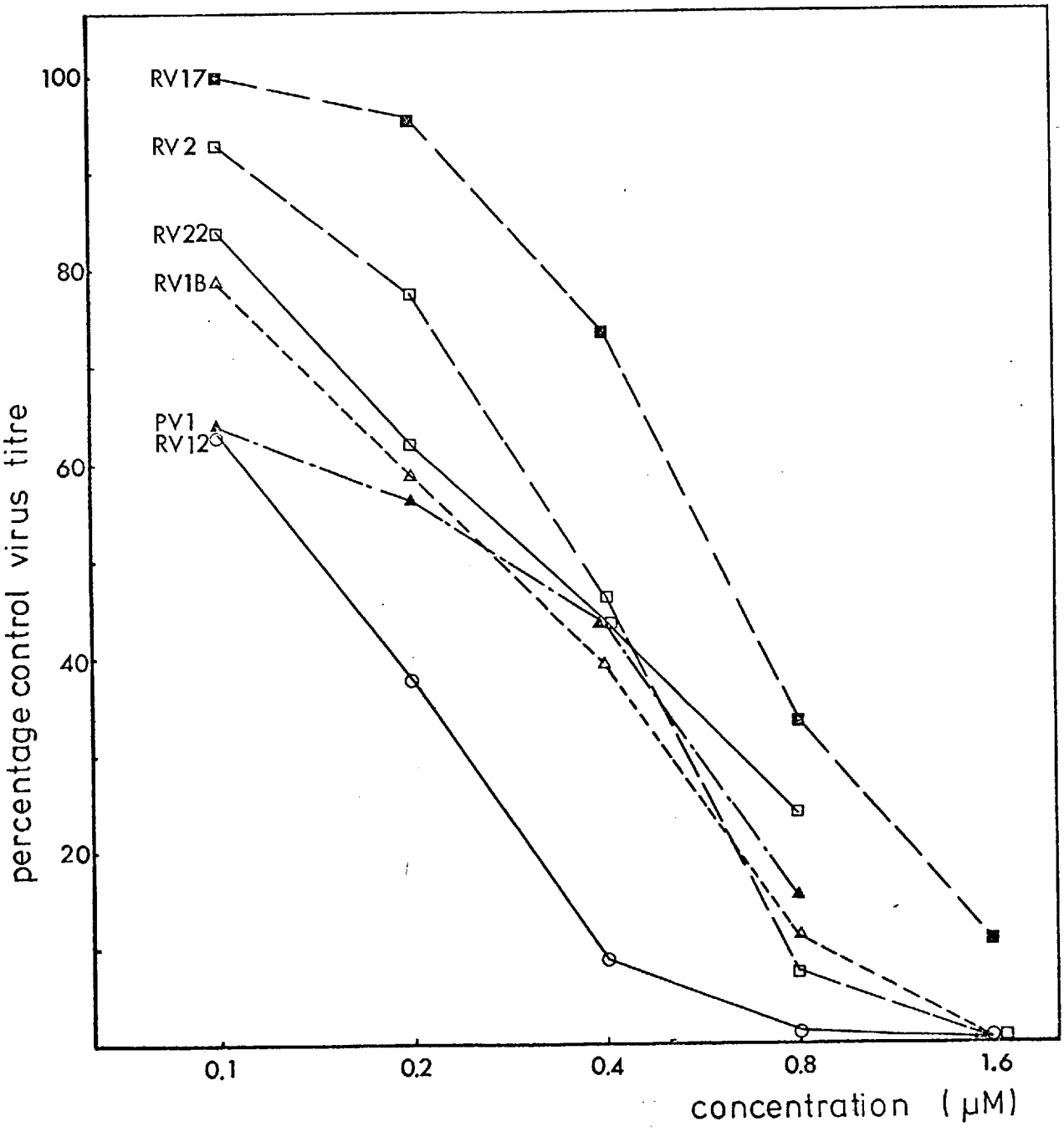


Fig. 3.41. Yield reduction dose-response lines for Abbott 36683 against 5 rhinovirus serotypes and poliovirus type 1. Percentage control yield produced in treated cultures plotted against the concentration of compound in the culture medium ($\log_2$ scale).

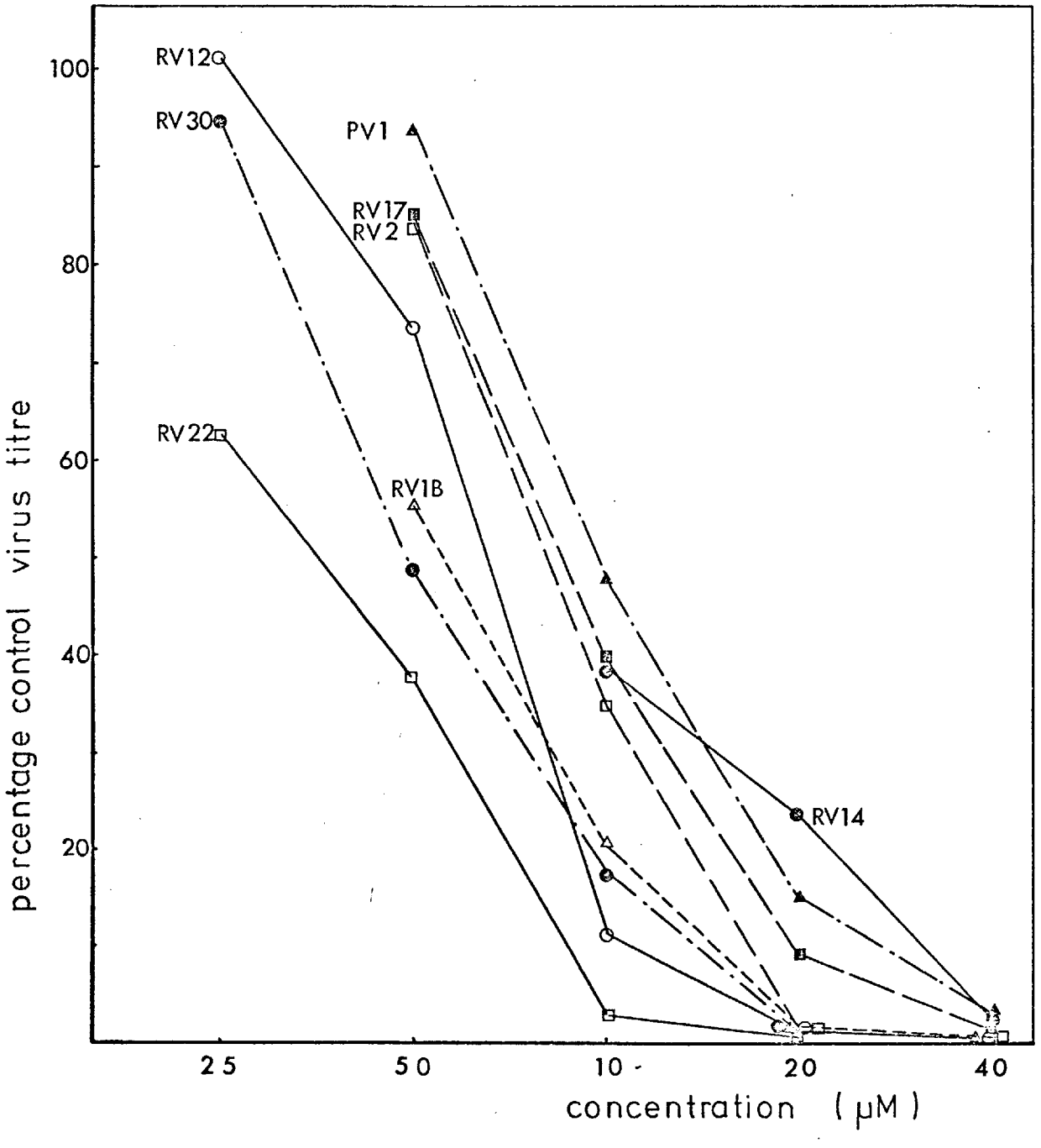


Fig. 3.42. Yield reduction dose-response lines for SK+F 40491 against 7 rhinovirus serotypes and poliovirus type 1. Percentage control yield in treated cultures plotted against the concentration of compound in the culture medium ($\log_2$ scale).

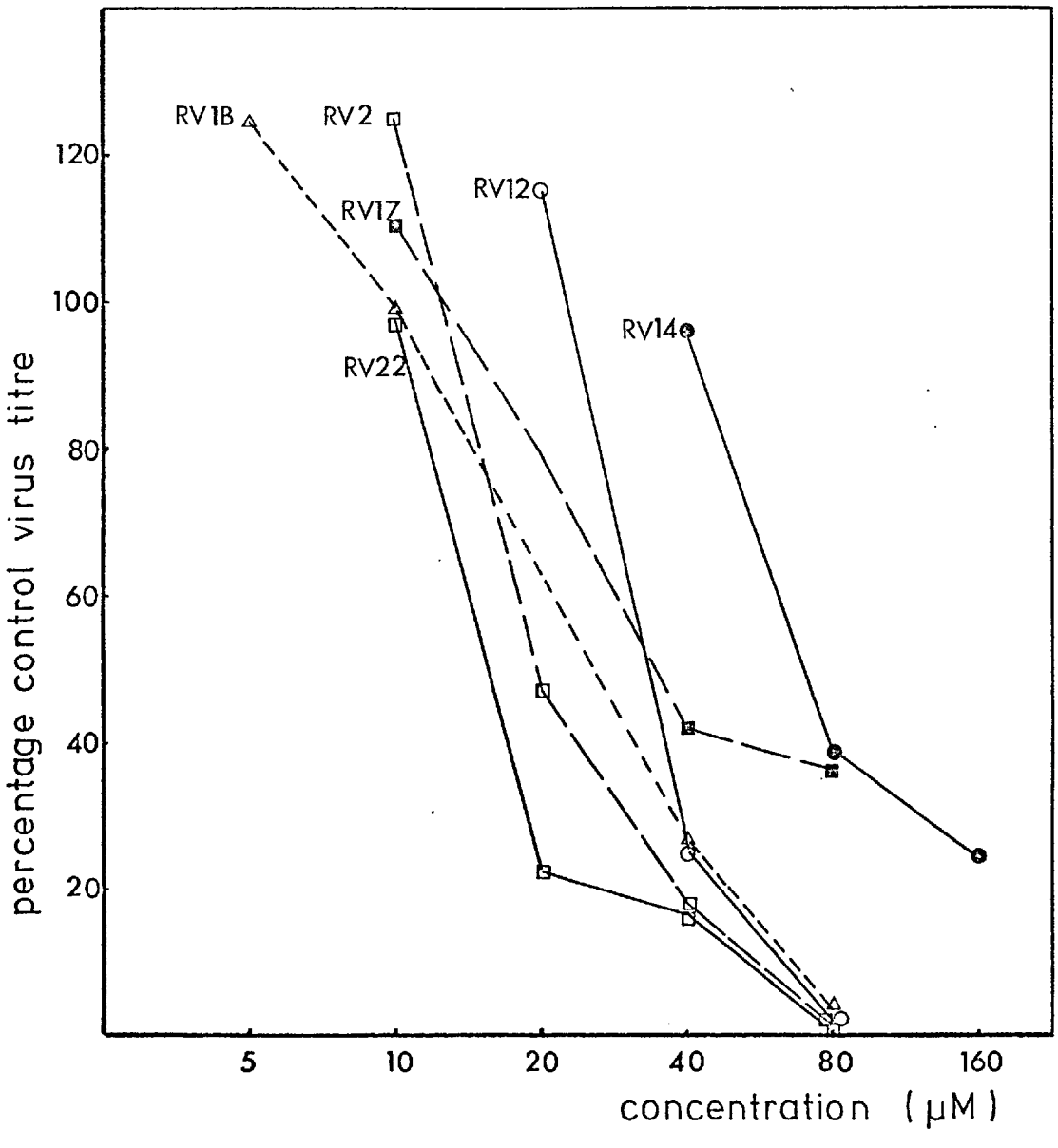


Fig. 3.43. Yield reduction dose-response lines for SK+F 30097 against 6 rhinovirus serotypes. Percentage control yield produced in treated cultures plotted against the concentration of compound in the culture medium ($\log_2$ scale).

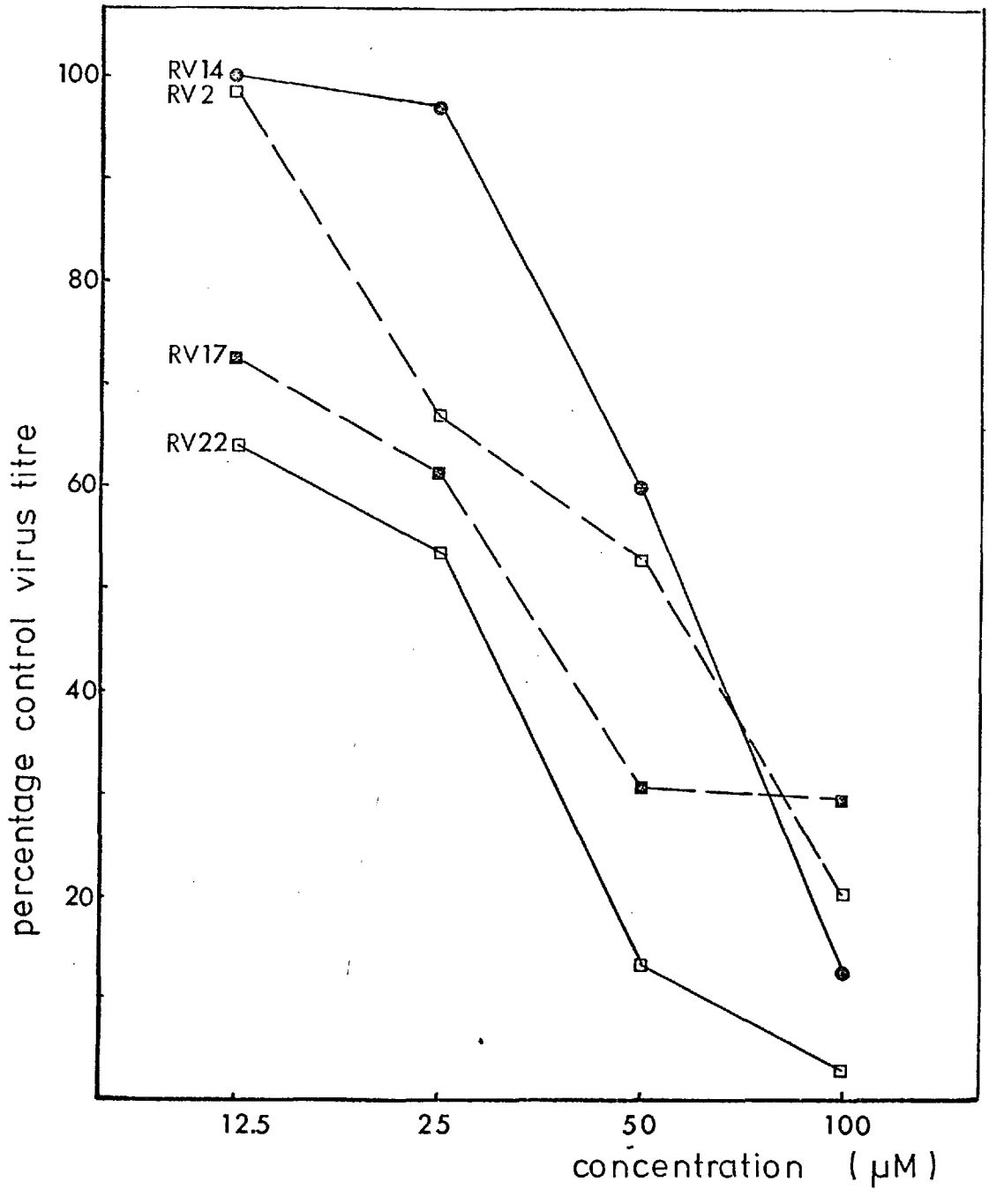


Fig. 3.44. Yield reduction dose-response lines for Wellcome 71-123 against 4 rhinovirus serotypes. Percentage control yield produced in treated cultures plotted against the concentration of compound in the culture medium ($\log_2$ scale).

Table 3.12

Rhinovirus serotypes and poliovirus type 1 listed in order of increasing sensitivity to Abbott 36683, SK&F 40491, SK&F 30097 and Wellcome 71-123 as determined by the yield reduction method

Abbott 36683	SK&F 40491	SK&F 30097	Wellcome 71-123
12	22	22	22
PV1	30	1B=2	17
1B	1B=2	12	2
22	2	17	14
2	17	14	
17	PV1		
	14		

A detailed comparison of yield reduction and plaque reduction dose-response lines will be given later. However, it can be noted here that rhinovirus type 12 generally produces yield reduction dose-response lines steeper than those produced by other serotypes. This is consistent with the plaque reduction dose-response lines.

It must again be stressed that a determination of the relative sensitivities of different rhinovirus serotypes by the mathematical method described can not be carried out on account of the non-parallelism of the dose-response lines.

3.2.3(b) Effect of Size of Multiplicity of Infection on the Virus Inhibitory Activity of Compounds as determined by the Yield Reduction Test.

It is of great importance to determine whether a change in the multiplicity of infection used in a yield reduction test has any effect on the measured activity of a compound as determined by this method.

This was initially tested with Abbott 36683 and poliovirus type 1. Yield reduction assays were carried out as previously described. Cell monolayers were infected with 1 ml of virus inoculum at multiplicities of infection (MOI) of 0.1, 1.0, 10, and 100 pfu per cell. After virus adsorption the remaining inoculum was removed and the cell monolayers were washed in PBS A. The monolayers were then treated with maintenance medium containing Abbott 36683 at 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 or 3.2 μM. Virus yields were harvested and titrated after 10 hours incubation at 36°.

Dose-response lines (virus yield, pfu per cell against compound concentration on a $\log_2$ scale) for each MOI were then plotted. They are shown in Fig.3.45.

From the 4 dose-response lines shown it can be seen that with increasing MOI the slope of the dose-response line increases. The concentration of Abbott 36683 required to reduce virus yield by 50% was determined from Fig.3.45 for each dose-response line. The MIC_{50}'s were 0.04, 0.14, 0.25 and 0.25 μM at MOI's of 0.1, 1.0, 10 and 100 respectively. The measured activity of Abbott 36683 against poliovirus type 1 therefore decreases with increasing MOI up to 10 pfu per cell. At 10 and 100 pfu per cell the measured activity is a constant.

The 4 dose-response lines were then plotted as percentage control virus titre against the compound concentration, this being the normal method for plotting dose-response lines (Fig.3.46). The dose-response lines again show a shift in position towards higher concentration values

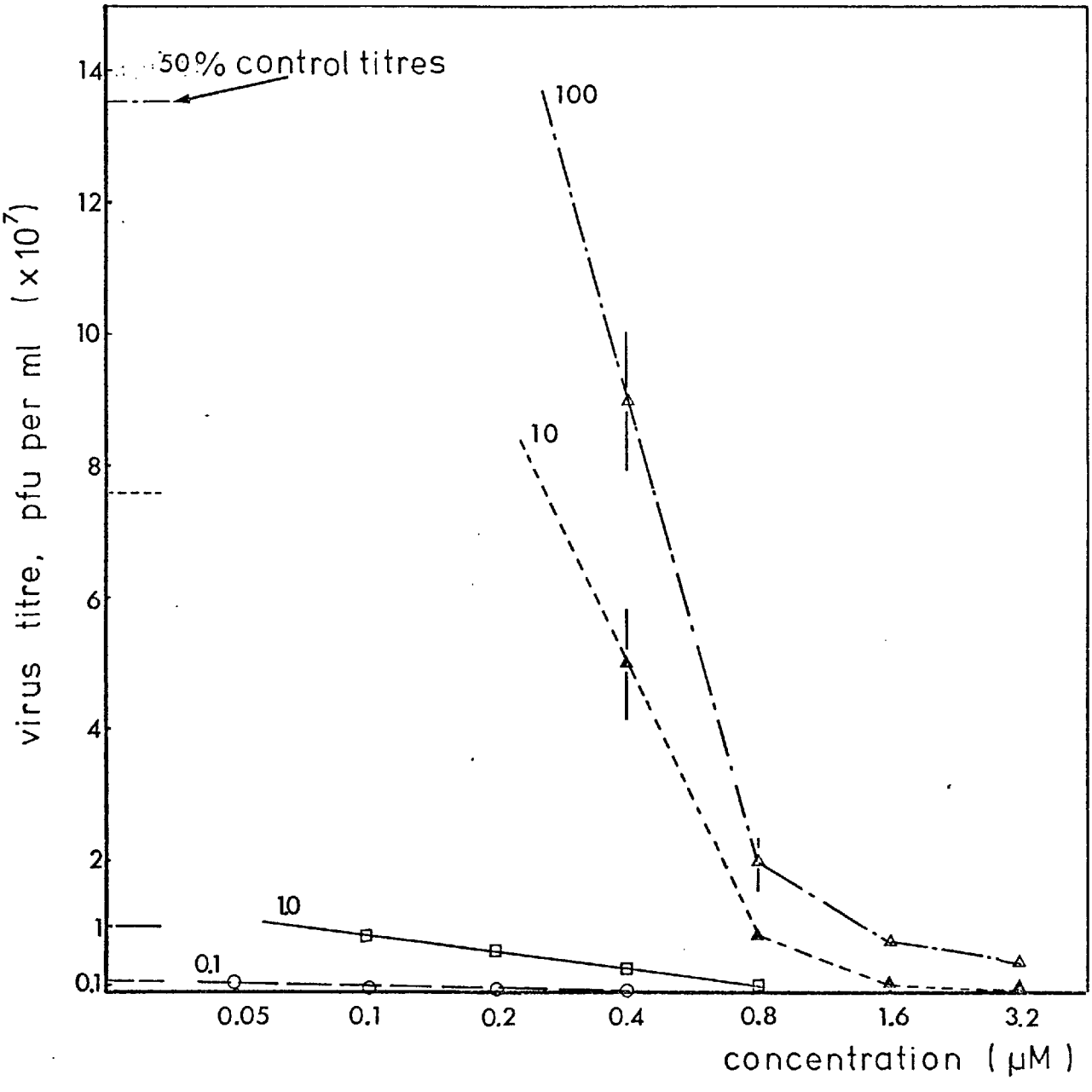


Fig. 3.45. Effect of changes in the multiplicity of infection on the dose-response lines obtained in yield reduction studies with Abbott 36683 and poliovirus type 1. Virus titre plotted against the concentration of Abbott 36683 in the medium (log_2 scale), for 4 multiplicities of infection, 0.1 (○——○), 1.0 (□——□), 10 (▲-----▲) and 100 pfu per cell (△—△). 50% of the control virus titres for each multiplicity of infection also indicated.

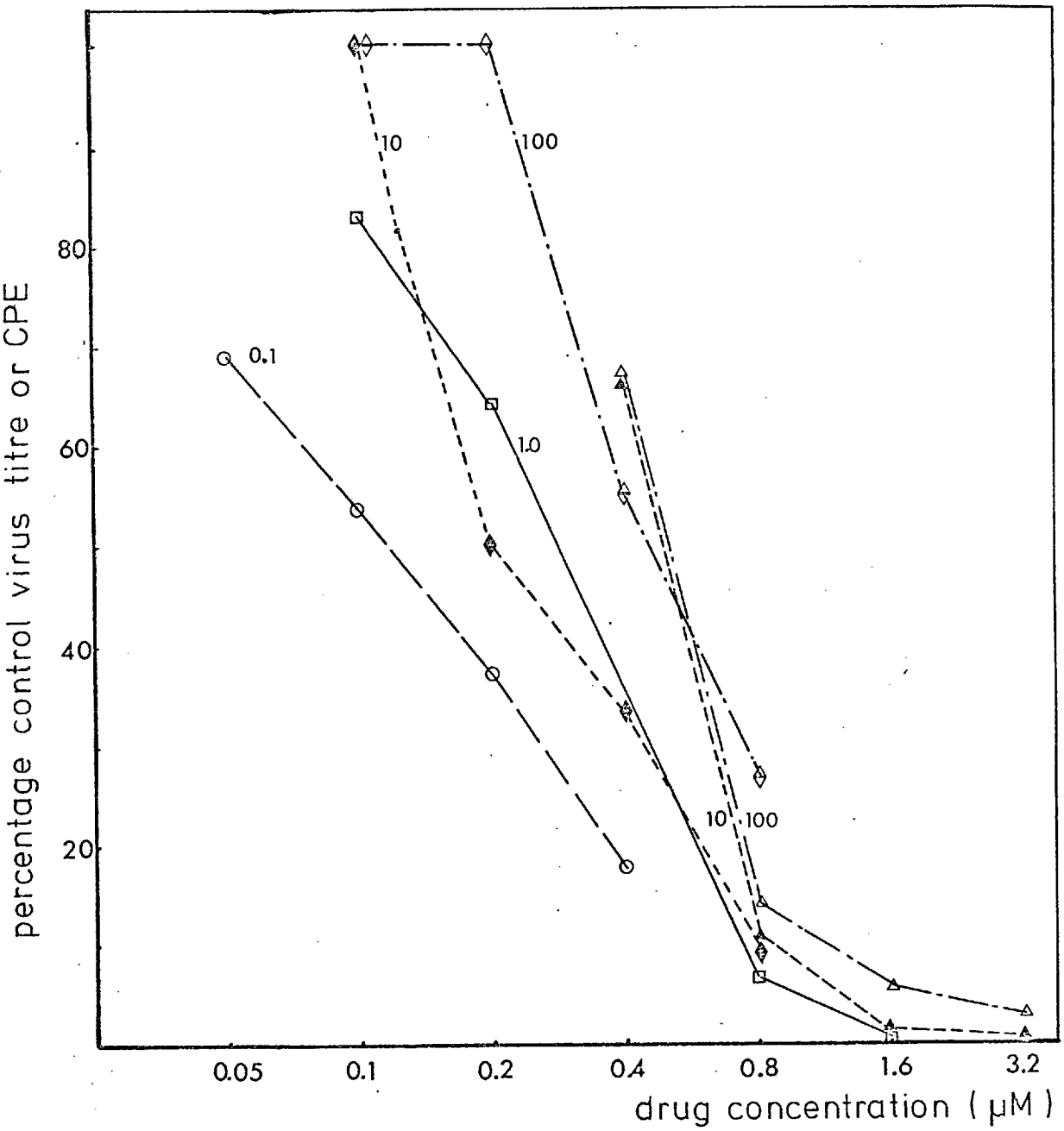


Fig. 3.46. Effect of changes in the multiplicity of infection on the dose-response lines obtained in yield reduction studies with Abbott 36683 and poliovirus type 1. Percentage of the control virus titres in treated cultures is plotted against the concentration of Abbott 36683 in the medium ($\log_2$ scale) for 4 multiplicities of infection, 0.1 (○——○), 1.0 (□——□), 10 (△----△) and 100 pfu per cell (△—·—△). Percentage of the control virus induced cytopathic effects is also plotted for the 2 highest multiplicities, 10 (◇----◇) and 100 pfu per cell (◇—·—◇).

with increasing MOI up to 10 pfu per cell. However, the dose-response lines for MOI's of 10 and 100 pfu per cell are similarly positioned. This fact does not necessarily follow from the finding that the MIC_{50} is the same at these 2 MOI's, since the dose-response lines might well have had different slopes. Hence it would appear from this that the activity of Abbott 36683 against poliovirus type 1 decreases with increasing multiplicity of infection but tends towards a constant at high multiplicities of infection.

However, Fig.3.46 also shows the CPE reduction dose-response lines at the 2 highest MOI's. These 2 lines are seen to differ considerably despite the fact that the yield reduction dose-response lines are similar, and Abbott 36683 appears to be more active against a MOI of 10 than against a MOI of 100. Hence although a certain concentration of compound may inhibit CPE to a varying extent in 2 cultures the same concentration may inhibit yield production equally in both cases (as has been shown already with rhinovirus type 22 and poliovirus type 1, Fig.3.33). This therefore emphasises the point that CPE reduction and yield reduction tests are not comparable.

In order to confirm that the results shown in Figs. 3.45 and 3.46 were not merely due to biological variation, the experiment was repeated with MOI's of 0.5, 2.5, 10 and 50 pfu per cell. The 4 dose-reponse lines obtained (percentage control titre against compound concentration) are shown in Fig.3.47. The pattern exhibited is the same as that in Fig.3.46. The dose-response lines show an apparent decrease in virus inhibitory activity with increasing MOI's from 0.5 to 10 pfu per cell. Again, however, the dose-response lines at 10 and 50 pfu per cell are similarly positioned and the activity of Abbott 36683 as determined from these 2 lines is a constant.

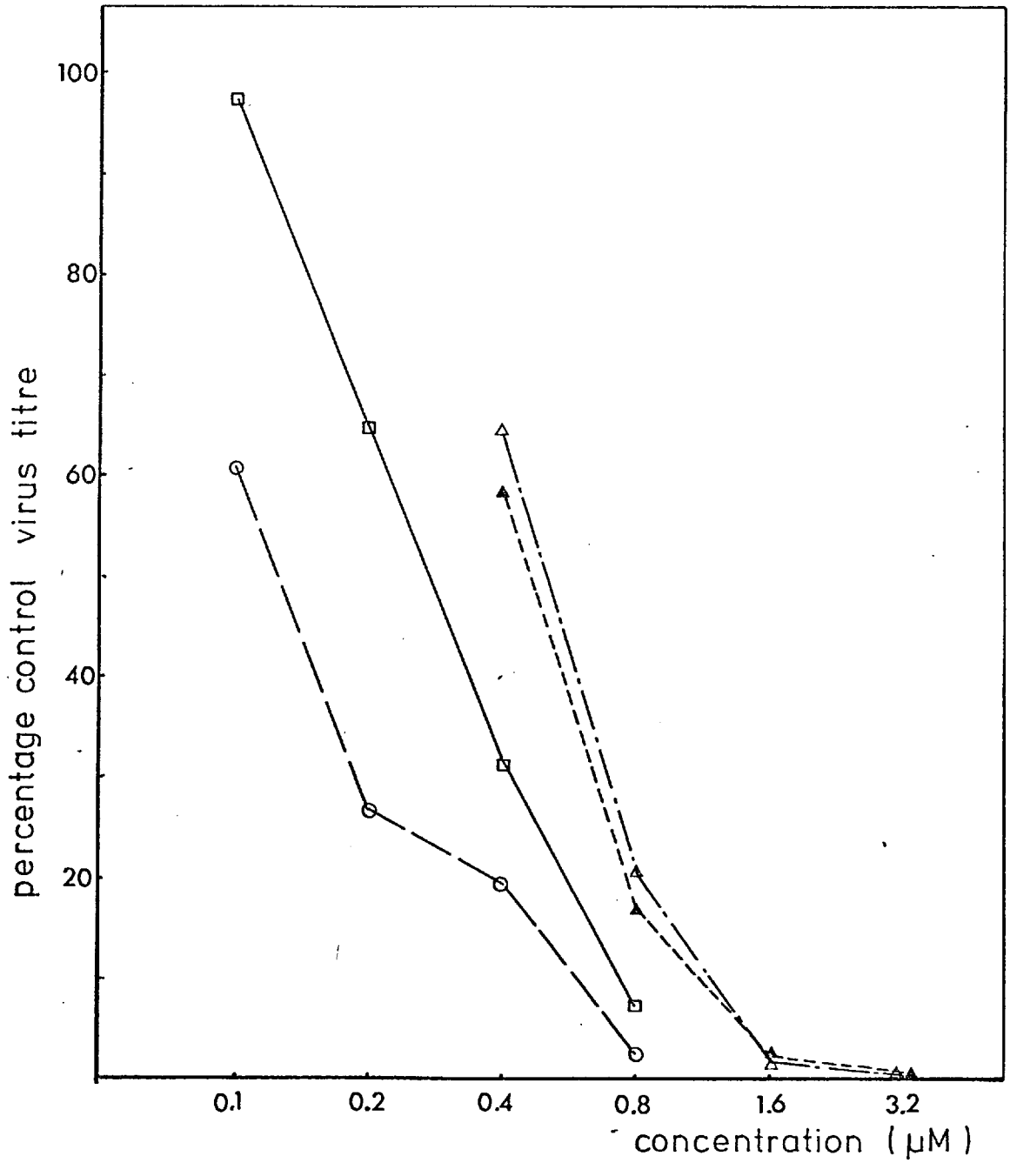


Fig. 3.47. Effect of changes in the multiplicity of infection on the dose-response lines obtained in yield reduction studies with Abbott 36683 and poliovirus type 1. Percentage of the control virus titre in treated cultures plotted against the concentration of compound in the culture medium ($\log_2$ scale) for 4 multiplicities of infection, 0.5 (○——○), 2.5 (□——□), 10 (▲----▲) and 50 pfu per cell (△—·—△).

Having shown this relationship between MOI and the position of the dose-response lines (and hence the measured virus inhibitory activity) for Abbott 36683 and poliovirus type 1, it was decided to determine whether the same relationship existed for Abbott 36683 and a rhinovirus. Rhinovirus type 17 was chosen for this study, which was carried out as for poliovirus type 1, using 4 MOI's, 0.1, 1.0, 10 and 30. It was not possible to obtain MOI's of greater than 30 pfu per cell, since the maximum titre of rhinovirus type 17 stocks obtained was 8.0×10^7 pfu per ml. However, it was thought that a multiplicity of 30 pfu per cell was sufficient for this study. Results of the yield reduction experiments are shown in Fig.3.48.

It can be seen that the same relationship between MOI and the position of the dose-response line is exhibited. Abbott 36683 appears to be increasingly less active when tested at multiplicities of 0.1, 1.0 and 10 pfu per cell. However, the activity measured at 10 and 30 pfu per cell is the same.

It has been reported by Matsumoto et al. (1972) that the degree of viral inhibition obtained with SK&F 30097 in yield reduction studies in WI-38 cells is not obviously dependent on the concentration of challenge virus, in that 50µg/ml provided a 1.3 to 1.8 $\log_{10}$ inhibition of rhinovirus in WI-38 cells at input multiplicities of virus varying from 0.001 to 10.

In view of this report and of the results obtained here with the compound Abbott 36683 the effect of input multiplicity on the measured activity of SK&F 40491 was studied. Fig.3.49 shows dose-response lines obtained with SK&F 40491 and rhinovirus type 17 at 4 multiplicities of infection (0.1, 1.0, 10 and 30 pfu per cell). The relationship which exists between MOI and measured activity for rhinovirus type 17 and Abbott 36683 is not apparent for rhinovirus type 17 and SK&F 40491.

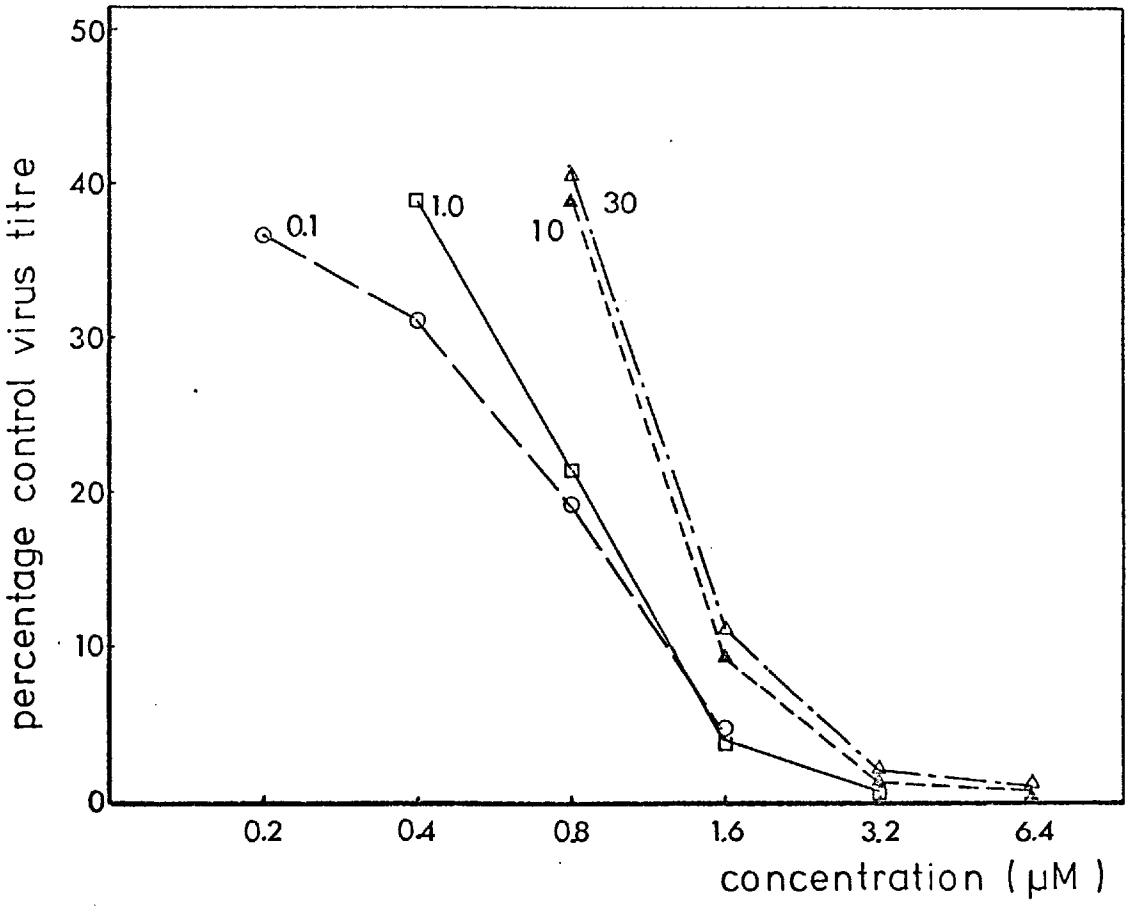


Fig. 3.48. Effect of changes in the multiplicity of infection on the dose-response lines obtained in yield reduction studies with Abbott 36683 and rhinovirus type 17. Percentage of the control virus titre in treated cultures plotted against the concentration of compound in the culture medium (log_2 scale) for 4 multiplicities of infection, 0.1 (○——○), 1.0 (□——□), 10 (▲- - -▲) and 30 pfu per cell (△—·—△).

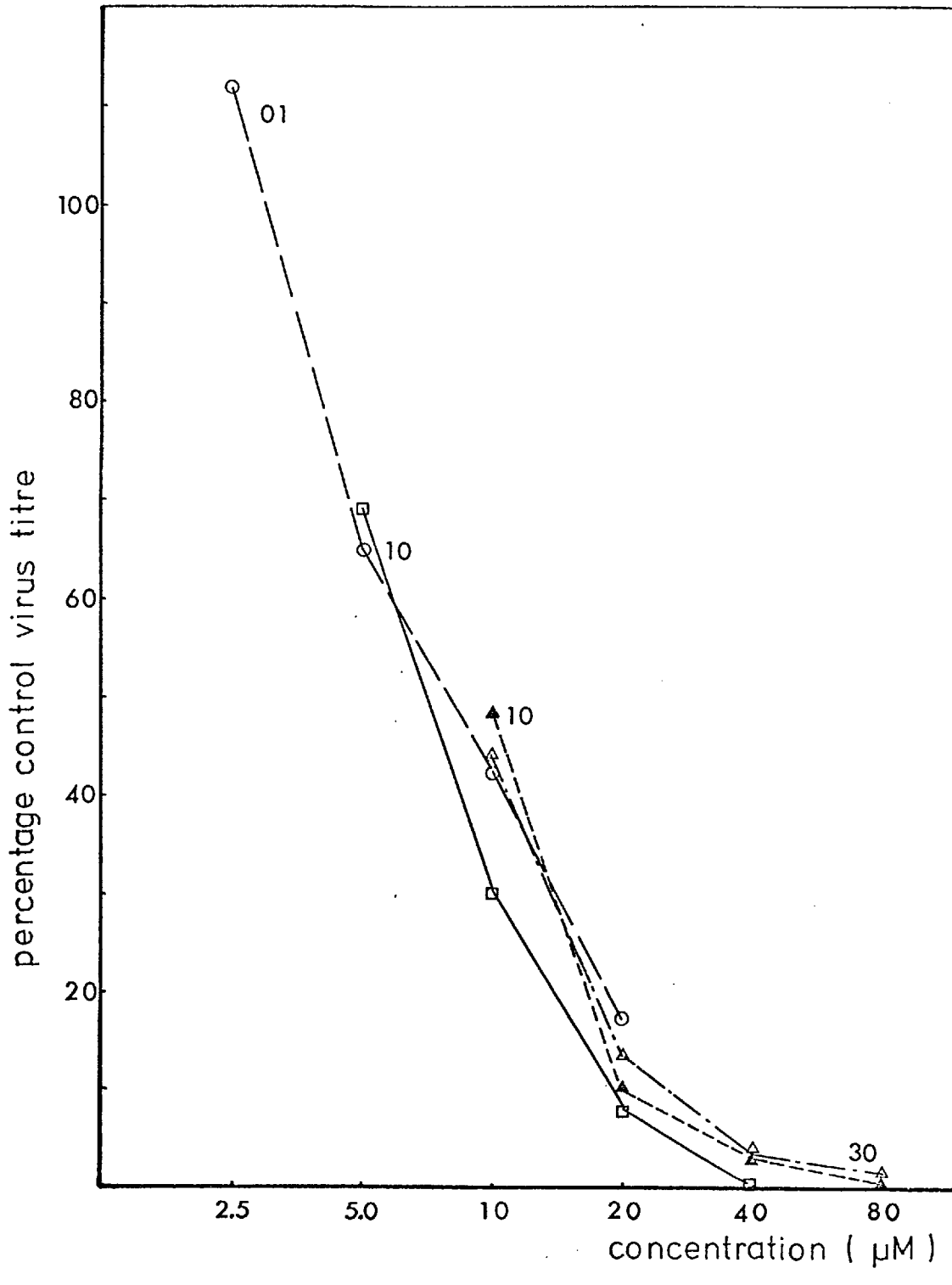


Fig. 3.49. Effect of changes in the multiplicity of infection on the dose-response lines obtained in yield reduction studies with SK+F 40491 and rhinovirus type 17. Percentage of the control virus yield in treated cultures plotted against the concentration of compound in the culture medium (log_2 scale) for 4 multiplicities of infection, 0.1 (○——○), 1.0 (□——□), 10 (▲——▲) and 30 pfu per cell (△——△).

There is a slight shift in the dose-response lines towards higher compound concentrations with increasing MOI's but this shift is in no way as marked as that obtained with Abbott 36683. At concentrations of 10 and 20μM the dose-response lines for MOI's of 0.1, 10 and 30 are very close (although that for an MOI of 0.1 is less steep that the others). It can therefore be concluded that the virus inhibitory activity of SK&F 40491 as determined by the reduction of rhinovirus yield in M-HeLa cells is not affected to any significant extent by an alteration in the size of the virus input inoculum. This is in agreement with results obtained by Matsumoto et al. (1972) with SK&F 30097. However, when attempting to quantitate the virus inhibitory activity of compounds the multiplicity of infection should be taken into account unless it is known not to affect the results.

3.2.4 Comparison of Dose-Response Lines obtained in Plaque Reduction and Yield Reduction Studies

The dose-response lines obtained by the plaque reduction and yield reduction methods were compared. Figs. 3.50 to 3.52 show the plaque reduction and yield reduction dose-response lines for Abbott 36683, SK&F 40491, SK&F 30097 and Wellcome 71-123 against rhinovirus types 22, 14 and 17. Similar plots were examined for all other rhinovirus serotypes tested but are not shown, since the relation between the 2 types of dose-response lines is generally the same as that shown for rhinovirus type 22 (Fig.3.50). This shows that the concentrations at which the response is dose-related are higher in yield reduction studies than in plaque reduction studies. Also for those virus serotypes which were tested in both plaque reduction and yield reduction experiments the order of increasing activity of the 4 compounds is the same in both types of experiment.

However, for rhinovirus types 14 and 17 the results do not follow the above general pattern (Figs. 3.51 and 3.52 respectively). Firstly with rhinovirus type 14 it is found that the plaque reduction and yield reduction dose-response lines for SK&F 40491 and Wellcome 71-123 are not widely separated. Secondly by the plaque reduction method SK&F 30097 is found to be more active than 71-123, whereas by the yield reduction method SK&F 30097 is found to be less active than Wellcome 71-123. With rhinovirus type 17 the dose-response lines for Wellcome 71-123 show the compound to be more active by yield reduction than by plaque reduction, and again SK&F 30097 is found to be more active than Wellcome 71-123 by the plaque reduction method but the reverse is found by the yield reduction method.

In order to obtain a quantitative comparison of the plaque reduction and yield reduction results, the concentrations of each of the 4 compounds which were required to produce a 50% reduction in plaque count, ED_{50}(plaque),

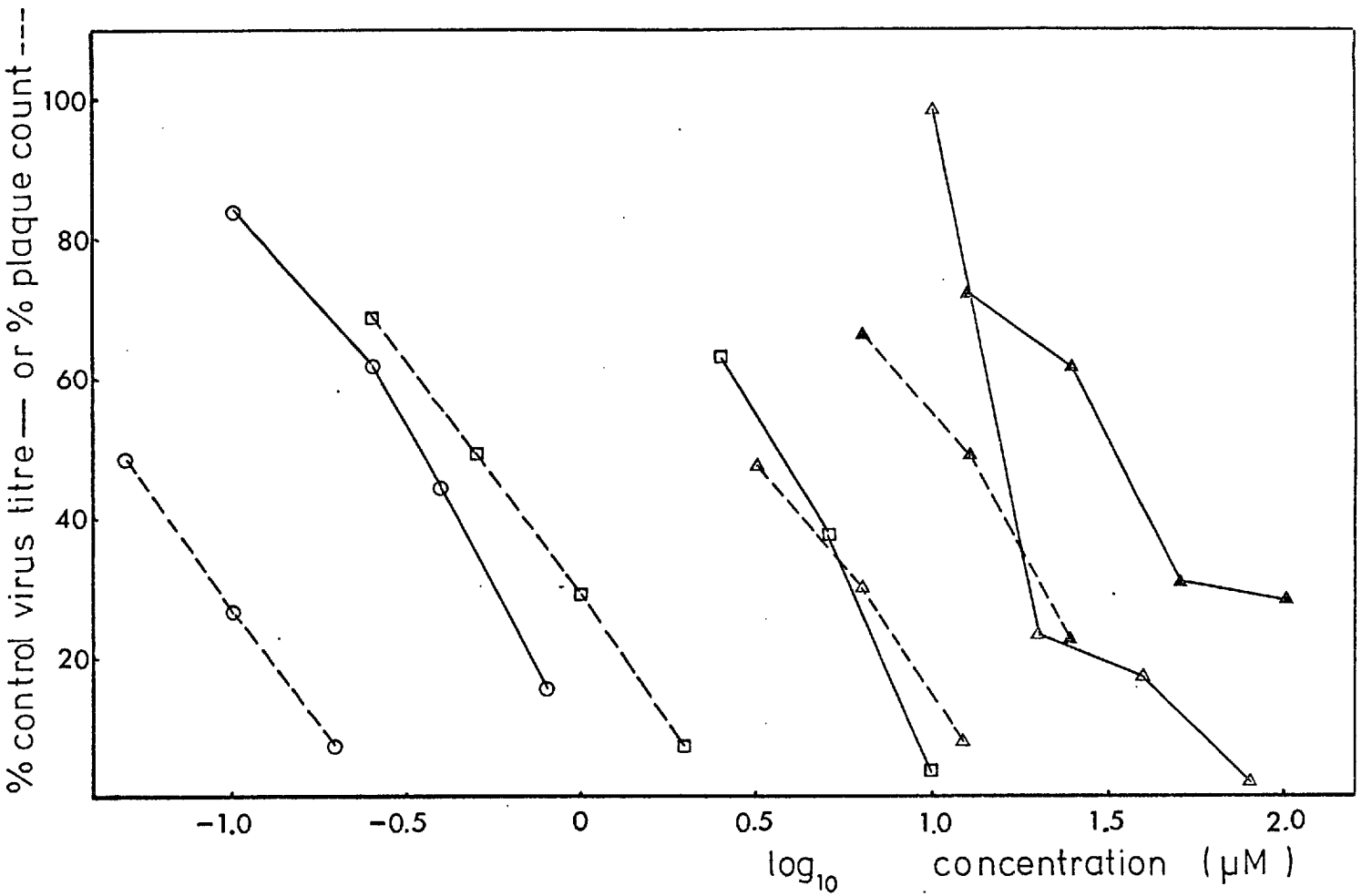


Fig. 3.50. Plaque reduction and yield reduction dose-response lines for Abbott 36683 (○——○), SK+F 40491 (□——□), SK+F 30097 (△——△) and Wellcome 71-123 (▲——▲) against rhinovirus type 22. Percentage of the control plaque count (– – –) or virus titre (——) plotted against the $\log_{10}$ of the concentration of compound in the medium.

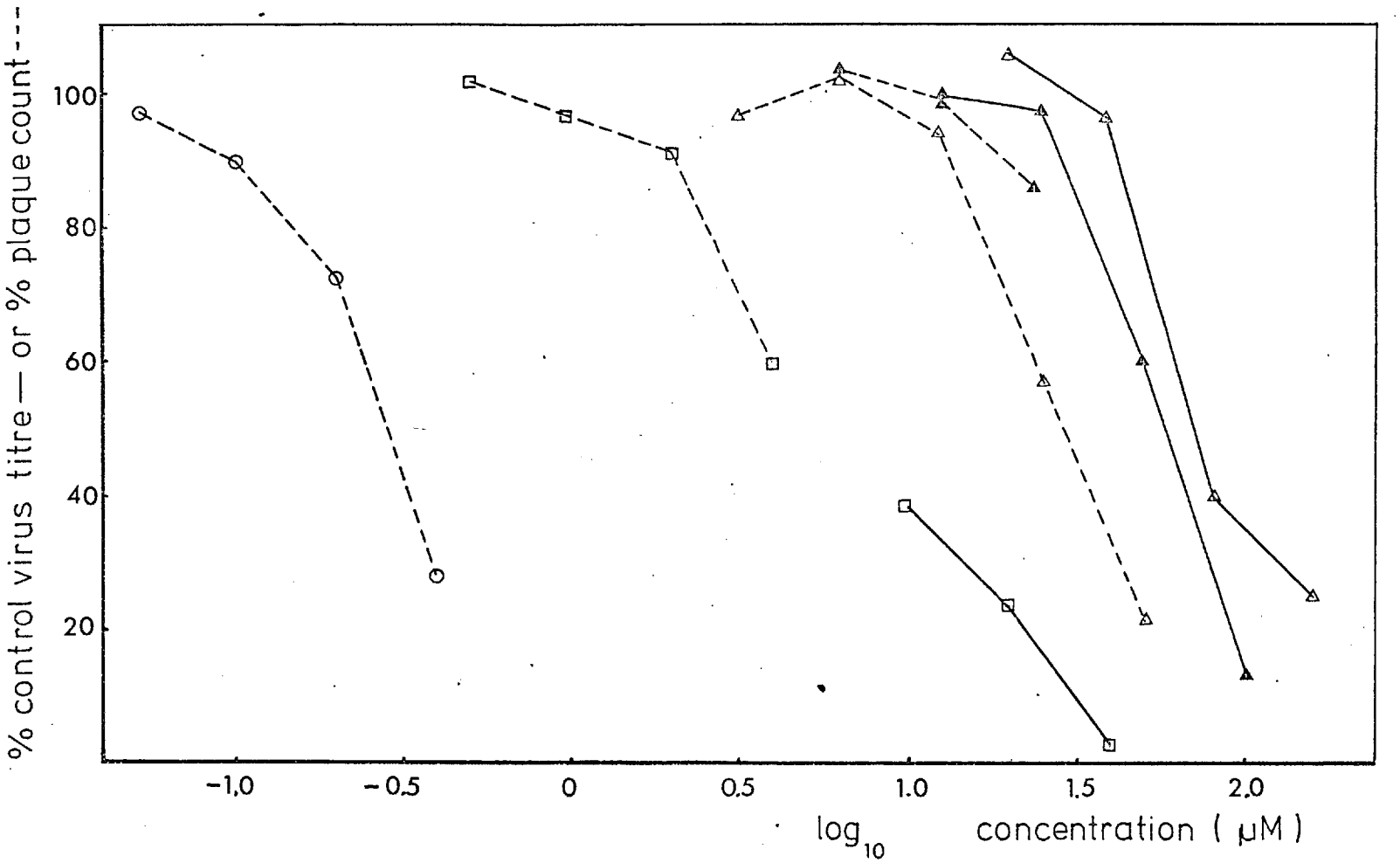


Fig. 3.51. Plaque reduction and yield reduction dose-response lines for Abbott 36683 (○——○), SK+F 40491 (□——□), SK+F 30097 (△——△) and Wellcome 71-123 (▲——▲) against rhinovirus type 14. Percentage of the control plaque count or virus titre plotted against the $\log_{10}$ of the concentration of compound in the medium.

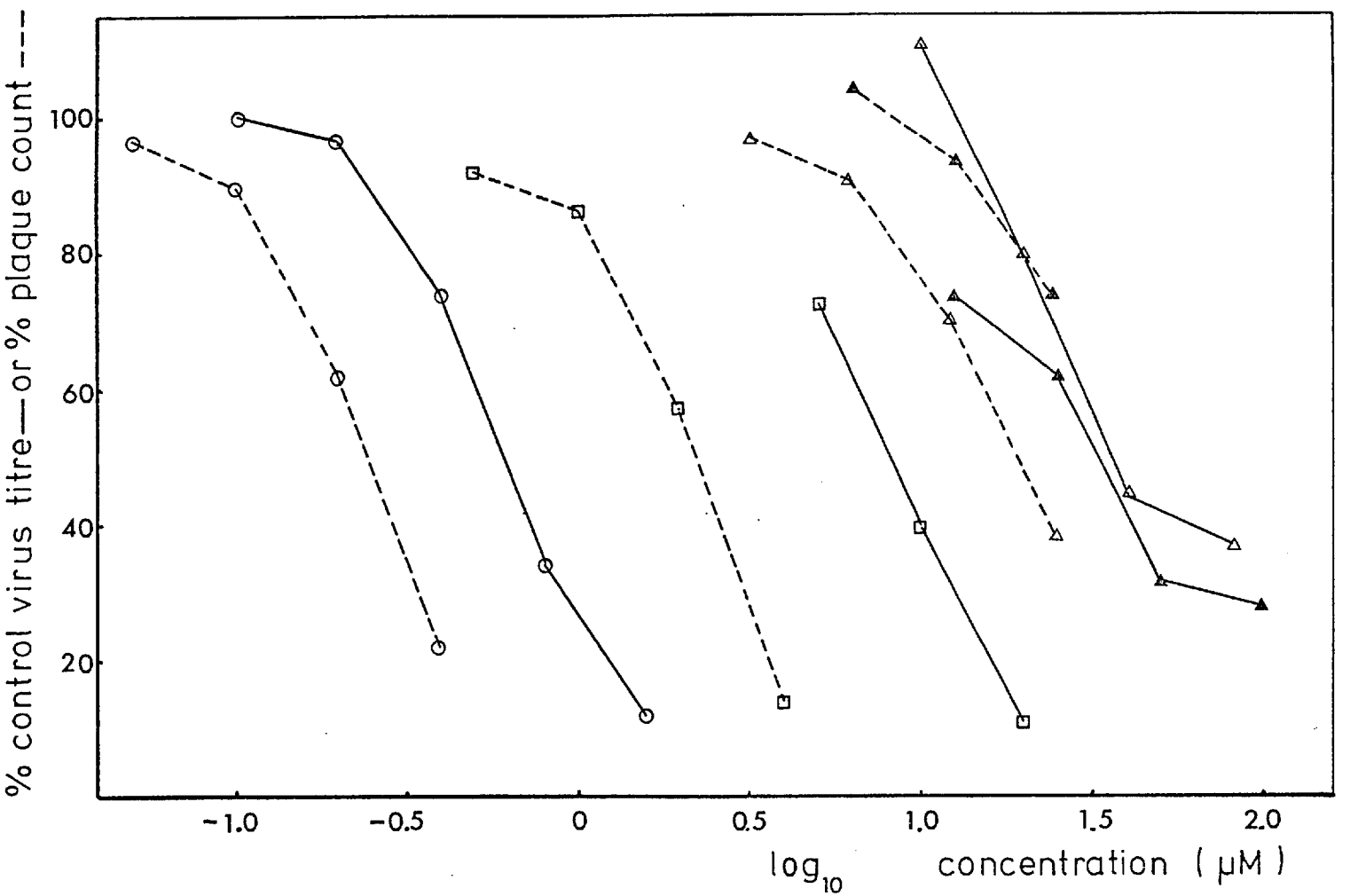


Fig. 3.52. Plaque reduction and yield reduction dose-response lines for Abbott 36683 (○——○), SK+F 40491 (□——□), SK+F 30097 (△——△) and Wellcome 71-123 (▲——▲) against rhinovirus type 17. Percentage of the control plaque count or virus titre plotted against the $\log_{10}$ of the concentration of compound in the medium.

Table 3.13 Concentrations of Abbott 36683, SK&F 40491, SK&F 30097 and Wellcome 71-123 required to produce a 50% reduction in plaque count or yield of rhinvorus types 1B, 2, 12, 14, 17, 19, 22 and 30 and poliovirus type 1

Compound	ED_{50}(plaque) or ED_{50}(yield) μM																	
	1B		2		12		14		17		19		22		30		PV1	
	PR	YR	PR	YR	PR	YR	PR	YR	PR	YR	PR	YR	PR	YR	PR	YR	PR	YR
Abbott 36683	0.083	0.282	0.224	0.372	0.096	0.145	0.282		0.246	0.603	0.071		0.046	0.339	0.145		0.282	0.282
SK&F 40491	1.072	5.623	1.230	8.128	0.977	6.607	3.890		2.291	7.943	0.832		0.490	3.548	1.349		3.802	9.550
SK&F 30097	13.49	26.92	16.60	19.50	16.22	33.11	28.18	70.79	19.05	35.48	19.50		2.88	15.85	12.59		14.79	
Wellcome 71-123	24.55		23.44	54.95	14.79		> 25	57.54	48.98	32.36	7.41		11.75	32.36			42.66	

Notes: PR, plaque reduction ED_{50} YR, yield reduction ED_{50}

or virus yield, ED_{50}(yield), was determined from the dose-response lines (Figs. 3.16 to 3.24 and 3.34 to 3.40). These values (the ED_{50}) are tabulated (Table 3.13). Also the ratio ED_{50}(yield):ED_{50}(plaque) was determined for every virus serotype and compound tested (Table 3.14).

In all cases except for Wellcome 71-123 against rhinovirus type 17 the concentration of compound required to reduce yield production by 50% is greater than that required to reduce plaque production by 50% (Table 3.13). The ratio ED_{50}(yield):ED_{50}(plaque) is not found to be a constant for the virus serotypes and compounds used. However, if this ratio is averaged for each virus against all compounds and vice versa it is found to be generally greater for the more active compounds and the more sensitive virus serotypes (Table 3.14).

Table 3.14

Ratio ED_{50}(yield):ED_{50}(plaque) for Abbott 36683, SK&F 40491, SK&F 30097 and Wellcome 71-123 against 6 rhinovirus serotypes

	Serotype						
Compound	1B	2	12	14	17	22	Average
Abbott 36683	3.39	1.66	1.51		2.46	7.41	3.28
SK&F 40491	5.25	6.61	6.76		3.47	7.24	5.87
SK&F 30097	2.00	1.18	2.04	2.51	1.86	5.50	2.52
Wellcome 71-123		2.34			0.60	2.75	1.89
Average	3.55	2.95	3.44	2.51	2.10	5.72	

3.2.5 Tests of Hypotheses to explain the Varying Sensitivities of Rhinovirus Serotypes to a Particular Virus Inhibitory Compound

It has clearly been shown in this quantitative study of rhinovirus inhibitory compounds that the variation in sensitivity of 8 rhinovirus serotypes to each of 4 compounds is the same in nearly all cases. This consistent trend in sensitivity has been determined by 3 different test methods, that is, the inhibition of virus-induced CPE in tube cultures, plaque reduction assays and yield reduction assays.

It is obviously of great interest and importance to discover the reason for varying compound sensitivity amongst rhinovirus serotypes. Basically, varying sensitivity may be due either to the variable inhibition of some specific compound-sensitive process involved in virus multiplication which is inhibited to a greater or lesser extent in different serotypes or more simply varying sensitivity may be dependent upon some characteristic of the virus replicative cycle.. Three things which, it was considered, could possibly affect the sensitivity of a virus to a compound were, the time taken for one normal virus growth cycle, the virus yield (pfu per cell) obtained in a normal virus growth cycle and the normal plaque size of a particular virus serotype. The last of these 3 possibilities is probably dependent on the former two. A serotype with a short growth cycle or a large virus yield might well prove to be less sensitive to virus inhibitory compounds than a serotype with a shorter growth cycle or smaller virus yield.

The possible relationship between virus sensitivity and plaque size has already been examined (Figs. 3.29, 3.30 and 3.31). It appeared that virus sensitivity might be dependent upon plaque size but results were not conclusive.

The size of virus yields obtained in single cycle growth experiments and the growth cycles of 2 rhinovirus serotypes were next investigated.

The control yields determined in yield reduction experiments were collated and are shown in Table 3.15. There appears to be no correlation between virus yield and virus sensitivity, but it must be made clear that the values listed in Table 3.15 are based on plaque counts and the efficiency or ease of plaquing often varies from experiment to experiment. Therefore, whilst it is quite in order to compare the yields in compound treated cultures as 'percentage control yield', since the percentage values would be the same whatever the plaquing efficiency in a particular experiment, it may not be correct to compare control yields from different experiments and expect these to be absolute values.

Table 3.15

Virus yields obtained in control cultures in yield reduction experiments

Serotype	Compound			
	Abbott 36683	Wellcome 71-123	SK&F 40491	SK&F 30097
1B	1.89×10^6		1.49×10^6	3.7×10^6
2	1.29×10^6	1.22×10^6	1.47×10^7	1.3×10^6
12	1.78×10^7		2.0×10^7	1.6×10^6
14		1.47×10^6	1.62×10^7	9.0×10^6
17	2.97×10^6	1.11×10^7	7.6×10^6	4.5×10^6
22	4.5×10^6	7.5×10^5	1.17×10^7	2.0×10^6
	4.9×10^7			
30			4.18×10^6	
PV1	8.43×10^7		3.7×10^7	
	6.3×10^7			
	3.61×10^7			

Finally the growth cycles of 2 rhinovirus serotypes (types 14 and 22) were studied. These were carried out as described (section 2.6.1). The virus yields in infected cultures were harvested at 0.5, 2.0, 4,25, 5.5, 7.0, 8.75 and 9.75 hours. Results are shown in Fig.3.53. It can be seen that there is no difference in the growth cycle times of types 14 and 22. Since these 2 serotypes represent the least and most sensitive types to inhibition by the virus inhibitory compounds studied it seems unlikely that the growth cycle time can be in any way connected with the variation in virus sensitivity to each of the 4 compounds.

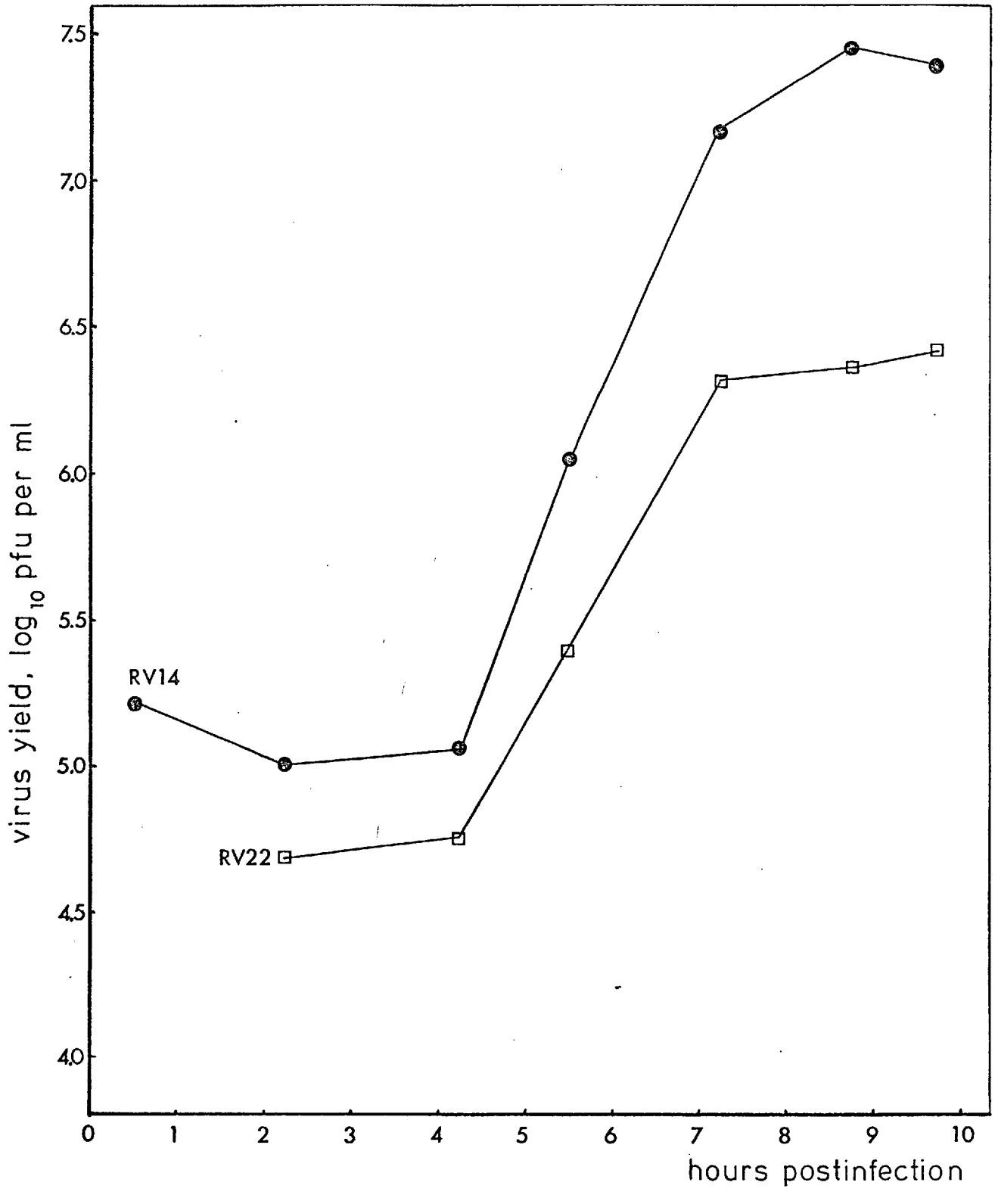


Fig. 3.53. Growth cycles of rhinovirus types 14 and 22 in M-HeLa cell cultures. Virus yield, $\log_{10}$ pfu per ml, plotted against the time of incubation (hours postinfection).

3.3 MODE OF ACTION STUDIES

Mode of action studies were carried out with SK&F 30097 as representative of the triazinoindoles. Some mode of action studies have already been reported for Abbott 36683, SK&F 30097 and SK&F 40491; however, it was wished here to carry out similar experiments with all 3 types of compound (i.e. benzimidazole, triazinoindole and adenine derivative) in order to compare the results obtained in one particular experimental system. Hence it was necessary to carry out experiments similar to those already described by other workers.

3.3.1 Virus Inactivation

Tests for the virucidal activity of the 2 triazinoindoles have already been reported (Haff et al., 1972; Matsumoto et al., 1972; Schleicher et al., 1972) and all compounds have been reported to be non-virucidal. However since such tests have involved only a fairly short period of incubation of the compound with the virus (generally up to 8 hours, although Matsumoto and co-workers incubated SK&F 30097 with poliovirus for 18 hours to test its possible virucidal activity) it was thought that they should be repeated with longer periods of incubation. This was thought to be of importance in view of the fact that in a tube culture test the virus is in contact with the compound for periods of much longer than 8 hours.

Tests for the virucidal activity of the 3 compounds Abbott 36683, SK&F 30097 and Wellcome 71-123 were carried out. Abbott 36683 and SK&F 30097 were tested against rhinovirus type 12 and Wellcome 71-123 was tested against poliovirus type 1 and rhinovirus type 2.

Virus diluted in maintenance medium (about 10^5 pfu per ml) was incubated at 36° in the absence or presence of compound (Abbott 36683, 4μM; SK&F 30097 80μM; Wellcome 71-123 100μM; and samples (3ml) were

withdrawn immediately after mixing the virus and compound and after various periods of incubation up to 90 hours. Six samples in all were taken for each virus-compound mixture. When all samples had been taken they were thawed and the residual virus which they contained was titrated by plaque assay up to dilutions beyond the limit of activity of the compound in the plaque reduction test. Initially only 4 samples were titrated, including the zero time and 2 hour samples. for each virus-compound mixture, since it was thought that the titration of all samples taken might prove unnecessary if the results showed the compounds to be non-virucidal. The residual virus titre was plotted against the time of incubation of the virus in the normal and test media and these graphs are shown in Figs. 3.54 and 3.55. The graphs show that both Abbott 36683 and SK&F 30097 are non-virucidal to rhinovirus type 12 (Fig.3.54) and that Wellcome 71-123 is non-virucidal to both poliovirus and rhinovirus type 2(Fig.3.55), since the rate of thermal inactivation in the presence of compound was in all cases equal to that in the controls.

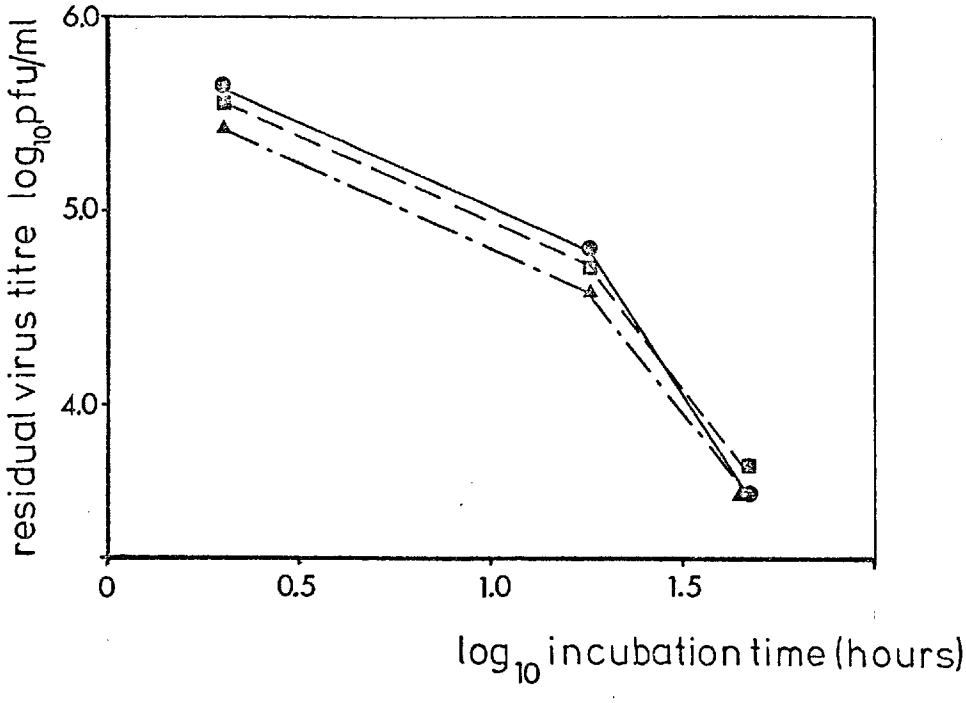


Fig. 3.54. Effect of Abbott 36683 and SK+F 30097 on thermal inactivation of rhinovirus type 12. Residual virus titre in samples incubated at 36° with Abbott 36683 (■——■), SK+F 30097 (▲——▲) or normal medium, control (●——●).

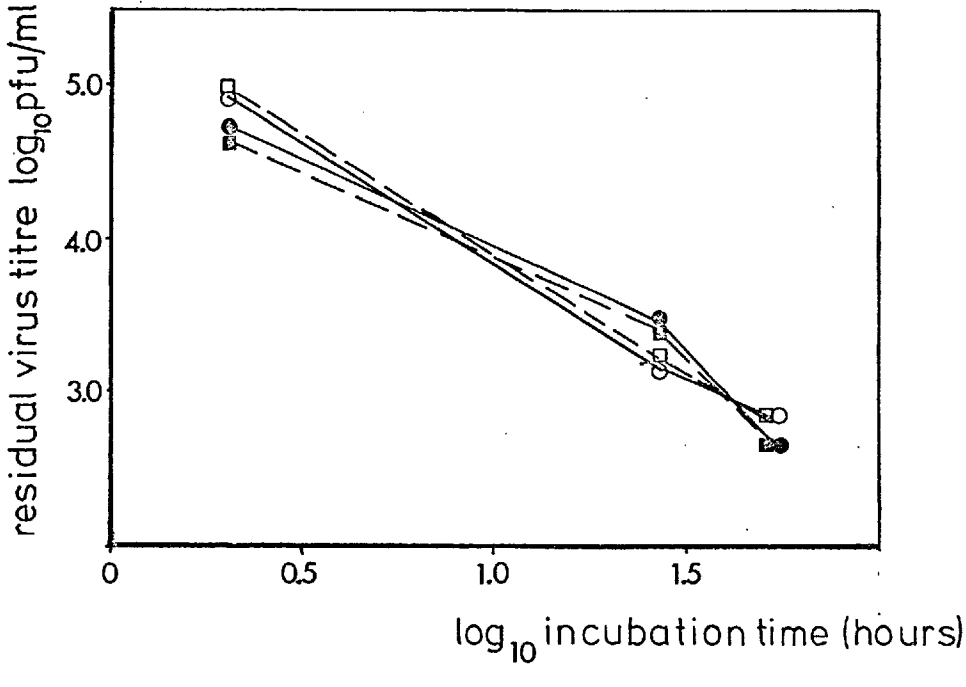


Fig. 3.55. Effect of Wellcome 71-123 on thermal inactivation of poliovirus type 1 and rhinovirus type 2. Residual virus titre in samples incubated at 36°.

●——● RV 2 control, ■——■ RV 2 incubated with Wellcome 71-123

○——○ PV 1 control, □——□ PV 1 incubated with Wellcome 71-123

3.3.2 Virus Adsorption

The effect of the 3 compounds on the kinetics of adsorption of poliovirus to OH3 cells was examined. Virus was allowed to adsorb to OH3 cell monolayers (as for normal plaque assay) in the absence or presence of compound (71-123,100μM; SK&F 30097, 80μM; Abbott 36683, 4μM). The kinetics of adsorption in each case were followed by removing the residual virus inoculum and overlaying the cells at 30, 60, 90 and 120 minutes after the addition of the inoculum, each test being carried out in duplicate. The plaques produced after incubation of the plates at 36° for 2 days were counted and the average plaque counts were plotted against the period of virus adsorption.

Fig.3.56 shows such plots for the adsorption to OH3 cells of poliovirus diluted in maintenance medium with or without the addition of any of the 3 compounds. The curves show that there is no change in the rate of adsorption of the virus to the cells when any of the compounds is present in the medium. None of the compounds therefore has any effect on the rate of adsorption. Hence it can be concluded that the compounds produce no modifications of the virus receptors on the cells.

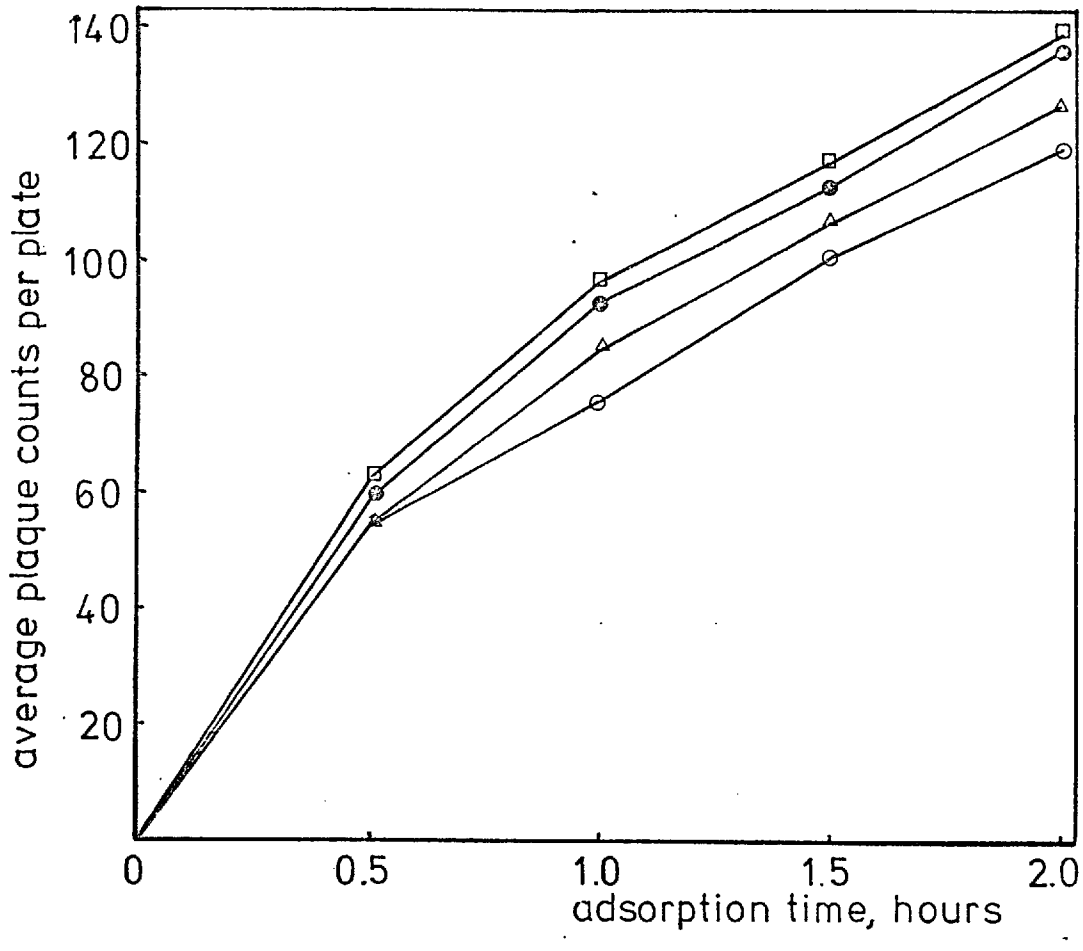


Fig. 3.56. Adsorption of poliovirus type 1 to OH3 cell monolayers. Average plaque count resulting from different periods of adsorption of virus inoculum. Adsorption in normal medium (●——●) or medium containing Abbott 36683 (○——○), SK+F 30097 (△——△) or Wellcome 71-123 (□——□).

3.3.3 Preincubation of the Cells with Compound

The effect of incubating cell monolayers with each of the compounds before virus infection, on subsequent plaque formation, was examined. Monolayers of OH3 cells (80 to 90% confluent) in 6.0cm tissue culture dishes were incubated with maintenance medium containing each of the compounds (at the concentrations used previously) or control maintenance medium containing no compound for 2 hours at 36°. After this time the medium was removed and the monolayers were infected with poliovirus (150 pfu), which was allowed to adsorb for 1 hour at 36°, each test being carried out in triplicate. Monolayers were then drained and overlaid as normal, and the number of plaques formed after incubation of the cultures for 2 days at 36° was counted.

Table 3.16 shows the plaque counts per plate for monolayers which had been overlaid with maintenance medium in the presence or absence of any of the compounds. There is no significant difference between the plaque counts obtained after incubating monolayers with any of the compounds for 2 hours and those obtained with no such pretreatment of cells. Additionally there was no visible difference in the general plaque sizes observed on control and treated plates. This therefore shows that preincubation of cells with any of the compounds has no effect on subsequent virus infection and plaque formation by poliovirus type 1.

Table 3.16

Effect of incubation of cell monolayers with Wellcome 71-123, Abbott 36683 or SK&F 30097 on subsequent infection and Plaque Formation by Poliovirus type 1. Plaque counts per plate obtained when monolayers had been variously treated before infection.

Compound in medium	Plaque counts per plate		
none	119	119	92
Wellcome 71-123	110	117	93
Abbott 36683	112	120	101
SK&F 30097	121	92	115

3.3.4 Effect of Compounds on Uncoating of Virus Particles

Experiments were carried out to determine whether any of the compounds had an effect on the uncoating of poliovirus particles. This was done by following the number of infections poliovirus particles present intracellularly for four hours after infection of the cultures.

Confluent monolayers of M-HeLa cells in 30ml tissue culture flasks were infected with poliovirus (a 1ml inoculum containing 10^6 pfu). Virus was allowed to adsorb for 1 hour at 36°. After this time the remaining inoculum was removed and replaced with 10ml of normal maintenance medium or maintenance medium containing one of the 3 compounds at the normal concentrations used. The cultures were then incubated at 36° and samples were taken at half hourly intervals for 4 hours, by freezing down 2 flasks each of control and treated cultures. Samples were then frozen and thawed 3 times and duplicate samples were pooled. The infectious virus titre of the samples was then plotted against the time of incubation after infection.

Fig.3.57 shows the plot of infectious virus titre against time of incubation for control cultures and cultures treated with Abbott 36683. There is a parallel reduction in the virus titre in both cultures over the first 3 hours of incubation. Similar results were also found for SK&F 30097 and Wellcome 71-123 (Figs.3.58 and 3.59). The results therefore indicate that there is no inhibition of virus uncoating in cultures treated with any of the 3 compounds. After 3 hours incubation the virus titre rises steeply in the control cultures while in the treated cultures there is no infectious virus production.

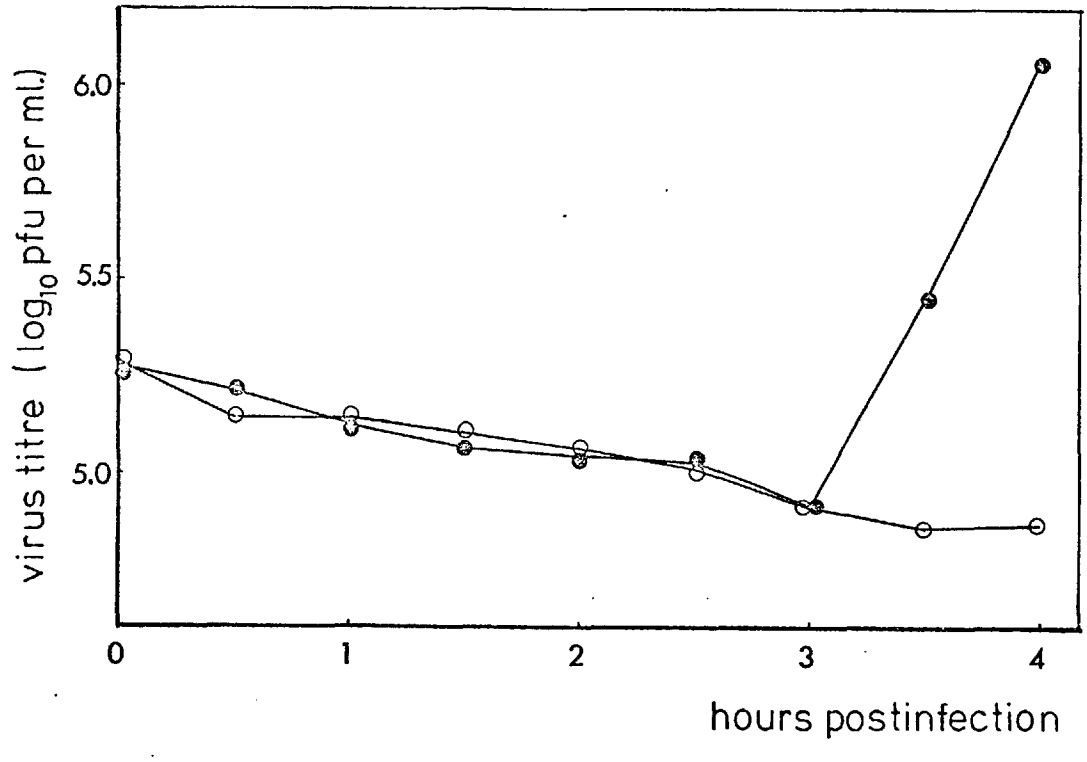


Fig. 3.57. Abbott 36683.

Figs. 3.57, 3.58 and 3.59. Early part of growth cycles of poliovirus type 1 in M-HeLa cells in the presence or absence of Abbott 36683, SK+F 30097 or Wellcome 71-123.

Virus titre, $\log_{10}$ pfu per ml, plotted against the time of incubation , hours postinfection, for control (●——●) or treated (○——○) cultures.

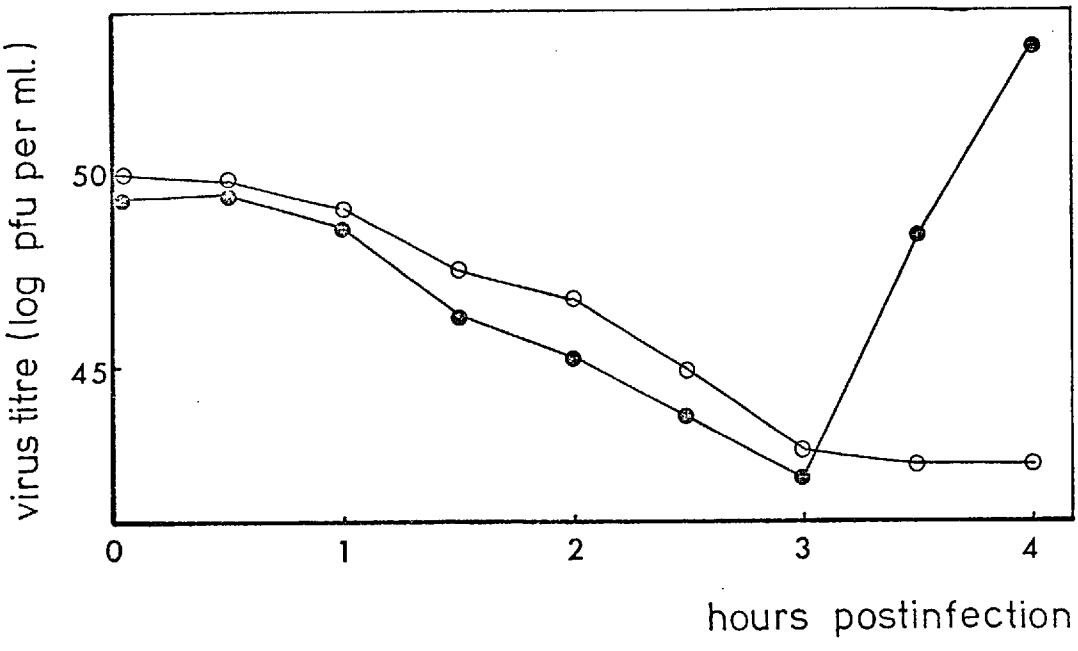


Fig. 3.58. SK+F 30097.

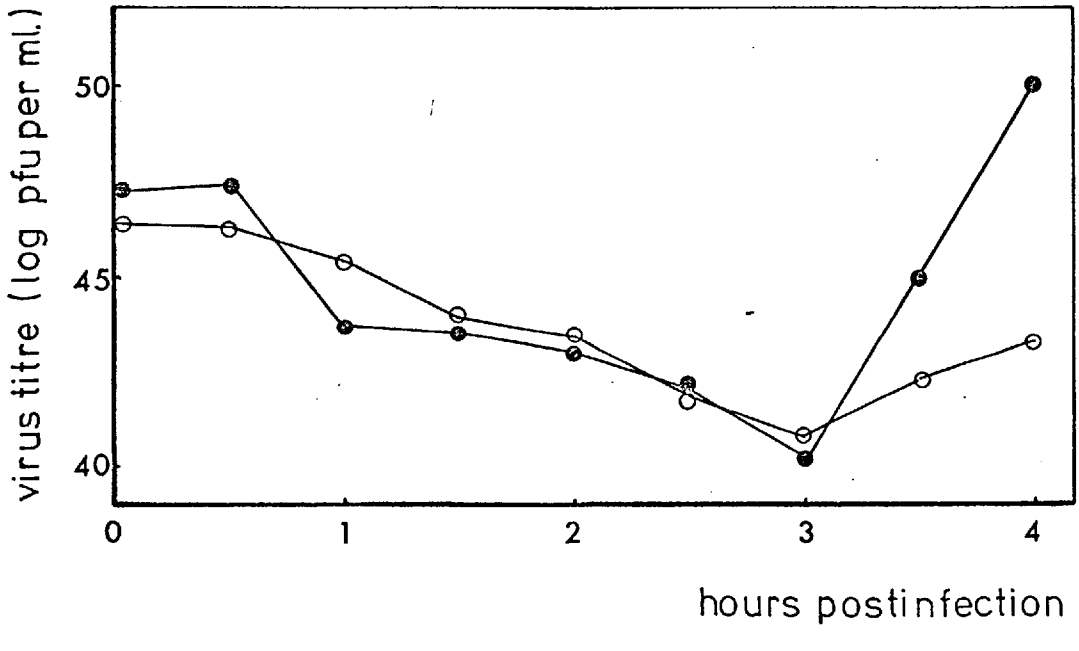


Fig. 3.59. Wellcome 71-123.

3.3.5 Effect of Wellcome 71-123 on release of Virus from Cells

By following the cell-associated and extracellular virus titres of normal cultures and cultures treated with Wellcome 71-123 (100μM) it was hoped to be able to determine whether this compound affected virus release from cells.

Confluent monolayers of OH3 cells in 30ml tissue culture flasks were infected with poliovirus type 1 (approximately 1 pfu per cell). After virus adsorption the cultures were washed in 5ml of PBS A, half were given normal maintenance medium (10ml) and the other half were given medium containing Wellcome 71-123. Cultures were incubated at 37°. At 2-hourly intervals after infection (from 0 to 8 hours) duplicate control and test cultures were harvested. Cell-associated virus and extracellular virus were harvested separately by removing and retaining the culture medium containing the extracellular virus and replacing it with fresh medium (10ml) in which to harvest the cell associated virus. The samples were titrated by plaque assay.

The virus titre for both cell associated and extracellular virus in normal and compound treated cultures was plotted against the period of incubation (Fig.3.60). It can be seen that the proportion of extracellular virus in the cultures is not decreased by the addition of 71-123 to the cultures. At 4, 6 and 8 hours postinfection extracellular virus comprised 1.69, 1.84 and 3.65% of the total virus titre in control cultures and 25.0, 7.75 and 4.25% of the total virus titre in treated cultures. In fact, at 4 hours and 6 hours, extracellular virus comprises a significantly higher proportion of the total virus titre in treated cultures than in control cultures. This is probably due to the fact that at these times extracellular virus is mainly virus left over from the inoculum which had adsorbed onto the cell sheet but which had possibly not penetrated the cells, rather than newly released virus,

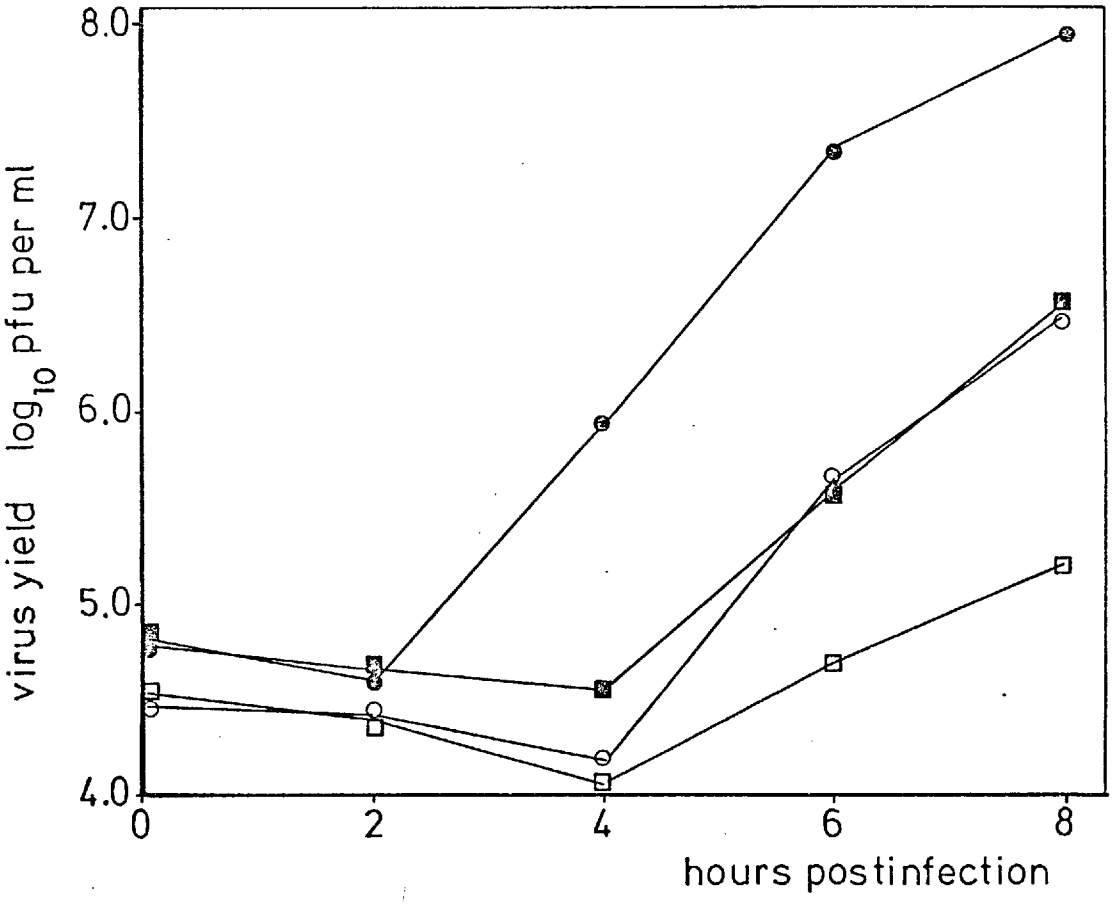


Fig. 3.60. Growth cycles of poliovirus type 1 in OH3 cells in the presence or absence of Wellcome 71-123.

○——○ Extracellular virus, control culture.

□——□ Extracellular virus, treated culture.

●——● Cell associated virus, control culture.

■——■ Cell associated virus, treated culture.

of which in treated cultures there is little. However, results from this experiment do demonstrate that 71-123 does not act by inhibiting the release of virus particles from cells.

3.3.6 Time of Addition of Compound to Virus Cultures under Single Cycle Conditions

Experiments so far carried out have shown that none of the compounds has any effect on virus adsorption, penetration or uncoating. Additionally none of the compounds is virucidal.

In order to define the period of time within the virus growth cycle at which the compounds are acting, time of addition studies were carried out.

Confluent M-HeLa monolayers in 30ml tissue culture flasks were infected with poliovirus or rhinovirus type 12 at a multiplicity of infection of 1.0 pfu per cell. Virus was allowed to adsorb for 1 or 2 hours (for poliovirus and rhinovirus respectively). The virus inoculum was then removed and replaced with normal medium (10ml) in all but 6 cultures, which received medium containing one of the compounds at the concentrations normally used (2 cultures per compound). Similarly duplicate cultures received medium containing compound at 1½, 3, 4½, 5¼ and 6 hours after infection. Six cultures (controls) received no compound at all during the period of the growth cycle.

All cultures were incubated for 7 hours after infection and at this time the infectious virus in each culture was harvested by freezing and thawing 3 times. Duplicate cultures were pooled and the samples then titrated by plaque assay. The virus titre of each sample, as a percentage of the control titre, was then plotted against the time of addition of the compound to that culture (hours after infection). Fig.3.61 shows the curves for Abbott 36683, SK&F 30097 and Wellcome 71-123. It can be seen that both Abbott 36683 and SK&F 30097 will produce a 90% inhibition of virus production if added up to 3 hours after infection. Wellcome 71-123, even when added at 0 hours, only produces a 93.0% inhibition of virus replication. However, this maximal inhibition is also maintained if 71-123 is added up to 3 hours

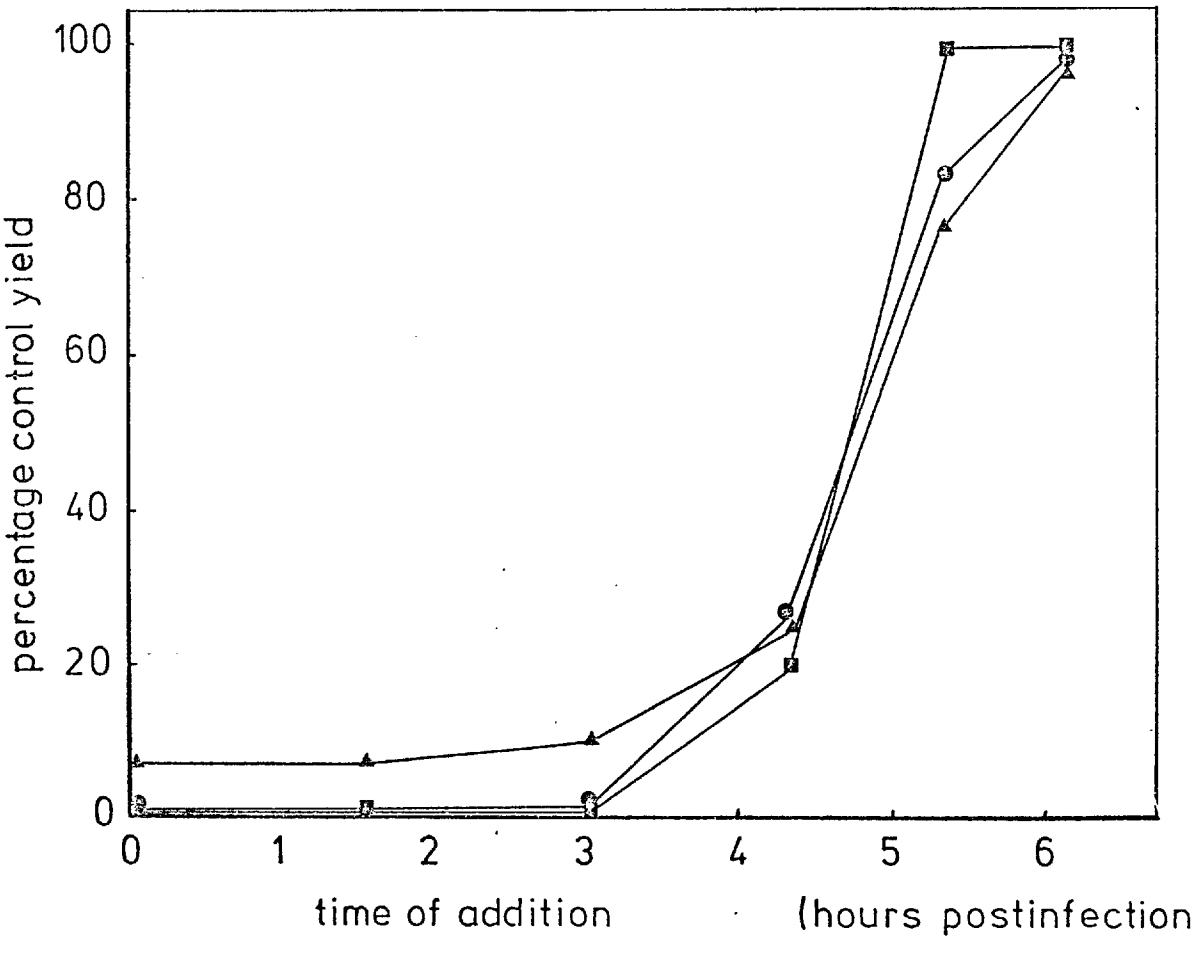


Fig. 3.61. Effect of delayed addition of compounds to poliovirus infected cultures, on the reduction in virus yield. The final yield of virus obtained in cultures treated with Abbott 36683, SK+F 30097 or Wellcome 71-123, at various times after infection is calculated as a percentage of the yield in untreated cultures and plotted against the time of addition of the compound. Abbott 36683 (●——●), SK+F 30097 (■——■) and Wellcome 71-123 (▲——▲).

after infection.

Adding the compound at 4⅓ hours in each case results in a decreased inhibition of virus replication. This inhibition becomes increasingly smaller the later the compound is added. The fact that one observes only a gradual decline in antiviral activity here is probably indicative of the fact that the virus infection is not synchronised, i.e. virus replication is at slightly different stages in different cells, since virus adsorption and penetration do not occur simultaneously in all cells.

The results therefore show that all compounds act a stage (or stages) late in the replicative cycle.

3.3.7 Timed removal of Abbott 36683 from Virus Cultures under Single Cycle Growth Conditions

To substantiate the results obtained in the above experiment (timed addition of compound to infected cultures) the reverse experiment was carried out where confluent monolayers of M-HeLa cells were similarly infected with poliovirus but were treated with compound-containing medium immediately after infection. At hourly intervals from 0 to 6 hours the compound-containing medium was removed from duplicate cultures and replaced with normal maintenance medium. All cultures were incubated for 7 hours after infection and the samples harvested and titrated as already described.

This experiment was carried out with Abbott 36683 (4μM). Since the results gave little additional information to that obtained from the study of the timed addition of compound, it was thought unnecessary to test SK&F 30097 and Wellcome 71-123 in this way.

However, the results obtained for Abbott 36683, shown in Fig.3.62, do substantiate the results obtained from the time of addition study. The experiment does show that the antiviral effect of the compound is reversible, since when the compound is removed so is its inhibitory effect. Again the results indicate that the compound acts at a stage late in the replication cycle of poliovirus since when the compound is removed at 4 hours postinfection 30% of the control virus yield is still obtained. Hence in 3 hours (from the time of removal of the compound, 4 hours, until the time when the cultures are harvested, 7 hours) the culture is able to produce 30% of the normal virus yield. If the virus replication was inhibited at an early stage, 3 hours would be insufficient for it to produce even 30% of the normal control yield. For example, in the normal growth cycle (see Fig.3.60) the total yield (extracellular plus cell associated virus) at 4 hours is 8.85×10^5 pfu per ml, which is only 0.98% of the total virus yield at 8 hours (9.03×10^7 pfu per ml). However, if virus replication is inhibited at

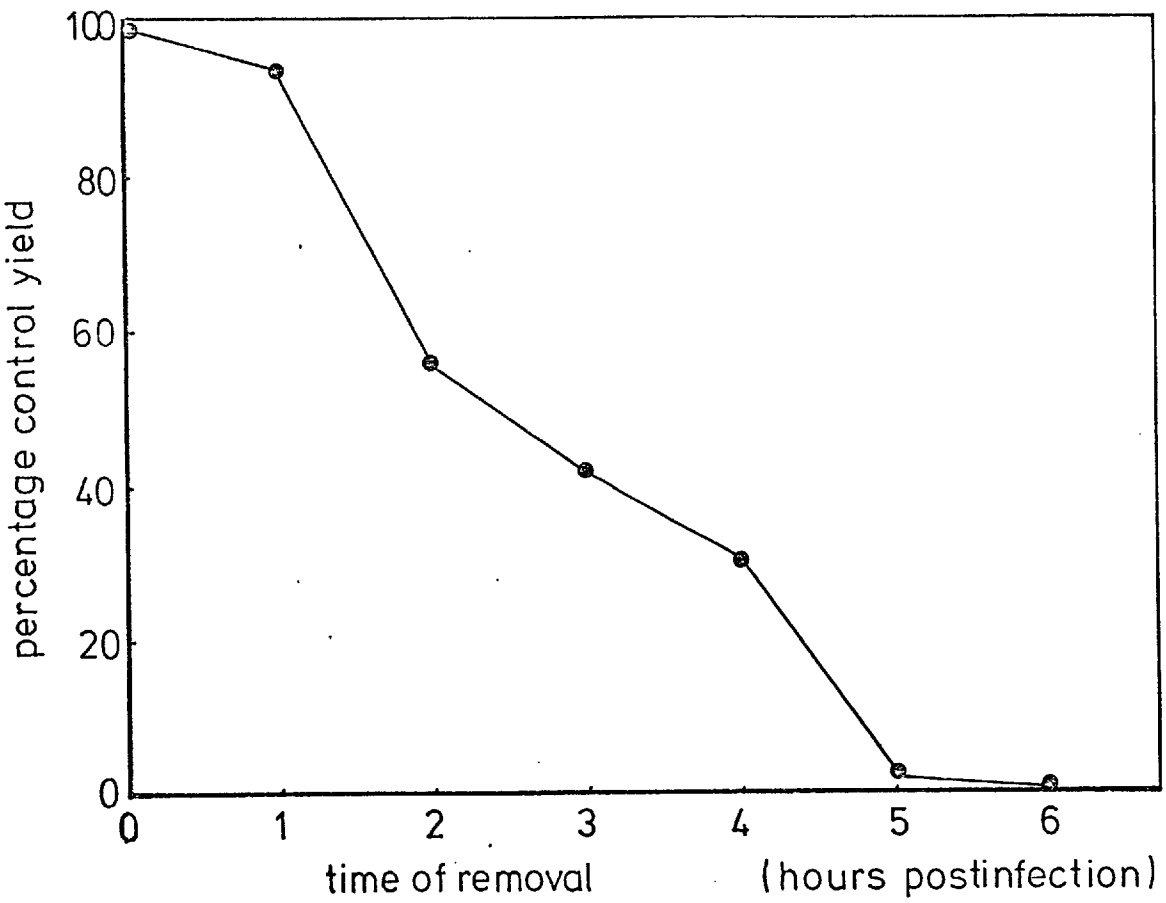


Fig. 3.62. Effect of timed removal of Abbott 36683 from poliovirus infected cultures. The final yield of virus obtained in cultures from which compound was removed at various times after infection and replaced by normal medium, is expressed as a percentage of the yield in completely untreated cultures and plotted against the time of removal of the compound.

a stage late in the growth cycle it requires only a short time in which to produce near maximal virus yields.

3.3.8 Effect of Actinomycin D on the inhibitory activity of Abbott 36683, SK&F 30097 and Wellcome 71-123

It was thought that some further indication to the mechanism of action of Abbott 36683 and SK&F 30097 might be obtained by determining whether actinomycin D reverses their virus inhibitory effect. Appleyard and co-workers (1965) reported that the activity of isatin 3-thiosemicarbazone (a compound to which the triazinoindole SK&F 30097 is related) could be reversed by the treatment of cell cultures with actinomycin D for 3 hours before infection with rabbitpox virus. The concentration of actinomycin D used was too low to cause any direct inhibition of virus multiplication. The inference from the results was that DNA-dependent RNA synthesis is essential for the virus inhibitory activity of isatin 3-thiosemicarbazone. Presumably isatin 3-thiosemicarbazone induces the production of an RNA species which in turn directs the synthesis of a protein which is possibly itself the antiviral agent.

Soon after the infection of cells with a picornavirus the rate of protein synthesis and RNA synthesis begins to decline (Baltimore 1969). The kinetics of inhibition of host protein and RNA synthesis by picornaviruses vary from one system to another. However, it is reported that in general the rate of protein synthesis falls to 25 to 50% of normal by the time virus-induced protein synthesis becomes evident. The kinetics of decline of cell RNA synthesis also vary from system to system, but generally RNA synthesis switches smoothly from host to virus-specific with a slow decline in overall RNA synthesis as infection proceeds. Although this inhibition of host cell biosynthetic processes does occur in infected cells it does not rule out the possibility of a virus-inhibitory compound acting by inducing the production of a virus-inhibitory substance *via* the cellular biosynthetic processes.

Hence it was thought desirable to carry out this study using actinomycin D.

It is well-known that actinomycin D does not inhibit the replication of polioviruses (Reich et al., 1961), since the multiplication of picornaviruses does not involve the synthesis of RNA on a DNA template. It was thought advisable, however, to determine a suitable non-toxic concentration of actinomycin D to use in this experiment.

Confluent monolayers of M-HeLa cells in 30ml tissue culture flasks were either infected with poliovirus type 1 at a multiplicity of infection of 1.0 pfu per cell, or left uninfected. The cultures were then treated with medium containing actinomycin D at a range of concentrations (0.8, 0.4, 0.2, and 0.1 µg per ml). Control cultures were given normal medium and all cultures were in duplicate. Cultures were incubated for 10 hours at 36°, after which time they were observed for virus-induced CPE or drug-induced cytotoxic effects. The infectious virus titre of infected samples was titrated by plaque assay.

The results of this test are shown in Table 3.17. It can be seen that all concentrations of actinomycin D used were cytotoxic. However, in none of the drug-treated cultures was the virus titre reduced. It was still thought advisable to carry out the tests with a non-toxic concentration of actinomycin D. A concentration of 0.025µg per ml was found to be non-toxic to cells in later experiments using the conditions described above.

Table 3.17

Effect of Actinomycin D on the Yield of Poliovirus type 1 in HeLa OH3 cells

Actinomycin D conc. µg/ml	Cytotoxicity in uninfected cells (% cell sheet)	Poliovirus Yield pfu/ml
0	0	9.6×10^7
0.1	25	9.6×10^7
0.2	50	8.7×10^7
0.4	100	9.1×10^7
0.8	100	9.4×10^7

Table 3.18

Effect of Actinomycin D on Inhibitory Activity of Abbott 36683, SK&F 30097 and Wellcome 71-123

Additions to medium	CTE %	CTE + CPE %		Virus Yield % Control Yield	
		Rhino	Polio	Rhino	Polio
Act.D (0.1µg/ml)	25.0	100	100	100	100
(0.025 µg/ml)	0	100	100	100	100
Abb. 36683 (4µM)	0	0	0	0.045	0.085
Abb. + Act.D (0.1)	25.0	-	25.0	-	0.055
Abb. + Act.D (0.025)	0	0	0	0.055	0.065
SK&F 30097 (40µM)	0	50	-	23.0	-
SK&F + Act.D (0.025)	0	50	-	18.9	-
71-123 (100µM)	0	50	-	21.7	-
71-123 + Act.D (0.025)	0	50	-	21.7	-

Tests were then performed to determine the effect of actinomycin D (0.025 µg/ml) on the rhinovirus inhibitory activity of Abbott 36683, SK&F 30097 and Wellcome 71-123. The effect of actinomycin D at non-toxic and toxic concentrations (0.025 and 0.1 µg/ml) on the poliovirus inhibitory activity of Abbott 36683 was also determined.

Duplicate cultures of cells (normal or infected with rhinovirus type 12) were treated with maintenance medium containing one of the following: actinomycin D, 0.025 µg/ml; Abbott 36683, 4µM; actinomycin D plus Abbott 36683; no additions. Cultures were treated similarly for SK&F 30097 (40µM) and Wellcome 71-123 (100µM). All cultures were incubated for 10 hours at 36°, after which time they were observed for cytopathic and cytotoxic effects, and the virus titre in infected cultures was titrated by plaque assay (4µM) and 2 concentrations of actinomycin D (0.025 and 0.1 µg/ml).

Table 3.18 shows the observed cytopathic and cytotoxic effects (CPE and CTE), expressed as a percentage of the total cell sheet affected, observed and the virus titres obtained after incubation of the cultures for 10 hours.

It can be seen that actinomycin D (0.025 µg/ml) does not reverse the inhibitory effect of any of the compounds tested, as shown by the fact that the inhibition of virus titre is the same in the presence of test compound alone as in the presence of test compound and actinomycin D. Additionally a toxic concentration of actinomycin D (0.1 µg/ml) does not reverse the poliovirus inhibitory effect of Abbott 36683.

From this it can be concluded that none of the 3 test compounds acts by inducing the production of a virus inhibitory protein via the cellular biosynthetic processes.

4.0 DISCUSSION

4.1 Plaque inhibition and tube culture techniques

4.1.1 Plaque Inhibition Technique

The plaque inhibition method was used in this study for the detection and rough quantitation of rhinovirus inhibitory activity.

The method was found to be an easy and quick method for the detection of antiviral activity. Of the compounds screened by this method 121 were found to be inactive, 6 were found to have doubtful activity and 11 were active against poliovirus type 1. Fewer compounds were found to be active against rhinovirus types 1B and 2. The method was found to be very reproducible.

The method was further used to obtain a rough estimation of the comparative activities of Abbott 36683, SK&F 40491, SK&F 30097, Wellcome 71-123 and Wellcome 71-329 against 8 rhinovirus serotypes. In order to obtain measurable inhibition zones on 60mm tissue culture plates in the test the amount of compound administered on each disc varied for each compound (Table 3.4). SK&F 30097, Wellcome 71-123 and Wellcome 71-329 were tested at the same compound dose (0.2μM). SK&F 30097 was found to be the most active of these 3 compounds, producing inhibition zones ranging from 19.0 to greater than 50 mm in diameter. Wellcome 71-123 produced zones of 20 mm against all serotypes tested and Wellcome 71-329 was seen to be less active than 71-123, producing inhibition zones ranging from 9.5 to 18.5 mm for the serotypes tested. Abbott 36683 and SK&F 40491 when tested at 0.009 and 0.075 μM amounts respectively gave inhibition zones of similar diameters against rhinovirus serotypes tested and the inhibition zones produced by SK&F 40491 were roughly twice the diameter of those produced by 0.1 μM SK&F 30097. It is not possible to compare the activities of these compounds accurately by the plaque inhibition method since the

relationship between activity and the diameter of the inhibition zone has not been determined. However, the results demonstrate that Abbott 36683 is approximately 10 times as active as SK&F 40491. SK&F 30097, Wellcome 71-123 and Wellcome 71-329 show successively less activity.

The comparative sensitivity of the rhinovirus serotypes to each of the compounds except Wellcome 71-123 could also be determined. The rhinovirus serotypes are listed in order of increasing sensitivity to each of the compounds (Table 3.5). No conclusion can really be drawn from these results but they will be compared with results obtained by other test methods.

4.1.2 Tube Culture Technique

Tube tests were also used in this study for the detection and quantitation of rhinovirus inhibitory activity. Abbott 36683 (2 μM and 4 μM), SK&F 40491 (20 μM and 40 μM), and SK&F 30097 (20 μM and 40 μM) were tested for activity against 9 rhinovirus serotypes and Wellcome 71-123 (100 μM) was tested for activity against 9 rhinovirus serotypes. The criterion for activity was a delay in virus-induced CPE by 2 days.

The results (Table 3.2) showed that Abbott 36683 and SK&F 40491 were active at both concentrations used against nearly all serotypes. One exception to this was the activity of SK&F 40491 against rhinovirus type 16 which varied according to compound concentration and virus input inoculum. The results obtained with Abbott 36683 therefore agreed with results of other workers (Schleicher et al., 1971), who showed this compound to be active against the first 55 rhinovirus serotypes. Also the results obtained with SK&F 40491 confirm and extend those of Haff and co-workers (1972), who found this compound to be active against all of 8 rhinovirus serotypes tested.

Wellcome 71-123 was found to be inactive against all of the 9 rhinovirus serotypes tested by the tube culture technique (types 1B, 2, 5, 9, 14, 17, 19, 22 and 30). Eight of these serotypes, all but type 5, had been previously tested against this compound by the plaque inhibition method, and positive activity had been observed in each case. The plaque inhibition method would therefore seem to be a more sensitive technique for detecting rhinovirus inhibitory activity than the tube culture technique. However, the inactivity of Wellcome 71-123 observed with the tube culture method cannot be explained completely on these grounds, since SK&F 30097, which shows only slightly greater activity than Wellcome 71-123 in the plaque inhibition test, is found to be active against many rhinovirus serotypes by the tube culture method, when tested at only 2/5ths of the concentration at which Wellcome 71-123 was tested.

The results obtained in tube culture tests with SK&F 30097 extend those of Gwaltney (1970), who found that SK&F 30097 (350 μM) gave maximum suppression of CPE with 12 rhinovirus serotypes. Also, at a concentration of 175 μM the compound inhibited 17 of 18 serotypes tested. By Gwaltney's method the tests were judged complete when the virus control tubes showed 75% or greater destruction of the cell sheets. Observed differences in the extent of CPE in experimental and control tubes of 75% or more were then graded as '+', 75 to 50% as '±', and less than 50% as '-' inhibition. Virus serotypes were tested at various input inocula, 3, 10, 30 and/or 100 $TCID_{50}$ per tube. From Gwaltney's results the serotypes tested have been placed into 3 groups depending on their sensitivity to 175 μM SK&F 30097 (Table 4.1).

Table 4.1

Sensitivity of rhinovirus serotypes to SK&F 30097 in the tube culture test. Results from Gwaltney (1970). Tests were carried out in WI-38 cells and numbers in brackets indicate the size of virus input inoculum

Activity	Serotypes		
+	40 (100)	50 (100)	18 (100)
	42 (3)	52 (30)	16 (10)
	45 (30)	54 (100)	20 (10)
	14 (10)	24 (100)	
	48 (100)	14 (100)	
±	44 (30)	46 (30)	
	53 (100)	14 (100)	
	43 (100)	55 (100)	
−	8 (100)		

It is difficult to conclude anything from these results for 2 reasons. Firstly it can be seen that 4 tests were carried out with rhinovirus type 14 and the results obtained are not consistent i.e. ± (30), + (10), ± (100) and + (100). Also the serotypes were not all tested at one particular virus input inoculum and it is known that the activity of a compound will vary depending on the size of the inoculum. Rhinovirus type 8 was only tested at 100 $TCID_{50}$ per culture and found to be insensitive to inhibition by SK&F 30097, whereas at a lower input it may well have been found to be sensitive.

However, where the same virus serotypes have been tested at equal virus input inocula both by Gwaltney and in the present study the results have been compared (Table 4.2).

Table 4.2

Sensitivity of rhinovirus serotypes to SK&F 30097 in the tube culture test. Results from Gwaltney (1970) and present study

		Gwaltney (1970)	Present study
	cell line	WI-38	M-HeLa
Virus Serotype	Virus input inoculum $TCID_{50}$ per culture	ACTIVITY	
8	100	-	-
14	10	+	-
16	10	+	-
18	100	+	-
40	100	+	-
43	100	±	-
53	100	±	-

Table 4.2 in fact shows almost total disagreement between the two sets of results, with the exception of the test of rhinovirus type 8. Nearly all serotypes tested by Gwaltney exhibited some sensitivity to SK&F 30097 whereas the same serotypes tested at the same virus input inoculum were found in the present study to be insensitive. Some sensitivity, however, was observed when these serotypes were tested at lower input inocula. The reason for this discrepancy in the results is due probably to the different cell lines used. It has

been shown in this study (section 3.2.1) that the appearance of rhinovirus induced CPE in WI-38 cells is slower than in M-HeLa cells. Additionally the delay in the appearance of CPE produced by a certain concentration of a virus inhibitory compound is longer in WI-38 cells than in M-HeLa cells and hence compounds would appear more active when tested in the former cell line if activity was based upon the same criterion in each case.

It is therefore not possible to make a meaningful comparison between the results obtained by Gwaltney and those of the present study. In this study tests were carried out in a more logical fashion and conclusions could be drawn from the results. On the basis of the results obtained in tube tests the serotypes tested could be placed into 3 groups on the basis of varying sensitivity at different virus inputs (Table 3.3). From this it was apparent that the sensitivity of the serotypes decreased with increasing virus input inoculum. This fact must in part be the reason for the variation in the results of different workers and its importance must not be understated.

No attempt has previously been made to compare the sensitivities of a large number of rhinovirus serotypes to any compound by the tube culture test since Boyle and co-workers (1970) determined the minimal inhibitory concentrations of SK&F 30097 against only 6 rhinovirus serotypes by this method. This study has shown that the tube culture technique can be used for such a purpose and give meaningful results.

For this study the concentrations of each of the test compounds which would delay virus-induced CPE by approximately the same time were determined. These were found to be 0.5, 5.0 and 80 μM for Abbott 36683, SK&F 40491 and SK&F 30097 respectively. Hence by this method Abbott 36683 is about 10 times more active than SK&F 40491 and 160 times more active than SK&F 30097. Plots of the delay in the appearance of CPE in treated cultures compared with control cultures against the size of

the virus input inoculum showed again that the compounds appeared to be less active at higher input inocula, i.e. the delay is shorter at higher inocula . From the plots the virus serotypes were placed in order of increasing sensitivity to each of the compounds (Table 3.5). This order was found to be generally the same for each of the compounds. This new finding was obviously thought to be of great practical significance and it was therefore hoped to be able to confirm the results by using other methods for the determination of rhinovirus inhibitory activity.

4.1.3 Comparison of Plaque Inhibition and Tube Culture Techniques

The order of increasing sensitivity to each of the compounds as determined by the tube culture technique was compared with that obtained by the plaque inhibition technique (Table 4.3).

Table 4.3

Rhinovirus serotypes listed in order of increasing sensitivity to Abbott 36683, SK&F 40491 and SK&F 30097 as determined by the tube culture and plaque inhibition techniques

Abbott 36683		SK&F 40491		SK&F 30097	
P.I.	T.C.	P.I.	T.C.	P.I.	T.C.
14	14	14	14	14	14
17	17	17	2	2	17
12	2	2=12	17	17	1B
30	30	30	1B	12	30
22	22	22	30	1B	2
2	1B	1B	12	30	22
1B	12		22	22	12

Notes: P.I. - plaque inhibition test

T.C. - tube culture test

The correlation between the results obtained in the 2 tests is not very close. The only 2 serotypes which maintain a fairly consistent position in both tests are types 14 and 17, of which type 14 is the least sensitive to all compounds in all cases. However, taking each test method individually the order of increasing sensitivity of the serotypes to each of the compounds is fairly similar, although neither shows a correlation obviously better than the other.

The comparative sensitivities of rhinovirus serotypes to each of the compounds will become clearer when the results obtained with other test methods are compared.

A comparison of the plaque inhibition and tube culture techniques as methods for the detection and quantitation of rhinovirus inhibitory activity should be made here.

For the purpose of detection of virus inhibitory activity the plaque inhibition method is easier, less time-consuming and more sensitive than the tube culture method. Many tests can be carried out on one tissue culture plate (for example on one 90 mm diameter plate 4 compound-containing discs can easily be accommodated), whereas for each test in tube cultures 3 cultures are ideally required. Secondly, after infecting and overlaying the plates in plaque inhibition tests they need only be fixed and stained in order to obtain the results. Tube cultures, however, need to be observed twice daily for 4 to 5 days. The plaque inhibition method also has the advantage that cytotoxicity can be observed in infected culture plates, whereas for tube culture tests employing M-HeLa cells separate uninfected treated control cultures must be included. It is true that if WI-38 cells are used, cytotoxicity can also be observed in infected cultures, since the spread of virus-induced CPE is not as fast in these cells as in M-HeLa cells, hence negating the need for controls. However, it is possibly not a safe assumption that cytotoxicity will always be distinct from

virus-induced CPE, as is the case in the plaque inhibition test.

Finally, tube culture tests suffer from the disadvantage of possible false positive or false negative results. In the former case a virucidal compound will give a false positive result in tube culture tests since the compound is in direct contact with the virus in the medium. Gladych *et al.* (1972) used both the plaque inhibition and tube culture tests to determine structure activity relationships amongst 64 substituted 5H-*as*-triazino[5,6-*b*]indoles. They found that the results obtained in the 2 tests did not always coincide and certain compounds were found to be active by the tube culture test but inactive by the plaque inhibition method. This result could possibly have been due to direct virucidal activity of the compounds.

False negative results may be obtained in tube culture tests when a partially resistant virus serotype is being used. In tube cultures the resistant portion of the virus population will multiply in the presence of the test compound and produce cytopathic effects. Using the plaque inhibition method, however, inhibition of the sensitive portion of the population will give rise to a zone of partial inhibition. This was in fact noted by Gwaltney (1968) in tests carried out with HBB-HCl.

4.1.4 Comparison of WI-38 and M-HeLa Cells in the Tube Culture Test

The use of WI-38 and M-HeLa cells for the determination of rhinovirus inhibitory activity was compared (section 3.2.1a). The relative sensitivities of rhinovirus types 2, 17 and 22 to Abbott 36683, SK&F 40491 and SK&F 30097 were generally found to be the same in both cell lines; the serotypes in order of increasing sensitivity to each of the compounds were 17<2<22. One exception to this rule was with SK&F 30097, where type 17 was found to be more sensitive than type 2 in WI-38 cells. The relative potencies of the compounds (at the concentrations used) against

the 3 virus serotypes was not, however, generally found to be the same in both cell lines. The results were more consistent in WI-38 cells, showing that the compounds, in order of increasing activity, were SK&F 30097 < SK&F 40491 < Abbott 36683 (at the concentrations used). The more consistent results obtained with WI-38 cells could be due to the fact that there is greater separation between each of the dose-response lines obtained in WI-38 cells than those obtained in M-HeLa cells.

It had been hoped to test a far greater number of rhinovirus serotypes in WI-38 cells but this was not possible because the culture of these cells was found to be extremely difficult, due to their age. This is one of the main drawbacks in the use of WI-38 cells in that they will undergo only a finite number of population doublings, usually about 50, and after approximately 30 doublings their rate of growth begins to slow down. Also, as has been reported, the delay of appearance of CPE in treated cultures is generally far longer in WI-38 cells than in M-HeLa cells, and for this reason it was found that the time required for a test with WI-38 cells was 1 to 2 days longer than that with M-HeLa cells.

Generally M-HeLa cells were found to be much easier to work with than WI-38 cells. It has been reported by many workers that CPE is more clearly observed in WI-38 cells since the rounded shape of an infected cell is so obviously different from the normal fibroblast shape. However, with a small amount of experience CPE was also easily observed in M-HeLa cells, although perhaps the early stages of cell rounding are not as clear as in WI-38 cells.

Certain workers have reported that tests for the inhibition of rhinovirus CPE in WI-38 cells have proved to be insensitive and variable (Haff et al., 1969). Hamparian and co-workers (1963) reported that WI-26 cells gave variable results whereas HeLa cells gave more

consistent results. It has clearly been demonstrated in this study that consistent results can be obtained with tube culture tests, although they are not as sensitive as the plaque inhibition test for the detection of antiviral activity.

4.1.5 Effect of the multiplicity of infection on the minimal inhibitory concentration as determined by tube culture tests

The present study and those of many other workers have shown that the activity of a compound when measured by the tube culture technique decreases with an increase in the size of the virus input inoculum. For example, Boyle and co-workers (1970) reported that the minimal effective dose increased with relatively small increments in viral challenge and with time of incubation. However, they gave no results to substantiate this statement. In view of this fact the relationship between the minimal inhibitory concentration (MOI) and the size of the input multiplicity of infection (MIC) was studied in detail (section 3.2.1b).

The results with Abbott 36683 and poliovirus type 1 demonstrated a linear increase in the MIC with MOI's ranging from 0.006 to 2.5 pfu per cell. With MOI's ranging from 2.5 to 50 pfu per cell the increase was again linear but more rapid than before. At MOI's of 50 pfu per cell and greater the MIC was toxic to the cells. This study therefore provided evidence for the statement that the virus inhibitory activity of a compound decreases with an increase in the size of the virus challenge.

The reason for the change in slope of the MIC versus MOI plot is not known, but it seems reasonable to assume that two different compound-sensitive processes are involved in the two portions of the graph (or that a second sensitive process is involved in the steeper portion of the graph). At the lower multiplicities of infection CPE is produced only by the normal multiplication of virus particles and the associated

inhibition of cell biosynthetic processes. However, at higher multiplicities CPE may also be the result of an additional virus-specific process or even a direct toxic effect of the high input of virus particles.

4.2 Plaque Reduction Technique

There have been no reports on the use of the plaque reduction technique, in particular the use of dose-response lines, for the quantitation of the rhinovirus inhibitory activity of compounds.

Gwaltney (1970) used the gradient plate plaque reduction technique to determine the minimal inhibitory concentration of SK&F 30097 against 11 rhinovirus serotypes. For 5 serotypes the MIC was found to be less than 42 μM and for the remaining 6 types the MIC was found to range between 21 and 32 μM. Akihama and co-workers (1968) compared the poliovirus inhibitory activity of 1,2-bis(2-benzimidazolyl)1,2-ethanediol derivatives, including Abbott 36683, using the plaque reduction method. However, their results were presented in tabular form and they showed no dose-response lines. Abbott 36683 was found to be the most active of the derivatives tested, producing 100% suppression of plaques at all the concentrations tested (10^{-4}, 10^{-5} and 10^{-6} molar).

4.2.1 Effect of various parameters on plaque reduction results

In this study tests with Abbott 36683 against poliovirus type 1 demonstrated that the incubation time of plaque cultures affects the results obtained in plaque reduction tests. Plaque count or plaque diameter dose-response lines were found to vary at 27, 43 and 49 hours, the compound appearing to be most active when cultures had been incubated for only 27 hours (Figs. 3.5 and 3.7). The concentrations of Abbott 36683 required to reduce plaque counts to 50% of the control value were found to be 0.071, 0.118 and 0.135 μM at 27, 43 and 49 hours respectively. Additionally, after 27 hours (but not 43 or 49 hours) incubation of the cultures at 0.2 μM concentration of the compound was found to be sufficient to cause complete inhibition of plaque formation. This fact demonstrates the importance of standardisation of incubation times.

These studies also demonstrated that plaque count and plaque diameter increased linearly with time between 27 and 49 hours. The distribution of plaque sizes was also shown to alter with incubation time and with increasing concentrations of Abbott 36683 in the overlay (Fig.3.9).

The size of the virus input inoculum was found to affect the measured activity of a compound as determined by the plaque reduction method, if dose-response lines were plotted as the percentage control plaque count against the log of the compound concentration. The actual reduction in plaque count produced by a certain concentration of Abbott 36683 in cultures infected with 150 pfu of virus was found to be less or the same as that in cultures infected with 250 pfu. However, when the values were expressed as a percentage of the control plaque count the reduction was found to be greater in the 150 pfu cultures than in the 250 pfu cultures (Fig.3.11). Since it is necessary to express plaque counts as percentage counts for plaque reduction dose-response lines this experiment demonstrated that the plaque count in control cultures should be the same in experiments where dose-response lines are to be compared.

The activity of Abbott 36683 against poliovirus type 1 was shown to depend on the cell line in which the compound was tested. With the plaque reduction technique Abbott 36683 was found to have greater activity against poliovirus type 1 when tested in Vero cells than when tested in HeLa OH3 cells. This was despite the fact that poliovirus plaques are much larger in Vero than in HeLa OH3 cells.

4.2.2 Quantitation of Rhinovirus Inhibitory Activity by the Plaque Reduction Test

The plaque reduction dose-response lines for Abbott 36683, SK&F 40491, SK&F 30097 and Wellcome 71-123 against rhinovirus types 1B, 2, 12, 14, 17, 19, 22, 30 and 53 are shown in Figs. 3.16 to 3.28.

One fact particularly noticeable from plaque reduction experiments was that low concentrations of SK&F 40491 and SK&F 30097 (0.25 and 6.25 μM respectively) were found to enhance plaque formation. It is in fact a well-known phenomenon that pharmacologically active substances may, at lowered concentrations, demonstrate an enhancement of the process which they normally inhibit.

Plaque reduction results were found to be very reproducible and it was found possible to determine the relative potencies of SK&F 40491, SK&F 30097 and Wellcome 71-123 compared with Abbott 36683 against each of the serotypes tested, and to determine the relative sensitivities of the serotypes compared with type 1B to each of the 4 compounds. In the former case 3 points on each of the dose-response lines for the 4 compounds against each individual serotype gave lines whose slopes did not differ significantly from one another. Hence the values of relative potency determined were fairly meaningful and gave a good quantitative assessment of the relative potency of each compound compared with Abbott 36683.

These results showed that SK&F 40491 was 4.85 to 18.5 times less active than Abbott 36683, SK&F 30097 55.5 to 200 times less active and Wellcome 71-123 91.0 to 333 times less active than Abbott 36683. The drawback in this calculation of potency therefore, is that a relative value only is obtained, and one is therefore dependent upon the use of one compound as a standard.

The determination of the relative sensitivities of the rhinovirus serotypes tested (compared with type 1B) was found to be more difficult

than the determination of the relative potencies of the compounds. This was due to the fact that the slopes of the dose-response lines of rhinovirus serotypes against one particular compound varied greatly and in most cases it was only possible to find 2 points on each of the dose-response lines which would give lines which did not differ significantly in their slopes. For this reason the points chosen were not necessarily typical of the complete dose-response lines and therefore the calculated values for relative sensitivities were often misleading. However, the use of this calculation here, although not very satisfactory, is the only way in which to obtain quantitative values of relative sensitivities.

From observations of the dose-response lines one can only place the serotypes in order of increasing or decreasing sensitivity to each of the compounds. The order determined for plaque reduction dose-response lines will be compared with those determined by other methods at a later stage in this discussion.

The fact that plaque reduction dose-response lines of one particular virus serotype to each of the compounds have more homogeneous slopes than the dose-response lines of all virus serotypes to one compound, demonstrates that the slop of a plaque reduction dose-response line is characteristic more of the virus serotype than of the compound. One particular example of this is rhinovirus type 12, which consistently gave dose-response lines steeper than those of all other serotypes tested.

4.2.3 Correlation of plaque diameter with sensitivity of virus serotype

It was most noticeable from the results obtained in plaque reduction experiments that the serotypes least sensitive to inhibition by each of the compounds were those which produced the largest plaques (that is types 14 and 17). Plaque diameter dose-response lines were constructed

for Abbott 36683 and SK&F 40491 (Figs. 3.29, 3.30 and 3.31a and b) against 5 rhinovirus serotypes. From these the serotypes were placed in order of increasing sensitivity to each of the compounds and the order compared with that determined from the plaque count dose-response lines. The order compared well provided that in the calculation of average plaque diameter in treated cultures, all those plaques which were completely suppressed by the compound (i.e. the number less than in the control culture) were considered as plaques of diameter 0.0 mm.

4.2.4 Gradient Plate Plaque Reduction

The gradient plate plaque reduction technique was compared with the normal plaque reduction technique as a method for the determination of rhinovirus inhibitory activity. This method has been reported by many workers to be a simple and quick method for the determination of the minimal inhibitory concentraion, toxic concentration and hence the therapeutic index of antiviral compounds. However, the present study did not find this to be the case. It was not easy to obtain a clear or straight cut-off line between plaque and no plaque zones and the few results obtained were not consistent and did not compare well with the results established by the normal plaque reduction method. The technique was not therefore used extensively, since the normal plaque reduction technique and the use of dose-response lines was thought to be a far superior method.

4.3 Yield Reduction Technique

4.3.1 Quantitation of rhinovirus inhibitory activity

The dose-response lines for the 4 test compounds against 7 rhinovirus serotypes and poliovirus type 1 were also constructed from the results obtained in yield reduction experiments.

The homogeneity of the slopes of these dose-response lines was found to be even poorer than that of the plaque reduction dose-response lines, and relative potency or sensitivity values could not therefore be calculated from the results.

The results were compared with those obtained by other workers. This is shown in Figs. 4.1 and 4.2 Fig.4.1 shows 3 dose-response lines for SK&F 30097 against rhinovirus type 2 obtained in 3 independent studies (Matsumoto *et al.*, 1972; Haff *et al.*, 1969 and the present study) with 3 different cell lines, WI-38, primary human embryonic kidney and M-HeLa respectively, and different multiplicities of infection, 1.0, 0.01 and 2.0 respectively. Considering these facts the dose-response lines are seen to compare fairly well.

Fig.4.2 compares the dose-response lines for SK&F 30097 against rhinovirus types 2, 14 and 17 obtained in the present study and those of Haff *et al.* (1969). There seems to be in fact no correlation between the 2 sets of lines. Those of Haff *et al.* do not show the increase in sensitivity of serotypes from type 14 to type 2 which has been fairly consistently exhibited in this study. This is possibly due in part to the different cell lines used, although the present study has also shown tube culture tests with WI-38 and M-HeLa cells to be comparable.

Haff *et al.* (1972) also tested the *in vitro* activity of SK&F 40491 using yield reduction studies. They found the ED_{50} values for rhinovirus type 2 and poliovirus type 1 to be 6.0 and 10.45 μM respectively compared with values obtained here of 8.128 and 9.55 μM.

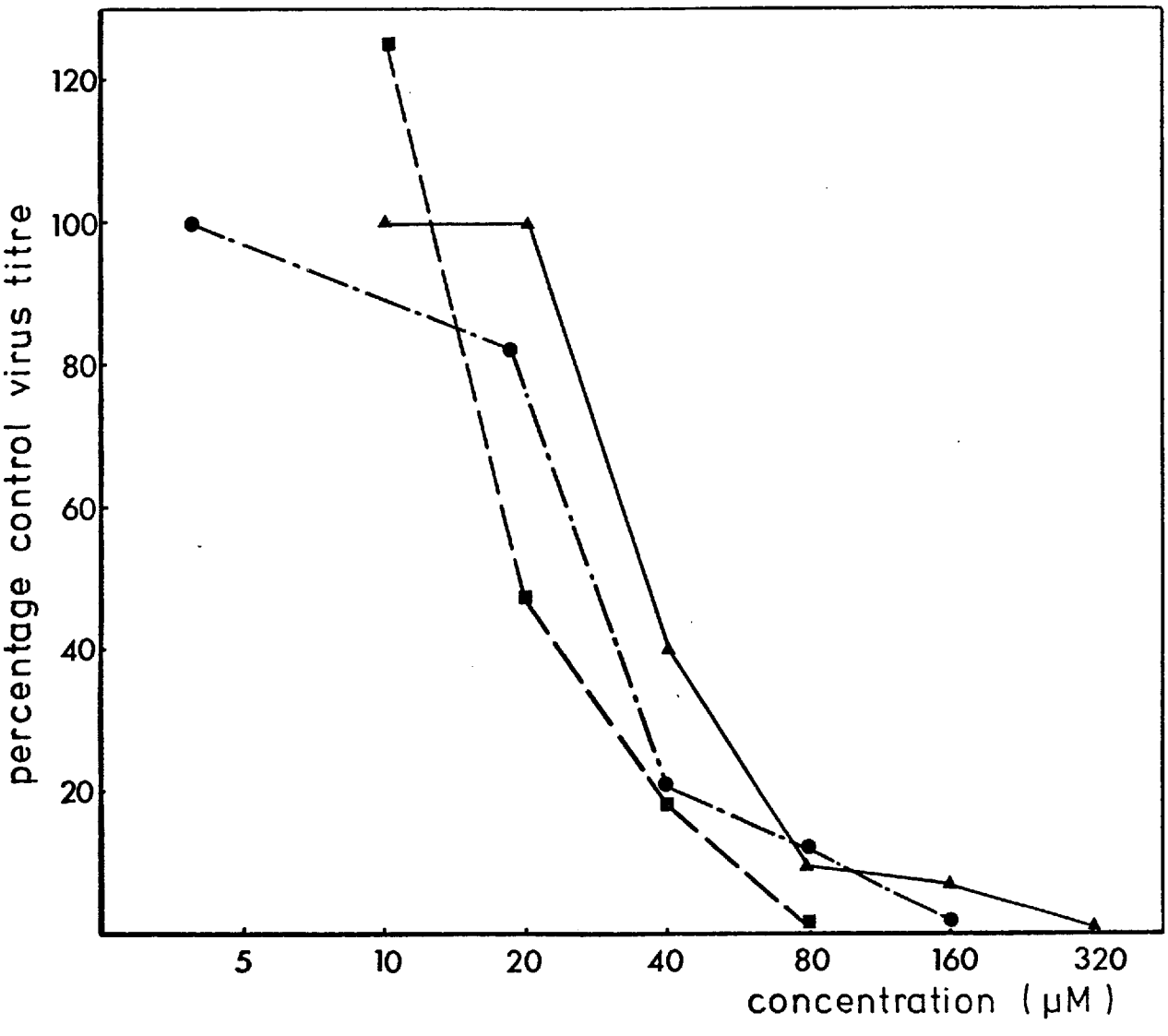


Fig. 4.1. Yield reduction dose-response lines for SK+F 30097 against rhinovirus type 2 obtained with 3 different cell lines (3 independent studies); WI-38 cells (Matsumoto et al., 1972) ▲——▲, primary human embryonic kidney cells (Haff, 1969) ●-·-● and M-HeLa cells (present study) ■——■.

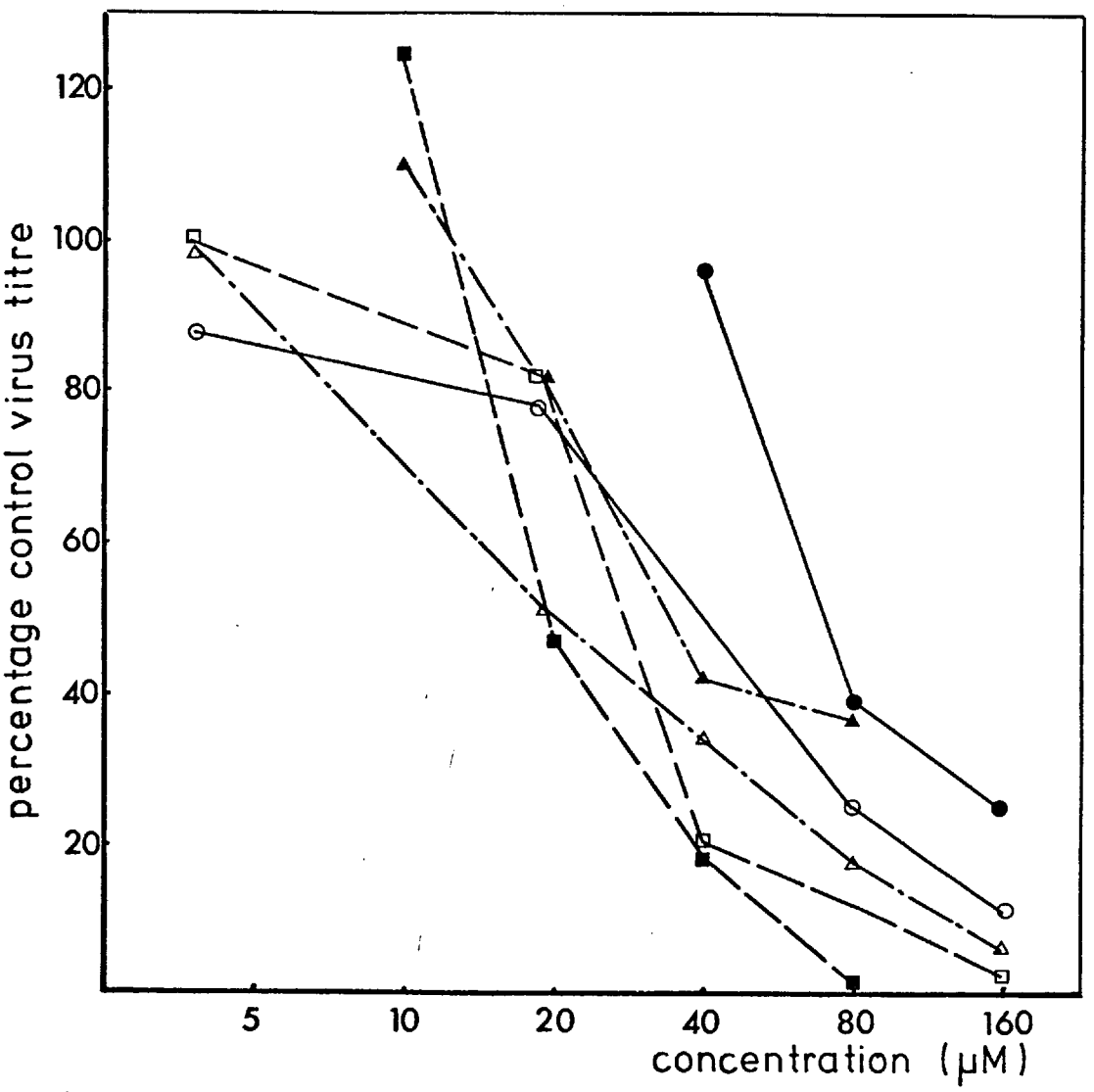


Fig. 4.2. Yield reduction dose-response lines for SK+F 30097 against rhinovirus types 2 (□——□), 14 (○——○) and 17 (△·—·△) in primary human embryonic kidney cells (Haff, 1969) open symbols or M-HeLa cells (present study) closed symbols.

4.3.2 Effect of Multiplicity of Infection on results obtained in yield reduction studies

Yield reduction experiments with Abbott 36683 against poliovirus type 1 and rhinovirus type 17 have shown that there is an increase in the ED_{50} of this compound with an increase (over a certain range) of the multiplicity of infection of virus. At MOI's between 0.1 and 10 pfu per cell the ED_{50} was found to increase but there was no difference between the ED_{50}'s at 10 and 100 pfu per cell (for poliovirus) or 10 and 30 pfu per cell (for rhinovirus). However, although the yield reduction dose-response lines were the same at these high multiplicities of infection the CPE reduction dose-response lines were not. The percentage reduction in CPE in cultures infected with poliovirus at a MOI of 100 was less than that in cultures infected with only 10 pfu per cell. Hence from observations of CPE alone the compound appears to be more active against a challenge inoculum of 10 pfu per cell.

The effect of multiplicity of infection on the activity of SK&F 30097 against rhinovirus type 17 was also studied. Here it was found that the yield reduction dose-response lines did not alter as markedly with increase in MOI as had been the case with Abbott 36683, and the conclusion from the results could only be that multiplicity of infection had no effect on the measured activity of the compound, in agreement with the report by Matsumoto et al. (1972) that there was no apparent change in the activities of the compound at multiplicities of 0.001 to 10 (measured in WI-38 cells against rhinovirus type 2). However, in tests of the inhibition of CPE in WI-38 cells Haff (1969) reported that the activity of SK&F 30097 diminished with increasing amounts of virus used for infection. The difference between the results obtained in tube tests and yield reduction tests could be accounted for in either or both of two ways. Firstly, at lower input inocula there may be a reduction in both yield and CPE whereas at higher input inocula there

may be a reduction in yield only (and the percentage reduction may be similar to that obtained at lower input inocula) as has already been demonstrated and discussed. Secondly, since tube culture tests are multicycle experiments (compared with yield reduction tests which are single cycle) it is possible that the effect of altering the multiplicity of infection on the measured activity not obviously noticeable in a single cycle experiment is magnified in successive cycles of virus replication.

Generally it was observed that the concentrations of each of the compounds required to obtain a reduction in virus yield was greater than that required to obtain a reduction in plaque count. The ratio ED_{50}(yield):ED_{50}(plaque) was found to vary for the different compounds and different serotypes (Table 3.14). However, the average for each compound individually (against all serotypes tested) was found to be 3.28, 5.87, 2.52 and 1.89 for Abbott 36683, SK&F 40491, SK&F 30097 and Wellcome 71-123 respectively and for each virus serotype individually (against all compounds tested) to be 3.55, 2.95, 3.44, 1.88, 2.10 and 5.72 for types 1B, 2, 12, 14, 17 and 22 respectively. Hence it would appear that this ratio is generally greater for the more active compounds and the more sensitive serotypes.

However, although the concentration of a compound required for a reduction in yield was greater than that required for a reduction in plaques, the compounds showed the same order of activity by the yield reduction method as by the plaque reduction, plaque inhibition and tube culture methods. That is, the compounds in order of decreasing activity are Abbott 36683 $\gg$ SK&F 40491 $\geqslant$ SK&F 30097 $>$ Wellcome 71-123 (except that against types 12 and 19 Wellcome 71-123 is slightly more active than SK&F 30097 in plaque reduction experiments). Also, Wellcome 71-123 appeared more active than SK&F 30097 against types 14 and 17 in yield reduction experiments. This was thought to be an

extremely odd result and is possibly due to the fact that Wellcome 71-123 is toxic at a concentration of 200 μM, and therefore in yield reduction experiments the compound is being used at a concentration which is not far below the toxic concentration.

4.4 Comparison of Sensitivity of Serotypes as determined by all methods used

The virus serotypes tested were placed in order of increasing sensitivity to each of the compounds and the orders determined by each of the test methods (i.e. plaque inhibition, plaque reduction, tube culture tests and yield reduction tests) were compared. This will be made clear if Tables 3.5, 3.6, 3.9 and 3.12 are summarised (Table 4.4).

Observation of Table 4.4 shows that there is a definite similarity in the order of increasing sensitivity to each of the compounds as determined by each of the 4 methods.

Further, closer similarity is found when taking each method individually and comparing the order obtained for each compound (e.g. the order for each of the compounds by the plaque inhibition method). Also, there is a closer similarity between the orders obtained in plaque inhibition and plaque reduction experiments and in those obtained in tube culture and yield reduction experiments than between the results obtained in plaque inhibition and tube culture experiment or plaque reduction and yield reduction experiments. This could be due to a number of factors. Firstly, both the plaque inhibition and plaque reduction experiments depend basically upon the suppression of plaque formation and it could be this which governs the sensitivity of the virus serotypes in these tests, as has been discussed previously. However, the tube culture and yield reduction tests are dependent upon two different criteria, that is the inhibition of virus-induced CPE and the inhibition of virus yield, which are not necessarily related directly, so this cannot account for the close similarity in the results obtained in the 2 tests.

However, another factor which might account for the similarity in the results obtained in plaque reduction and plaque inhibition or in tube culture and yield reduction experiments is the concentration of compound required in each of the tests. Generally the concentrations

of compounds required for plaque inhibition and plaque reduction experiments are far lower than those required for yield reduction and tube culture experiments.

Table 4.4 Rhinovirus serotypes placed in order of increasing sensitivity to Abbott 36683, SK&F 40491, SK&F 30097 and Wellcome 71-123 as determined from results obtained in plaque inhibition, tube culture, plaque reduction and yield reduction experiments

Abbott 36683				SK&F 40491				SK&F 30097				Wellcome 71-123			
PI	TC	PR	YR	PI	TC	PR	YR	PI	TC	PR	YR	PI	TC	PR	YR
14	14	PV1	17	14	14	14	14	14	14	14	14			14	14
17	17	14	2	17	2	PV1	PV1	2	17	17	17			17	2
12 8	2	17	22	2=12	17	17=53	17	17	1B	2	12			2	17
30	30	2	1B	30	1B	30	2	12	30	12	1B=2			PV1	22
19=22	22	30	PV1	19=22	30	2	1B=12	1B	2	PV1	22			30	
2	1B	1B	12	1B	12	1B	30	30	22	1B				1B	
1B	12	19			22	12	22	19	12	30				12	
		12				19		22		22				22	
		22				22								19	

Notes:
PI - plaque inhibition test
TC - tube culture test
PR - plaque reduction test
YR - yield reduction test

4.5 General considerations of all techniques for the quantitation of antiviral activity

The plaque inhibition test is not generally considered as a method for the quantitation of antiviral activity. It has been shown to give a rough estimation of the relative potencies of the 4 test compounds and of the relative sensitivities of the rhinovirus serotypes tested. However, the diameter of the zone of inhibition produced in a test is dependent not only upon the intrinsic activity of the compound but also upon its solubility and diffusibility. The plaque inhibition technique is a quick and easy method for the detection of antiviral activity, but it can only be considered as a very rough method for the quantitation of the virus inhibitory activity. However, it must be emphasised that this test has given very consistent results for the relative sensitivities of rhinovirus serotypes to Abbott 36683, SK&F 40491 and SK&F 30097, and the results agreed with those obtained with the plaque reduction technique.

The tube culture technique also, although generally thought to be a fairly insensitive technique for the determination of antiviral activity, has in fact proved to be remarkably sensitive and consistent. The main use of the tube culture technique in this study was to compare the sensitivities of many different rhinovirus serotypes to a small number of compounds. The results obtained compared well with those obtained by the more cumbersome yield reduction assay.

The consistency obtained in the results could be due to a number of points. Firstly, in observing tube cultures the time for the appearance of CPE, rather than the time for 50% or 75% involvement of CPE, was noted. It is thought that the first appearance of CPE can be far more accurately defined than a certain percentage of CPE as used by many other workers in tube culture tests. Secondly, a comparison of

the virus serotypes and compounds used was made by the drawing of dose-response lines (in this case the delay in CPE produced by a particular concentration of compound against the size of the challenge inoculum).

Other types of dose-response line have been used to express the results of tube tests. For example, Tamm and co-workers (1969) in structure activity studies on benzimidazole derivatives plotted the reduction in CPE (percentage of control) in treated cultures against the concentration of compound used, and from these plots the ED_{50} was determined. O'Sullivan and Wallis (1963) plotted results in a way similar to the present study, but plotted the reciprocal of the delay in the appearance of CPE (rather than the actual delay) against virus input inoculum.

However, it seems most important that in order to compare activities or sensitivities some sort of dose-response line must be used. One cannot compare the activities of compounds or the sensitivities of virus serotypes on a '+', '-' or '$\pm$' basis simply by testing the activity of one compound at one or two concentrations against one virus at one input inoculum. It is certainly not surprising that when tests are carried out in this fashion the results do in fact prove to be variable.

The tube test technique has 2 obvious advantages over both the plaque reduction and yield reduction methods for the quantitation of rhinovirus inhibitory activity. It is easily applicable to all rhinovirus serotypes, not just those which will produce plaques consistently. Secondly, it is relatively less time consuming than either of the latter two methods. The plaque reduction test depends on obtaining a countable number of plaques in control cultures and the test often fails due either to the fact that poor plaques are formed, thus making the numbers uncountable. The general inconsistency in plaque formation is probably typical of rhinoviruses, and although many authors have reported the formation of plaques with all the first 55 rhinovirus serotypes, in

practice plaque formation is consistently obtained with only a small number of serotypes. It was not found possible to standardise the size of plaques (even of the same serotype) although culture plates were carefully seeded at the same cell input and cultures were incubated for the same length of time. Variability in plaque formation generally and the great difficulty in obtaining any plaques with certain serotypes are therefore large drawbacks in the use of the plaque reduction technique.

The plaque reduction technique is in certain ways too sensitive a technique for the evaluation of antiviral activity, since plaque formation may be inhibited by compounds which are not truly antiviral. The concentrations of various ions (e.g. calcium and magnesium) in the overlay medium is critical to the production of plaques, although not to virus multiplication itself, and it is possible that certain compounds might affect the concentrations of these ions and therefore inhibit plaque formation. Hence the activity of a compound determined by plaque reduction should be confirmed by the use of some other technique which is not dependent upon the inhibition of plaque formation. However, for a true virus inhibitory compound the plaque reduction technique is an excellent tool for the quantitation of activity.

Yield reduction tests are even more time consuming than plaque reduction tests, since each sample obtained has to be titrated. Titration is most easily carried out by plaque assay (since the tube dilution technique is less accurate and again more time consuming) and this makes the yield reduction technique also dependent upon the ability to obtain plaques with the virus serotypes used.

However, having pointed out the disadvantages of the plaque reduction and yield reduction techniques it must be emphasised that these techniques give more quantitative results of activity than the tube culture or plaque inhibition tests.

Therefore, in conclusion, the quantitation of the activity of a large number of compounds or of the sensitivity of a large number of virus serotypes is best carried out with the tube culture technique. However, more accurate results for the quantitation of activity or sensitivity are obtained with the plaque reduction and yield reduction techniques. Finally, the activity of a compound exhibited by either of the plaque techniques should be confirmed by some other method.

MODE OF ACTION STUDIES

The results have shown that Abbott 36683, SK&F 30097 and Wellcome 71-123 do not act by directly inactivating virus particles nor by inhibiting or reducing the rate of virus adsorption to cell monolayers. The results with Abbott 36683 and SK&F 30097 agree with those already obtained by Schleicher and co-workers (1971), Matsumoto and co-workers (1972) and Boyle and co-workers (1970).

It has also been shown that incubation of HeLa cell monolayers with Abbott 36683 (4 μM), SK&F 30097 (80 μM) and Wellcome 71-123 (100 μM) for 2 hours before infection with poliovirus type 1 had no effect on subsequent plaque formation. This effect had not previously been studied with any of the compounds used here, although Haff and co-workers (1972) treated cell monolayers for 1 hour with different concentrations of SK&F 40491 before infection with rhinovirus type 1A. They found that in cells treated with 50 μg/ml plaque formation was reduced by 85-100% and the observed plaques were reduced in size, and in cells treated with 250 μg/ml no plaques at all were formed. Treatment of cells with concentrations of 2 and 10 μg/ml had no effect on subsequent plaque formation. Thus cells treated with SK&F 40491 before infection remained refractory to viral replication after the removal of all free compound by washing. By the use of ^{14}C-labelled compound, the effect at higher concentrations was found to be due to the binding of the compound to the cells. At concentrations of 250, 50, and 10 μg/ml the cell fractions retained 83, 20 and 2 μg/ml respectively and this can be compared with the minimum concentration required for plaque reduction or plaque formation (when the compound is contained in the overlay medium during the entire incubation period) which is 0.167 μg/ml (0.5 μM). Assuming that the same cell binding occurs with SK&F 30097 it can be seen that no effect on plaque formation could have been observed with the concentration employed in this study (80 μM) since

this is only approximately 20 times the minimum concentration required for plaque reduction, whereas the experiments with SK&F 40491 showed an effect with 300 times (50 μg/ml) the concentration required for plaque reduction (0.167 μg/ml). However, although the concentration employed was too low to demonstrate this cell binding effect the present study has shown that SK&F 30097, Abbott 36683 and Wellcome 71-123 do not act by altering the cell virus receptors in any way to prevent subsequent adsorption of the virus particles.

Studies were also carried out to determine whether the compounds acted by preventing uncoating of the virus particles after adsorption. Each compound was found to have no such effect, since the infectious virus titre in poliovirus-infected cultures was found to decrease similarly in control cultures and cultures treated with any of the compounds after infection. In every case the infectious virus titre decreased by about 50% during the first three hours after infection.

The release of poliovirus particles from infected cells was found to be unaffected by the presence of Wellcome 71-123 (100 μM). This was demonstrated by following both the cell-associated and extracellular virus production during a single growth cycle in control and treated cultures. Although the production of both cell-associated and extracellular virus is reduced in treated cultures the proportion of extracellular virus is not reduced, therefore demonstrating that Wellcome 71-123 does not affect the release of poliovirus from infected cells.

Delayed addition of the compounds Abbott 36683, SK&F 30097 and Wellcome 71-123 to poliovirus infected cultures demonstrated that each of the compounds acts at a similar stage in the growth cycle (Fig.3.61). Addition of the compounds at up to 3 hours after infection produces a

maximum inhibition of yield production in cultures. After this time, however, the inhibitory effect is reduced and compound added at 5 hours produces less than 15% of the maximum inhibition in each case. This demonstrates that the compounds act at a late stage (or stages) in the virus growth cycle since at 3 hours after infection all early events in virus replication have taken place and newly synthesised virus is beginning to appear.

Timed removal of Abbott 36683 from poliovirus-infected cultures confirmed the results from the delayed addition study in that the compound was shown to act at a late stage in viral synthesis.

This was previously demonstrated by Matsumoto and co-workers (1972). These workers also showed, by the use of radioactively labelled uridine, that poliovirus RNA synthesis in HeLa cells was inhibited by the compound SK&F 30097. They therefore postulated that this effect is responsible for the antiviral action of the compound. This is a fair conclusion to make since they had proved that the event sensitive to inhibition was in fact a late stage in viral synthesis.

The results in this study have demonstrated no additional points about the mechanism of action of SK&F 30097 to those previously reported by Matsumoto and co-workers (1972), except that the compound was found not to inhibit the uncoating of virus particles. This had been indirectly shown by Matsumoto, however, in showing that the compound acted at a late stage in viral synthesis. However, the present study has furthered the understanding of the mode of action of the compounds Abbott 36683 and Wellcome 71-123.

The time of addition studies carried out with all 3 compounds demonstrate that each acts at about the same stage in the virus replicative cycle. It would be of interest to determine whether Abbott 36683 and Wellcome 71-123 inhibit virus RNA synthesis, since this has not been shown here. However, it is quite possible that these compounds do act by inhibiting the same process. If this were found to be the

case the reason for the varying sensitivity amongst the rhinovirus serotypes could be rethought. Certainly, if all three compounds were found to inhibit the same process, i.e. RNA synthesis, this would add weight to the argument that the variation in sensitivity between rhinovirus serotypes is due to the fact that this compound-sensitive process does itself vary in sensitivity to inhibition by the compounds.

The present study has therefore demonstrated a narrow but fairly consistent variation in the sensitivity of rhinovirus serotypes to inhibitory compounds. This situation is very different from that demonstrated with herpes viruses, where the two serotypes fall mainly into distinct groups of high and low sensitivity to inhibitory compounds (Fiala, 1970; Person et al. 1972). This differential susceptibility is of importance in the treatment of patients with herpes infections. However, it seems that since no such marked difference in sensitivity to compounds is shown by the rhinovirus serotypes which were tested, this factor should not constitute a problem in the possible future specific treatment of rhinovirus infections.

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5.0 REFERENCES

Abo-Ahmed, H.S., Behbehani, A.M. and Wenner, H.A. (1967). Plaques of rhinoviruses in human diploid cells. Proc. Soc. exp. Biol. Med. 126, 463-464.

Ackerman, W.W. and Maassab, H.F. (1954). Growth characteristics of influenza virus: influences of a sulfonic acid. J. exp. Med. 99, 105-117.

Acornley, J.E., Chapple, P.J., Stott, E.J. and Tyrrell, D.A.J. (1968). Selection of variants of rhinoviruses. Arch. ges. Virusforsch. 23, 284-287.

Akihama, S., Okude, M., Sato, K. and Iwabuchi, S. (1968). Inhibitory effect of 1,2-bis(2-benzimidazolyl)-1,2-ethanediol derivatives on poliovirus. Nature, Lond. 217, 562-563.

Almeida, J.D. and Tyrrell, D.A.J. (1967). The morphology of three previously uncharacterized human respiratory viruses that grow in organ culture. J. gen. Virol. 1, 175-178.

Anderson, T.W., Reid, D.B.W. and Beaton, G.H. (1972). Vitamin C and the common cold: a double-blind trial. Can. med. Ass. J. 107, 503-508.

Andrewes, C.H., Chaproniere, D.M., Gompels, A.E.M., Pereira, H.G. and Roden, A.T. (1953). Propagation of the common cold virus in tissue cultures. Lancet ii, 546-547.

Appleyard, G., Hume, V.B.M. and Westwood, J.C.N. (1965). The effect of thiosemicarbazones on the growth of rabbitpox virus in tissue culture. Ann. N.Y. Acad. Sci. 130, 92-104.

Armstrong, C. (1939). The experimental transmission of poliomyelitis to the eastern cotton rat, Sigmodon hispidus hispidus. Publ. Hlth. Rep. 54, 1719-1721.

Bablanian, R., Eggers, H.J. and Tamm, I. (1965). Studies on the mechanism of poliovirus-induced cell damage. II. The relation between poliovirus growth and virus-induced morphological changes in cells. Virology 26, 114-121.

Bablanian, R., Eggers, H.J. and Tamm, I. (1966). Inhibition of enterovirus cytopathic effects by 2-(α-hydroxybenzyl)benzimidazole. J. Bact. 91, 1289-1294.

Bachrach, H.L. and Schwerdt, C.E. (1952). Purification studies on Lansing poliomyelitis virus: pH stability, CNS extraction and butanol purification experiments. J. Immun. 69, 551-561.

Baltimore, D. (1969). The replication of picornaviruses, pp. 101-176. In: The Biochemistry of viruses. New York and London, Academic Press (Ed.) Hilton Levy.

Baltimore, D., Eggers, H.J., Franklin, R.H. and Tamm, I. (1963). Poliovirus induced RNA polymerase and the effects of virus-specific inhibitors on its production. Proc. natl. Acad. Sci. U.S.A. 49, 843-849.

Bauer, D.J. (1972). Introduction to antiviral chemotherapy. In: Chemotherapy of Virus Diseases, vol. 1, Pergamon Press.

Bauer, D.J. (1973). Antiviral chemotherapy: the first decade. Br. med. J. ii, 275-279.

Bauer, D.J. and Apostolov, K. (1966). Adenovirus multiplication: inhibition by methisazone. Science, N.Y. 154, 796-797.

Bauer, D.J., Dumbell, K.R., Fox-Hulme, P. and Sadler, P.W. (1962). The chemotherapy of variola major infection. Bull. Wld. Hlth. Org. 26, 727-732.

Bauer, D.J. and Sadler, P.W. (1960). The structure-activity relationships of the antiviral chemotherapeutic activity of isatin β-thiosemicarbazone. Brit. J. Pharmac. Chemother. 15, 101-110.

Bauer, D.J., St. Vincent, L., Kempe, C.H. and Downie, A.W. (1963). Prophylactic treatment of smallpox contacts with N-methylisatin β-thiosemicarbazone (compound 33T57, Marboran). Lancet ii, 494-496.

Beare, A.S., Bynoe, M.L. and Tyrrell, D.A.J. (1968). Prophylaxis of influenza with a synthetic isoquinoline. Lancet i, 843-845.

Behbehani, A.M., Melnick, J.L. and DeBakey, M.E. (1965). Continuous cell strains derived from human atheromatous lesions and their viral susceptibility. Proc. Soc. exp. Biol. Med. 118, 759-763.

Bondi, A. Jr., Spaulding, E.H., Smith, D.E. and Dietz, C.C. (1947). Routine method for rapid determination of susceptibility to penicillin and other antibiotics. Am. J. med. Sci. 213, 221-225.

Boyle, J.J., Raupp, W.G., Stanfield, F.J., Haff, R.F., Dick, E.C., D'Alessio, D. and Dick, C.R. (1970). Progress in rhinovirus chemotherapy. Ann. N.Y. Acad. Sci. 173, 477-491.

Brammer, K.W., McDonald, C.R. and Tute, M.S. (1968). Antiviral properties of 1-phenoxymethyl-3,4-dihydro and 1,2,3,4-tetrahydroisoquinolines. Nature, Lond. 219, 515-517.

Brown, E.E. (1945). Common cold not caused by a virus. NW.Med. 44, 39-42.

Brown, P.K., Tyrrell, D.A.J. and Jacobs, J.P. (1963). Proc. Symposium on characterization and uses of human diploid cell strains. Opatija, 251-254.

Bucknall, R.A., Swallow, D.L., Moores, H. and Harrad, J. (1973). A novel substituted guanidine with high activity in vitro against rhinoviruses. Nature, Lond. 246, 144-145.

Bürki, F. (1965). Picornaviruses of cats. Arch. ges. Virusforsch. 15, 690-696.

Burnham, W.S., Sidwell, R.W., Tolman, R.L. and Stout, M.G. (1972). Synthesis and antiviral activity of 4-hydroxy-5,6,7,8-tetramethoxy-flavone. J. med. Chem. 15, 1075.

Buscho, R.F., Perkins, J.C., Knopf, H.L.S., Kapikian, A.Z. and Chanock, R.M. (1972). Further characterization of the local respiratory tract antibody response induced by intranasal instillation of inactivated rhinovirus type 1B vaccine. J. Immun. 108, 169-177.

Bynoe, M.L., Hobson, D., Horner, J., Kipps, A., Schild, G.C. and Tyrrell, D.A.J. (1961). Inoculation of human volunteers with a strain of virus isolated from a common cold. Lancet i, 1194-1196.

Caliguiri, L.A. and Tamm, I. (1968a). Action of guanidine on the replication of poliovirus RNA. Virology 35, 408-417.

Caliguiri, L.A. and Tamm, I. (1968b). Distribution and translation of poliovirus RNA in guanidine treated cells. Virology 36, 223-231.

Cate, T.R., Couch, R.B. and Johnson, K.M. (1964). Studies with rhinovirus in volunteers: production of illness, effect of naturally acquired antibody, and demonstration of a protective effect not associated with serum antibody. J. clin. Invest. 43, 56-67.

Chang, T. and Weinstein, L. (1973). Antiviral activity of isoprinosine in vitro and in vivo. Am. J. med. Sci. 265, 143-146.

Chapple, P.J., Head, B. and Tyrrell, D.A.J. (1967). A complement fixing antigen from an M rhinovirus. Arch. ges. Virusforsch. 21, 123-126.

Charleston, S.S. and Clegg, K.M. (1972). Ascorbic acid and the common cold. Lancet i, 1401-1402.

Committee on Enteroviruses. (1962). Classification of human enteroviruses. Virology 16, 501-504.

Conant, R.M. and Hamparian, V.V. (1968). Rhinoviruses: basis for a numbering system. I. HeLa cells for propagation and serologic procedures. J. Immun. 100, 107-113.

Conant, R.M., Hamparian, V.V., Stott, E.J. and Tyrrell, D.A.J. (1968a). Identification of rhinovirus strain D.C. Nature, Lond. 217, 1264-1265.

Conant, R.M., Somerson, N.L. and Hamparian, V.V. (1968b). Plaque formation by rhinoviruses. Proc. Soc. exp. Biol. Med. 128, 51-56.

Connelly, A.P.Jr.,and Hamre, D. (1964). Virologic studies on acute respiratory disease in young adults. II. Characteristics and serologic studies of three new rhinoviruses. J. Lab. clin. Med. 63, 30-43.

Coombes, J.D., Brammer, K.W., Herbst-Laier, R.H., Larin, N.M., McDonald, C.R., Tute, M.S., Wickham, E.A. and Williamson, G.M. (1970). Laboratory studies on two dihydroisoquinolines (Pfizer UK 2054 and 2371). Ann. N.Y. Acad. Sci. 173, 462-476.

Cooney, M.K. and Kenny. (1970). Reciprocal neutralizing cross-reaction between rhinovirus types 9 and 32. J. Immun. 105, 531-533.

Cowan, D.W., Diehl, H.S. and Baker, A.B. (1942). Vitamins for the prevention of colds. J. Am. med. Ass. 120, 1268-1271.

Darrell, R.W. and Howe, C. (1964). The neuraminidase of parainfluenza virus (type 2). Proc. Soc. exp. Biol. Med. 116, 1091-1094.

Davies, W.L., Grunert, R.R., Haff, R.F., McGahen, J.W., Neumayer, E.M., Paulshock, M., Watts, J.C., Wood, T.R., Herrmann, E.C. and Hoffmann, C.E. (1964). Antiviral activity of 1-adamantanamine (amantadine). Science, N.Y. 144, 862-863.

Dick, E.C. (1968). Experimental infections of chimpanzees with human rhinovirus types 14 and 43. Proc. Soc. exp. Biol. Med. 127, 1079-1081.

Dick, E.C. and Dick, C.R. (1968). A subclinical outbreak of human rhinovirus type 31 infection in chimpanzees. Am. J. Epidemiol. 88, 267-272.

Dimmock, N.J. and Tyrrell, D.A.J. (1962). Physicochemical properties of some viruses isolated from common colds (rhinoviruses). Lancet ii, 536-537.

Dimmock, N.J. and Tyrrell, D.A.J. (1964). Some physicochemical properties of rhinoviruses. Brit. J. exp. Path. 45, 271-280.

Dochez, A.R., Mills, K.C. and Kneeland, Y.Jr. (1931). Study of the virus of the common cold and its cultivation in tissue medium. Proc. Soc. exp. Biol. Med. 28, 513-516.

Dochez, A.R., Mills, K.C. and Kneeland, Y.Jr. (1936). Studies on the common cold; VI. Cultivation of the virus in tissue medium. J. exp. Med. 63, 559-579.

Dochez, A.R., Shibley, G.S. and Mills, K.C. (1929). A study of acute infection of the respiratory tract in the ape. Proc. Soc. exp. Biol. Med. 26, 562-565.

Dochez, A.R., Shibley, G.S. and Mills, K.C. (1930). Studies in the common cold; IV. Experimental transmission of the common cold to anthropoid apes and human beings by means of a filterable agent. J. exp. Med. 52, 701-716.

Dolan, T.M., Fenters, J.D., Fordyce, P.A. and Holper, J.C. (1968). Rhinovirus plaque formation in WI-38 cells with methylcellulose overlay. Appl. Microbiol. 16, 1331-1336.

Douglas, R.G. Jr., Cate, T.R. and Couch, R.B. (1966a). Growth and cytopathic effect of H type rhinoviruses in monkey kidney tissue culture. Proc. Soc. exp. Biol. Med. 123, 238-241.

Douglas, R.G. Jr., Cate, T.R., Gerone, P.J. and Couch, R.B. (1966b). Quantitative rhinovirus shedding patterns in volunteers. Am. Rev. resp. Dis. 94, 159-167.

Douglas, R.G. and Couch, R.B. (1972). Parenteral inactivated rhinovirus vaccine; minimal protective effect. Proc. Soc. exp. Biol. Med. 139, 899-902.

Dulbecco, R. (1952). Production of plaques in monolayer tissue cultures by single particles of an animal virus. Proc. natl. Acad. Sci. U.S.A. 38, 747-752.

Easterbrook, K.B. (1963). Conservation of vaccinial DNA during an abortive cycle of multiplication. Virology 21, 508-510.

Edmond, J.D., Johnston, R.G., Kidd, D., Rylance, H.J. and Somerville, R.G. (1966). The inhibition of neuraminidase and antiviral action. Brit. J. Pharmac. Chemother. 27, 415-426.

Eggers, H.J., Ikegami, N. and Tamm, I. (1965). Comparative studies with selective inhibitors of picornavirus reproduction. Ann. N.Y. Acad. Sci. 130, 267-281.

Eggers, H.J., Reich, E. and Tamm, I. (1963). The drug-requiring phase in the growth of drug-dependent enteroviruses. Proc. natl. Acad. Sci. U.S.A. 50, 183-190.

Eggers, H.J. and Tamm, I. (1961a). Spectrum and characteristics of the virus inhibitory action of 2-(α-hydroxybenzyl)benzimidazole. J. exp. Med. 113, 657-682.

Eggers, H.J. and Tamm, I. (1961b). 2-(α-Hydroxybenzyl)benzimidazole (HBB) as an aid in virus classification. Virology 13, 545-546.

Eggers, H.J. and Tamm, I. (1962). On the mechanism of selective inhibition of enterovirus multiplication by 2-(α-hydroxybenzyl)-benzimidazole. Virology 18, 426-438.

Eggers, H.J. and Tamm, I. (1963). Inhibition of enterovirus ribonucleic acid synthesis by 2-(α-hydroxybenzyl)benzimidazole. Nature, Lond. 197, 1327-1328.

Ehrlich, J., Sloan, B.J., Miller, F.A. and Machamer, H.E. (1965). Searching for antiviral materials from microbial fermentations. Ann. N.Y. Acad. Sci. 130, 5-16.

Enders, J.F., Weller, T.H. and Robbins, F.C. (1949). Cultivation of the Lansing strain of poliomyelitis virus in cultures of various human embryonic tissues. Science, N.Y. 109, 85-87.

Fenters, J.D., Fordyce, P.A., Gerin, J.L. and Holper, J.C. (1967). Propagation of rhinovirus on WI-38 cell monolayers in rolling bottles. Appl. Microbiol. 15, 1460-1464.

Fiala, M. (1968). Plaque formation by 55 rhinovirus serotypes. Appl. Microbiol. 16, 1445-1450.

Fiala, M. (1969). A study of the neutralisation of rhinoviruses by means of a plaque reduction test. J. Immun. 103, 107-113.

Fiala, M., Chow, A. and Guze, L. (1972). Susceptibility of herpes viruses to cytosine arabinoside: standardization of susceptibility test procedure and relative resistance of herpes simplex type 2 strains. Antimicrob. Ag. Chemother. 1, 354-356.

Fiala, M. and Kenny, G.E. (1966). Enhancement of rhinovirus plaque formation in human heteroploid cell cultures by magnesium and calcium. J. Bact. 92, 1710-1715.

Fiala, M. and Kenny, G.E. (1967). Effect of magnesium on replication of rhinovirus HGP. J. Virol. 1, 489-493.

Fletcher, R.D. and Platt, D. (1968). Growth characteristics of rhinoviruses in human gingival cells. Arch. ges. Virusforsch 25, 1-7.

Galabov, A., Shindarov, L., Vassilev, G. and Vassileva, R. (1972a). Inhibitory effect of N-phenyl-N'-aryl or alkylthiourea derivatives on poliovirus multiplication in cell cultures. Chemotherapy 17, 161-174.

Galabov, A., Shindarov, L., Vassilev, G. and Vassileva, R. (1972b). Inhibitory effect of N-phenyl-N'-aryl or alkylthiourea derivatives on the multiplication of some picornaviruses (Coxsackie B1, ECHO 19 and Foot-and-Mouth disease virus) in cell cultures. Arch. ges. Virusforsch. 38, 159-166.

General practitioner trial (1968). Ineffectiveness of vitamin C in treating coryza. Practitioner 200, 442-445.

Gey, G.O., Coffman, W.D. and Kubicek, M.T. (1952). Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. Cancer Res. 12, 264.

Gladych, J.M.Z., Hornby, R., Hunt, J.H., Jack, D., Boyle, J.J., Ferlauto, R.J., Haff, R.F., Kormendy, C.G., Stanfield, F.J. and Stewart, R.C. (1972). Antiviral agents. 5H-as-Triazino[5-6,b] indoles. J. med. Chem. 15, 277-281.

Gladych, J.M.Z., Hunt, J.H., Jack, D., Haff, R.F., Boyle, J.J., Stewart, R.C. and Ferlauto, R.J. (1969). Inhibition of rhinovirus by isatin thiosemicarbazone analogues. Nature, Lond. 221, 286-287.

Glazebrook, A.J. and Thomson, S. (1942). Administration of vitamin C in a large institution and its effect on general health and resistance to infection. J. Hyg. 42, 1-19.

Gwaltney, J.M. (1966). Micro-neutralization test for identification of rhinovirus serotypes. Proc. Soc. exp. Biol. Med. 122, 1137-1141.

Gwaltney, J.M. (1968). The spectrum of rhinovirus inhibition by 2-(α-hydroxybenzyl)benzimidazole and D-(-)-2-(α-hydroxybenzyl) benzimidazole HCl. Proc. Soc. exp. Biol. Med. 129, 665-673.

Gwaltney, J.M. (1970). Rhinovirus inhibition by 3-substituted triazinoindoles. Proc. Soc. exp. Biol. Med. 133, 1148-1154.

Haff, R.F. (1969). Rhinovirus - main target for antiviral screening. Progress in antimicrobial and anticancer chemotherapy. Proc. 6th Int. Congress of Chemotherapy 2, 818.

Haff, R.F., Flagg, W.B., Gallo, J.J., Hoover, J.R.E., Miller, J.A., Pinto, C.A. and Pagano, J.F. (1972). The in vitro antiviral activity of a triazinoindole (SK+F 40491). Proc. Soc. exp. Biol. Med. 141, 475-478.

Halperen, S., Eggers, H.J. and Tamm, I. (1964a). Complete and coreless haemagglutinating particles produced in ECHO 12 virus infected cells. Virology 23, 81-89.

Halperen, S., Eggers, H.J. and Tamm, I. (1964b). Evidence for uncoupled synthesis of viral RNA and viral capsids. Virology 24, 36-46.

Hamparian, V.V., Hilleman, M.R. and Ketler, A. (1963). Contributions to characterization and classification of animal viruses. Proc. Soc. exp. Biol. Med. 112, 1040-1050.

Hamparian, V.V., Ketler, A. and Hilleman, M.R. (1961). Recovery of new viruses (coryzavirus) from cases of common cold in human adults. Proc. Soc. exp. Biol. Med. 108, 444-453.

Hamre, D. (1968). Rhinoviruses. In: Monographs in Virology (Ed.) Melnick, J.L., vol. 1, Basel, S Karger AG.

Hamre, D., Bernstein, J. and Donovick, R. (1950). Activity of p-aminobenzaldehyde, 3-thiosemicarbazone on vaccinia virus in the chick embryo and in the mouse. Proc. Soc. exp. Biol. Med 73, 275-278.

Hamre, D., Connelly, A.P. Jr., and Procknow, J.J. (1964). Virologic studies of acute respiratory disease in young adults. III. Some biologic and serologic characteristics of seventeen rhinovirus serotypes isolated Oct. 1960 to June 1961. J. Lab. Clin. Med. 64, 450-460.

Hamre, D. and Procknow, J.J. (1961). Virological studies on acute respiratory disease in young adults. I. Isolation of ECHO 28. Proc. Soc. exp. Biol. Med. 107, 770-773.

Haskell, T.H., Peterson, F.E., Watson, D., Plessas, N.R. and Culbertson, T. (1970). Neuraminidase inhibition and viral chemotherapy. J. med. Chem. 13, 697-704.

Hayflick, L. (1961). The establishment of a line (WISH) of human amnion cells in continuous cultivation. Expl. Cell. Res. 23, 14-20.

Hayflick, L. (1965). The limited in vitro lifetime of human diploid cell strains. Expl. Cell Res. 37, 614-636.

Hayflick, L. and Moorhead, P.S. (1961). The serial cultivation of human diploid cell strains. Expl. Cell. Res. 25, 585-621.

Herbst-Laier, R. (1970a). A novel technique for in situ demonstration of cell viability and cell injury on the respiratory surface of trachea explants. Arch. ges. Virusforsch. 30, 248-251.

Herbst-Laier, R. (1970b). Organ cultures in evaluation of antiviral drugs. I. Virus infection in trachea explants as potential model systems for evaluation of antiviral drugs against respiratory viruses. Arch. ges. Virusforsch. 30, 379-396.

Herrmann, E.C. Jr. (1961). Plaque inhibition test for detection of specific inhibitors of DNA containing viruses. Proc. Soc. exp. Biol. Med. 107, 142-145.

Herrmann, E.C. Jr., Gabliks, J., Engle, C. and Perlman, P.L. (1960). Agar diffusion method for detection and bioassay of antiviral antibiotics. Proc. Soc. exp. Biol. Med. 103, 625-628.

Higgins, P.G. (1966). The isolation of viruses from acute respiratory infections. Part V. The use of organ cultures of human embryonic nasal and tracheal ciliated epithelium. Mon. Bull. Minist. Hlth. 25, 282-288.

Hobson, D., Flockton, H.I. and Gregory, M.G. (1969). The inhibitory activity of an isoquinoline derivative on the growth of an influenza A virus in tissue culture. Brit. J. exp. Path. 50, 494-501.

Holland, J.J., McLaren, L.C. and Syverton, J.T. (1959). Mammalian cell-virus relationship. III. Poliovirus production by non-primate cells exposed to poliovirus ribonucleic acid. Proc. Soc. exp. Biol. Med. 100, 843-845.

Hollinshead, A.C. and Smith, P.K. (1958). Effects of certain purines and related compounds on virus propagation. J. Pharm. exp. Ther. 123, 54-62.

Holper, J.C., Fenters, J.D. and Marquis, G.S. Jr. (1963). Studies to develop rhinovirus vaccines in human diploid cells. Symposium on the characterization and use of human diploid cell strains. Proc. int. Ass. microbiol. Soc. 291-301.

Hoorn, B. and Tyrrell, D.A.J. (1966). A new virus cultivated only in organ cultures of human ciliated epithelium. Arch. ges. Virusforsch. 18, 210-225.

Howe, C., Lee, C.T. and Rose, H.M. (1960). Influenza virus sialidase. Nature, Lond. 188, 251-252.

Huffman, J.H., Sidwell, R.W., Khare, G.P., Witkowski, J.T., Allen, L.B. and Robins, R.K. (1973). In vitro effect of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole, ICN 1229) on deoxyribonucleic acid viruses. Antimicrob. Ag. Chemother. 3, 235-241.

Hume, R. and Weyers, E. (1973). Changes in leucocyte ascorbic acid during the common cold. Scott. med. J. 18, 3-7.

International Enterovirus Study Group (Melnick, J.L. et al.)(1963). Picornavirus group. Virology 19, 114-116.

Isaacs, A. and Lindenmann, J. (1957). Virus interference. I. The interferon. Proc. R. Soc. (Biol.) 147, 258-267.

Jackson, G.G. and Dowling, H.F. (1959). Transmission of the common cold to volunteers under controlled conditions. IV. Specific immunity to the common cold. J. clin. Invest. 38, 762-769.

Jackson, G.G., Dowling, H.F. and Anderson, T.O. (1958). Neutralization of common cold agents in volunteers by pooled human globulins. Science, N.Y. 128, 27-28.

Jackson, G.G. and Muldoon, R.L. (1973). Viruses causing common respiratory infections in man. J. infect. Dis. 127, 328-355.

Johnson, K.M., Bloom, H.H., Mufson, M.A. and Chanock, R.M. (1962). Acute respiratory disease associated with Coxsackie A21 virus infection. I. Incidence in military personnel: observations in a recruit population. J. Am. med. Ass. 179, 112-119.

Johnson, K.M. and Rosen, L. (1963). Characteristics of five newly recognised enteroviruses recovered from the human oropharynx. Am. J. Hyg. 77, 15-25.

Kadin, S.B., Eggers, H.J. and Tamm, I. (1964). Synthesis and virus inhibitory activity of D- and L- isomers of 2-(α-hydroxybenzyl) benzimidazole. Nature, Lond. 201, 639-640.

Kapikian, A.Z., Almeida, J.D. and Stott, E.J. (1972). Immune electron microscopy of rhinoviruses. J. Virol. 10, 142-146.

Kapikian, A.Z., Conant, R.M., Hamparian, V.V. and 15 others (1967). Rhinoviruses: a numbering system. Nature, Lond. 213, 761-763.

Kapikian, A.Z., Conant, R.M., Hamparian, V.V. and 13 others (1971). A collaborative report: Rhinoviruses - extension of the numbering system. Virology 43, 524-526.

Kapikian, A.Z., Mufson, M.A., James, H.D. Jr., Kalica, A.R., Bloom, H.H. and Chanock, R.M. (1966). Characterization of two newly recognized rhinovirus serotypes of human origin. Proc. Soc. exp. Biol. Med. 122, 1155-1162.

Kärber, G. (1931). L'interprétation collective des expériences pharmacologiques faites en série. Arch. exp. Path. Pharmak. 162, 480-485.

Kaufman, H.E. (1962). Clinical cure of herpes simplex keratitis by 5-iodo-2'-deoxyuridine. Proc. Soc. exp. Biol. Med. 109, 251-252.

Kaufman, H.E., Martola, E.L. and Dohlman, C. (1962). The use of 5-iodo-2'-deoxyuridine (IDU) in the treatment of herpes simplex keratitis. Archs. Ophthal. N.Y. 68, 235-239.

Kenny, G.E., Cooney, M.K. and Thompson, D.J. (1970). Analysis of serum pooling schemes for identification of large numbers of viruses. Am. J. Epidemiol. 91, 439-445.

Ketler, A., Hamparian, V.V. and Hilleman, M.R. (1962). Characterization and classification of ECHO 28 - rhinovirus - coryzavirus agents. Proc. Soc. exp. Biol. Med. 110, 821-831.

Khare, G.P., Sidwell, R.W., Witkowski, J.T. Simon, L.N. and Robins, R.K. (1973). Suppression by 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole, ICN 1229) of influenza virus-induced infections in mice. Antimicrob. Ag. Chemother. 3, 517-522.

Kilbourne, E.D., Laver, W.G., Schulman, J.L. and Webster, R.G. (1968). Antiviral activity of antiserum for an influenza virus neuraminidase. J. Virol. 2, 281.

Kinlen, L. and Peto, R. (1973). Vitamin C and colds. Lancet i, 944.

Kisch, A.L., Webb, P.A. and Johnson, K.M. (1964). Further properties of five newly recognised picornaviruses (rhinoviruses). Am. J. Hyg. 79, 125-133.

Kneeland, Y. Jr., Mills, K.C. and Dochez, A.R. (1936). Cultivation of the virus of the common cold in the chorio-allantoic membrane of the chick embryo. Proc. Soc. exp. Biol. Med. 35, 213-215.

Kriel, R.L., Wulff, H. and Chin, T.D.Y. (1969). Microneutralization test for determination of rhinovirus and coxsackievirus A antibody in human diploid cells. Appl. Microbiol. 17, 611-613.

Kruse, W. (1914). Die Erreger von Husten und Schnupfen. Munch. med. Wschr. 61, 1547.

Kucera, L.S. and Herrmann, E.C. Jr. (1966). Gradient plate technique applied to the study of antiviral substances. Proc. Soc. exp. Biol. Med. 122, 258-262.

Lamden, M.P. and Chrystowski, G.A. (1954). Urinary oxalate excretion by man following ascorbic acid ingestion. Proc. Soc. exp. Biol. Med. 85, 190-192.

Landsteiner, K. and Popper, E. (1909). Übertragung der Poliomyelitis acuta auf Affen. Z. Immunitatforsch., Orig. 2, 377-390.

Larin, N.M., Beare, A.S., Copping, M.P., McDonald, C.R., McDougall, J.K., Roberts, B. and Smith, J.B. (1967). Spectrum and characteristics of the virus inhibitory action of two isoquinoline derivatives. Antimicrob. Ag. Chemother. 7, 646-653.

Lim, K.A. and Beynesh-Melnick, M. (1960). Typing of viruses by combinations of antiserum pools. Application of typing to enteroviruses (Coxsackie and ECHO). J. Immun. 84, 309-317.

Long, P.H., Doull, J.A., Bourn, J.M. and McComb, E. (1931). The etiology of acute upper respiratory infection (common cold). J. exp. Med. 53, 447-470.

Longley, S.R., Dunning, L. and Waldman, R.H. (1973). Effect of isoprinosine against challenge with A(H_3N_2)/Hong Kong influenza virus in volunteers. Antimicrob. Ag. Chemother. 3, 506-509.

Loring, H.S. and Schwerdt, C.E. (1944). Studies on purification of poliomyelitis virus; pH stability range of MVA strain. Proc. Soc. exp. Biol. Med. 57, 173-175.

Lowry, O.H., Bessey, O.A. and Burch, H.B. (1952). Effects of prolonged high dosage with ascorbic acid. Proc. Soc. exp. Biol. Med. 80, 361-362.

Lwoff, A. and Lwoff, M. (1964). Remarques sur l'isatin β-thiosemicarbazone et sur quelques inhibiteurs du developpement viral. CR Acad. Sci. (Paris) 258, 1924-1927.

Maassab, J.F. and Cochran, K.W. (1964). Rubella virus; inhibition in vitro by amantadine hydrochloride. Science. N.Y. 145, 1443-1444.

Mascoli, C.C., Leagus, M.B., Weigel, R.E., Stokes, J. Jr., Reinhart, H. and Hilleman, M.R. (1966). Attempt at immunization by oral feeding of live rhinovirus in enteric coated capsules. Proc. Soc. exp. Biol. Med. 121, 1264-1268.

Matsumoto, M. (1963). Newer respiratory disease viruses in Japan and some Far Eastern countries. Am. Rev. resp. Dis. 88, (Suppl) 46-55.

Matsumoto, S., Stanfield, F.J., Goore, M.Y. and Haff, R.F. (1972). The antiviral activity of a triazinoindole. Proc. Soc. exp. Biol. Med. 139, 455-460.

Meenan, P.N. and Hillary, I.B. (1969a). Prophylaxis of influenza B with UK 2054. Lancet ii, 614-615.

Meenan, P.N. and Hillary, I.B. (1969b). Field study of UK 2054 against natural infection with A_2-Hong Kong-68 influenza virus. Lancet ii, 641-642.

Mental, R. and Schmidt, J. (1973). Investigations on rhinovirus inactivation by hydrogen peroxide. Acta. Virol. 17, 351-154.

Mogabgab, W.J. (1962). 2060 virus (ECHO 28) in KB cell cultures. Characteristics, complement fixation and antigenic relationship to some other respiroviruses. Am. J. Hyg. 76, 15-26.

Mufson, M.A., Kawana, R., James, H.D., Gould, L.W., Bloom, H.H. and Chanock, R.M. (1965). A description of six new rhinoviruses of human origin. Am. J. Epidemiol. 81, 32-43.

Mufson, M.A., Ludwig, W.M., James, H.D., Gould, L.W., Rourke, J.A., Holper, J.C. and Chanock, R.M. (1963). Effect of neutralizing antibody on experimental rhinovirus infection. J. Am. med. Ass. 186, 578-584.

Mufson, M.A., Webb, P.A., Kennedy, H., Gill, V. and Chanock, R.M. (1966). Etiology of upper-respiratory-tract illnesses among civilian adults. J. Am. med. Ass. 195, 1-7.

Muldoon, R.L., Mezny, L. and Jackson, G.G. (1972). Effect of isoprinosine against influenza and some other viruses causing respiratory diseases. Antimicrob. Ag. Chemother. 2, 224-228.

Newman, J.F.E., Rowlands, D.J. and Brown, F. (1973). A physico-chemical sub-grouping of the mammalian picornaviruses. J. gen. Virol. 18, 171-180.

Noble, W.C. Jr. and Brainard, D.H. (1935). A note on the susceptibility of ferrets to the virus of the common cold. J. Bact. 29, 407-409.

O'Sullivan, D.G. and Ludlow, C.M. (1972). Antiviral properties of 1-allyl and 1-crotyl derivatives of 2-(α-hydroxybenzyl) benzimidazole. Experientia 28, 889-890.

O'Sullivan, D.G. and Ludlow, C.M. (1973). Relation between the time at which cytopathic endpoint occurs in infected cultures and the initial infective virus dose, with application to the study of inhibitors of cytopathic effect. Arch. ges. Virusforsch. 41, 295-309.

O'Sullivan, D.G., Ludlow, C., Pantic, D. and Wallis, A.K. (1969). Antiviral activity and molecular features of benzimidazoles. Antimicrob. Ag. Chemother. 153-159.

O'Sullivan, D.G., Pantic, D. and Wallis, A.K. (1964a). Action of D-1-alkyl-2-(α-hydroxybenzyl)benzimidazoles on tissue culture cells infected with poliovirus. Nature, Lond. 201, 378-379.

O'Sullivan, D.G., Pantic, D. and Wallis, A.K. (1964b). Protection of poliovirus-infected tissue culture cells using the 1-benzyl, 1-pentyl and 1-isopropyl derivatives of 2-(α-hydroxybenzyl) benzimidazole. Nature, Lond. 203, 433-434.

O'Sullivan, D.G., Pantic, D. and Wallis, A.K. (1965). Protection offered to poliovirus-infected tissue culture cells by methoxy- and hydroxymethyl compounds related to 2-benzylbenzimidazole. Nature, Lond. 205, 262-264.

O'Sullivan, D.G., Pantic, D. and Wallis, A.K. (1967). New, 1,2-disubstituted benzimidazoles with high inhibiting effects on poliovirus replication. Experientia 23, 704-706.

O'Sullivan, D.G. and Sadler, P.W. (1961). Agents with high activity against type 2 poliovirus. Nature, Lond. 192, 341-343.

O'Sullivan, D.G. and Wallis, A.K. (1963a). New benzimidazole derivatives with powerful protective action on tissue cells infected with types 1, 2 and 3 poliovirus. Nature, Lond. 198, 1270-1273.

O'Sullivan, D.G. and Wallis, A.K. (1963b). 2-(α-Hydroxybenzyl) benzimidazole salts and their influence on cultured cells infected with poliovirus. Nature, Lond. 200, 1101-1103.

Parsons, R. and Tyrrell, D.A.J. (1961). A plaque method for assaying viruses isolated from common colds. Nature, Lond. 189, 640-642.

Pauling, L. (1970). Vitamin C and the common cold. San Francisco, W.H. Freeman and Co. Ltd. (paperback).

Pearson, G.D. and Zimmerman, E.F. (1969). Inhibition of poliovirus replication by N-methylisatin-β-4':4'-dibutylthiosemicarbazone. Virology 38, 641-650.

Pelon, W. (1961). Classification of the '2060' virus as ECHO 28 and further study of its properties. Am. J. Hyg. 73, 36-54.

Pelon, W., Mogabgab, W.J., Phillips, I.A. and Pierce, W.E. (1957). A cytopathogenic agent isolated from naval recruits with mild respiratory illnesses. Proc. Soc. exp. Biol. Med. 94, 262-267.

Perkins, J.C., Tucker, D.N., Knopf, H.L.S., Wenzel, R.P., Kapikian, A.Z. and Chanock, R.M. (1969). Comparison of protective effect of neutralizing antibody in serum and nasal secretions in experimental rhinovirus type 13 illness. Am. J. Epidemiol. 90, 519-526.

Person, D.A., Sheridan, P.J. and Herrmann, E.C. Jr. (1970). Sensitivity of types 1 and 2 herpes simplex virus to 5-iodo-2'-deoxyuridine and 9-β-D-arabinofuranosyladenine. Infect. Immunity 2, 815-820.

Pfau, C.J. and Camyre, K.P. (1968). Inhibition of lymphocytic choriomeningitis virus multiplication by 2-(α-hydroxybenzyl) benzimidazole. Virology 35, 375-380.

Phillips, C.A., Melnick, J.L. and Grim, C.A. (1965a). Human aorta cells for isolation and propagation of rhinoviruses. Proc. Soc. exp. Biol. Med. 119, 843-845.

Phillips, C.A., Melnick, J.L. and Sullivan, L. (1970). Characteristics of four new rhinovirus serotypes. Proc. Soc. exp. Biol. Med. 134, 933-935.

Phillips, C.A., Riggs, S., Melnick, J.L. and Grim, C.A. (1965b). Rhinoviruses associated with common colds in student population. J. Am. med. Ass. 192, 277-280.

Pinto, C.A., Bahnsen, H.P., Ravin, L.J., Haff, R.F. and Pagano, J.F. (1972). The antiviral effect of a triazinoindole (SK+F 40491) in rhinovirus infected gibbons. Proc. Soc. exp. Biol. Med. 141, 467-474.

Pinto, C.A. and Haff, R.F. (1969). Experimental infection of gibbons with rhinoviruses. Nature, Lond. 224, 1310-1311.

Plotkin, S.A., Carp, R.I. and Graham, A.F. (1962). The polioviruses of man. Ann. N.Y. Acad. Sci. 101, 357-389.

Plummer, G. (1965). The picornaviruses of man and animals: a comparative review. Prog. med. Virol. 7, 326-361.

Pollard, M. and Caplovitz, C.D. (1947). Experimental studies with the agent of the common cold. Science, N.Y. 106, 243-244.

Porterfield, J.S. (1962). Titration of some common cold viruses (rhinoviruses) and their antisera by a plaque method. Nature, Lond. 194, 1044-1047.

Poser, E. (1972). Large ascorbic acid intake. New Eng. J. Med. 287, 412-413.

Price, W.H. (1956). The isolation of a new virus associated with respiratory clinical disease in humans. Proc. natl. Acad. Sci. U.S.A. 42, 892-896.

Price, W.H., Emerson, H., Ibler, I., Lachaine, R. and Terrell, A. (1959). Studies of the 'JH' and '2060' viruses and their relationship to mild upper respiratory disease in humans. Am. J. Hyg. 69, 224-249.

Prinzie, A., Schonne, E. and DeSomer, P. (1960). Inactivation et spécificité cellulaire de l'acide ribonucléique du poliovirus. (Inactivation and cellular specificity of the ribonucleic acid of the poliomyelitis virus.) Arch. ges. Virusforsch. 10, 153-166.

Prusoff, W.H. (1959). Synthesis and biological activities of iododeoxyuridine, an analog of thymidine. Biochem. biophys. Acta 32, 295-296.

Rada, B., Blaškovič, D., Šorm, F. and Škoda, J. (1960). The inhibitory effect of 6-azauracil riboside on the multiplication of vaccinia virus. Experientia 16, 487-488.

Ravin, L.J., Shami, E.G. and Rattie, E. (1970). Physico-chemical evaluation of 3-(3-hydroxy-3-methylbutylamino)-5-methyl-as-triazino[5,6-b]indole (SK+F 30097). J. pharm. Sci. 59, 1290-1295.

Reed, S.E., Beare, A.S., Bynoe, M.L. and Tyrrell, D.A.J. (1970). Studies of the isoquinoline derivatives (UK 2371 and UK 2054) in respiratory infections of volunteers, using influenza viruses, a parainfluenza virus and a rhinovirus. Ann. N.Y. Acad. Sci. 173, 760-769.

Reed, S.E. and Boyde, A. (1972). Organ cultures of respiratory epithelium infected with rhinovirus or parainfluenza virus studied in a scanning electron microscope. Infect. and Immun. 6, 68-76.

Reed, S.E. and Bynoe, M.L. (1970). The antiviral activity of isoquinoline drugs for rhinoviruses in vitro and in vivo. J. med. Microbiol. 3, 346-352.

Reed, L.J. and Muench, H. (1938). A simple method of estimating 50 per cent endpoints. Am. J. Hyg. 27, 493-497.

Reich, E., Franklin, R.M., Shatkin, A.J. and Tatum, E.L. (1961). Effect of actinomycin D on cellular nucleic acid synthesis and virus production. Science, N.Y. 134, 556-557.

Rhead, W.J. and Schrauzer, G.N. (1971). Risks of long-term ascorbic acid overdosage. Nutr. Rev. 29, 262-263.

Ritzel, G. (1961). Critical evaluation of vitamin C as a prophylactic and therapeutic agent in colds. Helv. med. Acta. 28, 63-68.

Robinson, L.K. (1950). Effect of heat and of pH on strains of Coxsackie virus. Proc. Soc. exp. Biol. Med. 75, 580-582.

Roderick, W.R., Nordeen, C.W. Jr., von Esch, A.M. and Appell, R.N. (1972). Bisbenzimidazoles. Potent inhibitors of rhinoviruses. J. med. Chem. 15, 655-658.

Rosen, L. (1965). Subclassification of picornaviruses. Bact. Rev. 29, 173-184.

Rosenthal, M.S. (1965). Contamination of rhinovirus seed pools revealed in HEp2 cell suspension cultures. J. gen. Microbiol. 38, 409-416.

Rott, R. and Drzeniek, R. (1963). Abspaltung einer Neuraminidasehaltigen Komponente auf Newcastle disease Virus (NDV). Z. Naturf. 18b, 1127.

Rowe, W.P., Murphy, F.A., Bergold, G.H., Casals, J., Hotchin, J., Johnson, K.M., Lehmann-Grube, F., Mims, C.A., Traub, E. and Webb, P.A. (1970). Arenaviruses: Proposed name for a newly defined virus group. J. Virol. 5, 651.

Ruck, B.J., Brammer, K.W., Page, M.G. and Coombes, J.D. (1969). The detection and characterization of an induced RNA polymerase in the chorioallantoic membranes of embryonated eggs infected with influenza A_2 virus. Virology 39, 31-41.

Sabin, A. (1967). Amantadine hydrochloride: Analysis of data related to its proposed use for prevention of A_2 influenza virus disease in human beings. J. Am. med. Ass. 200, 943-950.

Sato, N., Matsumoto, S., Tsunoda, A. and Ishida, N. (1966). In vitro activity of some 2-N^3-guanidinobenzimidazole derivatives against poliovirus. J. Antibiot. Ser. A. 19, 172-174.

Schleicher, J.B., Aquino, F., Rueter, A., Roderick, W.R. and Appell, R.N. (1972). Antiviral activity in tissue culture systems of bis-benzimidazoles, potent inhibitors of rhinoviruses. Appl. Microbiol. 23, 113-116.

Seto, J.T. and Rott, R. (1966). Functional significance of sialidase during influenza virus multiplication. Virology 30, 731-737.

Shibley, G.S., Hanger, F.M. and Dochez, A.R. (1926). Studies in the common cold. I. Observations of the normal bacterial flora of the nose and throat with variations occurring during colds. J. exp. Med. 43, 415-431.

Shibley, G.S., Mills, K.C. and Dochez, A.R. (1930). Studies of the etiology of the common cold. J. Am. med. Ass. 95, 1553-1556.

Shindarov, L., Galabov, A., Neykova, N., Simov, D., Davidkov, K. and Kalcheva, V. (1971). Inhibitory action of N-2-hydroxyphenylthiourea derivatives on picornavirus multiplication in cell cultures. Acta Virol. 15, 404-410.

Shindarov, L., Galabov, A., Vassileva, V., Simov, D. and Davidkov, K. (1970b). Inhibitory effect of N-methyl-N(2-hydroxyphenyl)N'-benzylthiourea on multiplication of poliovirus in cell cultures. Acta Virol. 14, 401-404.

Shindarov, L., Galabov, A., Vassileva, V., Simov, D., Kalcheva, V. and Davidkov, K. (1970a). Studies on the antiviral activity of N-2-hydroxyphenylthiourea and N-2-hydroxyphenyl-urea. Proc. Postgr. Med. Inst. (Sofia) 17, 43-48.

Shinkai, K. and Nishimura, T. (1972). Inability of isoquinoline derivatives to inhibit virus neuraminidase activity. J. gen. Virol. 16, 227-229.

Shipkowitz, N.L., Bower, R.R., Schleicher, J.B., Aquino, F., Appell, R.N. and Roderick, W.R. (1972). Antiviral activity of a bis-benzimidazole against experimental rhinovirus infections in chimpanzees. Appl. Microbiol. 23, 117-122.

Sidwell, R.W., Allen, L.B., Khare, G.P., Huffman, J.H., Witkowski, J.T., Simon, L.N. and Robins, R.K. (1973). Effect of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Virazole, ICN 1229) on herpes and vaccinia keratitis and encephalitis in laboratory animals. Antimicrob. Ag. Chemother. 3, 242-246.

Sidwell, R.W. and Huffman, J.H. (1971). Use of disposable micro tissue culture plates for antiviral and interferon induction studies. Appl. Microbiol. 22, 797-801.

Sidwell, R.W., Huffman, J.H., Khare, G.P., Allen, L.B., Witkowski, J.T. and Robins, R.K. (1972). Broad-spectrum antiviral activity of Virazole: 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide. Science, N.Y. 177, 705-706.

Skinner, M.S., Halperen, S. and Harkin, J.C. (1968). Cytoplasmic membrane-bound vesicles in echovirus 12-infected cells. Virology 36, 241-253.

Smith, L.H., Fromm, H. and Hofmann, A.F. (1972). Acquired hyperoxaluria, nephrolithiasis, and intestinal disease. Description of a syndrome. New Eng. J. Med. 286, 1371-1375.

Soto, A.J., Hall, T.S. and Reed, S.E. (1973). Trial of the antiviral action of isoprinosine against rhinovirus infection of volunteers. Antimicrob. Ag. Chemother. 3, 332-334.

Stanfield, F.J., Haff, R.F. and Stewart, R.C. (1967). The antiviral activity of caprochlorane. Proc. Soc. exp. Biol. Med. 125, 297-303.

Stott, E.J. (1967). Applications of tissue culture to the study of the epidemiology of respiratory viruses. Ph.D. thesis. Univ. Glasgow.

Stott, E.J. and Heath, G.F. (1970). Factors affecting the growth of rhinovirus 2 in suspension culture in L132 cells. J. gen. Virol. 6, 15-24.

Stott, E.J. and Killington, R.A. (1972a). Haemagglutination by rhinoviruses. Lancet, i, 1369-1370.

Stott, E.J. and Killington, R.A. (1972b). Rhinoviruses. Ann. Rev. Microbiol. 26, 503-524.

Stott, E.J. and Tyrrell, D.A.J. (1968). Some improved techniques for the study of rhinoviruses using HeLa cells. Arch. ges. Virusforsch. 23, 236-244.

Stott, E.J. and Walker, M. (1967). Human embryo kidney fibroblasts for the isolation and growth of rhinoviruses. Brit. J. exp. Path. 48, 544-551.

Stott, E.J. and Walker, M. (1969). Antigenic variation among strains of rhinovirus type 51. Nature, Lond. 224, 1311-1312.

Szybalski, W. (1952). Gradient plate technique for study of bacterial resistance. Science, N.Y. 116, 46-47.

Tamm, I., Bablanian, R., Nemes, M.M., Shunk, C.H., Robinson, F.M. and Folkers, K. (1961). Relationship between structure of benzimidazole derivatives and selective virus inhibitory activity. Inhibition of poliovirus multiplication and cytopathic effects by 2-(α-hydroxy benzyl)benzimidazole and its 5-chloro derivative. J. exp. Med. 113, 625-656.

Tamm, I. and Caliguiri, L.A. (1972). 2-(α-Hydroxybenzyl)benzimidazole and related compounds. In: Chemotherapy of Virus Diseases, vol. 1 (Ed.) D.J. Bauer, Pergamon Press.

Tamm, I. and Eggers, H.J. (1962). Differences in the selective virus inhibitory action of 2-(α-hydroxybenzyl)benzimidazole and guanidine HCl. Virology 18, 439-447.

Tamm, I. and Eggers, H.J. (1963a). Specific inhibition of replication of animal viruses. Science, N.Y. 142, 24-33.

Tamm, I. and Eggers, H.J. (1963b). Unique susceptibility of enteroviruses to inhibition by 2-(α-hydroxybenzyl)benzimidazole and derivatives. International symposium on chemotherapy, 2nd, Naples, 1961, Part II. S. Karger, Basel, New York, p. 89.

Tamm, I., Eggers, H.J., Bablanian, R., Wagner, A.F. and Folkers, K. (1969). Structural requirements of selective inhibition of enteroviruses by 2-(α-hydroxybenzyl)benzimidazole and related compounds. Nature, Lond. 223, 785-788.

Tamm, I., Folkers, K. and Horsfall, F.L. Jr. (1952). Inhibition of influenza virus multiplication by 2,5-dimethylbenzimidazole. Yale J. Biol. Med. 24, 559-567.

Tamm, I., Folkers, K., Shunk, C.H., Heyl, D. and Horsfall, F.L. (1953). Inhibition of influenza virus multiplication by alkyl derivatives of benzimidazole. III. Relationship between inhibitory activity and chemical structure. J. exp. Med. 98, 245-259.

Tamm, I. and Horsfall, F.L. (1952). A mucoprotein derived from human urine which reacts with influenza, mumps and Newcastle disease viruses. J. exp. Med. 95, 71-97.

Tamm, I. and Nemes, M.M. (1959). Selective inhibition of poliovirus multiplication. J. clin. Invest. 38, 1047-1048.

Taylor-Robinson, D., Hucker, R. and Tyrrell, D.A.J. (1962). Studies on the pathogenicity for tissue cultures of some viruses isolated from common colds. Brit. J. exp. Path. 43, 189-193.

Taylor-Robinson, D., Johnson, K.M., Bloom, H.H., Parrott, R.H., Mufson, M.A. and Chanock, R.M. (1963). Rhinovirus neutralizing antibody responses and their measurement. Am. J. Hyg. 78, 285-292.

Taylor-Robinson, D. and Tyrrell, D.A.J. (1962a). Serotypes of viruses (rhinoviruses) isolated from common colds. Lancet i, 452-454.

Taylor-Robinson, D. and Tyrrell, D.A.J. (1962b). Serological studies on some viruses isolated from common colds (rhinoviruses). Brit. J. exp. Path. 43, 264-275.

Thomas, D.C., Conant, R.M. and Hamparian, V.V. (1970). Rhinovirus replication in suspension cultures of HeLa cells. Proc. Soc. exp. Biol. Med. 133, 62-65.

Thompson, R.L. (1964). Chemoprophylaxis and chemotherapy of viral diseases. Advanc. Chemother. 1, 85-131.

Thompson, R.L., Minton, S.A. Jr., Officer, J.E. and Hitchings, G.H. (1953). Effect of heterocyclic and other thiosemicarbazones on vaccinia infection in the mouse. J. Immun. 70, 229-234.

Togo, Y., Schwartz, A.R. and Hornick, R.B. (1973). Failure of a 3-substituted triazinoindole in the prevention of experimental human rhinovirus infection. Chemotherapy 18, 17-26.

Topping, N.H. and Atlas, L.T. (1947). The common cold: a note regarding isolation of an agent. Science, N.Y. 106, 636-637.

Tyrrell, D.A.J. (1963a). Further experiments on viruses isolated from common colds (rhinoviruses). In: Perspectives in Virology III, (Ed.) M. Pollard, Hoeber, New York, pp. 238-256.

Tyrrell, D.A.J. (1968). Rhinoviruses. Virology monographs 2.

Tyrrell, D.A.J. and Blamire, C.J. (1967). Improvements in a method of growing respiratory viruses in organ culture. Brit. J. exp. Path. 48, 217-227.

Tyrrell, D.A.J. and Bynoe, M.L. (1961). Some further isolations from common colds. Br. med. J. i, 393-397.

Tyrrell, D.A.J. and Bynoe, M.L. (1965). Cultivation of a novel type of common-cold virus in organ cultures. Br. med. J. i, 1467-1470.

Tyrrell, D.A.J. and Bynoe, M.L. (1966). Cultivation of viruses from a high proportion of patients with colds. Lancet i, 76-77.

Tyrrell, D.A.J., Bynoe, M.L., Hitchcock, G., Pereira, H.G. and Andrewes, C.H. (1960). Some virus isolations from common colds. I. Experiments employing human volunteers. Lancet i, 235-237.

Tyrrell, D.A.J., Bynoe, M.L. and Hoorn, B. (1968). Cultivation of 'difficult' viruses from patients with common colds. Br. med. J. i, 606-610.

Tyrrell, D.A.J. and Parsons, R. (1960). Some virus isolations from common colds. III. Cytopathic effects in tissue cultures. Lancet i, 239-242.

Virus Subcommittee of International Nomenclature Committee (Andrewes et al.). Recommendations on virus nomenclature. Virology 21, 516-517.

Votava, M. and Tyrrell, D.A.J. (1970). The possible value of certain cells for the propagation of respiratory viruses. Arch. ges. Virusforsch. 29, 253-256.

Wagner, R.B., Steinberg, B.A. and Ruchman, I. (1967). Method for determining antineuraminidase activity. Appl. Microbiol. 15, 239-242.

Walker, G.H., Bynoe, M.L. and Tyrrell, D.A.J. (1967). Trial of ascorbic acid in the prevention of colds. Br. med. J. i, 603-606.

Ward, T.G. and Proctor, D.F. (1950). Isolation of a common cold virus in chick embryos and the clinical manifestations it produces in volunteers. Am. J. Hyg. 52, 91-106.

Warren, L. (1959). The thiobarbituric acid assay of sialic acids. J. biol. Chem. 234, 1971-1975.

Wawrkiewicz, J., Smale, C.J. and Brown, F. (1968). Biochemical and biophysical characteristics of vesicular exanthema virus and the viral ribonucleic acid. Arch. ges. Virusforsch. 25, 337-351.

Webb, P.A., Johnson, K.M. and Mufson, M.A. (1964). A description of two newly-recognised rhinoviruses of human origin. Proc. Soc. exp. Biol. Med. 116, 845-852.

Webster, R.G. and Laver, W.G. (1967). Preparation and properties of antibody directed specifically against the neuraminidase of an influenza virus. J. Immun. 99, 49-55.

Wenner, H.A. and Behbehani, A.M. (1968). ECHO viruses. Virology monographs, 1, 1-72.

Wildy, P. (1971). Classification and nomenclature of viruses. Monographs in Virology, 5.

Williamson, G.M. and Jackson, D. (1969). The antiviral activity of the isoquinolines famotine and memotine in respiratory infections in man. Bull. Wld. Hlth. Org. 41, 665-670.

Wilson, C.W.M. and Loh, H.S. (1969). Ascorbic acid and the common cold. 4th International Congress of Pharmacology, abstract, p. 458.

Wilson, C.W.M. and Loh, H.S. (1973). Common cold and Vitamin C. Lancet i, 638-641.

Witkowski, J.T., Robins, R.K., Sidwell, R.W. and Simon, L.N. (1972). The design, synthesis and broad spectrum antiviral activity of 1-β-D-ribofuranosyl-1,2,4-carboxamide and related nucleosides. J. med. Chem. 1150-1154.

Woodson, B. and Joklik, W.K. (1965). The inhibition of vaccinia virus multiplication by isatin-β-thiosemicarbazone. Proc. natl. Acad. Sci. U.S.A. 54, 946-953.