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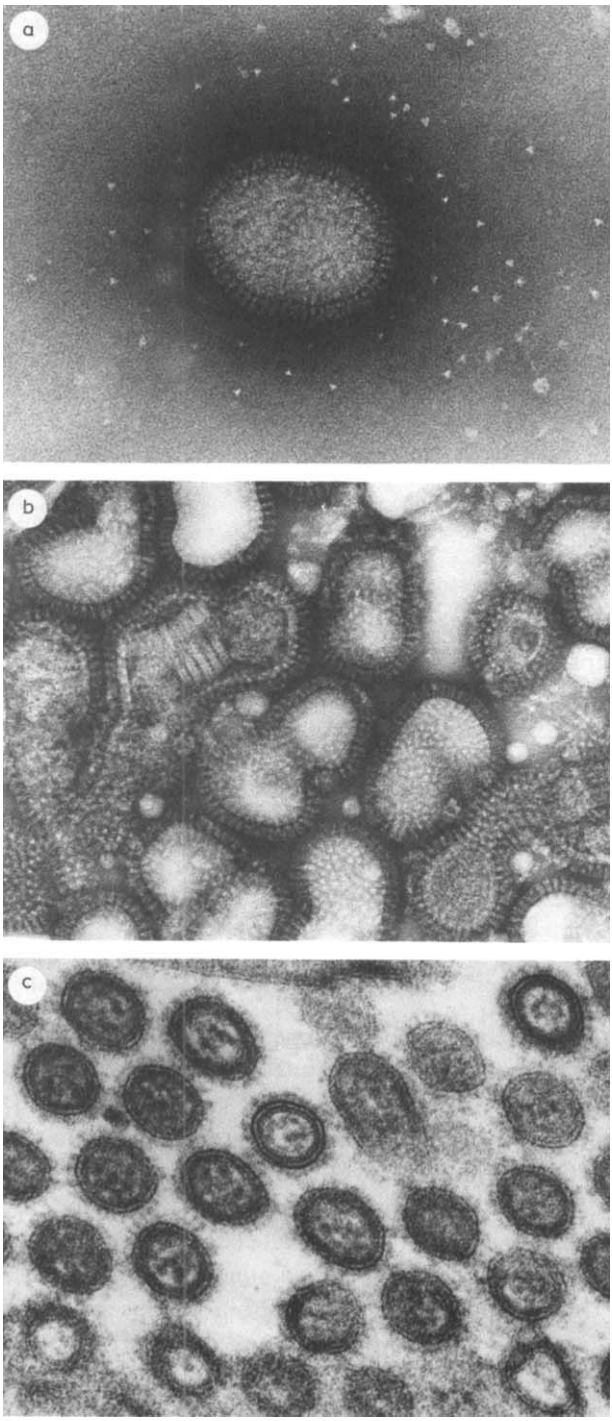
CHAPTER 7

Orthomyxovirus infections

The earth is a unity for influenza A virus in a manner not yet found for probably any other parasite (Hope-Simpson, 1979, Stuart-Harris, 1981, Kilbourne, 1975, Pereira, 1979) and epidemics occur in all inhabited parts of the globe regardless of latitude, longitude, altitude, climate, rainfall, temperature, humidity, race and sex. Influenza A is the classic pandemic virus infection of man (although now not the only pandemic virus, since enterovirus type 70 is essentially similar in epidemiology, see Chapter 4) and influenza B virus also can cause sharp outbreaks, resulting in significant mortality.

7.1 Structure of influenza A virus and protein composition

The influenza A virion is a rather pleomorphic enveloped virus, often taking a 'doughnut' shape when visualized using negative staining techniques (Fig. 7.1). The lipid envelope (which is derived from the host cell in which the virus has replicated) is studded with the two morphologically distinguishable glycoprotein spikes, namely the haemagglutinin (HA) and the neuraminidase (NA). The latter takes the morphological form of a mushroom but with a narrow extended stalk, while the HA is triangular in cross section rather like a Toblerone chocolate bar. Both spikes penetrate through the lipid bilayer via 'tails' of hydrophobic amino acids and contact the underlying layer of matrix (M) protein. The M protein forms a monomolecular layer and probably contributes in a major way to the structural integrity of the virion. Enclosed in the matrix shell is the nucleoprotein and the P1, P2 and P3 complex, closely assocciated with the ss RNA of the virus. The three polymerase pro-


a
b
c

teins constitute an RNA transcriptase enzyme (Kawakami and Ishihama, 1983, reviewed by McCauley and Mahy, 1983) responsible for initiating RNA replication in the infected cell. The virion RNA exists as 8 separate single stranded fragments which are replicated independently as described below.

We shall examine in some detail the structure and function of some of these structural proteins and RNAs of influenza virus (Table 7.1). More is known about the composition and replication of influenza than probably any other human virus. We can therefore use the influenza virus as a model and safely conclude that future scientific investigations of other viruses will follow a similar pattern.

7.1.1. Haemagglutinin (HA)

The haemagglutinin (HA) of influenza A virus is a trimer of 224 K M.W. It may be solubilized from the viral membrane by bromelain enzyme digestion, which removes a 5406 M.W. C-terminal hydrophobic (anchoring) peptide from each subunit. The haemagglutinin is a typical integral membrane protein, characterized by a three-domain structure with a large hydrophilic, carbohydrate-containing domain on the external surface of the membrane, a small, uncharged hydrophobic peptide of 24–28 amino acids spanning the virus membrane, and a small, hydrophilic domain (10–55 amino acids) on the internal side of the membrane (reviewed in Webster et al., 1982, Palese and Kingsbury, 1983).

Typical of membrane glycoproteins, the haemagglutinin chain is initially synthesized to include an N-terminal hydrophobic 'signal' peptide, which is subsequently removed as part of the process by which the protein is transported across and anchored into the membrane. Each polypeptide chain of the trimer is glycosylated at seven sites with a total carbohydrate of 13 000 M.W. (19% by weight).

The HA protein is a major determinant of virulence of influenza virus (Bosch et al., 1979) and also contains important antigenic determinants which stimulate the production of neutralizing antibody following infection or immunization. The HA protein has important biological functions of attachment to host cell receptors and, once inside the cell mediates fusion events which result in release of RNA and cell infection. Therefore, the HA represents an important target protein both from the point of view of vaccines and also chemotherapy and hence it is probably the most well studied human virus protein. An important characteristic of the HA is the mosaic characteristic of its antigenic determinants ('antigenic drift', resulting from single or a few amino acid substitutions) and its ability also to undergo major antigenic changes ('antigenic shift'). Amino acid sequence analysis of the HA of different subtypes shows, however, that certain stretches are conserved and this raises

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Fig. 7.1. Electron micrograph of influenza A virions. a, Single virion surrounded by HA spikes (viewed end); b, Partially disrupted particles showing matrix layer and NP-RNA complex; c, Sections of virus showing matrix layer and lipid bilayer. (Courtesy of Dr. D. Hockley.)

the possibility that these could be used as specific targets for new antivirals and vaccines. This is discussed in more detail below when we refer to the work on oligopeptides as inhibitors of virus replication. From the point of view of vaccines, earlier studies in animals showed cross-protection (Schulman and Kilbourne, 1965, Werner, 1966) between subtypes of influenza A virus (but not between influenza A and B viruses) and this was thought to be HA mediated. Therefore shielded antigenic determinants could exist which are recognized more easily in certain animals than in humans. Again this emphasizes the possibility of obtaining immunogens which induce cross-reactive antibody against a wide variety of strains. At present antigenic drift and shift pose major problems for vaccine efficacy.

Detailed studies have now been carried out on the HA of influenza A viruses using X-ray crystallography (Wilson et al., 1981) which together with earlier sequencing data (Porter et al., 1979, Gething et al., 1980) has allowed the construction of accurate 3 dimensional models of the HA and the precise localization of the cell binding site, antigenic determinants (Fig. 7.2) and fusion sequences.

As regards the binding site, the distribution of conserved amino acids on the 3 Å structure reveals a highly conserved region in a surface pocket on the distal end of the molecule that seems suited for a binding to host-cell oligosaccharide. This tentative receptor binding pocket includes conserved residues Tyr 98, His 193, Glu 190, Trp 153 and Leu 194.

The most highly conserved sequence in the haemagglutinin is the amino terminus of HA2, where 1 substitution in the first 11 and only 5 in the first 23 amino acids have been observed. This sequence is associated with the activity by which the virus penetrates a host-cell internal lysosomal membrane to initiate infection. Cleavage of HA into HA1 and HA2 at this point is required for infections penetration and for in vitro membrane fusion (An homologous sequence is present at the cleavage-activation site on the Sendai virus fusion protein, where cleavage is required both for infectivity and membrane fusion activity.) The HA2 N-terminal sequence is strongly nonpolar, the first charged side chain occurring at position 11 (Glu). It is unexpectedly rich in glycines (1, 4, 8 and 13, 16, 20, 23) which could indicate flexibility or the presence of an unusual conformation.

As regards antigenic binding sites, four (A, B, C and D) have been identified (Fig. 7.2) by analyzing antigenic mutants selected in the laboratory under immunological pressure from monoclonal antibodies (Yewdell et al., 1970). Site A is an unusual protruding loop from amino acids 140 to 146 which projects 8 Å from the local molecular surface, and forms the centre of the most obvious antibody-binding site. The haemagglutinin of each antigenically distinct virus of epidemic significance has a mutation in this region. Site B comprises the external residues 187–196 of an α helix, and adjacent residues along the upper edge of a pocket tentatively implicated in virus receptor binding. Two substitutions in the haemagglutinin of the 1972 virus, A/Memphis/103/72: 188, Asn to Asp, and 155, Thr to Tyr; and two more in that of the 1975 virus, A/Victoria/3/75: 189, Gln to Lys and 193, Ser to Asn charac-

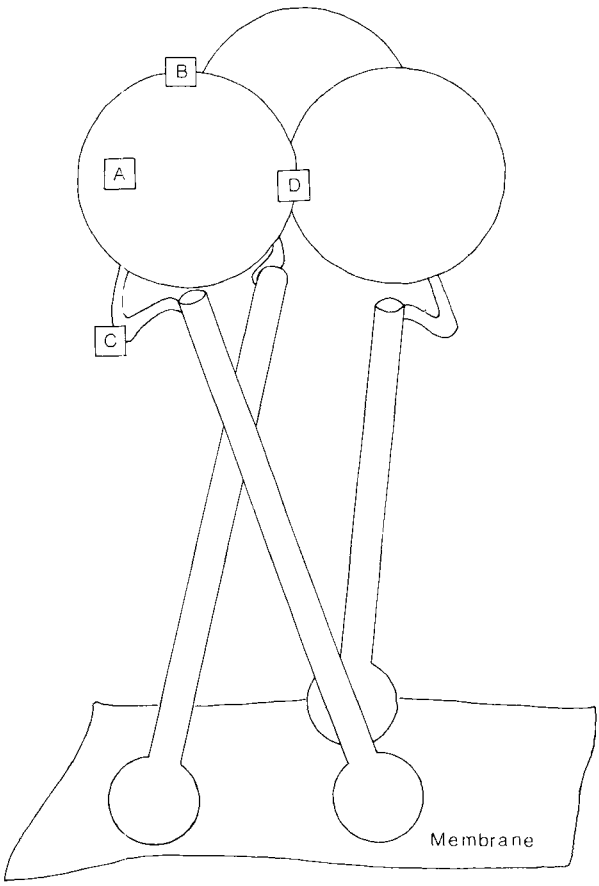


Fig. 7.2. Schematic diagram of the HA trimer of A/Aichi/68 virus. Each trimer contains a stem and a globular domain which contains the variable antigenic determinants A, B, C and D. It is not known at present whether the influenza B HA molecule is identical, but it is assumed to be similar.

terize the site, but the lack of sequence information for this region of the haemagglutinins of several variants leaves the comparison incomplete. Site C is a bulge in the tertiary structure at the disulphide bond between Cys 52 and Cys 277, 60 Å from the distal tip of the molecule and comprises another antibody-binding site (see Fig. 7.2). The haemagglutinins of the viruses from both epidemic periods have substitutions clustered in this region.

In the first three sites noted above, the amino acid substitutions noted as causing antigenic variation are external. Site D departs from this. Several amino acid substitutions in the haemagglutinins of both natural and laboratory-selected antigenic mutants occur in the interface region between subunits in the haemagglutinin trimer. These amino acids may be recognized as a result of a relative movement of the globular regions of HA1 to expose the interface regions. However, it is possible that

the actual antibody binding site is remote from amino acids but affected by the exact fit at the interface, and might be disturbed by the substitutions listed.

7.1.2. The matrix protein (M)

The complete sequence of RNA segment 7 (coding for the group specific matrix antigen) of two strains of A/PR8/34 (H1N1) and of A/Udorn/72 (H3N2) has been reported as well as partial sequences of a number of other strains (reviewed in Palese and Kingsbury, 1983). Following the first AUG codon in the positive strand RNA, a 252-residue protein, rather hydrophobic and rich in arginine, is encoded. Comparison of the sequences of RNA segment 7 of the H3N2 (Udorn) and H1N1 (PR8) strains shows that the sequences coding for the matrix (M) of these viruses isolated 38 years apart are highly conserved, in keeping with antigenic studies. Comparison of 230 nucleotides of RNA segment 7 from five human H1N1, H2N2 and H3N2 strains isolated over a 43 year period suggests that the same segment 7 was retained throughout the antigenic shifts of HA and NA type (H1N1 to H2N2 to H3N2). In addition, the complete sequences contain a second open reading frame which overlaps the M protein sequence by 68 nucleotides.

Three mRNAs transcribed from RNA segment 7 have been isolated. One (M1 mRNA) consists of an uninterrupted, nearly full-length, copy of RNA segment 7 and is responsible for the production of the M protein. An M2 protein is generated from a spliced product of the M1 mRNA, such that after the nucleotides encoding the N-terminal nine amino acids, nearly 600 nucleotides are spliced out and the reading frame is changed; a protein product corresponding to this has been identified in infected cells. In addition, a third mRNA has been found, which would code only for an 8-residue peptide identical to the C terminus of M1. Such a product has not yet been isolated.

7.1.3. Nucleoprotein (NP)

The nucleoprotein is one of the group-specific antigens of influenza viruses that distinguishes between influenza A, B and C viruses (Stuart-Harris and Schild, 1979). It probably constitutes the backbone of the helical internal complex that is associated with the RNA segments and the three different polymerase proteins.

The NP gene of A/PR/8/34 virus is 1,565 nucleotides long and is capable of encoding a protein of 498 amino acid residues (M.W. 56 106) rich in arginine. Double immunodiffusion tests showed antigenic differences between the NPs of H1N1 and the H3N2 strains and recent studies with monoclonal antibodies to the NP of A/WSN/33 (H1N1) viruses have shown that antigenic variation occurs in this molecule (Schild et al., 1979). The NP molecule possesses at least three non-overlapping antigenic areas, one area being the same on all strains tested. Monoclonal antibodies to this conserved domain inhibited in vitro transcription of viral RNA, suggest-

ing that this region of the NP is involved in RNA transcription (Van Wyke et al., 1980).

7.1.4. The non-structural proteins

RNA segment 8 codes for at least two non-structural polypeptides, NS1 and NS2, which are translated from separate mRNAs (reviewed by Palese and Kingsbury, 1983, Lamb et al., 1980, Inglis et al., 1979). Mapping and sequence studies have shown that NS1 and NS2 overlap by 70 amino acids that are translated from different reading frames. Polypeptides NS1 and NS2 share 9 amino acids at their N termini, but after this sequence the mRNA for NS2 has a deletion of 423 nucleotides, then rejoins the rest of the mRNA in the +1 reading frame. The function of NS1 or NS2 has not been established. NS1 is made in large amounts and accumulates in the nucleus, whilst NS2 is made late in infection and is found predominantly in the cytoplasm.

Because NS1 and NS2 are internal proteins of infected cells and hence less available to antibodies, they would be expected to show less sequence variation than the surface glycoproteins (HA and NA). Accordingly, comparison of the sequences of the NS genes of fowl plague and the two human influenza strains A/Udorn/72 (H3N2) and A/PR/8/34 shows only 8-11% differences (see Palese and Kingsbury, 1983). An open reading frame potentially coding for an extra polypeptide has been noted in the noncoding, virion RNA of the NS genes of A/PR/8/34, Udorn/72 and FPV, but is not present in the NS gene of duck/Alberta/60/76. No protein corresponding to this extra 'gene' has yet been identified.

7.1.5. Polymerase proteins

The three largest proteins of the virion (P1, P2, P3) with molecular weights of 96 000, 87 000 and 85 000, respectively, are found in association with the nucleoprotein and virion RNA and carry the polymerase activity which transcribes the invading viral RNA. Proteins P1 and P3 are probably required for complementary RNA synhesis and P2 and NP for virion RNA synthesis. The complete nucleotide sequences of two of the three polymerase genes of the A/PR/8/34 strain have been determined, but the extent of variation in the polymerase genes is unknown, although these may play an important part in host range and virulence (Almond, 1977).

Defective interfering (DI) influenza virus particles are generated by high multiplicity passage in permissive cells. These particles are of interest because they facilitate the establishment of persistent infection in cell cultures and could therefore be concerned in latency. They contain new small RNA molecules which are absent from infectious virus and which are generated predominantly by massive internal deletion from the three P genes, although three sequences of one small RNA is a mosaic of several segments from at least two of the polymerase genes.

7.1.6. Neuraminidase (NA)

The NA is the second glycoprotein on the surface of the virion. Several roles have been suggested for the neuraminidase. The enzyme catalyses cleavage of the α–ketosidic linkage between terminal sialic acid and an adjacent sugar residue. This reaction permits transport of the virus through mucin and destroys the haemagglutinin receptor on the host cell, thus allowing elution of progeny virus particles from infected cells. The removal of sialic acid from the carbohydrate moiety of newly synthesized haemagglutinin and neuraminidase is also necessary to prevent self-aggregation of the virus. In general, then, the role of neuraminidase may be to facilitate mobility of the virus both to and from the site of infection.

The protein exhibits both antigenic shift (a major change in amino acid sequence) and antigenic drift (minor changes in amino acid sequence). Two serologically distinct subtypes of neuraminidase are known in human influenza viruses. The N1 subtype was associated with virus isolated between 1933 and 1957, after which time the N2 subtype appeared in 'Asian' influenza. No major change in the structure of the neuraminidase has occurred since, although the haemagglutinin subtype changed from H2 to H3 in 1968 in the Hong Kong pandemic.

The neuraminidase of influenza virus is an integral membrane glycoprotein. It is a tetramer of molecular weight 240 K, reducing to 200 K when solubilized from the virus with pronase. Sequences of neuraminidases from several strains of N1, N2 and B subtypes are known. A hydrophobic N-terminal region serves to anchor the neuraminidase in the membrane. The N2 enzyme subunit contains 469 amino acids, with carbohydrate attached in four places. The crystallizable product of the pronase digestion begins at residues 74 or 77. These so called neuraminidase 'heads' carry the full antigenic and enzymatic capability of the membrane-associated neuraminidase. Varghese et al. (1983) have now described the detailed structure of the NA at 2.9 Å resolution. The NA structure is unusual and is a 4-fold symmetric oligomer of identical polypeptide chains with a box-shaped 'head' connected to the virus membrane by a long slender stalk. For crystallization, the stalk was removed by pronase digestion, liberating the square head which retains full enzymatic and antigenic capabilities. The fold of the polypeptide chain is unique. Each monomer contains six β-sheets; each sheet contains four strands with the topology of a 'W'. Viewed from above the head, each monomer consists of six of the four-stranded sheets arrayed like the petals of a flower but twisted like the blades of a pinwheel. (reviewed by Wiley, 1983):

"Although the three-dimensional structure of the stem is unknown, it may be even more unusual. Amino acids 1–6 are on the cytoplasmic side of the membrane followed by an uncharged and primarily hydrophobic peptide (7–35) which spans the membrane, anchoring the protein to the lipid. The anchor also acts as a signal peptide during membrane translocation but is not removed by signal peptidase. The slender stem (residues 36–73) is unusual in containing 50 per cent of the oligosac-

charides of the molecule (four sites) and in exhibiting absolutely no amino acid sequence homology among NAs from various virus strains – although the remainder of the molecule shows highly significant (> 50 per cent) homology. Thus, both viral membrane glycoproteins, the NA and HA, exhibit novel structural features: the NA, a β-sheet pinwheel and a slender stalk; and the HA, a loop-like topology beginning and ending at the membrane, and a fibrous stem centred on an 80 Å long triple-stranded coiled-coil of α-helices."

The four carbohydrate chains in crystalline NA are distributed two on the top and two on the bottom of the box-shaped head. One oligosaccharide is found at a subunit-subunit interface. Both on the NA and on the HA most of the oligosaccharides are attached on the lateral surfaces of the glycoprotein in positions where the glycoproteins might be expected to contact other proteins embedded in the same membrane.

The catalytic site of the NA has been located by difference Fourier analysis of crystals soaked in sialic acid. The site is surrounded by 14 conserved charged residues and contains three hydrophobic residues, Tyr, Trp and Leu.

By examining the location of amino acid substitutions in a series of 'drifted' field strains and in three antigenic variants selected by growth in monoclonal antisera, Colman and colleagues (1983) have been able to propose the location of antigenic determinants on the NA. The antigenic sites are composed of loops connecting strands of β-sheet. They form a nearly continuous surface that encircles the catalytic site on the top of the NA. Although residues in the active site itself are conserved from strain to strain, the proximity of the variable loops to the site suggests that a significant part of the variable loops could interfere with any antibody contacting the active site cavity (reviewed by Wiley, 1983).

7.2. Early influenza virus–cell interactions

Very little is known in precise terms about the molecular details of interaction between human viruses, including influenza, and cell receptors. However, with the advent of rapid sequencing of virus glycoproteins involved in these interactions our knowledge of sequences involved should increase rapidly. We should recognize the pitfalls of studies of early interactions between viruses and cells, an important one being whether the interaction we observe will lead to infection, or whether we are simply observing non-infectious interaction. Nevertheless more studies are urgently required and data obtained could easily be applied, for example, to the design of specific inhibitors for chemoprophylaxis and also help to understand important problems of antibody-virus interaction and neutralization, virus cell fusion and virus host range. Sialic acids are known to be essential components of the cell surface receptors for influenza, paramyxoviruses, polyoma and encephalomyocarditis virus, but the viruses nevertheless appear to exhibit strict and varied specificities for

the precise sialyloligosaccharide sequences that serve as their receptor determinants. Probably the best characterized virus receptor is glycophorin, a sialo glycoprotein binding site for influenza virus on erythrocytes. Sialic acid containing glycolipids (gangliosides) occur naturally in the plasma membrane of cells and act as receptors of cholera toxin, interferon and some glycoprotein hormones as well as certain viruses. Of course, both gangliosides and glycoprotein may be involved in the adsorption of a single virus because on approaching a cell the virus firstly would interact with glycoprotein before more intimate interaction with the plasma membrane containing gangliosides.

We have referred above to the impossibility of making generalized statements about virus receptors and an excellent example of the reason for this, namely, marked differences in virus cell receptor interaction by even genetically closely related viruses is described by Carroll et al. (1981). These authors describe the different adsorption properties of the RI/5$^-$ and RI/5$^+$ viruses with erythrocytes and attribute this to the different sialyloligosaccharide receptor specificities of their HAs. Erythrocytes were treated with *Vibrio cholera* neuraminidase to remove sialic acids and then modified with CMP–NeuAc and three purified sialyltransferases to contain either the NeuAcα2, 3 Gal, NeuAcα2, 6 Gal or NeuAcα2, 6 Gal NAc linkages on cell surface glycoproteins. The HAs of the two viruses RI/5$^-$ and RI/5$^+$ had totally different specificities, binding respectively to NeuAcα2, 3 Gal and NeuAcα2, 6 Gal linkages. A further implication of this study is that the receptor site and functional properties of the HA may change independently of the overall antigenic properties. Two antigenic sites B and D on the influenza HA molecule (Fig. 7.2) are near the receptor site and therefore it is possible that variations in receptor specificity might arise from natural selective pressure placed by host specified sialyloligosaccharide present on secreted or cell surface glycoproteins. Indeed, more recently, we have described the selection of subpopulations of influenza A and B viruses by cultivation of virus in MDCK cells or in embryonated hens eggs (Schild et al., 1983). The latter host system is extremely selective and clones out a population of virions with different antigenic composition, as detected with both monoclonal and polyclonal antibodies, compared to the same parental virus cultivated in MDCK cells. The novel implication is that a non-immune selective mechanism involving host cell receptor sites may operate to select and generate antigenic variants of influenza virus, thus contributing towards antigenic drift and evolution of the virus.

Influenza virus is redistributed on the cell surface soon after infection (Patterson and Oxford, unpublished data) but, in contrast to adenovirus, inhibition of redistribution is not paralleled by a block in virus uptake. These differences may reflect the number of membrane receptors available to the two viruses. If a sufficient number of suitable receptors are within close proximity of a bound influenza virus particle there would be no requirement for active movement or extensive diffusion of receptors. There is now evidence that receptor mediated uptake of enveloped viruses can occur at specialized sites on the plasma membrane known as 'coated pits'.

Ultrastructurally the sites are recognized by thickening on the cytoplasmic side of the membrane which is due to a 170K protein called clathrin. Receptor bound ligands are taken up at coated pits into coated vesicles (Dourmashkin and Tyrrell, 1970, Patterson et al., 1979) and then transported to lysosomes or other intracellular sites (Fig. 7.3). The clathrin coat dissociates soon after pincing off and is recycled back to the plasma membrane.

Until recently membrane fusion as a mechanism of entry and uncoating was thought to be mainly restricted to the paramyxovirus group (see Chapter 8) but now there is evidence that fusion plays a role in the uncoating of many enveloped viruses. What is not clear for many viruses is the *site* of fusion. Whether there is fusion

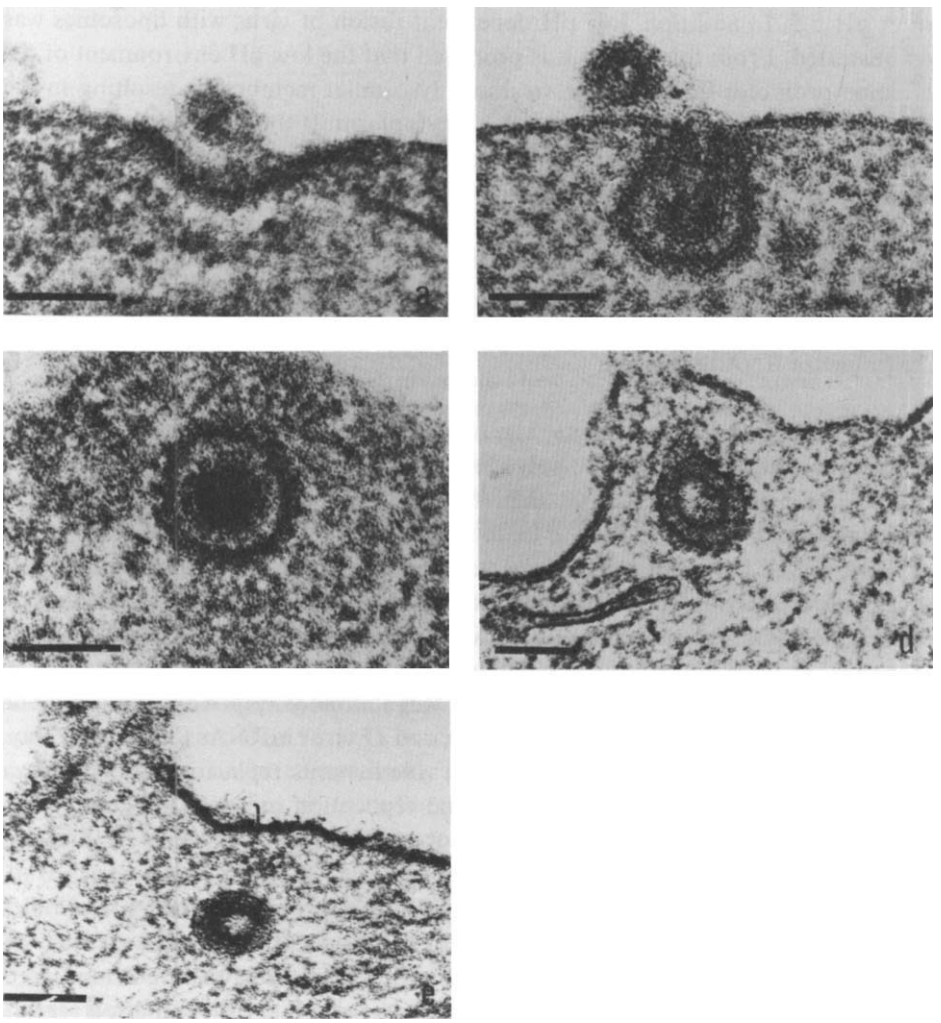


Fig. 7.3. Stages of infection of cell with influenza A virus. (Courtesy of Dr. S. Patterson.)

at the plasma membrane or uptake by pinocytosis into a cytoplasmic vacuole followed by fusion has been disputed for several different virus groups. The finding that the N-terminus of HA2 is highly conserved throughout the different influenza serotypes and is almost identical to the N-terminus of Sendai virus F1 protein has strengthened the fusion argument.

Thus, after uptake into a cytoplasmic vacuole the influenza virus still faces the problem of penetrating a cellular membrane and releasing its nucleic acid in order to initiate infection. In their studies on SFV, Helenius and his colleagues (1980) observed that virions entering via coated pits ended up in lysosomes. In parallel studies SFV was induced to fuse with the plasma membrane of cells by adsorbing cells with virus at 4°C and then briefly exposing the preparations to warm (37°C) medium at pH 5.5. In addition, low pH dependent fusion of virus with liposomes was demonstrated. From this data it was proposed that the low pH environment of the lysosome promotes fusion of the viral and lysosomal membrane, resulting in the release of the viral nucleic acid into the cell cytoplasm. It was claimed that the antiviral action of lysosomotropic agents such as chloroquine, amantadine and NH_4^+ was mediated by an ability to raise lysosomal pH (Ohkuma and Poole, 1978) and block virus infection at an early stage prior to transcription. We shall return to this later in this Chapter.

7.3. Influenza RNA replication

Two types of genome transcript are synthesised during infection, the virus mRNAs which are incomplete transcripts, polyadenylated at their 3′ ends and with additional non viral primer sequences at their 5′ ends, and unpolyadenylated complete transcripts which are considered to be the templates for genome replication (Smith and Hay, 1982). Different mechanisms are responsible for the production of these two classes of cRNAs but surprisingly little is known about the interrelationships between their synthesis and that of vRNA for example. More recent data, however, indicates that vRNA synthesis is regulated throughout infection and this may involve selective transcription of its template. Regulation of vRNA synthesis may be largely responsible for the controlled production of virus mRNAs (Smith and Hay, 1982). The cell nucleus plays an important role in virus replication for influenza is unable to replicate in enucleated cells and replication in nucleated cells is prevented by actinomycin D or mitomycin C treatment before, or early in infection (See Fig. 7.4. for extra details).

In certain respects the initiation of influenza virus RNA transcription resembles the initiation of protein synthesis on cellular messenger RNAs in eukaryotic systems. Affinity labelling and affinity chromatography experiments have demonstrated that a polypeptide of M.W. 24 K, a component of eukaryotic initiation factors 3 and 4B, is involved in cap binding in host cells. More recently it was shown that

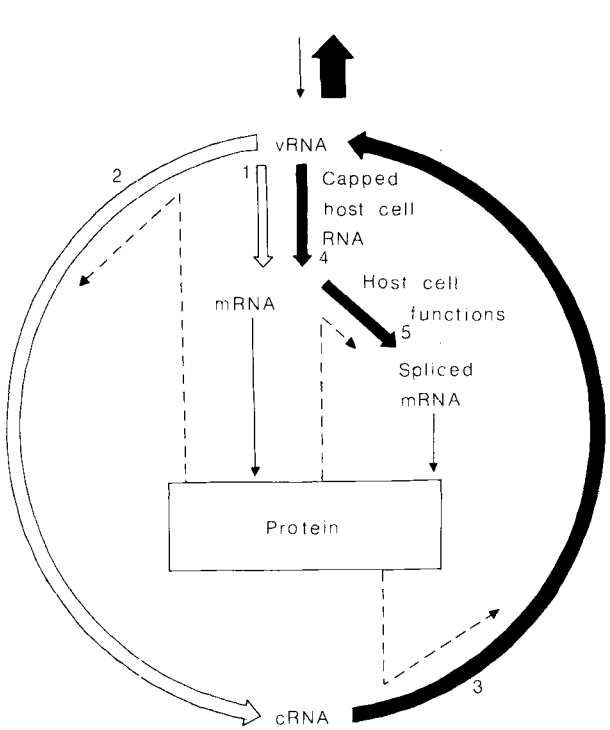


Fig. 7.4. A scheme for the replication of influenza virus genome (after McCauley and Mahy, 1983). ⇒, transcription from input genome RNA; →, transcription from cRNA or newly synthesized vRNA; ——→, necessary interactions. Step 1: primary transcription, which occurs in the absence of protein synthesis but requires host cell transcription. Step 2: cRNA synthesis from input genome vRNA . Step 3: selective and amplified vRNA synthesis directed by cRNA. Both require ongoing protein synthesis. Step 4: transcription of newly synthesized vRNA to form mRNA requires host cell caps. Step 5: modification of mRNAs 7 and 8 by splicing requires protein synthesis and undefined host cell functions.

in the presence of ATP and Mg ions oxidized reovirus mRNA is crosslinked to two proteins of M.W. 28 K and 50 K which may be structurally related to the 24 K polypeptide (Sonenberg, 1981). Studies with monoclonal antibodies indicate that the 24 K cap-binding polypeptide may be contained in even larger protein precursors (Sonenberg et al., 1981). It has also been demonstrated that translation of capped mRNAs can be competitively inhibited by various cap analogues (Both et al., 1976, Roman et al., 1976). Influenza virus has the unique feature to use host cell-coded capped RNAs as primers for the synthesis of its own mRNA (Fig. 7.5). In the priming reaction the cap together with some 10 to 15 nucleotides is cleaved from host RNA by a viral endonuclease and is subsequently used to initiate viral RNA transcription (Krug et al., 1981). As a result a host RNA-derived capped sequence is found to be linked to the 5′-end of the viral mRNA. This cap transfer reaction can also be performed in vitro using a variety of mRNAs such as globin mRNA or reovirus mRNAs as primers. The structural requirements for an RNA

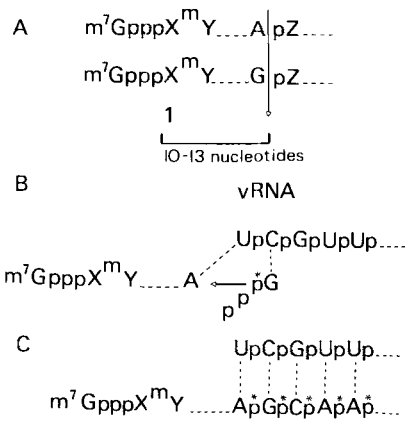


Fig. 7.5. Mechanism for the priming of influenza viral RNA transcription by capped RNAs (after Krug et al., 1981). A, cleavage; B, initiation; C, elongation.

to be active as a primer have been investigated and only RNAs containing a capped 5′-terminus can efficiently initiate viral mRNA synthesis. In addition to natural mRNAs, synthetic polynucleotides such as capped poly (A) and capped poly (AU) can prime viral transcription. Thus, binding of the capped RNA to the viral transcription complex is clearly caused by interactions other than base pairing with the viral template RNA. This leaves the cap structure as the only common signal of recognition of the primer RNA. Therefore it seems likely that some cap recognition site must be present within the polymerase complex and recently the cap binding protein has been established as the virion polymerase protein P2 (Blaas et al., 1982).

In more detail, Krug et al. (1981) identified those bases of a representative primer, β-globin mRNA, that were transferred to the viral RNA transcripts. Using ^{125}I-labelled globin mRNA as primer for in vitro transcription, he found that the predominant sequence at the 5′ end of each viral mRNA segment was identical to the first 13 nucleotides (plus the cap) at the 5′ terminus of β-globin mRNA, which has the sequence: m^{7}Gpppm^{6}1AmC(m)ACUUGCUUUU13GAC....[8]. Because only the C residues were labeled with ^{125}I, these results indicated that possibly the first 12, 13 or 14 5′-terminal bases of β-globin mRNA were transferred to the viral mRNAs (Fig. 7.5). Analysis of the minor ^{125}I-labeled oligonucleotides found in the viral mRNAs indicated that shorter, 5′-terminal fragments of β-globin mRNA (8–11, or 2–3 bases in length) were sometimes transferred and that the transferred pieces were most likely linked to G as the first base incorporated by the transcriptase.

The priming mechanism involves recognition of the 5′-terminal methylated cap structures (m^{7}GpppXm), because only RNAs containing a cap are active as primers. Removal of the m^{7}G of the cap by chemical or enzymatic treatment eliminates all priming activity, and this activity can be restored by enzymatically recapping the RNA. The absence of either methyl group from the cap of an RNA greatly re-

TABLE 7.1.
Influenza virus-coded proteins

Designation	Approximate no. of molecules per virus particle	Molecular weight estimated by:			Remarks
		Gel electrophoresis	Gene sequence	Gene+protein sequence	
P1 polymerase 1		96K			Internal proteins associated with RNA transcriptase activity. Cap binding
P2 polymerase 2	30–60	87K			
P3 polymerase 3		85K			
HA haemagglutinin	500				
HA1				36 074+11 500 (Mem/102/72)	Surface glycoprotein responsible for attachment of virus to cells.
HA2				27 368+1400 (Mem/102/72)	Trimer composed of two polypeptides HA1 and HA2 formed by post-translational cleavage of the primary translation product.
NP nucleoprotein	1000	50–60K	56 106		Internal protein associated with RNA and polymerase proteins, helical arrangement.
NA neuraminidase	100	48–63K	50 087		Surface glycoprotein, with enzyme activity. Tetramer molecule with a 200 000-*M*, head.
M1 matrix	3000			27 861	Major virion component surrounding the core, involved in assembly and budding.
M2 matrix		15K	11 000		Coded from the same gene segment as M1 in a second reading frame, a non-structural protein, function unknown.

TABLE 7.1. *(continued)*

Designation	Approximate no. of molecules per virus particle	Molecular weight estimated by:			Remarks
		Gel electrophoresis	Gene sequence	Gene+protein sequence	
NS1 non-structural protein		25K	26 815		Non-structural protein, function unknown.
NS2 non-structural protein		12K	14 216		Non-structural protein coded for in a second reading frame from same gene segment as NS1, function unknown, synthesized late in infection.

For additional references see McGeoch et al., 1976, Skehel, 1972, Lamb et al., 1980, Webster et al., 1982.

duces its priming activity and the absence of both methyl groups completely eliminates activity.

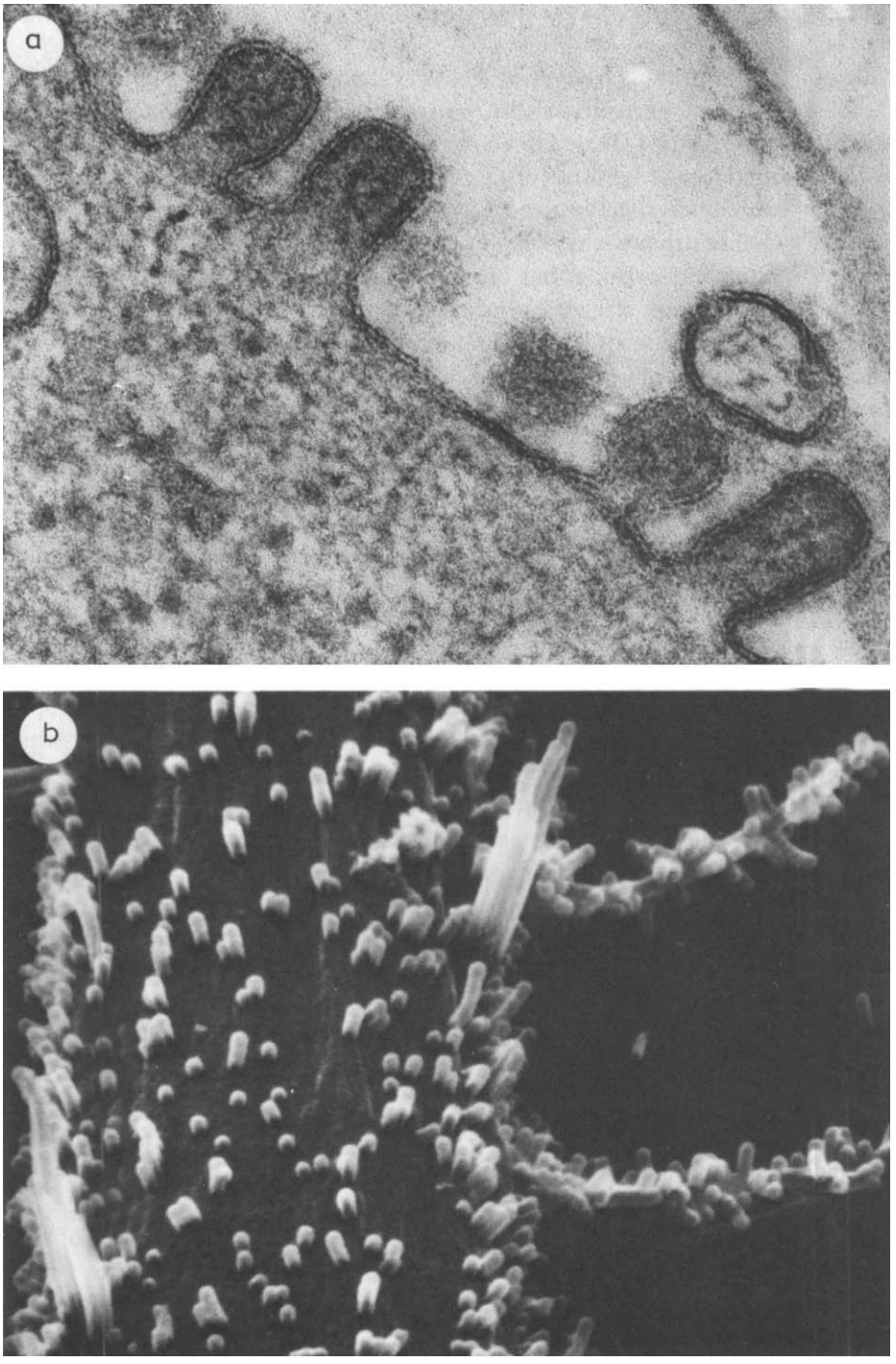
Because similar priming by capped RNAs also apparently occurs in the uninfected cell, the α-amanitin-sensitive (RNA polymerase II) step required for viral RNA transcription in vivo and alluded to above, is explained, i.e. the host RNA polymerase II is required for the synthesis of capped RNA primers. Only those capped cellular RNAs made after infection, and not those pre-existing before infection, serve as primers. One possibility why only newly synthesized capped cellular RNAs serve as primers is that pre-existing, but not newly synthesized, capped RNAs are tied up in ribonucleoprotein structures (including polyribosomes) and cannot be used by the viral transcriptase. If so, the viral transcriptase would be expected to function near the site of synthesis of capped cellular RNAs in the nucleus. Several lines of evidence have suggested that some steps in viral RNA transcription occur in the nucleus, but this has not yet been directly demonstrated. The above sequence of events represents a further target for the development of new inhibitors of influenza virus.

7.4. Later events in influenza virus replication

Much less is known about the later events of influenza virus replication. The virion proteins are synthesized and transported to sites of virus budding. The glycoproteins NA and HA are inserted in the plasma membrane, as detailed above, whilst presumably because of trans-membrane crossing of 'ends' of these glycoproteins, matrix protein aligns itself on the internal side of the plasma membrane. It is assumed that there are specific sites of interaction between NP (containing the RNA and P protein complex) and M, which results in spontaneous formation of complete particles which then bud from the cell surface (Fig. 7.6). Obviously, many questions remain to be answered including the mechanism (if any) for assuring the correct amount of RNA per virion, the triggering process for budding and details of budding itself. It is considered that enzymatic activity of NA ensures release of virions (since inhibition of NA activity results in aggregates of virus at the cell surface) and absence of self aggregation via NA–HA bonding.

7.5. Epidemiology of influenza virus

Influenza viruses are classified into three types – A, B and C – on the basis of their type-specific nucleoprotein and matrix protein antigens although the three viruses also show differences in biological and epidemiological properties. The majority of pandemics so far recorded have been caused by influenza A virus, which is also the type associated with influenza of avian, equine and swine species (Easterday, 1975,


a
b

Laver and Webster, 1979). Type B influenza causes more restricted epidemics and has only been isolated from humans, whereas type C causes even fewer clinical problems in humans, but has also been isolated in pigs (Guo et al., 1983). Type A influenza viruses are further classified into subtypes based on the antigenic characteristics of their surface antigens, haemagglutinin (HA) and neuraminidase (NA). Twelve distinct HA subtypes and nine NA subtypes are now recognized in the nomenclature system for influenza A viruses recommended by the World Health Organization, and a thirteenth HA subtype has recently been identified (Table 7.2). It is implicit in this system that all viruses with common H or N subtype have HA or NA antigens shown to be related by conventional laboratory tests, while such relationships do not occur between subtypes. However, included among viruses of a common subtype designation will be strains showing considerable degrees of antigenic variation ('antigenic drift'). Thus, the human virus A/Bangkok/1/79 (H3N2) contains HA and NA antigens demonstrably related to those of A/Hong Kong/68 (H3N2) virus but showing considerable degrees of difference from them.

Within strains of a single subtype, the differences in deduced amino acid sequence are generally less than 10% but recent extensions of the data have revealed differences in the N-terminal region of HA1 from two strains each of H7 and H10 viruses close to 20%, almost equal to the difference between the amino acid sequences of the two closest subtypes (H2 and H5). Thus, the change from H2 to H3 must involve a more drastic mechanism. Several possibilities could be mentioned to explain this 'antigenic shift': (1) the 'new' virus may have caused an epidemic in man many years previously and have remained hidden ever since. Evidence for this kind of event has been obtained. Thus, the strain of 'Russian flu' (H1N1) which reappeared in Anshan in northern China on 4 May 1977 and subsequently spread to the rest of the world, seems to be identical, in all genes, to the virus which caused an influenza epidemic in 1950. This 'hidden place' could include an animal reservoir, refrigerator or even a persistent or latent infection of humans. (2) Some of the 'new' viruses may be derived from animal or avian viruses. One strain of human influenza has been shown to be such a recombinant (reassortant). The Hong Kong (H3N2) virus contains the NA (and other) genes from an Asian (H2N2) strain of human influenza and an HA which is antigenically related to that of A/Duck/Ukraine/63 (H3N8) and A/equine/2/Miami/63 (H3N8) viruses. The amino acid sequence homology between the HAs of A/Duck/Ukraine/63 and A/Aichi/2/68 viruses (both of subtype H3) is 96%. The donating virus could have been one which contained Hong Kong HA that had persisted from a much earlier human influenza epidemic maintained unchanged in the same way as the Russian 'flu. (3) An animal or bird virus could become infectious for man (Easterday, 1975, Laver and Webster, 1979). This unpre-

←

Fig. 7.6. Influenza A viruses budding from infected cells. a, sectioned cell with budding viruses (×195000) b, scanning electron micrograph of influenza virus budding from human diploid cells (×26000). (Courtesy of Dr. Hockley.)

TABLE 7.2.
Classification of influenza viruses: proposed grouping of haemagglutinin and neuraminidase antigens

Proposed new designation	Previous subtype designation	Proposed prototype strains
H1	H0	A/PR/8/34
	H1	A/FM/1/47
	Hsw1	A/swine/Wisconsin/15/30
H2	H2	A/Singapore/1/57
H3	H3	A/Hong Kong/1/68
	Heq2	A/Eq/Miami/1/63
	Hav7	A/duck/Ukraine/1/63
H4	Hav4	A/duck/Czech/56
H5	Hav5	A/tern/South Africa/61
H6	Hav6	A/turkey/Mass/3740/65
H7	Heq1	A/equine/Prague/1/56
	Hav1	A/FPV/Dutch/27
H8	Hav8	A/turkey/Ontario/6118/68
H9	Hav9	A/turkey/Wisconsin/66
H10	Hav2	A/chick/Germ/N/49
H11	Hav3	A/duck/England/56
H12	Hav10	A/duck/Alberta/60/76
N1	N1	A/PR/8/34
		A/FM/1/47
		A/swine/Wisconsin/15/30
N2	N2	A/Singapore/1/57
		A/Hong Kong/1/68
N3	Nav2	A/tern/South Africa/61
	Nav3	A/turkey/England/63
N4	Nav4	A/turkey/Ontario/6118/68
N5	Nav5	A/shearwater/E.Aust/72
N6	Nav1	A/duck/England/56
N7	Neq1	A/equine/Prague/1/56
N8	Neq2	A/equine/Miami/1/63
N9	Nav6	A/duck/Memphis/546/74

dictability is one of the major worrying features about influenza, together of course with its demonstrated virulence for humans and explains why so much work is carried out with the virus.

Mortality data from 1200 million people living in 88 of the 156 or so WHO member states (approximately one quarter of the world's population) show that more than two million deaths from acute respiratory disease occur annually (reviewed by Lennette, 1981) and that the majority of acute respiratory disease is attributed to viruses of 5 families. Influenza A and B viruses cause a very significant number

of these deaths. The highest mortality during an influenza epidemic is always noted among certain groups which are identified as 'special risk groups'. We shall see the significance of this group of persons later when we discuss prevention of influenza by vaccines and chemoprophylaxis, because special risk persons constitute the main target groups. Mass vaccination and chemoprophylaxis against influenza is not generally practiced because of the absence of broad spectrum antivirals and the less than 100% efficacy of the current vaccines. Persons in these special risk groups are old people (generally over 65 years of age (see Table 7.3)), babies under 18 months of age, persons with diabetes or chronic heart, kidney and respiratory ailments. As early as 1847, William Farr estimated the impact of influenza in London by estimating excess mortality, viz. he subtracted the number of deaths in an influenza-free winter from the number of deaths in an epidemic winter. Stepwise multiple regression models are now used (Tillett et al., 1980) to obtain these estimates using, in the UK, data from the office Population Censuses and Surveys and generally figures over an 8–11 year period are analyzed. Factors known to influence the 4 weekly returns include seasonal variation and time trends in morbidity and mortality. The results of such an analysis are shown in Table 7.3. The influenza outbreak in 1975–76 was caused by two influenza A (H3N2) variants and also influenza B viruses and 22 250 excess deaths were recorded, most of them due to respiratory causes. This represents the deaths of approximately 1 in 275 of the elderly population in the UK. In the same winter there were 908 000 excess sickness benefit claims, equivalent to an illness in approximately 1 in 20 of those persons insured (viz. 56% of the 16–64 age group). So the total morbidity in the adult population was 1.6 million. This analysis does not include children.

It is widely recognized therefore that influenza A and B viruses form very worth-

TABLE 7.3.
Estimates of excess deaths and sickness benefit claims in England and Wales attributable to influenza (after Tillett et al., 1980)

Winter	Excess total deaths all causes	Excess deaths respiratory causes	Excess deaths 65+ age group all causes	Excess new claims for sickness benefit (×1000)
1975–76	22 250	17 210	19 590	908
1976–77	4060	5790	NA	322
1977–78	5910	6080	NA	364
1978–79	5430	5500	NA	237
Approx. standard error of estimates	5000	2500	4300	225

NA, data not available

while 'targets' for new drugs and vaccines. Although influenza A virus is one of the most studied viruses, particularly from the point of view of molecular biology of virus replication, morphology and antigenic structure, many important questions concerning the biochemical basis of virulence and even the epidemiology of the virus remain unanswered. Specific preventive measures are often undertaken haphazardly and with no very great enthusiasm in many countries.

7.6. Influenza – clinical features

Characteristically the illness begins suddenly (incubation period 2–3 days) with shivering, malaise, headache and aching of limb muscles and a pain in the back (Christie, 1980). The temperature rises rapidly and the afflicted person takes to bed. The main feeling is one of general malaise, pain and discomfort. The temperature ranges from 38.3 to 40.5°C and commonly cheeks may be flushed, eyes infected, throat congested. Fortunately the illness is most often short lived, the temperature drops by day 3 and the patient is out of bed by day 5 or 6 and working on day 10. However, in older patients the recovery period can be quite prolonged and weakness and lassitude marked. In general, mortality figures suggest 1 death in 6000 cases. Young children tend to have a milder disease than older children or adults (reviewed in Stuart-Harris and Schild, 1976). Aspects of diagnosis of influenza are also considered in Fig. 7.10.

Chest complications tend to occur in patients previously suffering from chronic bronchitis or rheumatic, ischaemic or hypertensive heart disease. Pneumonia may result from infection by the virus itself, invasion by staphylococci after virus damage of epithelial cells or invasion by pneumococci. Viral pneumonia presents as an overwhelming infection with profound toxaemia – so much so that the respiratory signs may not be noticed. In staphylococcus pneumonia the clinical signs may be more noticeably respiratory and the person may have partially recovered from influenza when the recovery is interrupted by a sudden onset of dyspnoea. Cyanosis may be featured with signs of shock and collapse of blood pressure. Pneumococcal pneumonia may often be less severe and onset may be delayed until the patient appears to be at the post influenza convalescent stage. Classical signs of lobar consolidation are noted but the response to antibiotics is usually rapid. A rare complication, which is frequently fatal, of influenza B (and influenza A) infection is Reye's syndrome, a fatty degeneration of the viscera and especially the liver.

7.7. Pathogenicity

The main individual aspects of the pathogenicity (viz. virulence) of any virus are mucous surface interaction, entry to host tissues, virus replication, avoidance of

host defences spread, damage to the host, and tissue or organ specificity (reviewed by Sweet and Smith, 1980). The overall 'virulence' of the virus may depend on complex interactions between these varying factors, or, in some cases a single factor will predominate (Mims, 1982, Bosch et al., 1979, Burnet, 1979, Scholtissek et al., 1977, Rott et al., 1976).

Assessments of the degree of virulence of a pandemic and moreover an antigenically variable virus such as influenza is probably impossible, because it is difficult to establish if recorded differences in mortality, for example, between the 1918 influenza pandemic and the 1957 pandemic (estimated 16 times higher mortality in 1918) were caused by an increased virulence of the virus causing the former pandemic or whether the immune status of the population was different, or whether additional factors such as population movements, unusual foci of high population density (because of the world war) contributed. Nevertheless it is quite clear that variations in virulence of influenza viruses do occur in the laboratory.

Following an infection in humans, influenza virus invades the epithelial surfaces of the respiratory tract and many different types of cells can be infected including ciliated, intermediate, basal and goblet cells of the nasal epithelium or more rarely in the case of pneumonia, cells lining the alveoli (reviewed by Sweet and Smith, 1980). Humans may be infected initially by the settling of large particles containing virus in the upper respiratory tract or more fine particles in the lower respiratory tract. It is particularly interesting that, most commonly, influenza is an infection of the *upper* respiratory tract in humans. It can be speculated that in the lung, alveolar macrophages may be particularly efficient at removing and destroying virions. The presence of non-specific inhibitors such as mucoproteins can neutralize influenza virus infectivity by preventing attachment of virus to new susceptible cells. Influenza virus can be carried and hence spread downwards in the respiratory tract by mucociliary action or downward drainage and rarely (in humans) can infect lung tissue and cause death by primary virus pneumonia.

7.8. Inhibitors of influenza virus

Table 7.4 summarizes (although not exhaustively) attempts over the last 3–4 decades to obtain effective inhibitors of influenza A and B virus. Only two compounds (apart from interferon) have confirmed antiviral activity in man, namely amantadine and ribavirin, and therefore particular attention is devoted to these two molecules. Amantadine can be used as an excellent illustration of the problems and frustrations of developing an anti-influenza virus compound (Tables 7.5, 6).

We would like to emphasize two important points at this stage. Firstly, influenza virus may represent a rather unique virus where chemotherapy *and* vaccines may have to be used as a complete strategy for virus control in the field. Secondly, it should be realized that most of the past searches for new influenza inhibitors have

TABLE 7.4.
Inhibitors of influenza virus

Function	Inhibitor	Mode of action
Virion attachment and penetration	Sulphated polysaccharides	Inhibition of virus adsorption
	Amphotericin B methyl ester	Acts on lipid membranes
	Amantadine hydrochloride and derivatives	Inhibition of initiation of transcription by virion transcriptase, or fusion events
RNA transcription	Halogenated ribofuranosyl-benzimidazoles	Unknown
	Ribavirin triphosphate	Selective inhibition of viral RNA polymerase?
	Gliotoxin	Reacts with sulphydryl groups
	Aranotin	
	Selenocystine	
	2-Acetylpyridine-3-thiosemicarbazone (2-APTSC)	High activity against transcriptase enzyme in vitro but not in vivo
	Ca-EDTA and Ca-DTPA liposomes	Act against zinc metalloenzyme by formation of apoenzyme zinc-ligand complex
	Triphenylmethane compounds	Active against RNA transcriptase
Viral protein synthesis and later events of transport of viral proteins	Plant antiviral peptide (PAP)	Elongation of proteins. Effect on ribosomes?
	2-Deoxy-D-glucose	Inhibition of glycosylation
	Glucosamine	
	Tunicamycin	
	Mithramycin	Inhibited HA, NA and M protein protease inhibitors
	Canavanine	Inhibition of RNP formation
	Concanavalin A	Inhibits release of newly formed virions
	FANA	Inhibits virus budding, effective in tissue culture, but not in mice. Neuraminidase inhibitor

See also Oxford, 1977

been carried out using biological screening procedures and more or less random testing of synthesized molecules (Swallow, 1978). Of course, in retrospect, it is not possible to deduce whether the rather depressing lack of success has been due to the particular features of the virus itself, or alternatively to the 'randomness' of the search (Galasso et al., 1979). This question may be answered in the next few years as more groups utilize what appears to be more 'designed' approaches searching for inhibitors of RNA transcriptase, fusion, neuraminidase, mRNA capping etc. Sequence studies of HA, NA and other virus proteins have now provided a vast amount of hard biochemical data on which new more logical screening procedures

TABLE 7.5.
Biological activities of amantadine and recently synthesized analogues

Molecule[a]	Chemical structure	Biological activity
1	amino adamantane (amantadine)	Influenza A virus inhibitor in vitro, in vivo and in man. Effective in Parkinson's disease
2	α-Methyl-1-adamantane methylamine (rimantadine)	Influenza A virus inhibitor in vitro, in vivo and in man (no activity in Parkinson's disease)
3	1-amino-3,5,dimethyl-adamantane (memantine, DMAA)	More potent than amantadine as stimulator of motor activity. Parkinson's drug
4	1 substituted adamantyl hydrazine derivative	Anti mycoplasma and fungal and herpes (HSV) activity
5	N substituted 1 adamantyl carboxamidines	Antiviral activity versus influenza A, vaccinia and herpes virus. The 4 amino derivative had anti polio activity whilst the adamantyl acetamidine inhibited NDV
6	acylated adamantyl thio urea derivatives	In vivo anti influenza A activity (not active versus HSV, SFV or Sendai)
7	alkyl substituted thio ureas	In vitro activity versus HSV, vaccinia and adenovirus
8	amide derivatives: N-(1-adamantyl) cinnamide	In vitro versus influenza, HSV, vaccinia. In vivo versus influenza A
9	propyl carbamate	antifungal
10	4 homotwistane derivatives	Amino and amino ethyl analogues were active against NDV
11	aminospirane	In vitro against influenza A and B viruses. In vivo activity versus influenza A. The dialkyl aminoalkyl derivative inhibited rhinovirus type 14 in vitro
12	2 substituted 1-(aminoalkyl) adamantanes	Some molecules may have anti Parkinson's activity
13	phencyclidine analogues	Anti cholinergic activity
14	bicyclo-octyloxyaniline	Hypo beta lipoproteinaemic agent

[a]See Fig. 7.7 for molecular structure of the compounds.

can be based. An excellent example of such a new approach is the synthesis of short peptides, whose sequence is deduced from that of the known fusion sequence at the N terminus of HA2 (Richardson et al., 1980).

7.9 Amantadine

The cyclic primary amine Amantadine (Symmetrel) was the first, and remains at present the only, inhibitor of influenza A virus replication to be licensed in several

TABLE 7.6.
Brief history of the development of amantadine as an antiviral compound

Event	Reference
1. Influenza A and B viruses shown to be inhibited by amines and ammonium compounds	Eaton et al. (1962)
2. Amantadine selected as an amine with in vivo activity but only against influenza A viruses	Davies et al. (1964) Grunert et al. (1965)
3. Mode of action of amantadine established approximately as inhibiting an early event of virus replication	
4. Clinical efficacy of amantadine described in prophylactic studies in volunteers with H2N2 viruses	Jackson et al. (1963)
5. In vitro and in vivo drug resistant H2N2 viruses isolated	Oxford et al. (1970)
6. Clinical efficacy of therapeutic administration	Hornick et al. (1970)
7. 'Re-emerged' H1N1 viruses and also H3N2 viruses inhibited in vitro and in clinical studies	
8. Aerosol usage of amantadine investigated in experimental and clinical pneumonia	
9. Clinical comparison of amantadine and rimantadine show approximately comparable antiviral activity but rimantadine may be less toxic	Zlydnikov et al. (1981) Dolin et al. (1982)
10. Mode of action of amantadine reinvestigated to include fusion events and lysomotropic activity. Multiple modes of action?	White et al. (1981) Bukrinskaya et al. (1980)
11. General consensus that rimantadine (200 mg/day) is less toxic than amantadine (200 mg/day) although this may relate to different pharmacokinetics. Rimantadine is recommended therefore for prophylaxis and *either* compound for therapy.	Galasso et al. (1984)

Note that amantadine is also used in the treatment of Parkinson's disease (Timberlake and Vance, 1978, Schwab et al., 1972)

countries and to be used as an antiviral in the clinic and general community (reviewed by Oxford and Galbraith, 1980, Hoffman, 1980, Smorodintsev et al., 1970, Tables 7.5 and 7.6). Nevertheless, neither the original compound nor molecular derivatives (Table 7.5) such as rimantadine (Zlydnikov et al., 1981, Indulen and Kalninya, 1980) have gained universal acceptance among clinicians as fully effective compounds to be used prophylactically or therapeutically against influenza virus infections. There is little doubt that the relatively poor usage of amantadine has reduced interest in the search and development of new antivirals against influenza and other respiratory viruses, and it is of some importance to establish if the problems faced by amantadine are unique to this compound or would be similar for other (and hopefully even more effective) antivirals against these respiratory viruses. It could be added that in comparison to the dearth of antivirals against respiratory viruses, compounds acting against herpes virus infections have been developed (acyclovir, and PFA) and may soon be established as the first antivirals to be used on a large scale in the community (see Chapters 12, 13).

7.9.1. Inhibition of influenza A viruses by amantadine – do different influenza A virus subtypes vary in their degree of inhibition by the compound?

Laboratory studies of the inhibition of influenza viruses by amines (including amantadine) and ammonium compounds established quite early that the spectrum of antiviral activity of certain (but not all) of these compounds was somewhat restricted (Fig. 7.7). Thus, although certain simple primary amines such as methylamine inhibited both influenza A and B virus in vitro (Eaton et al., 1962, Oxford and Schild, 1968) amantadine had little or no effect against influenza B viruses and only inhibited influenza A viruses (Tables 7.7, 8). In addition, studies indicated some difference even between influenza A viruses of different subtypes as regards their degree of inhibition by amantadine. Certain viruses, such as A/PR/8/34 (H1N1), were poorly inhibited by the compound, whereas viruses of the H2N2 subtype, for example, were well inhibited (Grunert et al., 1965, Schild and Sutton, 1965). In retrospect this 'increased' resistance might have been caused by the multiple mutations undoubtedly accumulated by A/PR/8/34 virus during extensive laboratory passage.

This early laboratory data which showed an apparent heterogeneity of response of influenza A viruses to inhibition by amantadine gave rise to concern that new epidemic subtypes of influenza A might not be inhibited by amantadine. As a result the compound was only licensed initially in the USA for prophylaxis of influenza A viruses of the H2N2 subtypes, which were demonstrably sensitive to the com-

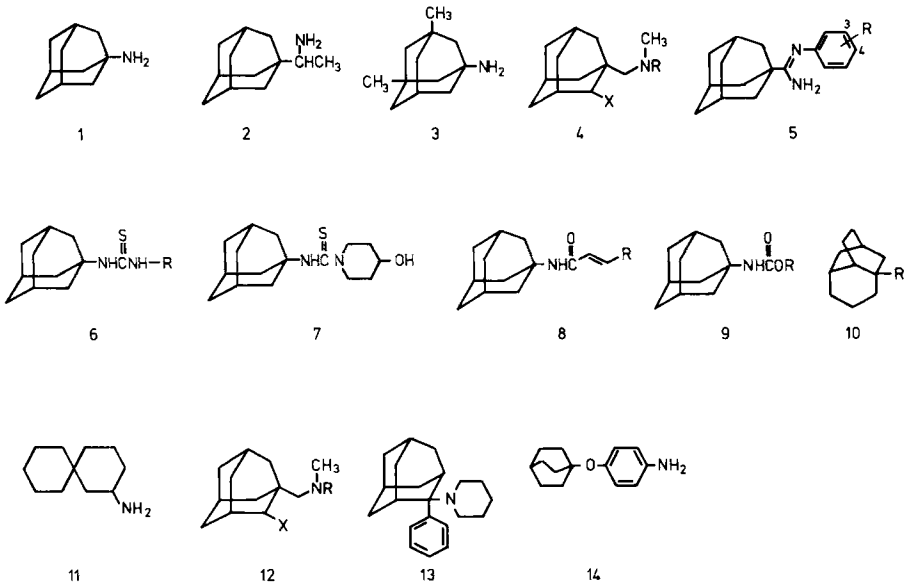


Fig. 7.7. Amantadine molecules tested for antiviral activity (see Table 7.5).

TABLE 7.7.
Inhibition of influenza A and B viruses by amantadine and ammonium ions

Virus	Reduction in virus end point titre ($log_{10}TCID_{50}$/ml)		
	Amantadine (25 μg/ml)	Ammonium acetate (100 μg/ml)	Rimantadine (25 μg/ml)
A/NWS(H1N1)	2.0	1.7	1.5
A/Singapore/1/57(H2N2)	2.5	3.5	Not tested
A/Scotland/49/57(H2N2)	6.0	4.5	5.5
B/England/13/65	0.5	2.6	0.5

MK cell cultures were infected with influenza viruses and incubated in the presence or absence of drugs for 3–4 days. Virus infectivity endpoints were determined by haemadsorption. A reduction of 1.0 $log_{10}TCID_{50}$/ml or 90 per cent inhibition of virus growth is considered significant in this test.

pound. When the new pandemic influenza A/Hong Kong/68 (H3N2) virus appeared, an opportunity was lost to use and test the compound on a large scale during the first wave of the epidemic at a time when no influenza vaccine was available. However, the compound is now licensed in the USA and UK for the prophylaxis and therapy of *any* human influenza A virus.

In summary, therefore, it would appear that most but not all (see below) recent and unpassaged influenza A viruses of the different human subtypes (H1N1), (H2N2) and (H3N2) are well inhibited by amantadine in vitro and in laboratory animal model systems. Furthermore, with the earlier H2N2 virus isolates a good correlation was established in most, but not all studies, between the in vitro inhibitory effect of amantadine and clinical efficacy and therefore it was anticipated that the recent H1N1 and H3N2 viruses would be inhibited in clinical practice. Recent clinical trials have confirmed this optimism (see below).

7.9.2. Amantadine-resistant influenza viruses

Earlier studies (Oxford et al., 1970) showed that amantadine-resistant influenza A viruses could be selected by passage of virus in mice treated with very high (150 mg/kg/day) doses of the compound. Before passage in vitro the influenza A virus was inhibited by 0.3 μg/ml amantadine, whereas after a single passage in the presence of the drug, 6 μg/ml of amantadine was required to inhibit replication of some virus isolates. After six passages in vivo most influenza strains were completely resistant to amantadine and the related drug rimantadine.

A number of laboratories have since investigated the genetic basis of amantadine resistance by producing virus recombinants between amantadine resistant and

TABLE 7.8.
Inhibition of polypeptide synthesis of influenza A and B viruses by amantadine

Virus	% Inhibition of virus induced polypeptide synthesis by 25 μg/ml amantadine		
	NP	NS1	M
Hswine1N1			
A/NJ/8/76	80.0	92.0	80.5
H1N1 subtype			
A/Jap/93NS/78	69.8±15.3	91.7±3.2	99.1±1.3
A/Lackland/AFB/3/78	90.5	96.6	94.0
A/Brazil/11/78	96.5	98.4	ND
A/Fukushima/78	95.0	95.0	ND
H2N2 subtype			
A/Leningrad/549/80	77.4	91.9	86.8
H3N2 subtype			
A/England/641/78	87.4	86.8	86.5
A/England/939/78	83.9	93.8	93.1
A/England/938/78	60.2	87.5	69.0
A/Alaska/78	73.9	88.2	99.2
A/Bangkok/1/79	74.3	90.1	93.6
Influenza B/Singapore/79	0	0	0

Vero cell cultures were infected with 10 EID_{50}/cell of virus and incubated in the presence or absence of amantadine overnight when the cells were pulsed with ^{35}S methionine for 30 min and cell lysates analysed by electrophoresis in polyacrylamide gels. Following autoradiography, the quantities of virus induced polypeptides were estimated by densitometer analysis of autoradiographs and analysis of the tracings using a Kontron MOP digiplan apparatus. Note that polypeptide synthesis of all the influenza A viruses is well inhibited by amantadine but no antiviral effect is detected against a representative influenza B virus.

amantadine susceptible influenza A viruses (Scholtissek and Faulkner, 1979, Lubeck et al., 1978). In this way transfer of drug resistance can be correlated with transfer of a particular gene or group of genes. At present the results from different laboratories are somewhat conflicting, although several groups agree that gene 7, coding for matrix protein, appears to co-segregate with amantadine resistance. The interpretations, however, are complicated to some extent by the observation that using different in vitro techniques the same influenza A virus may appear inhibited or resistant to the drug. In addition, data on determinants of influenza virulence suggest a multi-gene linkage with these biological properties. It is quite possible, however, that several gene products are involved in the mode of action of amanta-

dine and that the product of gene 7 may have 'helper' activity.

At present little extensive field work has been carried out to search for rimantadine or amantadine resistant viruses in contacts or in persons being treated for influenza. This is an important aspect to investigate since it is quite likely that amantadine resistance could spread among field viruses by genetic reassortment. Some influenza H3N2 or H1N1 viruses circulating at present in the community are known to be recombinants (see Chapter 17) containing genes of both virus subtypes and thus intra- or intertypic recombination is probably occurring with a relatively high frequency (Ghendon et al., 1981). Heider et al. (1981) have recently reported two relatively resistant influenza A H3N2 virus isolates in Berlin where rimantadine has not been used as a prophylatic. However, even the amantadine sensitive viruses in this work showed a rather poor dose response to amantadine and therefore the study needs to be extended and confirmed. Our own studies in the UK would suggest that occasional field isolates of H3N2 and H1N1 viruses are partially resistant to amantadine.

7.9.3. Inhibition of virus replication by amantadine — mode of action at the molecular level

Early biological studies demonstrated that amantadine acted at an early stage in influenza A virus infection and later, more detailed studies established the point of action at approximately the late stage of virus uncoating (reviewed by Oxford and Galbraith, 1980). More recently, recognition that the N terminus of the HA2 polypeptide of influenza haemagglutinin has an amino acid sequence similar to that of the fusion sequence of the F protein of Sendai viruses, and also the demonstration of fusion and haemolysis events between influenza viruses and cells at low pH (White et al., 1981) have led to the hypothesis of an important role of fusion during infection of cells with influenza A virus. A possible re-interpretation of the above data on the mode of action of amantadine at present would be that influenza viruses penetrate susceptible cells by viropexis and thus enter the cell cyptoplasm in coated vesicles (Fig. 7.8). Comparable pre-lysosomal cytoplasmic vacuoles have been shown to have a low pH and under these conditions influenza HA-mediated fusion could occur, since a configuration change in the HA could result in the N terminus of HA2, which is normally some distance from either end of the molecule contacting the membrane of the vacuole (Wilson et al., 1981). Fusion of the viral and vacuole membranes would then occur, resulting in release of viral RNA and subsequent transport to the cell nucleus, where initial viral RNA transcription is known to occur. Amantadine and other amines are known to increase the pH of intracytoplasmic vacuoles and so the drug could act by simply increasing the pH to 6.5 or 7.0 when fusion could not occur and viral infection would be blockaded. The hypothesis is most attractive, but some important observations remain contradicting at present. Thus, as we noted above, amantadine resistance is known to be cor-

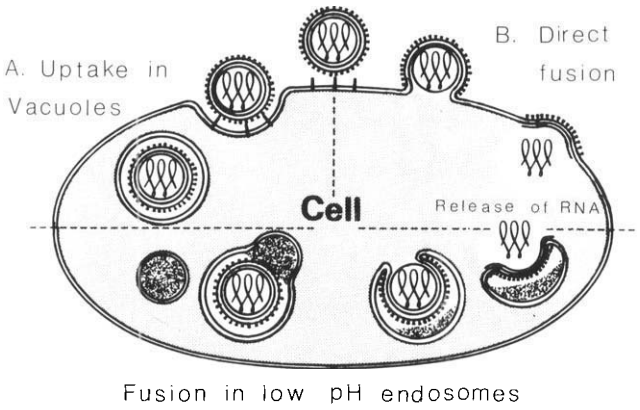


Fig. 7.8. A possible mode of action of amantadine in preventing fusion (drawing courtesy of Dr. D. Hockley).

related with gene 7 (coding for M protein) and not gene 4 (coding for HA). In addition, there is the problem also referred to above, of why some viruses such as influenza B would be resistant to amantadine. Finally, in a recent study Richman et al. (1981) showed that when amantadine-treated cells were washed in compound free medium, though relatively high concentrations of amantadine remained intracellularly, the cells were now *susceptible* to infection. Yet immediately before, with amantadine in the culture medium and equilibrated in the cytoplasm, the cells resisted infection. This implies an antiviral role of amantadine at the superficial external plasma membrane of the cell rather than an intracellular action. Earlier biological experiments with amantadine-treated cells showed that after incubation with trypsin (which removed surface adhering material and presumably amantadine) cells become susceptible to viral infection.

We have investigated the pH optimum of haemolysis (Fig. 7.9) of a number of A and B viruses as part of a separate study on biological characteristics of field isolates of influenza virus. If the pH optimum of fusion as shown by haemolysis for influenza B viruses and drug-resistant A viruses was higher than for amantadine sensitive influenza A viruses, fusion and hence infection would proceed even in the presence of amantadine at pH 6.6, whereas corresponding events would be blocked with most amantadine-sensitive influenza A viruses. Although a small but reproducible difference in pH optimum and pH maximum for haemolysis was noted between an amantadine-resistant virus (maximum pH 6.2) and the parental amantadine-sensitive virus (maximum pH 5.6) this was nevertheless close to the range of pHs shown by other influenza A viruses (Table 7.9). In addition, recombinants such as X-49 and NIB-4, NIB-7 and NIB-8 which are known to have inherited gene 7 and other genes from A/PR8/34 (H1N1) virus, and are thus relatively resistant to amantadine like the A/PR8/34 parent, show a similar pH optimum and maximum for haemolysis to the second parental strains which are all inhibited by amantadine.

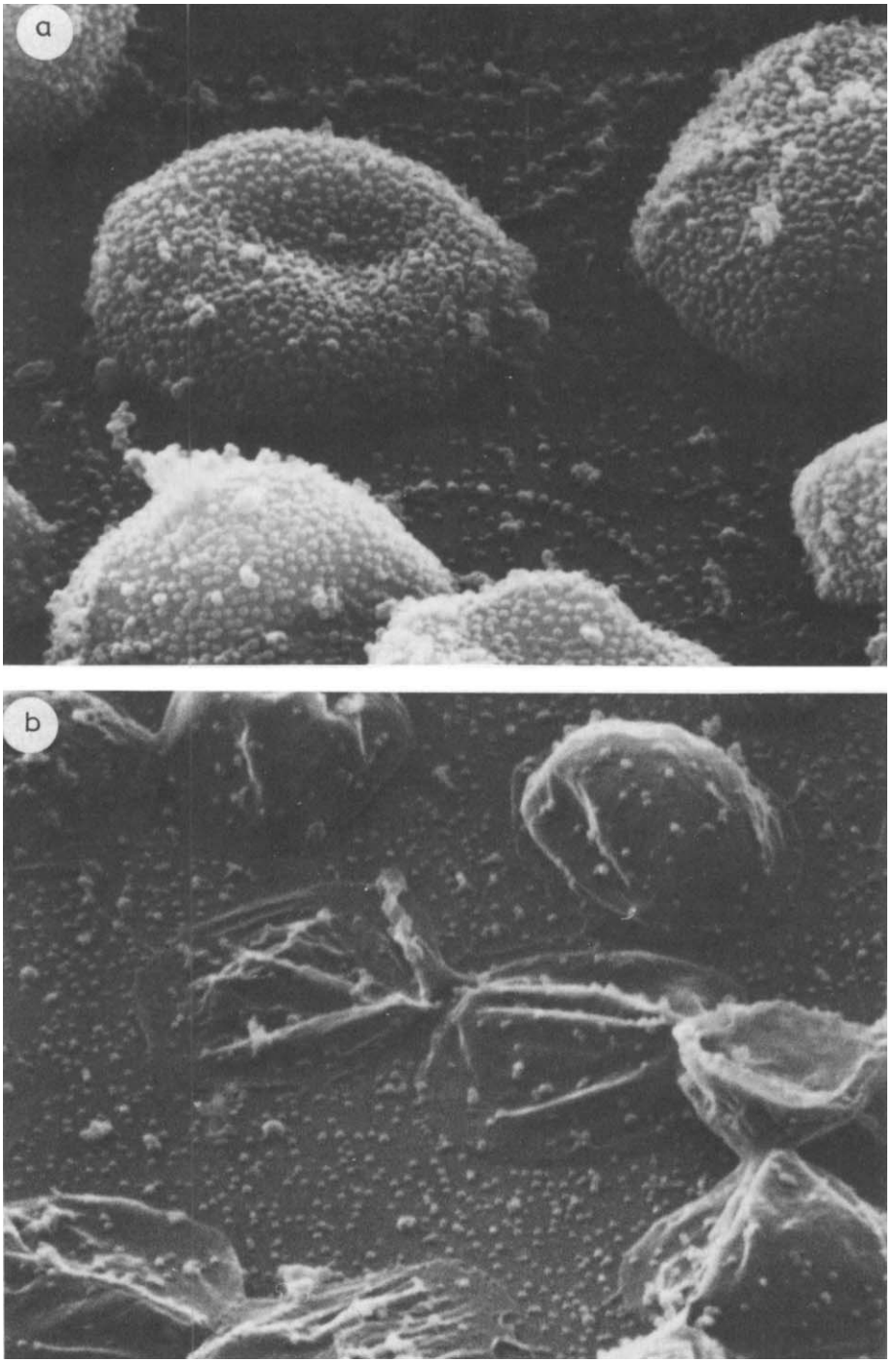

a
b

TABLE 7.9.
pH optimum and pH maximum of haemolysis for a range of influenza A and B viruses

Viruses		pH maximum	Amantadine resistance
B/HK/73		5.8	+
A/Leningrad/80	(H2N2)	5.2	0
A/Brazil/78	(H1N1)	5.4	0
A/PR/8/34	(H1N1)	5.3	+
A/USSR/77	(H1N1)	5.1	0
A/Bangkok/79	(H3N2)	5.6	0
Recombinants			
NIB-8		5.6	+
NIB-4		5.4	+
NIB-7		5.3	+
Passaged strains			
A/Krashnidor/59	(H2N2)	5.6	0
A/Krashnidor/59R	(H2N2)	>6.2	+

Note that A/Krashnidor/59/R had been derived from the amantadine-susceptible parent by in vitro passage in the presence of rimantadine (M. Indulen, personal communication). Approximately 10 μl of purified virus (10 mg/ml) was adsorbed at neutral pH to 0.1 ml of 10% suspension of erythrocytes. 2 ml of phosphate-citrate buffer was added at pH varying from 5.0 to 6.5 in 0.1 unit steps. After incubation at 37°C for 1 h the lysed cells were deposited by centrifugation and the optical density of the supernatant fluid determined using a Unicam SP 1750 spectrophotometer to estimate the degree of haemolysis compared with RBC incubated in the absence of virus. pH maximum is the higher pH at which significant haemolysis occurred.

Influenza B/HK/73 virus showed a high pH optimum and a high pH maximum of haemolysis, but B/Le/40 was similar to influenza A viruses in its pH profile although neither virus is inhibited by amantadine (Table 7.9). Finally, as anticipated, high concentrations of added amantadine did not inhibit haemolysis of red blood cells by amantadine-sensitive influenza A viruses.

Thus, further work is required to investigate and establish any role for a fusion event, to determine if low pH haemolysis of red blood cells is caused by a fusion process and to look for a correlation between pH optimum for this biological event and amantadine resistance or susceptibility.

←

Fig. 7.9. Low pH induced haemolysis of RBC studied by scanning electron microscopy. a, red blood cells at normal pH with virions adsorbed; b, red blood cells with virus attached at pH 6. – extensive lysis of cells has occurred. (Courtesy of Dr. D. Hockley.)

7.9.4. Possible influence of genetic and phenotypic variation among influenza viruses on amantadine-induced protection

Genetic and phenotypic heterogeneity of viruses may be of considerable practical importance in attempts to control certain virus diseases by chemo- or immunoprophylaxis (see Chapter 17). Thus, certain naturally circulating influenza A viruses may be resistant to antiviral drugs such as amantadine by virtue of mutations in gene 7 or may differ in virulence, antigenic or biological properties and thus may be able to circumvent drug induced protection. Studies of influenza A and B viruses circulating in the community have shown that quite extensive genetic and phenotypic variation occurs. Thus, a number of recently isolated influenza viruses of both H3N2 and H1N1 antigenic subtypes have a temperature sensitive (*ts*) phenotype and differ in virulence for volunteers (Chapter 17). Laboratory studies of artificially induced influenza *ts* mutants have demonstrated clearly that such mutants are attenuated for man and that the shut-off temperature is related to attenuation. The occurrence of non-*ts* and *ts* viruses in nature probably indicates that influenza A viruses of varying virulence are circulating in the community. In our studies, even viruses isolated from the same city varied considerably in the phenotypic *ts* character. Influenza A and B viruses circulating in the community may also differ in respect to the biological property of plaquing in MDCK cells and to the electrophoretic properties of structural and non-structural polypeptides and RNA. Finally, analysis of field isolates of influenza A and B viruses using panels of monoclonal antibodies to virus HA indicates a considerable degree of antigenic heterogeneity even among viruses isolated in rather circumscribed outbreaks in single towns or schools (Chapter 17). It is quite possible that biological techniques currently used to estimate the degree of inhibition of viruses by amantadine are too insensitive and that significant but small differences in drug resistance between viruses may be missed. These differences might, however, have a significant effect on the circulation of viruses in the community.

7.9.5. Pharmacology of amantadine

In summary, it has been established that, following oral administration of amantadine to man or mice, concentrations of amantadine could be attained in the tissues which would be expected to have influenza A virus inhibitory effects (around 1 μg amantadine/g tissue). However, what does *not* appear to be clearly established is the exact intracellular localization of administered amantadine and the correlation with antiviral activity (Richman et al., 1981).

Following single oral doses of amantadine hydrochloride, an average of 86% of the dose was recovered from the urine in five different subjects on nine occasions when urine collections were continued for 4 days or more. The average excretion in 24 hours was 56% of the dose based on 21 determinations in 16 subjects (Table 7.10).

TABLE 7.10.
Blood levels of amantadine in human subjects following single-oral doses of the hydrochloride (after Bleidner et al., 1965)

Time after dose	Blood level (μg/ml) dose		
	2.5 mg/kg	4.0 mg/kg	5.0 mg/kg
0	0	0	0
0.5	0	0.1	0.1
1	0.1	0.3	0.6
2	0.2	0.3	0.6
4	0.3	0.5	0.4
6	0.2	0.3	0.3
8	0.2	0.3	0.5
24	0.1	0.1	

The maximum blood level was generally reached in 1 to 4 hours after an oral dose (Table 7.11). There was no evidence of acetylated or methylated forms of amantadine in any of the human urine samples examined in spite of efforts to demonstrate their presence, and no extraneous peaks have been observed that can be attributed to metabolites of the drug (Bleidner et al., 1965).

Aoki et al. (1979) investigated the disposition of doses of 25, 100 and 150 mg amantadine taken every 12 h for 15 days in 13 healthy young adults. The authors detected a rather slower absorption of the drug compared to previous studies. The average time to peak plasma concentrations was 3–4 h. Almost complete oral bioavailability of amantadine was indicated by the recovery of approximately 80% of a single oral dose. The median ratio of plasma to renal clearance of amantadine approximated unity, which suggested that the compound was not extensively metabolized.

Distribution of an amantadine derivative (1-amino-3,5-dimethyl adamantane)

TABLE 7.11.
Urinary excretion of amantadine in human subjects (after Bleidner et al., 1965)

Time after dose (hr)	Dose range (mg/kg)	Recovery (% of dose)	Recovery range (% of dose)
0–24	2–7	56±13	27–78[a]
0–96	2–4	86± 9	62–93[b]

Human volunteers were given a single oral dose of amantadine and levels of amantadine in the urine were determined at various times thereafter.

[a] 21 determinations in 16 subjects

[b] 9 determinations in 5 subjects

was established in post-mortem tissues of a 77-year-old woman with Parkinson's disease (Wesemann et al., 1980). The patient had been treated with 2×10 mg of the amantadine molecule daily for 53 days. Levels of the compound (μg/g) in the tissues were as follows: kidney (0.18), lung (0.17), spleen (0.1), blood (0.07), cerebellum (0.22). Thus, it is of a particular interest that relatively high levels of the molecule were found in the lung.

Levels of amantadine in tissue specimens were also determined in a 5-month-old girl with influenza A virus pneumonia (Fishaut and Mostow, 1980). 2.5 mg/kg of amantadine each 12 h was administered and tissue specimens obtained $4\frac{1}{2}$ h after the final dose. Serum concentrations ranged from 0.8 to 1.64 μg/ml whilst higher concentrations of amantadine were found in the lung (21.4 μg/ml). This level of compound would significantly inhibit the replication of recent H3N2 and H1N1 influenza A viruses.

In other studies of pharmacology, volunteers were given aerosols of amantadine (Hayden et al., 1980). One hour after aerosol treatments with 1.0 g amantadine per 100 ml of solution in the glass nebulizer, amantadine levels in nasal wash samples (mean 30.3 μg/ml) greatly exceeded blood and nasal wash levels following oral administration.

Little detailed work has been published concerning the pharmacology of many of the derivatives of amantadine which have been synthesized and tested as antivirals. However, the varied biological activities of some of these molecules would suggest that differences in tissue distribution and adsorption might occur. As an example, rimantadine induces fewer CNS side effects in humans than amantadine and this might be related to reduced levels in the CNS since, for example, the compound has no anti-Parkinson's disease activity unlike amantadine itself which is active in this region.

7.9.6. Toxicology of amantadine

A central nervous stimulant effect of amantadine was described (Vernier et al., 1969) in acute and chronic toxicity studies in animals, but only at concentrations around 30 mg/kg orally, which is about 10 times the dosage in man. The predominant signs of central nervous stimulation were increased motor activity, tremors, anorexia, increased sensitivity to environmental stimuli or convulsions in some cases. The stimulant effects have also been described in volunteers given high doses of the compound. At relatively high doses in animals, other effects were transient vasodepressor effects, cardiac arrhythmias, weak ganglionic blocking effect, increase of myocardial contractile force or blocks of phenethylamine vasopressor response (Vernier et al., 1969).

7.9.7. Absence of subtle induced CNS effects of amantadine and rimantadine

Millet et al. (1982) have carried out a controlled comparison of amantadine and rimantadine on CNS effects and included a commonly used antihistamine compound (chlorpheniramine). The study was carried out at the University of California and 52 adult volunteers participated, with a mean age of 25 years. There was no significant difference among treatment groups with respect to age or sex of the subjects. Mild symptoms occurred with approximately equal frequency in all groups and therefore were excluded from further analysis. Table 7.12 shows the frequency and cumulative scores of moderate or severe symptoms, which were most commonly reported within each treatment group. The frequency of reported symptoms was low in the amantadine and placebo groups. Anti-histamine-like side effects, such as drowsiness and dry mouth, were less frequent and severe in the amantadine group than in the chlorpheniramine group. However, moderate to severe inability to concentrate, dizziness, and fatigue were reported more frequently by subjects who received the combination of amantadine and chlorpheniramine. Two subjects who received this combination reported additional symptoms of confusion and distorted depth perception as well as nausea and chills.

There were no significant differences between the group mean scores on the second practice trial and the pretreatment trial of the Critical Tracking Test among all treatment groups (Table 7.13).

The effects of amantadine or rimantadine on higher central nervous system functions, such as memory and attention, become more important when considering its use for prophylaxis of large populations. The data of Millet et al. (1982) shows that

TABLE 7.12.
Frequency and severity of subjective side effects analyzed by treatment group (after Millet et al., 1982)

Symptoms	No of subjects experiencing side effects in group treated with:[a]				
	Amantadine (10)	Amantadine-chlorpheniramine (11)	Chlorpheniramine (11)	Rimantadine (10)	Placebo (10)
Decreased concentration	1 (6)	4 (25)	2 (11)	0 (0)	0 (0)
Dizziness	0 (0)	2 (13)	0 (0)	1 (2)	2 (6)
Headache	0 (0)	1 (3)	1 (2)	1 (2)	0 (0)
Fatigue	0 (0)	2 (11)	1 (6)	2 (4)	0 (0)
Drowsiness	1 (9)	4 (13)	3 (14)	3 (12)	1 (6)
Dry mouth	0 (0)	2 (11)	1 (4)	1 (6)	0 (0)

[a] Numbers in parentheses represent the total symptom score (moderate complaint, 2; severe complaint, 3). Total scores within symptom categories for each treatment group represent the sum of the total daily moderate and severe complaints during the treatment period (4 days).

TABLE 7.13.
Psychomotor test performance in drug and placebo groups (after Millet et al., 1982)

Test	Group mean scores before and during treatment with:[a]				
	Amantadine	Amantadine-chlorpheniramine	Chlorpheniramine	Rimantadine	Placebo
Attentional					
Critical tracking	−0.085 (17.19)	−0.059 (12.68)	−0.039 (13.11)	+0.044 (9.81)	+0.027 (11.83)
Children's checking	+0.060 (1.35)	−0.027 (0.55)	−0.045 (0.77)	+0.800 (1.21)	−1.300 (0.89)
Cognitive					
Grammatical transformation	−1.000 (1.46)	−2.280 (3.65)	−0.166 (3.02)	+3.370 (2.07)	+7.800 (5.18)
Memory					
Memory for designs	+0.500 (0.83)	+0.090 (0.59)	+0.540 (0.59)	+0.400 (0.74)	+0.500 (0.42)
Symbol digit modalities					
Written	+6.400 (2.08)	+10.100 (2.99)	+10.200 (2.39)	+5.400 (2.08)	+6.000 (2.71)
Oral	+3.600 (1.47)	+8.800 (1.96)	+9.640 (2.41)	+8.700 (2.21)	+4.700 (2.13)

[a] Difference among group mean scores before and during treatment. Numbers in parentheses represent the standard error of the mean. −Value means decreased performance; +value means improved performance. In the amantadine, amantadine-chlorpheniramine, chlorpheniramine, rimantadine and placebo groups, there were 6, 7, 8, 8 and 8 subjects, respectively.

neither of these drugs had a significant effect upon performance of tasks which involved attention, cognition, and memory.

The early clinical studies of rimantadine in the USSR (Smorodintsev et al., 1970) and more recently in the USA have apparently indicated that the molecule is less toxic than amantadine (Dolin et al., 1982) and fewer CNS side effects are noted (although these effects are very mild with amantadine and are detected in no more than 10% of patients as described above – see Table 7.14). In a recent study Hayden et al. (1983) found that at similar plasma concentrations amantadine and rimantadine did not differ in the frequency or severity of their side effects. Oral dosing of volunteers with the same concentration (200 mg/day) of amantadine and rimantadine resulted in two fold different plasma levels (4 h after the initial dose of drug) of 300 ± 98 ng amantadine and 140 ± 68 ng rimantadine. Moreover, the plasma drug concentrations correlated significantly with total symptom score (Tables 7.15

TABLE 7.14.
Selected summary of reported side-effects of amantadine and rimantadine

Study	Virus	Dose (mg/day)	No. treated	No. given placebo	No. with side-effects[a]	
					Treated	Placebo
Amantadine						
Togo et al. (1968)	H2N2	200	29	29	0	0
Wingfield et al. (1969)	H3N2	100	23	48	0	0
Galbraith et al. (1969a)	H3N2	200	94	82	2	0
Galbraith et al. (1969b)	H3N2	200	102	100	3	0
Hornick et al. (1970)	H3N2	200	94	103	0	0
Knight et al. (1969)	H3N2	200	13	16	0	0
Togo et al. (1970)	H3N2	200	54	48	0	0
Smorodintsev et al. (1970)	H3N2	100	1313	512	94 (7.1%)	26 (5.2%)
Oker-Blom et al. (1970)	H3N2	200	192	199	— (8.7%)	— (3.4%)
Kitamoto (1971)	H3N2	200	182	173	18 (9.9%)	21 (12.1%)
					9 (4.9%)	9 (5.2%)
					5 (2.7%)	6 (8.5%)
O'Donoghue et al. (1973)	H3N2	200	50	61	0	0
Dolin et al. (1982)	H1N1	200	145	148	23 (15.9%)	7 (4.8%)
Bryson et al. (1980)	—	200	60	49	(33%)	(10%)
Rimantadine						
Zlydnikov et al. (1981)	H3N2	50	1647	1498	53 (3.2%)	32 (2.1%)
Zlydnikov et al. (1981)	H1N1	50	2998	1498	88 (2.9%)	32 (2.1%)
Dolin et al. (1982)	H1N1 and H3N2	200	147	148	10 (6.8%)	7 (4.8%)

[a] Side-effects in treated or placebo groups included insomnia, headache, nausea, vomiting, 'jitteriness' and diarrhoea.

TABLE 7.15.
Plasma concentrations of amantadine and rimantadine (after Hayden et al., 1983)

Drug	Initial dose (mg)	Daily dose (mg)	Plasma level[a] (ng/ml)	
			First	Second
Amantadine	100	200	300± 98	723±366
Rimantadine	100	200	140± 68	442±149
Amantadine	200	300	633±145	1405±437
Rimantadine	200	300	301± 75	913±270

[a]First plasma level was obtained at 4 h after initial dose. Second plasma level was obtained 4 h after ninth drug dose.

and 16). Thus, an important conclusion was that amantadine and rimantadine appeared to differ in their pharmacokinetics, but not in their potential for side effects at comparable plasma concentrations.

7.9.8. Effects of amantadine on pregnancy in animals

In a study by Kyo et al. (1970) amantadine hydrochloride was administered orally in two separate doses of 120 mg/kg (higher dose group) and 40 mg/kg once a day for 6 successive days from the 9th to the 14th day of pregnancy to nullipara rats of Wistar strain at the age of 3–4 months, in order to examine its effects upon the foetus during the final stage of pregnancy and their postnatal growths. The results indicated a slight retardation of increase in the body weight of dams in the higher dose group, but amantadine had no effect on the number of nidations at the end of the final stage of pregnancy. In the higher dose group, however, the mortality

TABLE 7.16.
Relationship between occurrence of moderate or marked side effects in amantadine and rimantadine recipients and plasma drug concentration after 4.5 days of drug administration (after Hayden et al., 1983)

Symptom type	Drug	No. of volunteers with adverse symptoms/total at plasma drug concentration /ng/ml)				
		⩽500	501–1000	1001–1500	1501–2000	>2000
CNS	Amantadine	0/5	2/20	8/32	7/19	3/8
	Rimantadine	0/12	2/48	4/17	0/2	0/0
Sleep	Amantadine	0/5	5/20	10/32	5/19	3/8
	Rimantadine	0/12	6/48	2/17	0/2	0/0
GI	Amantadine	0/5	4/20	4/32	4/19	0/8
	Rimantadine	0/12	5/48	2/17	1/2	0/0

rate of the foetus and the drop in body weight of surviving littermates showed a significant difference from those of the control group, although no malformation was observed in the group. Finally observations on the growth of the littermates up to the end of the 6th postnatal week in the spontaneous parturition group indicated that the parturition rate was significantly lower in the higher amantadine dose group than in the control group. Amantadine at the doses tested had no effect on suckling rate, external differentiation, survival rate, auditory senses, motility and development of gonadal functions or skeletal structure.

In contrast, in a study of Lamar et al. (1970), Holtzman rats and New Zealand white rabbits were dosed orally with amantadine (0, 50 and 100 mg/kg) from 5 days prior to mating until day 6 of pregnancy. In rats, but not in rabbits, results of autopsies performed on day 14 of gestation showed significant decreases in the number of implantations and increases in the number of resorptions at 100 mg/kg. Teratology studies were performed in rats (0, 37, 50 and 100 mg/kg) by administering the drug orally on days 7 and 14 of gestation. Autopsy was just before expected parturition. Increases in resorption and decreases in the number of pups per litter were noted at 50 and 100 mg/kg. Gross examination of rat pups at these dose levels revealed no malformations at 37 mg/kg. Malformation at 50 and 100 mg/kg included oedema, malrotated hindlimbs, missing tail, stunting and brachygnatha. Examination of cleared and alizarin-stained skeletal preparations of foetuses revealed cases of absent ribs and absence of the lumbar and sacral portions of the spinal column in the 50 and 100 mg/kg groups. Thus, in rats but not in rabbits, amantadine seems to be embryotoxic and teratogenic. Teratogenicity in rats occurs at 50 mg/kg/day, or about 12 times the usual human dose.

7.9.9. Prophylactic and therapeutic clinical trials with amantadine versus infection with influenza A (H_2N_2), (H_3N_2) and (H_1N_1) viruses: how effective is the compound as an antiviral?

Many but not all organized clinical trials conducted under vigorous double blind, placebo controlled conditions have established a prophylactic and therapeutic effect of amantadine and rimantadine against influenza A viruses (reviewed by Oxford and Galbraith, 1980). In contrast, only a few controlled trials have shown negative effects, but of course these are of considerable interest. There is general agreement in the literature that the protective effect of amantadine (used prophylactically) against influenza A virus would approximate to 70–80% (with a range from 0–100%). To place this figure in perspective, clinical trials with influenza vaccines have, over the same period of time shown similar results with protective effects varying from 0–100% with a mean approaching 70–80%. The death incidence in a moderate influenza outbreak approximates to 1 in 5000 whereas the incidence of death following vaccination is estimated at 1 in 2 million or lower. This yields a maximum benefit risk ratio of 400:1 in favour of immunization. For both amanta-

dine and vaccine with 70% protective efficacy and a 10% attack rate, 14 persons would have to be immunized or given amantadine to prevent influenza in one person. Assuming a 0.1% mortality rate then approximately 14 000 persons would have to be treated or immunized to prevent one death.

With this background, it is of some comparative interest to examine representative clinical trials carried out between 1965 and 1984 and to illustrate exactly how effective amantadine is in preventing or curing influenza A infection. We should then be able to assess whether the compound is active enough to warrant more extensive use in the future or whether amantadine or rimantadine should be considered only as the first anti-influenza compound and whether new compounds of a much higher degree of activity would be required. Alternatively, are expectations of the clinician based on several decades of experience with antibacterials, too high to be realised with antivirals? It might suitably be mentioned here that an important property of an anti-influenza compound, particularly to be used as a prophylactic, would be *not* to completely abort influenza virus infection, but rather to reduce virus replication and abort clinical signs. The patients would thus be able to produce a natural immunological response, so conferring protection against reinfection with the same virus immediately drug prophylaxis ceases.

Following satisfactory safety testing of amantadine in animals, and clearance by the Food and Drug Administration in the USA, human volunteer studies were begun using attenuated strains of influenza A virus as a challenge. We shall consider representative artificial and natural challenge studies of amantadine as a prophylactic and therapeutic agent. (We have not attempted to analyze the data from all clinical trials reported in the literature – rather the review is selective.)

7.9.10. Artificial challenge studies

The advantages of this method, involving as it does the challenge of human subjects with a known virus, are twofold. The first is administrative convenience in that results of such clinical investigation are generated immediately, as compared with the delay involved when waiting for a naturally occurring outbreak, or epidemic of influenza. The second concerns the use of a virus of known virulence to the host and sensitivity to the chemoprophylactic agent. The design can be planned in meticulous detail, including the screening of all volunteers for pre-existing antibody to the challenge virus. It is thus possible to involve groups of volunteers with low or absent antibody to the challenge virus, and to compare the response seen in volunteers who already possess a significant level of antibody to the virus at the time of challenge. A possible objection to this method of investigation of an anti-influenza agent may be its dissimilarity to the infection as it occurs in natural epidemic form and the use of high challenge doses of virus thus presenting an over vigorous testing (Beare and Reed, 1977).

Initially, following the discovery by the Du Pont Company of the antiviral activi-

ty of amantadine, in the United States, clinical studies were confined to America. However, as evidence of prophylactic action in influenza was disseminated, clinical investigations were set up in Europe and the Far East. In general, the dosage used was 100 mg every 12 h, but some studies, notably that by Smorodintsev in 1969, employed 100 mg daily. Clinical studies in the Soviet Union using rimantadine have been reviewed recently (Indulen and Kalninya, 1980, Zlydnikov et al., 1981) and therefore will not be discussed here in detail but summarized in Tables 7.23 – 7.25.

In initial challenge studies, Jackson et al. (1963) selected volunteers from amongst 735 college students who submitted blood specimens for determination of the influenza antibody titre. Two-thirds (497) of the students were considered to have a high serum antibody titre, 1:20 or greater. Subjects for placebo challenge were randomly selected from the high antibody group; 21 subjects served as placebo controls, and 18 were observed for drug toxicity. Among the 735 initial subjects, 199 were in the low antibody group (1:10 or less). These subjects, who were challenged with influenza virus, were placed in groups which received either placebo or 100 mg amantadine. One half of each group was given the capsule as pretreatment, beginning 18 h before virus challenge. The other half received treatment beginning 4 h after challenge. All treatment was continued for the next 6 days. After challenge, illness, virus recovery, exfoliative cytology and serology were studied. Among the 199 volunteers challenged with influenza virus, 89 (45 per cent) had a four-fold or greater rise in antibody in convalescent sera, indicating infection with the challenge virus. This represented an infection rate of 70 per cent among subjects with a pre-challenge antibody titre of 1:10 or less. Volunteers who had a pre-challenge antibody titre of 1:20 had an infection rate of only 22%. Among those with a higher serum level of antibody, only 9% became infected. Among the two placebo groups, 66% and 73% of the subjects with a low antibody titre became infected as judged by a serological rise in titre. Among those with higher antibody, 14% and 26% were infected. Among subjects *pre*-treated with the drug, a considerable reduction in infection was observed in volunteers of either antibody status. Low antibody subjects given amantadine had an infection rate of only 37% which was a statistically significant reduction ($P < 0.01$) compared with the combined groups. No therapeutic effect was detected (see below).

However, an early challenge study, carried out at the Common Cold Research Centre in England, using an attenuated strain of influenza A sensitive to amantadine in in vitro laboratory studies failed to demonstrate prophylactic action of the drug clinically or serologically (Tyrrell et al., 1965). Relatively large doses of egg-adapted virus were used in the volunteers and the numbers involved were small.

Togo et al. (1968) reported a study performed with the cooperation of volunteers at the Maryland House of Correction. Sixty-five men with titres of 1:2 or less of neutralizing antibodies were enrolled and, after full baseline evaluation, were housed in the research ward at the prison. Oral temperature, pulse rate and respiration rates were recorded at four-hourly intervals. Follow-up specimens were collected

at 7, 14, 21 and 28 days post challenge. Seven trials were conducted. In the preliminary potency-testing of the inoculum, seven men were challenged. In the following six drug-evaluation studies, viral challenge was performed on a total of 58 men, volunteers in each group numbering 14, 6, 12, 6, 8 and 12. Amantadine and lactose-containing placebo capsules were administered by double-blind technique from randomly numbered bottles. The drug was given in 100 mg doses twice daily for eight days in the first two studies and for nine days in the following four studies, starting about 26 h prior to the viral challenge. The drug-treated group received a total of 300 mg of amantadine before the virus dose of 64 000 $TCID_{50}$ was given nasopharyngeally. The virulence of the virus inoculum was examined in 7 men who received undiluted virus fluid containing 64 000 $TCID_{50}$. Clinical illness observed was classified according of the severity of signs and symptoms. The following criteria applied:

2+ = moderately ill with temperature above 38.3°C and occasional respiratory tract signs.
1+ = mild illness, significant symptomatology and temperature 37.8°C.
± = questionable illness, no fever, but comprising of pertinent symptoms.
0 = no suspected illness.

Subsequent experience with the 18 volunteers enrolled in the drug evaluation study showed similar clinical responses and the induced illness was generally milder than naturally occurring influenza. The prophylactic effectiveness of amantadine in volunteers with experimentally induced influenza A infection was assessed in six separate but consecutive double-blind trials. The most severe illnesses (24) occurred only in the placebo-treated subjects. The occurrence of six instances of 2+ illnesses in the placebo-treated subjects compared with none in the drug-treated group is a statistically significant difference ($P=0.011$). There was a striking disparity in the overall incidence of clinical illness between the two groups. Febrile illness, with ratings of 1+ or 2+ were observed in thirteen patients in the placebo-treated group, compared with five patients with 1+ febrile illness in the amantadine group, and this is statistically significant.

7.9.11. Prophylactic trials in the community

The first study of amantadine in the family environment was performed by Galbraith et al. (1969a) with the cooperation of family doctors, the majority of whom were members of the epidemic observation unit of the Royal College of General Practitioners. Each doctor, many of whom had experience in the conduct of serologically controlled clinical trials, was asked to include in the study the families of up to five index cases and five contact cases. In the study, 'the family' was defined as all occupants of a household over 2 years of age living in daily contact with each other. The 'index case' was defined as the first person over the age of 2 years to contract clinical influenza in a household, and 'contact cases' were defined as individuals living in the household and having contact with the index case. All index

cases received placebo medication in order not to influence the possible spread of influenza, while the families of index or contact cases received drug or placebo by random allocation. All the members of one family except the index cases, received the same treatment (drug or placebo). The diagnosis of influenza in the index case was made on clinical and epidemiological grounds. At the doctor's first visit, blood was taken from the index case and from as many of the other members of the family as practicable. A second blood sample was taken two or three weeks later, and tests for HI and CF antibody were performed. Daily records were kept of body temperature and the presence of a cough. A cough, accompanied by a rise of temperature to 37.8°C or higher, was taken as criterion for a diagnosis of clinical influenza.

Twenty-two family doctors studied 52 families comprising 208 contacts who were divided between treated and placebo groups. Of the 52 index cases, 35 (67 per cent) showed serological evidence of influenza A infection. In the 35 families in which there was serological evidence of influenza A infection in the index case, two of 55 (3.6%) contacts in the amantadine-treated group developed clinical symptoms of influenza, whilst 12 of 85 (14.1%) contacts in the corresponding placebo group developed an influenza-like illness. The difference in incidence in these two groups was of marginal significance ($P=0.07$). However, there was no serological evidence of infection with influenza A virus in the two individuals of the amantadine group who developed clinical illness. In contrast, in the placebo group, ten of the 69 contacts from whom paired sera were available developed antibody rises to influenza A (Table 7.17). When serologically confirmed cases of influenza are considered, the difference between the drug and placebo treated groups is significantly different statistically ($P=0.05$–0.01) The authors also observed that when the proportion of contacts with serological evidence of influenza A infection, irrespective of clinical

TABLE 7.17.
General practice (UK) study: effect of amantadine on incidence of clinical influenza in contacts of index-cases (after Galbraith et al., 1969)

Laboratory evidence of influenza A infection in index case	Treatment	No. of families	Contacts who developed clinical influenza within 10 days of entering the study					
			All cases			Confirmed serologically		
			No.	%	*P* value	No.	%	*P* value
Present	Amantadine	13	2/55	3.6		0/48	0	
	Placebo	22	12/85	14.1	0.07	10/69	14.5	0.05–0.01
Absent	Amantadine	11	1/45	2.2		0/43	0	
	Placebo	6	3/23	13.0	0.3–0.2	2/21	9.5	0.10

This study examined the prophylactic effect of amantadine by determining the effect of the compound on spread of influenza from an initial index case (which was left untreated) to the rest of the family.

illness, was compared for the placebo and drug-treated groups (Table 7.18), the difference between the groups was highly significant ($P = 0.001$–0.01).

The following winter, the investigators repeated the family study in the face of an epidemic of a new pandemic virus Hong Kong influenza – A/HK/1/68 (H3N2). Seventy-two general practitioners volunteered to take part and 58 families were included, comprising 176 individuals. The only difference in design between this study and the previous year's was that the index case in each family received amantadine or placebo in keeping with the rest of the family. As before, the results of cases suffering from clinical influenza and those with serological proof of influenza A infection, were submitted to statistical analysis. In this instance, the drug and placebo-treated individuals behaved similarly and amantadine failed to protect persons receiving the compound from influenza. The virus itself was equally sensitive to amantadine as the previous year's strain and the authors sought to explain this reversal by considering the initial antibody status of those cases under study. It was seen that during the 1967/68 study, the initial level of HI antibody to the current strain of influenza A was higher (40 per cent with initial HI antibody below 1:12) compared with the 1968/69 study when 90 per cent of the contacts studied possessed initial HI titres below 1:12. This may have been responsible for the difference but the administration of amantadine to half the number of index cases could have influenced the infectivity of these individuals so that a direct comparison of these two trials is not possible.

7.9.12. Therapeutic activity of amantadine

Studies in influenza infected mice indicated unexpectedly, that administration of amantadine could have a significant therapeutic effect. Clinical trials were set up

TABLE 7.18.
General practice (UK) study: effect of amantadine on the incidence of clinical and subclinical influenza A infections (after Galbraith et al., 1969)

Laboratory evidence of influenza A infection in index case	Treatment	Contacts with serological evidence of influenza infection					
		clinical and subclinical infections			Subclinical infections only		
		No.	%	*P* value	No.	%	*P* value
Present	Amantadine	7/48	14.6		7/48	14.6	
	Placebo	27/69	39.1	0.001–0.01	17/69	24.6	0.2
Absent	Amantadine	7/43	16.3		7/43	16.3	
	Placebo	5/21	23.8	0.7	3/21	14.3	0.8

This study examined the prophylactic effect of amantadine by determining the effect of the compound on spread of influenza from an initial index case (which was left untreated) to the rest of the family.

in the USA and the therapeutic effect of amantadine in man was clearly established (Togo et al., 1970).

A study of the therapeutic effect of amantadine amongst patients in the family environment was carried out by general practitioners in the United Kingdom and reported by Galbraith et al. (1971, 1975). Fifty-seven doctors took part and included 203 patients with clinically diagnosed influenza. Amantadine was provided in capsule form (100 mg) or as a syrup (50 mg in 5 ml) and patients received active or placebo medication by random number allocation on a double-blind basis. Adults received 100 mg every 12 h and children aged 10–15 years 100 mg daily, younger children (2–10) a proportional dose of syrup. Medication was started from the time the patient was first seen by the doctor and was continued for 7 days. A blood sample was taken at the doctor's first visit and a second 2–3 weeks later. These were tested for HI antibody with A/HongKong/68 virus and for CF antibody. A fourfold or greater rise in either or both these tests was taken as evidence of influenza A infection. Of the 203 patients entered, only 153 provided results which satisfied the criteria for analysis. Of the 153, 72 received amantadine and 81 placebo. The mean duration of fever is shown in Table 7.19 where the differences between drug and placebo-treated patients were significantly different. When symptomatology excluding fever was considered, no differences were demonstrated between the two groups, but this may have been due to the lack of sensitivity in the method of recording clinical illness.

7.9.13. More recent clinical trials with influenza A (H_3N_2) and (H_1N_1) viruses and amantadine or rimantadine

More recently a number of excellent double blind placebo controlled trials have been carried out and the data obtained has confirmed the data from earlier trials with H2N2 and H3N2 viruses. In such a prophylactic trial Dolin et al. (1982) reported the first well controlled comparison in the USA of the prophylactic effects of rimantadine and amantadine in an area where an active influenza surveillance

TABLE 7.19.
Therapeutic trial of amantadine in general practice (UK study) (after Galbraith et al., 1971)

	Duration of temperature (hrs)			*P* value
	Amantadine	Placebo	Difference	
Males	55.1	71.5	16.4	$0.05 > P > 0.02$
Females	37.7	80.6	42.8	< 0.01
Both sexes	46.6	75.1	28.5	< 0.01

In this double-blind placebo controlled trial, medication with amantadine was initiated within 24 hrs of onset of symptoms and continued for 7 days.

indicated early that an influenza A outbreak had commenced caused by H3N2 (20% of cases) and H1N1 viruses (80% of cases). A total of 450 volunteers enrolled with a mean age of 25.0 ± 0.5 years and with no significant differences in age, race, male: female ratios or level of pre-existing HI antibody. Throat swabs for virus isolation were taken 2 times per week and volunteers were assigned to amantadine, rimantadine or placebo groups. A 100 mg tablet was taken twice daily for 7 days and any symptoms were recorded. Each week the volunteers returned the symptoms diary to the co-ordinating centre and received a further 7 days supply of tablets. If any respiratory illness occurred, volunteers were asked to return at once to the centre, and were examined by a physician. Influenza-like illness was defined as a cough and/or fever greater than 37.7°C and two or more of the following symptoms: sore throat, headache and myalgia. The trial lasted six weeks and a serum sample was obtained at the beginning and again at the end of the study for serological analysis. Significantly more placebo recipients (40.9%) developed influenza-like illness compared to amantadine (8.9%) or rimantadine (14.3%) groups giving a reduction in the rate of illness of 78.2% and 65.0% respectively (Table 7.20). However, as noted in previous trials (reviewed by Oxford and Galbraith, 1980), the rates of laboratory confirmed influenza-like illness were reduced by 85.4% by rimantadine and 91.2% by amantadine suggesting that a proportion of influenza like illness observed during the study was not caused by influenza A virus.

Of a small group of amantadine patients sampled 89% had drug detectable in the urine (52–438 μg/ml) suggesting a good compliance rate. In total 62 volunteers left the study during the 6 weeks because of possible side-effects. The withdrawal

TABLE 7.20.
Effect of rimantadine and amantadine in preventing influenza-like illness and laboratory confirmed influenza among volunteers (after Dolin et al., 1982)

Group	Number	Number with influenza-like illness or influenza	Per cent
Influenza-like illness			
Placebo	132	54	40.9
Rimantadine	133	19	14.3
Amantadine	113	10	8.9
Laboratory confirmed influenza			
Placebo	132	27	20.5
Rimantadine	133	4	3.0
Amantadine	113	2	1.8

Illness was defined as cough and/or fever 37.7 °C p.o. and two or more of the following: sore throat, headache, myalgia.
Laboratory confirmed influenza was determined by virus isolation and/or serum antibody rises.

rates were 10.8% for placebo, 9.5% for rimantadine and 22.1% for amantadine and the excess rate in the latter group was mainly caused by CNS effects including insomnia, jitteriness and difficulty in concentrating, although symptoms generally cleared within 48 hours of the cessation of medication. Both amantadine and rimantadine were thus highly effective in preventing illness and/or infection with no statistically significant differences between the efficacy rates of the two compounds. However, amantadine treated persons had significantly more CNS side effects with an excess rate of 9.0% compared to placebo patients. The authors concluded that rimantadine might be the compound of choice for the chemoprophylaxis of influenza A infection in young volunteers, but further trials could be carried out in the elderly and high risk individuals.

Another recent clinical trial has compared the *therapeutic* effect of amantadine versus rimantadine against an H1N1 virus (Van Voris et al., 1981). This trial will also be described in some detail, because it illustrates both clinical and serological criteria which need to be applied in a comprehensive clinical therapeutic study of an anti-influenza compound. The study was carried out in a group of university students naturally infected with A/USSR/77 (H1N1) virus. Clinical criteria were used for the initial diagnosis of influenza and included the presence of a headache, fever, malaise and myalgia of less than 48 hours' duration. This is in accordance with the majority of the previously reported therapeutic studies, where benefit was not noted in persons entering treatment later than 48 hours after the initial symptoms. A complete history was taken on admission to the trial and thereafter clinical examinations were carried out at 4, 48 and 72 hours, 7 days and 3 weeks, and specific signs categorized and graded on a scale of 0 to 3. Oral temperatures were measured by the clinician daily. Nasal washings for virus isolation were taken at the time of admission to the study and at 48 to 72 hours. Acute and convalescent sera were obtained and tested for rising titres of HI antibody to confirm the clinical diagnosis. All students took a tablet (100 mg) each morning and night. After 48 h of therapy, blood was taken to test for levels of drug – this is an important factor to control that students were indeed taking the medication (levels ranged from 0.3 to 0.8 μg/ml, whilst the virus was inhibited by 0.2 μg/ml of drug). Of the 54 volunteers to enter the study, 45 were proven to have been infected with A/USSR/77 (H1N1) virus. This is a rather high rate of clinical diagnosis, since, for example, typical general practitioner trials in the UK normally record a 60% success in influenza diagnosis (Galbraith et al., 1969). When the total symptom scores were calculated for the three groups, an arbitrary 50% level of clinical improvement (compared with pretreatment) was reached in the amantadine and rimantadine groups at 48 hours. This was not reached until 72 hours by the placebo group ($P<0.25$). An improvement was noted in the drug treated groups in both respiratory and systemic signs. Mean temperatures for the drug treated groups were significantly lower ($P<0.01$) than the placebo group. An interesting finding (for students!) was that both at 48 and 72 hours after commencement of the study more students in the drug treated groups

were able to attend class compared to the placebo group. Although the initial titres of the excreted virus were similar in each group, by 48 hours the proportion of students shedding virus was significantly lower in the groups treated with amantadine or rimantadine. All students developed post-infection HI antibody to the infecting virus. Minor CNS side effects were noted in a proportion of students in the amantadine group by day 5, but these appeared to be less important than the relief of influenza symptoms, since students in this group returned to class more rapidly than students in the placebo group. The authors concluded that a therapeutic advantage in favour of amantadine occurred, and that the 24–48 hours benefit appeared to justify the therapeutic use of amantadine or rimantadine especially in comparison with the current usage of non specific drugs such as antibiotics, antihistamines and cough suppressants.

In summary, therefore, amantadine or rimantadine in most clinical trials with influenza A viruses have not resulted in 100% protection against infection or clinical disease. Its prophylactic efficacy would approximate to that of influenza vaccine but with the qualification that amantadine has no effect against influenza B viruses. The compound has been used successfully to prevent the spread of virus in families, hospitals, factories and closed communities such as prisons and antiviral effects are consistent with viruses of the different subtypes H1N1, H2N2 and H3N2. A quite clear therapeutic effect is also obtained resulting in a 1–2 day faster recovery, lower temperatures and fewer clinical symptoms with these viruses, and, perhaps more significantly from the point of view of virus spread in the community, reduced excretion of virus.

7.9.14. Could amantadine be used successfully on a wider scale?

At present health authorities in most countries recommend the routine use of influenza vaccine to prevent influenza infection in persons designated at special risk of mortality, e.g. older persons, diabetics, asthmatics and persons with chronic obstructive heart disease and bronchitis. Little attempt has been made to provide protection for the community at large or to abort an epidemic, in spite of the well documented economic and social disruption and mortality caused by influenza A and B viruses (reviewed by Stuart-Harris and Schild, 1976). Notable exceptions have been the attempt to immunize the population of the USA with A/Swine virus containing vaccine following the outbreak of influenza A/NJ/76 infection in the Fort Dix camp in the USA, and the necessarily (because of their size) uncontrolled trials of live attenuated influenza vaccine in cities in the USSR and China. It could be argued that general prophylactic measures could and should be instituted with more enthusiasm and vigour than at present and that such measures could include prophylaxis in certain groups with amantadine. A consensus meeting in the USA (Elliot, 1979) and also a more recent meeting in Vienna (Galasso et al., 1984) suggested that the following groups should be considered for amantadine prophylaxis. (See also Tables 7.21 and 22.)

1. Unvaccinated children and adults at high risk of serious mortality and mortality because of underlying diseases, which include pulmonary, cardiovascular, metabolic, neuromuscular, or immunodeficiency diseases.
2. Adults whose activities are vital to community function and who have not been vaccinated with an appropriate contemporary influenza vaccine: for example, policemen, firemen, selected hospital personnel. Such persons are in frequent contact with others who may have influenza and should be considered at higher risk of contracting influenza than the general population.
3. Persons in semiclosed institutional environments, especially older persons, who have not received the current influenza vaccine.

The groups for which the panel concluded the benefit-to-risk considerations are less clear include all elderly patients (65 years or older) who have not received influenza vaccine. In addition, the use of amantadine hydrochloride for prophylaxis in hospital patients in the presence of a demonstrated outbreak should take into consideration local and particular risk factors and conditions; for example, the patient who

TABLE 7.21.
Population groups in which amantadine or rimantadine has demonstrated prophylactic and therapeutic activity

Groups	Countries in which placebo controlled trials have shown efficacy	References
1. Families	(UK and USSR)	Galbraith et al., 1969 Zlydnikov et al., 1981
2. Hospitals	(USA and Hungary)	Nafta et al., 1970
3. Schools	(UK and USA, USSR)	Finklea et al., 1967 Smorodintsev et al., 1970
4. Prisons	(USA)	Bloomfield, 1970 Togo et al., 1968 Hornick et al., 1970
5. University campuses	(USA, Finland, USSR)	Jackson et al., 1963 Smorodintsev et al., 1970 Dolin et al., 1982 Oker Blom et al., 1970
6. Open studies (towns)	(USSR)	Zlydnikov et al., 1981
7. Army and Navy barracks and camps	(USSR, USA, Hungary)	Nafta et al., 1970
8. Factories	(Yugoslavia)	Likar, 1970

Note that the summary of countries involved with these trials is not exhaustive. The early licensing and usage of amantadine was subject to two critical reviews by Sabin (1967, 1978). However, the more extensive field use of the two compounds in the last 5 years has provided data to answer many of the earlier questions.

TABLE 7.22.
Population groups in which a more extended use of rimantadine and amantadine, versus influenza A virus, could be suggested

1. Persons (vaccinated or not) presenting with clinical influenza within 48 h of the onset of clinical signs. Future clinical trials may establish if amantadine or rimantadine used therapeutically can reduce mortality in persons at special risk, or reduce the rate of complications such as pneumonia.
2. Adults, such as hospital workers, public transport personnel etc. in the face of an epidemic when insufficient vaccine is available, or when contraindications exist to vaccine.
3. Households contacts of an index case of influenza.
4. Hospital patients and personnel, to prevent hospital spread when patients with influenza A virus infections are admitted. This would mainly be prophylactic use of the compound.
5. Persons in institutions, such as old persons homes and boarding schools. Prophylactic and therapeutic.
6. Unvaccinated adults, who nevertheless have serious underlying disease which place them in a potentially high mortality group following an attack of influenza e.g. persons with pulmonary, cardiac, metabolic or immunological deficiencies. Amantadine or rimantadine could be used prophylactically and persons vaccinated at the same time.
7. Vaccinated adults who are at high risk from an attack of influenza. Rimantadine or amantadine-supplemented protection would be expected to raise the basic 70% protective effect of vaccine alone. Single non reactogenic doses of vaccine against a new pandemic virus subtype would not be expected to give significant protection – two doses of vaccine would be required – particularly with subunit or split virus vaccines. Therefore amantadine could be administered prophylactically during the two–three week period of development of vaccine induced immunity.
8. In the event of the arrival of a new pandemic strain of influenza A virus *all* the above groups and additionally individuals in the general community who would like prophylaxis. (Excluded would be young children and pregnant women because of the limited amount of clinical and toxicological data in these two groups.)

See Galasso et al., 1984, Elliot, 1979.

is to undergo inhalation anaesthesia may be at higher risk of serious complications. Finally, an obvious possibility would be to supplement vaccine induced (partial) protection with amantadine (partial) protection. Only a few studies have investigated the possible additive effects of vaccine and amantadine in this approach and preliminary results have been encouraging (reviewed by Zlydnikov et al., 1981) raising protective efficacies from 32–37% in vaccine or rimantadine groups to 60% in the group given both vaccine and rimantadine.

7.9.15. Differential diagnosis of respiratory infections – a problem for all antivirals with a narrow antiviral spectrum of activity?

Figure 7.10 illustrates the complexity of the clinical diagnosis of influenza A from a number of other viruses causing a rather similar clinical picture. Also influenza

A infection itself in individuals can range from asymptomatic (there probably exists at least a 1:1 ratio of clinical : subclinical cases) through mild cold symptoms, to typical acute influenza and, in the extreme case, virus infection of the lower respiratory tract and pneumonia. A major limitation of amantadine in one respect has been the narrow spectrum of antiviral activity, limited to influenza A virus with only marginal activity (and here limited to in vitro data) against para-influenza viruses, VSV, rubella, arenaviruses and influenza B (reviewed by Oxford and Galbraith, 1980). On the other hand, in influenza A virus epidemic years, the clinical diagnosis rate of influenza by general practitioners can be relatively high, as is shown by the controlled clinical trials in the UK (Galbraith et al., 1969). It is apparent then that any extended use of amantadine must be accompanied and initiated by clear laboratory and virological data and establishment of influenza A virus spread in the community. This has been clearly recommended in the Vienna report

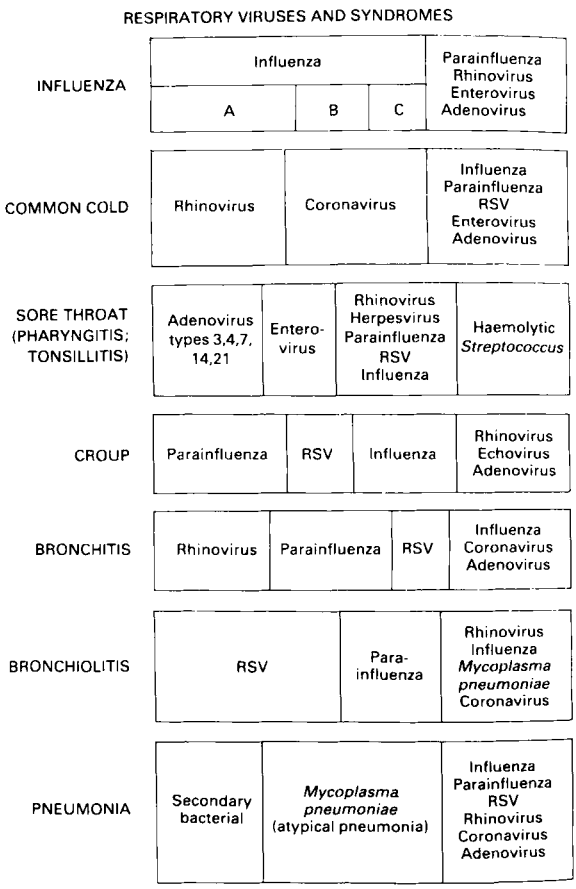


Fig. 7.10. Respiratory viruses and syndromes (after Stuart-Harris and Schild, 1976).

(1984). In the UK, the PHLS and the Royal College of General Practitioners have considerable experience in quickly and accurately estimating outbreaks of influenza A virus in the community. Similar monitoring is carried out in the USA and USSR and therefore in these three countries particularly, prophylactic measures could be initiated with some degree of virological certainty. In the USSR, for example, communities already are encouraged to use rimantadine prophylactically over a period of 3–6 weeks during a city outbreak of influenza A virus. Rimantadine can be purchased from pharmacies without prescription for approximately £1.20 for a three week course, but is also administered free of charge in certain organizations (M. Indulen, Latvian Academy of Sciences, personal communication). In the UK and USA the amantadine has to be prescribed by a medical practitioner and the cost for a 10 day course (in the UK) is approximately £3.50. A parallel situation is occurring with certain of the new anti-herpes compounds, where rapid self-prescription by patients at the first signs of a virus induced lesion is required to show beneficial clinical effects. As mentioned briefly above, certain concepts which have been established about the use of antibacterials may therefore have to be modified for antivirals to include more reliance on a patient's ability to initiate prophylaxis. It must be expected, however, that widespread usage of antivirals may quickly result in the selection and spread of drug resistant variants. Drug resistance is a major clinical problem with antibacterials but has, at the same time, encouraged the search for more effective molecules.

The Vienna report suggesting population groups for amantadine and rimantadine therapy, or prophylaxis using rimantadine, based its conclusions on more recently published data which has established that although rimantadine and amantadine have similar antiviral properties, rimantadine at 200 mg/day has no demonstrable toxic effects and therefore it is the choice for prophylaxis. On the other hand, the very mild side effects of amantadine (less, for example, than many nonspecific compounds used at present to treat the clinical effects of influenza) are not noticeable in persons already suffering from the clinical effects of influenza and so either rimantadine or amantadine can be used for therapy. It should be added that early studies in the USSR established the usefulness of rimantadine and these are summarized in Tables 7.23, 24 and 25.

7.9.16. Therapeutic effects of amantadine aerosols

Hayden et al. (1980) established that aerosols of amantadine in young adults experiencing acute illness caused by influenza A virus had a discernable but modest therapeutic and antiviral effect. (It could be added that aerosol administration has been used more recently with ribavirin and, in particular, against respiratory syncytial virus infections in both adults and children – Chapter 8). In the Hayden trial (1980) the effect of small-particle aerosol therapy with amantadine was assessed in a randomized, double-blind study of 20 patients with naturally acquired influenza A vir-

TABLE 7.23.
Summary of data on prophylactic efficacy of amantadine and rimantadine against infection with influenza (USSR trials) (after Zlydnikov et al., 1981)

Subjects no. in group	Strain of virus	Drug, dosage schedule	Results
Volunteers infected by inoculation	A/Hong Kong/68		
49		Amantadine, 100 mg per day, starting 24 h before virus inoculation, for 7 days	Protection index, 60.2%; fever rate, 30.6%
36		Rimantadine, 100 mg per day, starting 24 h before virus inoculation, for 7 days	Protection index, 87.2%; fever rate, 11.1%
47		Placebo	Fever rate, 80.9%
Volunteers infected by inoculation	A/Hong Kong/68		
109		Amantadine, 100 mg per day, starting 24 h before virus inoculation, for 7 days	Rate of clinical symptoms, 34.8%
90		Rimantadine, 100 mg per day, starting 24 h before virus inoculation, for 7 days	Rate of clinical symptoms, 17.7%
118		Placebo	Rate of clinical symptoms, 81.3%
Seven groups of industrial workers	Influenza epidemic 1975 A/Port Chalmers/1/73		
1881		Rimantadine, 50 mg per day, for 20–27 days	Efficiency index according to clinical diagnosis, 2.04; according to serologic diagnosis, 3.2; and compared with the outer control group, 4.8
1711		Placebo-treated group, with inner and outer control groups	
Industrial workers living in hostels	Influenza epidemic of 1976 (A/Victoria/3/75)		
402		Rimantadine, 50 mg per day, for 15 days	Efficiency index, 1.5; coefficient of efficacy, 32.9% (according to clinical data and serologic diagnosis, 2.4 and 58.3%, respectively)

Subjects no. in group	Strain of virus	Drug, dosage schedule	Results
425 Family contacts	Outbreak of influenza B in 1977	Placebo	
66		Rimantadine, 50 mg twice per day for adults and 50 mg per day for children 5–15 years of age (the drug was given for 7–10 days)	Morbidity rate, 27%
72 Four collectives of adults	Epidemic foci in influenza epidemic 1974–1975 (A/Port Chalmers/73) and 1975–1976 (A/Victoria/3/75)	Placebo	Morbidity rate, 72%
5935		Rimantadine, 50 mg per day, for 17–25 days	3.3-fold decrease in influenza morbidity in 1975 and 2.6-fold decrease in 1976

us infection. Aerosol treatments of 20 min with either distilled water or with amantadine hydrochloride (1.0 g/100 ml of distilled water) were given three times a day for four days. The amantadine-treated patients experienced a significantly more rapid resolution of clinical signs and symptoms when compared with placebo-treated patients. The resolution of fever was similar for both groups. Aerosol delivery of amantadine did not affect the frequency of viral isolation from upper respiratory tract secretions, but was associated with a trend toward reduced quantity of viral shedding. Serial pulmonary function tests found no important differences between the groups. Amantadine-treated patients experienced a greater frequency of mild local side effects (rhinorrhea, nasal irritation) during aerosol exposures but aerosol treatments did not cause any apparent decline in pulmonary function.

In summary, therefore, the amantadine group of molecules has had a long gestation of two decades but the accumulation of scientific data is now overwhelming in its indication of the clinical usefulness of the compounds against influenza A virus infections. Future studies must monitor closely for development of drug resistant strains, the usefulness of amantadine at lower dosage levels and the usefulness of both amantadine and rimantadine in preventing mortality in the special risk groups.

TABLE 7.24.
Adverse effects of rimantadine administered prophylactically during influenza epidemics (USSR trials) (after Zlydnikov et al., 1981)

Epidemic	Treatment of group, no. of subjects	Dosage	No. (%) of subjects reporting					Total no. (%) reporting adverse effects
			Dyspepsia	Sleep disorders	Headache	Malaise	Other complaints	
A/Victoria/75 (H3N2) 1975–1976	Rimantadine (1647)	50 mg per day for 20–27 days	15 (0.91)	0	14 (0.85)	1 (0.16)	23 (1.39)	53 (3.22)
	Placebo (1498)		2 (0.13)	0	12 (0.80)	4 (0.27)	14 (0.93)	32 (2.14)
A/USSR/77 (H1N1) 1977–1978	Rimantadine (1351)	50 mg per day for 15 days	11 (0.61)	1 (0.07)	9 (0.66)	2 (0.14)	12 (0.88)	35 (2.6)
Total	Rimantadine (2998)		26 (0.86)	1 (0.03)	23 (0.76)	3 (0.1)	35 (1.16)	88 (2.93)
	Placebo (1498)		2 (0.13)	0	12 (0.80)	4 (0.27)	14 (0.93)	32 (2.14)
	P		<0.05	>0.05			>0.05	>0.05

TABLE 7.25.
Summary of data on therapeutic efficacy of rimantadine in patients with influenza (USSR trials) (after Zlydnikov et al., 1981)

Subjects no. in group	Strain of virus	Drug, dosage schedule	Results
Adult outpatients with natural infection	A/Hong Kong/68		
164		Rimantadine, 150 mg per day for 5 days (100 patients treated with rimantadine, 64 with placebo)	Decreased intensity of toxic and catarrhal symptoms, reduction in the duration of clinical symptoms by 2–3 days as compared with control group
Inpatients with natural infections	A/Port Chalmers/1/73, influenza epidemic		
116		Rimantadine, 50 mg 4 times per day for 5 days	Decreased intensity and shorter duration of fever and toxicity; no influence on catarrhal symptoms
39		Rimantadine, 75 mg 3 times per day for 5 days	
43		Placebo	
Outpatients	Influenza epidemics (H3N2) 1973–1975		
240		Rimantadine for 6 days, according to the scheme: days 1–2, 100 mg 3 times per day; days 3–4, 100 mg 2 times per day; days 5–6, 50 mg 2 times per day; 120 patients treated with the drug, 120 with placebo	Shorter periods of toxicity and of catarrhal symptoms, faster clearing of virus as revealed by immunofluorescent technique, reduction (2 times) in the number of complications

7.10. Inhibitory effects of ribavirin

A synthetic nucleoside analogue 1-β-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide (ribavirin, virazole, Fig. 7.11) has been shown to inhibit the multiplication of both DNA- and RNA-containing viruses including influenza viruses (Sidwell et al., 1972, Witkowski et al., 1972, Smith and Kirkpatrick, 1980) (Tables 7.26 and 27). Influenza type A and B virus replication is inhibited in tissue culture cells by ribavirin and

the compound acts at an early stage in the influenza-infected cell, possibly by inhibiting the production of essential nucleotides and hence RNA synthesis. More recently, aerosols of ribavirin have been shown to have a clinical effect against influenza B virus infection in humans and this is considered in more detail below (McClung et al., 1983).

The inhibitory effect of ribavirin on the replication of a number of different influenza A and B viruses was measured in an early study using the egg piece system (Fazekas de St Groth and White, 1958). In these experiments ribavirin was added to egg pieces in WHO plates at a final concentration of 0.04 mM at the time of virus infection and the virus was titrated in ribavirin treated and in control untreated plates and the plates incubated for 3 days at 37°C (Oxford, 1975). The nucleoside analogue at 0.04 mM caused a very significant inhibition (>4.0 log ID_{50}/ml) of the replication of a range of influenza A strains of human, equine, avian and swine origin including A/Duck/England/62 (Hav3Navl), A/Swine/Cambrigde/39 (Hsw/inelN1), A/Equine/-Miami/63 (Heg2Neg2) and A/Port Chalmers/1/73 (H3N2) viruses. In addition, influenza B/Hong Kong/8/73 virus was inhibited to a similar degree to the influenza A viruses. The multiplication of fowl plaque virus A/FPV/Dutch/27 (Hav1Neq1) in CEF cells was also inhibited by ribavirin as determined by plaque inhibition tests. The effect of ribavirin against respiratory virus infections in mice is shown in Table 7.27.

7.10.1. Teratogenic effects of ribavirin

Kochhar et al. (1980) investigated the possible teratogenic activity of ribavirin in the mouse model. Pregnant ICR mice were injected at 10th–13th days of gestation

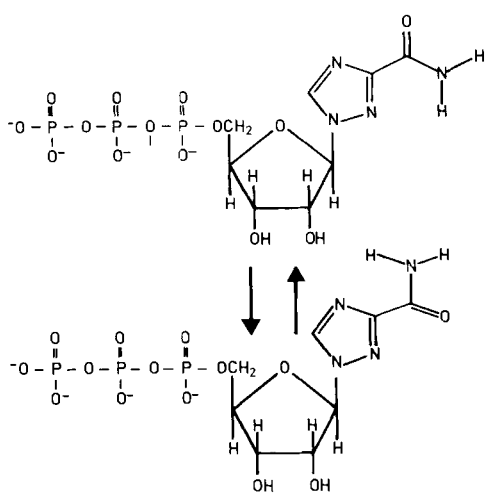


Fig. 7.11. Molecular structure of ribavirin triphosphate.

TABLE 7.26.
In vitro antiviral activity of ribavirin (after Sidwell et al., 1972)

Virus	Cell line	Virus rating
DNA viruses		
HS type 1 (HF)	KB	1.2
HS type 2 (MS)	KB	1.1
PR (RK17C24, derived from Aujeszky)	RK-13	0.0
MCM (Smith)	ME	0.9
Vaccinia (Lederle CA)	KB	1.0
Myxoma (Sanarelli)	RK-13	1.7
AV type 3 (GB)	KB	0.7
RNA viruses		
PI type 3 HA-1 (C243)	KB	0.8
PI type 1 (Sendai)	CE	$\geqslant 10$
Influenza A_2 (Jap/305)	CE	3.2
Influenza B (Lee)	CE	10
RV type 1A (2060)	KB	0.6
RV type 13 (353)	KB	0.8
RV type 56 (Phillips)	KB	0.7
Coxsackie B_1 (Conn.-5)	KB	0.4
PV type 2 (MEF-1)	KB	0.0
VS (Indiana)	KB	0.7
SF (original)	L-929	0.6

A virus rating of 1.0 or greater indicates a significant effect.
Virus abbreviations: HS, herpes simplex; PR, pseudorabies; MCM, murine cytomegalovirus; AV, adenovirus; PI, parainfluenza; RV, rhinovirus; PV, poliovirus; VS, vesicular stomatitis; SF, Semliki Forest. Cell abbreviations: KB, human carcinoma of the nasopharynx; RK-13, continuous rabbit kidney; ME, primary mouse embryo; CE, primary chick embryo; L-929, mouse fibroblast.

with a single i.p. dose of ribavirin in the range of 10–200 mg/kg. All dosages in excess of 25 mg/kg were teratogenic. The optimal teratogenic dose varied with the stage of development, being higher at advanced stages of development. Depending on the dose and stage of treatment virtually all parts of the skeleton including the craniofacial and limb bones were susceptible to ribavirin. Both the frequency and multiplicity of skeletal defects increased as the dose was raised. The stage dependency of defects in the orofacial bones was markedly apparent. Treatment on day 10.5 resulted in shortened maxilla in all survivors, while treatment on either day 11 or 11.5 resulted in a high frequency of reduction in the length of both upper and lower jaws. Treatment on the 12th day resulted in a very low incidence of effect on the maxilla (4%) but a high frequency (88–100%) of reduction and deformation of the mandible. Ribavirin, both in vivo and in vitro, inhibited embryonic DNA synthesis. The inhibition was transitory and did not seem to be directly related to the embryolethal activity of the drug. Although the role of metabolic inhibition in

TABLE 7.27.
Activity of ribavirin against lethal respiratory virus infections (10 to 20 LD_{50}) in mice (after Sidwell et al., 1972)

Ribavirin (mg/kg per day, p.o.)	Survivors (%)		P	Day of death (mean)		P
	T	C		T	C	
Influenza A (PR8)						
75	90	20	<0.001	20.1	10.2	<0.001
37.5	40	20	>0.3	12.4	10.2	<0.05
Influenza A (Jap/305)						
150	100	30	<0.001	>21.0	10.0	<0.01
75	70	30	<0.001	16.8	10.0	<0.001
Influenza B (Lee)						
30	40	0	<0.05	16.0	8.4	<0.001
15	20	0	>0.3	12.2	8.4	<0.05
Parainfluenza I (Sendai)						
75	90	0	<0.001	19.9	9.8	<0.001
37.5	70	0	<0.001	18.3	9.8	<0.001

Groups of 10 to 20 mice were used in each experiment (T, treated; C, control).

precipitating teratogenesis is not clear, cytotoxic action of ribavirin against proliferating limb bud mesenchymal cells is directly associated with the origin of limb deformities. A review of the toxicology of ribavirin by Hillyard (1980) summarizes data with the compound.

7.10.2. Clinical efficacy of ribavirin versus influenza A and B virus infection

Early clinical trials with ribavirin and influenza gave somewhat conflicting results (Table 7.28). Thus, Salido-Rengell et al. (1977) evaluated ribavirin in a natural outbreak of influenza A virus (H3N2) in a girls' school in Mexico City. Therapy begun very early in the course of illness and was associated with less severe illness and inhibition of viral shedding. Magnussen et al. (1977) also observed a beneficial effect of ribavirin in young adult volunteers experimentally challenged with influenza virus A/Victoria/3/75 (H3N2). The drug-treated group had significantly fewer individuals with severe illness, and an inhibitory effect on viral shedding was also observed. In contrast, Cohen et al. (1976) and Togo and McCracken (1976) were unable to demonstrate a beneficial effect of ribavirin in young adult volunteers experimentally infected with two strains of influenza A virus (H3N2) and one of influenza B virus. Differences in ribavirin dosage regimens and in the severity of illness produced by natural vs. experimental infections probably contributed to the

TABLE 7.28.
Double-blind controlled trials of ribavirin vs. influenza

Investigator	Type study	Virus	Ribavirin dose	Number studied	Effect of ribavirin
Salido-Rengell et al., 1977	Natural infection	A/England/72 (H3N2)	200 mg/day 5 days	61	↓ severity clinical illness ↓ virus isolations ↓ HI antibody response
Cohen et al., 1976	Challenge	A/Maryland/74 (H3N2) + A/Dunedin/73 (H3N2)	600 mg/day 10 days (starting −2 days)	37	None
Togo et al., 1976	Challenge	B/Georgia/74	600 mg/day 10 days (starting −2 days)	30	'Marginal' decreased severity of clinical illness
Magnussen et al., 1977	Challenge	A/Victoria/75 (H3N2)	1000 mg/day 5 days (start +6 hrs)	29	↓ severity clinical illness ↓ virus shedding

inconsistent results of these investigations. Except for mild transient rises in total serum bilirubin levels in approx. 25% of treated subjects, ribavirin appeared to be well tolerated.

More recently Smith et al. (1980) investigated the effect of ribavirin against naturally acquired influenza A (H1N1) virus infection in young adults. A double-blind placebo-controlled trial of ribavirin was conducted in 97 young adult males naturally infected with influenza virus similar to A/Brazil/11/78 (H1N1). Ribavirin was given orally at a dose of 1000 mg/day for five days beginning within 24 or 48 h after onset of clinical influenza. The clinical signs and symptoms of influenza and quantitative viral shedding were the same in ribavirin- and placebo-treated groups. Ribavirin treatment was associated with significantly fewer fourfold or greater rises in antibody to influenza A viral antigen by the complement-fixation test, while rises in haemagglutination-inhibiting antibody titres occurred with equal frequency in both groups. The ribavirin-treated group experienced significant increases in bilirubin and in reticulocyte counts after onset of therapy. This suggested that ribavirin treated subjects had decreased synthesis and/or increased destruction of erythrocytes during treatment but the effect was not of sufficient magnitude to suggest that haemolysis was involved.

7.10.3. Ribavirin aerosol treatment in influenza B infection

Following the successul demonstration of antiviral efficacy of aerosolised ribavirin

in animal models versus influenza A virus and also in volunteers (Knight et al, 1981), McClung et al. (1983) have recently investigated antiviral efficacy versus influenza B virus. This is an interesting approach because few antivirals apart from ribavirin has been shown to be active in animal models versus influenza B, which is a virus which can cause mortality and serious morbidity in some years (see above).

Students coming to a health centre with an oral temperature above 38.3°C and an illness of less than 24 h duration suggestive of influenza were included in the study. Treatment with ribavirin aerosol was commenced within 1 hour of admission and lasted for 16 h continuously. The following morning a regular schedule of 4 hours treatment at 7 am, 2 pm and 7 pm was initiated for 3 days (McClung et al., 1983).

On entry into the study the severity in clinical signs was similar in both groups but by 17 h illness in ribavirin treated patients had improved to a greater extent than in controls (Table 7.29). Similarly, as regards temperature, the maximum temperature was lower in treated patients and remained lower throughout the trial period (Table 7.30). After the first 8 hours a decline in virus titre in nasal washes was detected in the treated group which was significantly different from the control groups during the next 2 days. In conclusion, a more rapid recovery of ribavirin treated patients was noted, and the results were comparable to those obtained previously with influenza A infections (Knight et al., 1981). The estimated dose of ribavirin deposited in the respiratory tract was 55 mg/h. This represents the first report of successful treatment of influenza B infection in humans, since amantadine, for example, is only effective against influenza A viruses.

Amantadine and ribavirin represent the only classes of compounds to have demonstrable effects against influenza A and B viruses in the clinic. Each compound however has its weaknessess: amantadine is only active against influenza A viruses, whilst ribavirin, as a nucleoside analogue (Witkowski et al., 1972) is teratogenic and immunosuppressive in animals (Potter et al., 1976) and hence may have insur-

TABLE 7.29.
Mean maximum daily temperature in ribavirin aerosol treated and control patients infected with influenza B virus (after McClung et al., 1983)

	Temperature, °C			
	Day 0	Day 1	Day 2	Day 3
Treated (n=11)	39.2	38.5	37.8	37.3[a]
Control (n=10)	39.0	39.1	38.3	37.7[a]
t-test, one-tailed	N.S.	0.02	0.028	N.S.

[a] For treated group, n=6.
N.S., not significant.

TABLE 7.30.
Systemic illness in ribavirin aerosol treated and control patients infected with influenza B virus (after McClung et al., 1983)

	Mean symptom scores[a]						
	Day 0	Day 1		Day 2		Day 3	
		am	pm	am	pm	am	pm
Treated (n=11)	2.4	1.2	1.1	1.0	0.64	0.78[b]	0.78[b]
Control (n=10)	2.2	1.9	1.6	1.5	1.2	1.3[b]	1.0[b]
Wilcoxon's rank-sum test, one-tailed	N.S.	0.008	N.S. (0.084)	N.S. (0.096)	0.045	N.S.	N.S.

[a] Range of severity, 0 through 3+
[b] For treated group, n=6, for control group, n=6
N.S., not significant.

mountable problems of toxicity, particularly when used orally as a prophylactic. Work continues to find more effective inhibitors and more recently this has tended to centre around inhibitors of specific virus enzymes, such as the influenza RNA transcriptase and neuraminidase, and synthetic competitive inhibitors of the fusion sequence of influenza HA. Both the former and latter approaches have the theoretical advantages of broad specificity, since the RNA polymerase enzyme is constituted by NP and P polypeptides (shared by virtually all influenza A viruses), whereas the 'fusion' sequence at the N terminus of the HA_2 molecule is also shared by the HA of different subtypes of influenza. It is thought likely then that any inhibitors so developed would inhibit *all* influenza A viruses and, hopefully, influenza B viruses as well. In the next few pages we shall attempt to summarize some of this work and to emphasize approaches which could be exploited in the near future.

7.11. Inhibitors of influenza RNA polymerase

Influenza A and B viruses have RNA-dependent RNA polymerase activity associated with their cores (Chow and Simpson, 1971, Skehel, 1971). This RNA-polymerase activity has been detected in the microsomes and nuclei of influenza-infected cells, and the virus-associated enzyme can transcribe, in vitro, the influenza virus genome, suggesting that the enzyme is required for virus multiplication. Therefore inhibitors of the RNA polymerase enzyme have potential application as chemoprophylactic agents against RNA-containing viruses (Helgstrand and Öberg, 1978, see also Chapter 3). Ho and Walters (1971) described the inhibition of cell-associated RNA-dependent RNA polymerase of influenza A/PR8 (H1N1) virus by selenocys-

tine, and the related compound selenocystamine dihydrochloride inhibits the virus-associated RNA polymerase enzyme of a number of influenza A and B viruses (Oxford and Perrin, 1974). We have described the in vitro inhibition of influenza virus-associated RNA-dependent RNA polymerase by selenocystamine dihydrochloride, bathophenanthroline disodium disulphonate and certain heterocyclic thiosemicarbazones (Fig. 7.12). A property common to these compounds is the ability to chelate soft, heavy, metal ions such as zinc and copper. Conversely, similar types of compounds in which the possibility of chelation was diminished showed significantly (e.g. 3-acetyl-pyridine-thiosemicarbazone) less inhibitory activity against influenza virus RNA-dependent RNA polymerase (Table 7.31). Mass spectrometry and atomic absorption techniques have detected the association of zinc with purified influenza B virus and the hypothesis was advanced that the RNA-dependent RNA polymerase enzyme of influenza virus was a zinc-activated enzyme.

Table 7.31 shows the in vitro inhibition of RNA-dependent RNA polymerase of

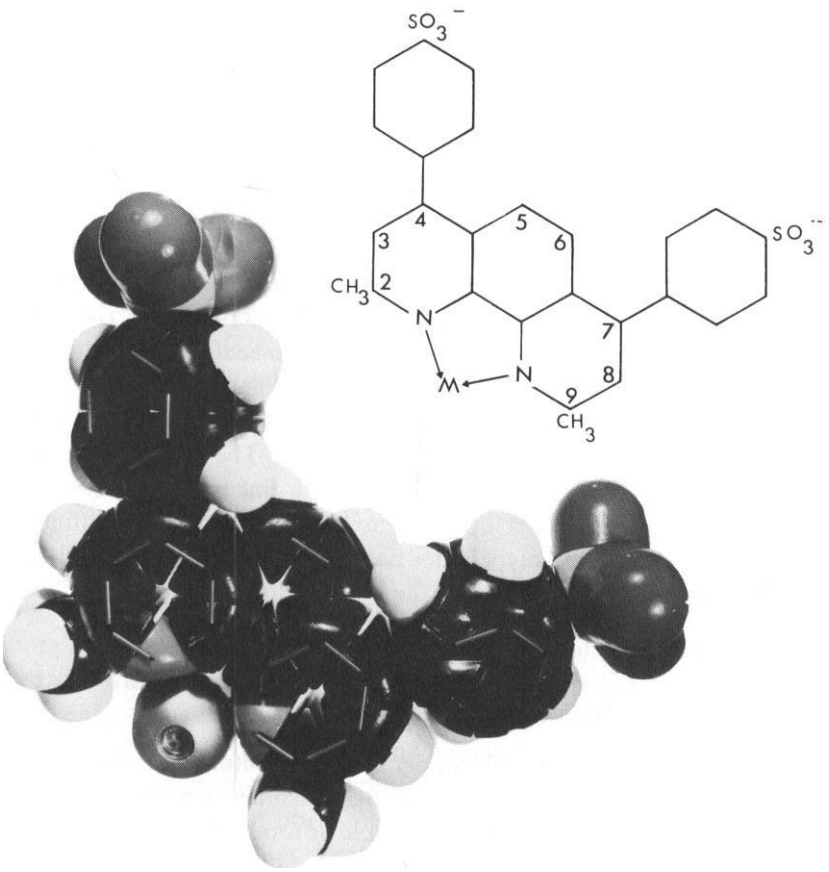


Fig. 7.12. Influenza RNA polymerase inhibitor (1:1 bathocuproine–zinc complex). Normally the zinc would be an integral part of a zinc RNA polymerase metalloenzyme.

TABLE 7.31.
Effect of chelating agents on influenza virus and bacterial RNA and DNA polymerases

	Concentration of compound (mmol) to inhibit incorporation of ^{3}H-UMP or ^{3}H-TMP by 50%			
	A/RI5$^+$ RNA polymerase	B/LEE RNA polymerase	*Escherichia coli* RNA polymerase	*Micrococcus lysodeikticus* DNA polymerase
Bathocuproine disodium disulphonate	0.02	0.08	0.08	0.30
2-acetylpyridine thiosemicarbazone	0.003	0.002	0.20	0.20
3-acetylpyridine thiosemicarbazone (control)	1.0	1.0	N.T.	N.T.
Isatin 3-thiosemicarbazone	0.06	0.02	0.20	0.20

N.T., not tested

influenza A and B viruses by selenocystamine dihydrochloride and other molecules. This former compound also inhibited the DNA-dependent RNA polymerase of *E. coli* at a concentration of 0.04 mmol, but had little effect on the DNA-dependent DNA polymerase of *Micrococcus lysodeikticus*. Thus, a certain degree of selectivity was detected, particularly for influenza B/LEE RNA polymerase and *E. coli* RNA polymerase, although the differences between inhibitory concentrations for the latter enzyme and the RNA polymerase of A/RI-5$^+$ were not significant.

If the RNA polymerase of influenza virus is a metallo-enzyme, more active and more selective chelating agents might be designed and tested. The compounds tested at present also inhibit *E. coli* RNA polymerase, which is a zinc metallo-enzyme but any inhibitory effect on mammalian cell polymerases would depend on configuration of RNA polymerase enzyme polypeptides near the zinc binding site and also on the relative stability constants of zinc for the polypeptide ligand and any competing ligand.

7.11.1. Targeting of inhibitors using liposomes

An additional problem is to target the RNA polymerase inhibitors into virus-infected cells. Attempts to do this have been made using liposomes, since certain compounds described above are either relatively insoluble or too highly charged to penetrate the plasma membrane of cells.

The use of liposome-encapsulated chelating agents for the selective delivery of chelating agents to the interiors of cells of the respiratory tract is an attractive possi-

bility (Perrin, 1977). Liposomes are finely dispersed phospholipid spherules, or vesicles around 1–10 μm in diameter, made up of concentric multiple bilayers that incorporate water and low-molecular-weight solutes in compartments between bimolecular lamellae (Fig. 7.13). Liposomes can be taken up into a cell by pinocytosis or can be engulfed by phagocytes. Once inside a cell, the liposome is broken down by lysosomal lipases and the chelating agent or other drug is liberated. Liposomes may protect drugs from metabolic modification and immunological reaction. The lipid composition may be varied considerably, giving a range of membrane structures, and charged liposomes can be formed by incorporating bases such as stearylamine or anionic species such as diacetyl phosphate or phosphatidic acid.

By using encapsulation in liposomes, the deposition and tissue-retention of highly charged inhibitors may be significantly increased. In model experiments Ca-EDTA or Ca-DPTA were used which are effective chelators of zinc but have a low and defined toxicity in experimental animals and man. The rationale of the experiments was to deplete the zinc levels in cells of the upper respiratory tract and hence to prevent the formation of, or inhibit the function of, the zinc containing influenza RNA transcriptase enzyme. However, in preliminary experiments in influenza virus-infected ferrets and mice, no antiviral activity was detected with the latter compounds, although only very low concentrations have been tested to date. In further experiments we selected the most active thiosemicarbazone (2-APTSC) for further in vivo studies in mice and ferrets. However, we were not able to detect any virus inhibitory effect or any effect of the compound on mouse mortality or mean day of death, or on the clinical signs of influenza in the ferret. Animals were given 5 mg/kg of 2-APTSC at –24, 0, 24, 48, 72 and 96 hours. For the above experiments we used A/Hong Kong/1/68 (H3N2) virus and included either amantadine or ribavirin as control compounds with demonstrable inhibiting effect in the particular model system.

7.12. Alternative animal models for assessment of anti-influenza virus activity

Potter et al. (1981) have described an interesting and relevant infant rat model. In essence when influenza virus replicates in the turbinates of infant rats they become more susceptible to challenge with *Haemophilus influenza*. Initial studies were carried out with amantadine and a polynucleotide (poly CS^4U). In the first study, 9 of 11 infant rats inoculated with influenza A/Victoria/75 virus and later given *H. influenza* developed bacteraemia and 6 of 11 developed meningitis; in contrast, virus-infected rats given amantadine at a concentration of 50 mg/kg by the subcutaneous, interperitoneal or oral route showed a significantly reduced incidence of bacteraemia and amantadine treatment by the intranasal route produced the most significant reduction in bacterial infection. These results were also shown in parallel studies using influenza A/Texas/77 virus. In a second, similar study, using influenza

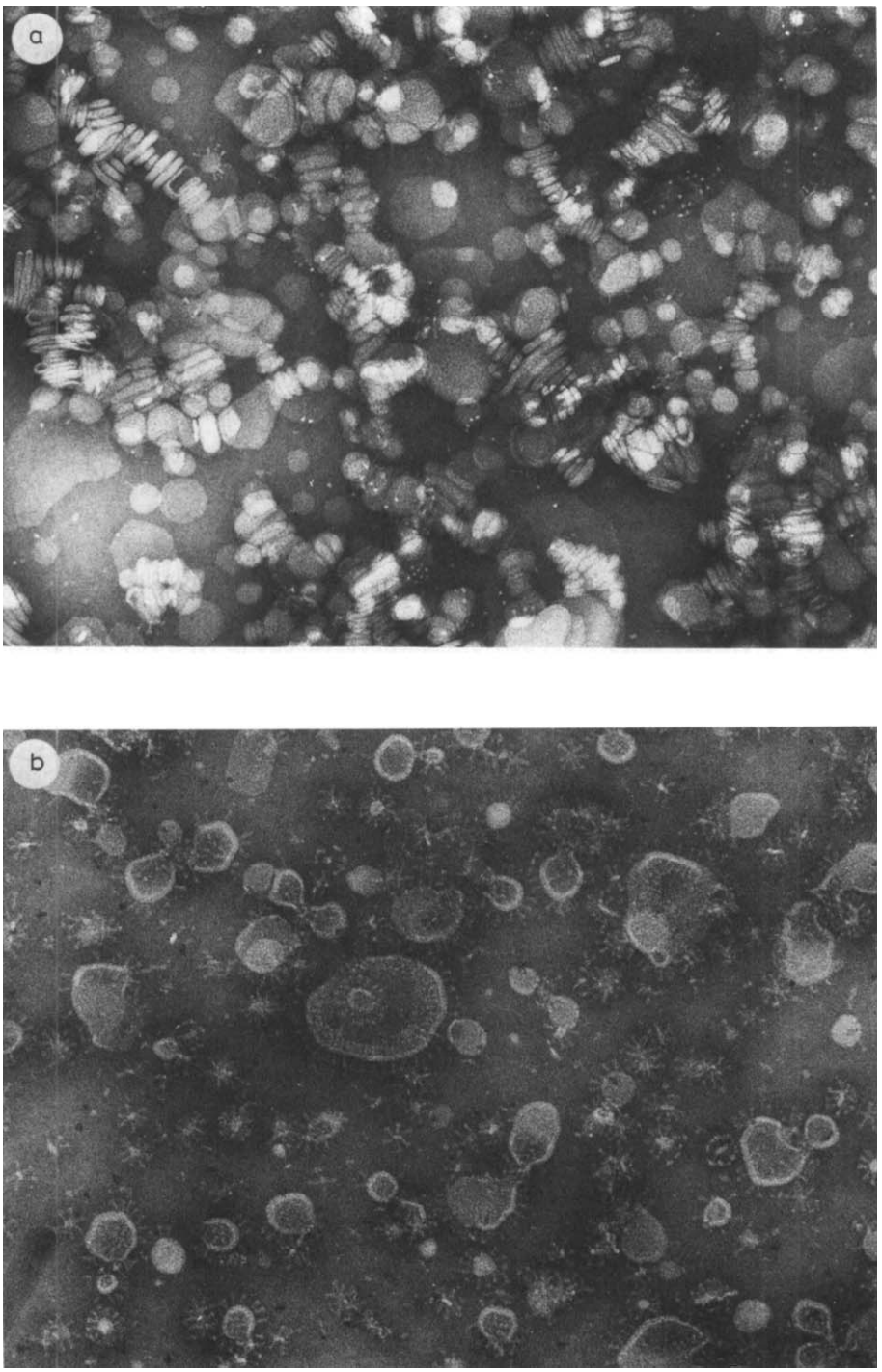

a
b

TABLE 7.32.
Incidence of bacteraemia and meningitis in rats following influenza virus infection and treatment with poly CS⁴U (after Potter et al., 1981)

Virus inoculation	Poly CS⁴U (30 μg/kg×4)[a]	Number of rats with bacterial infection[b]			
		Bacteraemia		Meningitis	
		No.	%	No.	%
A/Texas (10^4 EID_{50})					
+	–	8/11	73	5/11	45
–	–	2/13	15	0/13	—
+	+(i/n)	2/19	10[c]	1/19	5[c]
–	+(i/n)	1/10	10	0/10	—
+	+(i/p)	11/29	38	2/19	10
–	+(i/p)	6/20	30	1/20	5
A/Victoria (10^4 EID_{50})					
+	–	9/9	100	6/9	67
–	–	1/10	10	0/10	—
+	+(i/n)	8/15	53[c]	3/15	20[c]
–	+(i/n)	1/10	10	0/10	—

[a] Compound given i/n or i/p at 0, 6, 26 or 30 h following virus inoculation.
[b] Rats inoculated with 4.5×10^5 cfu of HIb at 48 h following infection with influenza virus; bacteraemia and meningitis assessed at 72 h after H.inf. infection.
[c] Significant protection ($P = < 0.05$).

virus A/Victoria/75 and A/Texas/77, poly (CS⁴U) also reduced the incidence of systemic infection (Table 7.32).

7.13. New compounds (and interferon) inhibiting influenza virus

Sodium 5-aminosulfonyl-2, 4-dichlorobenzoate (M12325) was evaluated for antiviral activity in tissue culture and infected mice (Ohnishi et al., 1982). At concentrations ranging from 2.5 to 75.8 μg/ml, the drug inhibited the cytopathic effects of 10 mean tissue culture infective doses of influenza virus A/WSN, A/FM, A/Kumamoto, and B/Great Lakes (Table 7.33). Concentrations up to 150 μg/ml did not inhibit the cyptopathic effects of herpes simplex virus, vaccinia virus, or adenovirus and concentrations up to 3160 μg/ml did not inhibit the growth of MDCK, Vero or HEL cells in culture, and hence a relatively large therapeutic index was estab-

←

Fig. 7.13. Influenza experimental virosome vaccine (Courtesy of Dr. D. Hockley). a, phospholipid vesicles; b, phospholid vesicles with HA attached by sonication ("virosomes").

TABLE 7.33.
Antiinfluenza activity, cytotoxicity, and therapeutic ratio of M12325 in culture (after Ohnishi et al., 1982)

Virus	Cell	MIC (μg/ml)		IC_{50} (μg/ml)		Therapeutic ratio	
		M12325	Amantadine	M12325	Amanta-dine	M12325	Amanta-dine
INFV-W	MDCK	2.5 (0.5–5.0)	3.7 (0.5–5.0)	>3160	102±9	>1260	27.6
INFV-W	MDCK	3.1 (0.5–5.0)	32.5 (15–50)	>3160	102±9	>1020	3.13
INFV-F	MDCK	13.3 (5.0–15)	7.8 (1.5–15)	>3160	102±9	>243	13.1
INFV-K	MDCK	3.8 (1.5–5.0)	3.3 (1.5–5.0)	>3160	102±9	>831	30.9
INFV-G	MDCK	7.0 (0.5–15)	32.5 (15–50)	>3160	102±9	>451	3.13

lished. Single oral doses of M12325, ranging from 10 to 300 mg/kg, administered 1 h before and 1 h after challenge, reduced mortality in mice inoculated intranasally with influenza A/WSN virus. Twice daily oral doses for 14 days effected significant reductions in the mortality of mice infected intranasally with influenza A/WSN, A/FM, A/Kumamoto, and B/Great Lakes, and parainfluenza virus, but they were not effective in mice infected with herpes simplex virus. Multiple doses of 10 and 30 mg/kg, administered intraperitoneally, reduced lung consolidation and virus titre whereas M12325 was well tolerated in multiple doses up to 1 g/kg orally.

Finally, in vitro and in vivo studies have clearly demonstrated that influenza A and B virus replication is inhibited by interferon (see also Chapter 3, Table 3.9). Moreover, the few experiments performed to date in controlled studies in volunteers have confirmed this activity (Merigan et al., 1973). Obviously further detailed studies are required with both influenza and other respiratory viruses, because interferon would be expected to have a broad spectrum of antiviral activity.

7.14. Influenza vaccines

Vaccination remains, at present, the main way of preventing epidemic influenza A and B viruses but, at least more recently vaccination has been confined to groups at special risk of mortality from influenza. The earlier widespread use of live attenuated vaccine in the USSR has now declined somewhat and most countries only produce enough vaccine to immunize some 10% of the population. Again, more recently, this vaccine has tended to be inactivated whole virus or subunit vaccine (reviewed by Selby, 1976). These (whole virus) vaccines have been produced for the past 4 decades and with improving technologies of ultra-centrifugation and gel filtration are now relatively pure virus proteins. HA antigen content is well controlled by single radial diffusion techniques (Wood et al., 1977) but it must be admitted

that many problems remain to be solved. Some of these are discussed in more detail below, whilst the reader is also referred to Chapter 2 where some more modern approaches of gene cloning are discussed and to Chapter 17 where some problems of antigenic and genetic variation are outlined (see also Table 7.34). Laboratory studies have established clearly that HA and NA antigens contain the main antigenic determinants of the virus responsible for inducing protective immunity (reviewed in Potter and Oxford, 1979). Passive antibody to HA and NA protects mice against lethal infection with influenza A virus, as well as immunization with HA and NA antigens. Immunization or passive immunity with antibody to M or NP for example has no protective effect (Virelizier et al., 1976, Fazekas de St. Groth and Graham,

TABLE 7.34.
Some currently used influenza vaccines and some experimental approaches

Vaccine	Comments or reference
Inactivated	
subunit	True subunit preparation with HA and NA removed by centrifugation
split virus	Contains all virion protein but lipids are removed and the virus disrupted
whole virion	The most immunogenic vaccine but also produced the most side reactions, particularly in children
Live attenuated recombinant with host range mutant A/PR/8/34 (H1N1) virus	Florent et al., 1977; Florent, 1980
	Occasionally such recombinants may be virulent for humans (Oxford et al., 1978)
ts or *ca* mutants made by recombination	Problems of 'revertants' in young children have yet to be overcome
Experimental vaccines	
virosomes	Liposomes are sonicated with HA and NA to reconstitute a synthetic virus. More immunogenic than HA alone
HA aggregates	A new approach to increase immunogenicity of HA (Morein et al., unpublished data)
Virus grown in human diploid cells	Cell grown virus may exhibit different antigenic determinants (Schild et al., 1983)
HA cloned in mammalian cells or bacteria	Chanock and Murphy, 1979; Chanock, 1982; Heiland and Gething, 1981
Genes from avian 'enteric' influenza viruses – and administration of live vaccine orally	Recombinants may be made with genes of avian duck influenza A viruses (Murphy et al., 1983). Russian workers have administered live vaccine orally.
Oligopeptides of antigenic determinants of 'fusion' sequence	Green et al., 1982; Atassi and Webster, 1982

1954). Although cell mediated immunity (see Chapter 2 and Wells et al., 1979) may aid in the recovery from influenza, nevertheless the most important correlates of protective efficacy are local nasal IgA and serum levels of IgG neutralizing antibody (reviewed in Stuart-Harris and Schild, 1976). Underpinning the mountain of difficulties is the major one of original antigen sin (Francis, 1953), whereby it was observed that the first attack of influenza leaves an indelible immunological memory to the HA of that virus. Subsequent stimulation of the immune system by infection or immunization more often than not leads to an increase in synthesis of antibody to the HA of this first virus, rather than to the virus actually in the current vaccine. It is difficult to imagine how any approach to influenza vaccine could overcome this difficulty and this again emphasizes that a combined approach with antivirals and vaccines may represent the best strategy.

7.14.1. Inactivated influenza virus vaccines

Inactivated influenza A and B virus vaccines are prepared nowadays most often by the purification of recombinant (Kilbourne, 1969; Schulman and Palese, 1978) influenza virions harvested from infected allantoic fluids of embryonated hens' eggs by rate-zonal centrifugation. The virus (which is approximately 98% pure) is then inactivated with formalin or β-propiolactone and standardized by radial immunodiffusion (Wood et al., 1977) or rocket immunoelectrophoresis to contain around 30 μg of protein per human vaccine dose. Using high growth 'recombinant' viruses (Kilbourne, 1969) which contain as many as 6 genes from the high yielding laboratory virus A/PR/8/34 (H1N1) and only 2 genes (coding for HA and NA) from the 'wild' virus, approximately 2–3 doses of virus vaccine may be obtained from one embryonated egg. But it is quite apparent that the application of a considerable technology and time span is required to produce enough vaccine to immunize a population of 200 million persons in the USA, for example (Table 7.35). A period of many weeks must necessarily elapse from the initial outbreak caused by a new

TABLE 7.35.
Use of influenza vaccine in the USA

Year	Total population		Population aged 65 and over	
	Number of persons immunized $\times 10^6$	Percentage of total immunized	Number of persons immunized $\times 10^3$	Percentage of total immunized
1968–69	21	10.7	3506	19.0
1972–73	15	7.7	3209	15.8
1973–74	17	8.3	3638	17.4

Source: US Immunization Survey, 1969, 1973, 1974

TABLE 7.36.
Production of inactivated vaccine for A/Hong Kong epidemic, 1968

Isolation	4 days
Identification, recombination, distribution of strain	8 weeks
From seed strain for first release	10 weeks
To production of first million doses	2 weeks
Total	20 weeks

5 million doses 22 weeks
20 million doses 29 weeks
Source: Murray, R. Bull. WHO 41, 495.
Note: there is little reason to suppose that this period would be significantly shorter today

influenza A virus and the production of vaccine against the antigenic variant (Table 7.36). (In contrast, the efficacy of existing antivirals such as amantadine can be established within days.) Large scale immunization (Table 7.37) has been attempted in the USA in the face of a threatened outbreak of virus caused by A/New Jersey/76 (H1N1), a virus genetically related to the causative virus of the 1918 pandemic which had remained in the pig population in the USA since the pandemic in humans. However, the mass vaccination campaign was halted when a hitherto unrecognized complication of vaccination was observed – Guillain-Barré syndrome, a progressive disorder of the central nervous system (Keenlyside et al., 1980). Nevertheless, the campaign was a considerable technological achievement, with rapid production of vaccine in quantity, and efficient administration on a large scale using jet guns.

The other problems of inactivated influenza vaccine relate to the rather low efficacy (approximately 70% protection), poor longevity of the immune response (1 year), evasion of the induced immune response by antigenic mutants of the virus, and residual toxicity of the vaccine itself. As regards the latter, most adult persons receiving current inactivated vaccines notice only local pain and stinging at the site of inoculation and although this may deter future acceptance of vaccine it is not a serious reaction. However, in children or unprimed adults, whole virus vaccine

TABLE 7.37.
Population groups in which a more extended use of inactivated influenza A and B vaccine could be recommended

1. Persons at special risk of mortality e.g. preexisting pulmonary, cardiac, metabolic and immunological deficiencies and older persons (over 60 years of age).
2. Persons in institutions and boarding schools, where attack rates can be very high.
3. Hospital patients and personnel, public transport personnel, police, factory workers.
4. In the event of a new pandemic of influenza A virus as many of the community who wish to be included in a vaccination campaign.

See also Smith et al., 1976

may induce more serious side reactions, including fever. To circumvent these adverse effects, more recently developed vaccines are 'subunit' preparations containing only the relevant antigens which induce protective immunity – namely the two surface glycoprotein spikes of the virus, haemagglutinin and neuraminidase (reviewed by Tyrrell and Smith, 1979; Webster and Laver, 1966). Such subunit vaccines are less reactogenic but unfortunately they are also less immunogenic in unprimed individuals. Studies are continuing in attempts to improve the immune response to HA subunits. For example, addition of synthetic adjuvants such as muramyl dipeptide (MDP or 6-stearoyl MDP) may increase the B cell response to the HA molecule, at least in animal models.

An exciting new approach has been the incorporation of a synthetic gene transcribed from gene 4 (coding for HA protein) by a reverse transcriptase into an *E.coli* plasmid system (Emtage et al., 1980). The result of this genetic engineering experiment was a bacterium which synthesized influenza HA in amounts detectable by immunoprecipitation (around 2 μg/ml) (see also Chapter 2 and the experiments of Gething and Sambrook, 1982). However, at this stage many obstacles remain. Firstly, the HA constitutes only 2–3% of the total protein synthesized by the bacterium and is not transported to the exterior. Thus, the HA has to be released by lysing the bacteria and purifying it from *E.coli* proteins and endotoxins. Although the HA clearly possesses some antigenic determinants, of prime importance for vaccination is retention of immunogenicity. It is probable that enzymatic degradation occurs in the presence of *E.coli* proteases and thus the HA molecule may be in monomer form, similar to HA removed from virus by the proteolytic enzyme bromelain. In this case the molecule may be poorly immunogenic and may need to be reconstituted onto a virosome, for example, to increase the immune response. On the other hand, the technology of bacterial cultivation on a large scale is well advanced and a single large fermentation batch could produce theoretically 100 mg of HA, enough antigen for 10 000 doses of vaccine at the current formulation.

7.14.2. Synthesis of an antigenic site on influenza HA

As noted above, the N-terminal region of HA2 (the fusion region) is believed to be involved in the initial infection and uncoating of the influenza virus and, from the X-ray structure, the first 10 residues of this region appear to be accessible to antibody (at least at low pH). Because of its involvement in viral infection, antibodies may arise against this region in the course of defence against viral infection. The elegant studies of Atassi and Webster (1983) with synthetic peptides comprising the HA2 region (residues 1–11) of influenza A virus and influenza B virus were carried out to investigate the antigenicity of this region of the HA molecule. Two peptides, comprising the fusion region (residues 1–11 of the HA2 part of HA) of strain A and strain B influenza virus, were synthesized and their abilities to bind rabbit, goat, and human anti-influenza antibodies were determined. In quantitative im-

munoadsorbent titrations, the two peptides bound considerable amounts of antibodies in rabbit and goat antisera against virus or HA of the A or B strain, as well as in several human sera from patients recovering from influenza A. Of the 30 anti-HA monoclonal antibodies, 5 bound completely and 4 bound partially to the peptides. Antibodies were raised in rabbits against the peptides by immunizing with peptide-bovine serum albumin conjugates, or with the free peptides. Anti-peptide antibodies were bound by HA and by the intact virus of the respective strain. However, these antisera failed to exhibit significant virus neutralizing activity. The finding that sera from several individuals after viral infection had large amounts of antibodies directed against the fusion region unequivocally established it to be an important antigenic site on virus HA for humans under conditions of natural influenza infection.

In comparison with the relatively strong immunogenic activity of this location, weak antigenicity has been reported in the region of residues 91–108 and in some of the carbohydrate side chains of HA1. Recently, 20 peptides comprising almost 75% of the HA molecule were synthesized (Green et al., 1982) and, even though most were antigenic when coupled to protein carrier, none of the peptides reacted with anti-HA antibodies, indicating that they did not contain any antigenic sites of HA. The antigenic site described by Atassi and Webster (1983) is not one of the four antigen regions suggested from examination of the X-ray structure of HA (see above). However, it is accessible and is involved in infectivity. Also, this site is not predictable from the empirical approach on the basis of hydrophilicity index. In fact, the fusion region is so hydrophobic that the synthetic peptides were insoluble in aqueous solvents.

Antigenic sites should be expected to reside in accessible surface regions, but not every surface region constitutes an antigenic site and thus, exposure is not a sufficient criterion for immunogenicity. Furthermore, antigenic sites are not necessarily highly hydrophilic regions, and hydrophobic interactions frequently provide major contributions to the binding energy. Also, as seen here and reported for haemoglobin α-chain, major antigenic sites could reside in surface regions that have very low hydrophilicity or are mostly hydrophobic. Such regions are rendered accessible by the three-dimensional and oligomeric characteristics of a particular protein.

In summary, the study is an excellent start but many potential peptides now remain to be investigated. It is also possible that the antibodies induced, although having no conventional neutralizing activity, may still inhibit virus release or infection in a biological system.

7.14.3. Problems and perspectives with inactivated influenza vaccines

Since we shall examine data in some detail of the *ca* live virus approach (see below) it will be of interest to examine some problems with conventional inactivated influenza vaccines. In an excellent study in the Post Office in the UK data was accrued

TABLE 7.38.
Post Office – Telecommunications: Main causes of sickness absence 1974–75

	Days (thousands)		Days/employee	
	Men	Women	Men	Women
Respiratory	514.4	421.4	3.3	5.2
(influenza)	(147.0)	(98.0)	(0.9)	(1.2)
Injuries	258.9	90.9	1.6	1.1
(at work)	(57.2)	(4.7)	(0.4)	(0.1)
Gastrointestinal	182.1	127.5	1.1	1.6
Musculoskeletal	162.9	109.4	1.0	1.3
Cardiovascular	154.6	52.5	0.9	0.6
Psychiatric	58.9	67.6	0.4	0.8
Malignant	8.3	6.5	0.1	0.1
Other	416.3	510.0	2.4	6.3
Unspecified	54.9	48.6	0.3	0.6
All causes	1838.5	1434.4	11.1	17.6

TABLE 7.39.
Subjective reactions in 247 influenza vaccinees (after Smith et al., 1976)

	Type of vaccine			
	Injected	Killed nasal	Attenuated nasal	
			First dose	Second dose
Number vaccinated	89	78	80	75
% with no reactions	3	36	43	67
% with general reaction	63	63	57	33
% with local reaction	92	—	—	—

which answered questions about vaccine efficacy, safety and acceptance by healthy persons working in industry. Firstly the data in Table 7.38 emphasizes the importance of influenza as a cause of sickness absence in this group of workers. Table 7.39 shows data on subjective reactions in some of the employees following inactivated or live influenza vaccines. Most vaccinees experienced some local or even general reactions to vaccination and this was, perhaps, reflected in the acceptance rates for vaccine in successive years which declined (Table 7.40). Of particular interest is the observation that over the 5 year period, days lost from sickness or absence were fewer in the vaccinated group compared with the unvaccinated group, although fluctuations were noted from year to year. Certificated sickness absences were also reduced significantly, both in the influenza and non-influenza period

TABLE 7.40.
Acceptance rate of influenza vaccine in industrial workers (after Smith et al., 1976)

Factory	Nature of factory	1971/2	1972/3	1973/4	1974/5
Post Office					
Telecomms.	Skilled technical	42%	34%	35%	32%
Posts.	Postmen and counter staff	—	36%	28%	23%
A	Light industry and office	40%	26%	22%	19%
B	Light industry and office	42%	27%	20%	14%
C	Office staff	—	32%	26%	27%

Based on 1973/4 total population

TABLE 7.41.
Influenza vaccination study in the Post Office. Days lost from sickness absence/week/100 employees (after Smith et al., 1976)

		1971/72		1972/73		1973/74	
		Vacc.	Unvacc.	Vacc.	Unvacc.	Vacc.	Unvacc.
Excess in influenza period compared with non-influenza period	Telecomms.	4.1	6.4	8.0	6.3	2.3	2.3
	Posts	—	—	3.6	5.1	3.4	4.4
Average over whole study period	Telecomms.	22	22	25	24	21	23
	Posts	—	—	29	31	30	32

TABLE 7.42.
Certificated sickness absence[a] in factory employees, 1972–73 (after Smith et al., 1976)

Category of employees	No. of employees	Average no. of days lost from sickness absence/100 employees/week		
		Influenza period	Non-influenza period	Excess in influenza period
Vaccinated A+B	646	4.6	2.4	2.2
Vaccinated B	524	6.9	2.8	4.1
Non-vaccinated	3280	7.9	3.9	4.0

[a] Excluding absentees in week of vaccination and absences over 9 weeks' duration.

(Tables 7.41, 42) (showing the placebo effect of medical attention on persons reporting ill!). It might be added that a recent analysis of economic savings from vaccinating old persons in the USA has shown a considerable economic benefit (Riddiough et al., 1983).

7.14.4. Live attenuated influenza virus vaccines

An alternative approach is the use of live attenuated influenza vaccines (Beare et al., 1975; Richman and Murphy, 1979). In theory, there should be a greater opportunity for rapid production since up to 1000 doses of live virus vaccine can be recovered from a single embryonated hen's egg. Alternatively, influenza A virus strains can now be cultivated in human diploid cells and this may also have theoretical advantages. (Herrero et al., 1983, Schild et al., 1983). There is also experimental evidence that the immunity resulting from the administration of live attenuated vaccines is broader than that induced by inactivated vaccines and this may help circumvent the perennial problem of antigenic drift (reviewed by Potter and Oxford, 1979).

In early studies two groups of virologists, one in Leningrad (Alexandrova and Smorodintsev, 1965) and one in Ann Arbor (Maassab, 1967) adapted influenza viruses to low temperatures (cold adapted mutants or *ca* mutants) and investigated the potential of these viruses as attenuated virus strains. The viruses will not replicate at 37°C and hence, at least in theory, would not replicate in the lower respiratory tract and would be expected, in view of the accumulated mutations, to show signs of attenuation. Laboratory 'recombinants' or viruses with reassorted genomes between the *ca* mutants and the most recent virulent antigenic variants are made, and assessed for virulence in volunteers. In this way a recombinant can be selected with *ca* genes from one parent and the HA and NA genes from the virulent parent, and such recombinant viruses are apparently attenuated for man.

An alternative and much investigated possibility is to induce a temperature sensitive (*ts*) lesion (or lesions) in a well characterized donor strain of influenza A virus (a 'master' or 'mistress' strain) and then transfer the genes responsible for the *ts* defect, together with genes coding for HA and NA from the current virulent virus, to make laboratory recombinants (Murphy et al., 1976). The rationale is similar to the *ca* approach – viruses which are unable to replicate at 37°C cannot infect cells in the lower respiratory tract of man, and hence would be expected to show reduced virulence properties. However, a complication of some of the earlier studies with *ts* mutants has been some tendency to revert back to a more virulent non *ts* phenotype. But this has been less of a problem with the *ca* mutants which, at present, look more hopeful for future studies.

In a third general approach to live attenuated influenza vaccine viruses, recombinants have been prepared between an old attenuated laboratory virus A/PR/8/34 (H1N1), which is probably a 'host range mutant' since it has been 'adapted' to ferrets, mice and eggs and thus 'de-adapted' to humans, and the recent virulent virus.

Viruses which have HA and NA genes from the virulent virus parent and the remaining genes from the attenuated A/PR/8/34 parent, are investigated in volunteers for attenuation (Beare et al., 1975). It has been shown quite clearly, however, that occasionally such recombinants still retain some virulence for man (Oxford et al., 1978) and such studies have, therefore, to proceed with caution. The present complex epidemiological situation with influenza A virus, with two antigenic subtypes co-circulating (H3N2 and H1N1) introduces a further complication with live vaccines. If two such laboratory attenuated recombinants are administered to man then additional recombination events may occur in naturo leading to 'new' viruses perhaps with more virulent properties (Rott et al., 1979, Scholtissek et al., 1979). However, such processes are probably occurring anyway in nature (Palese and Young, 1982). Two live vaccine viruses administered together may interfere with each other's replication (Potter et al., 1983) and so result in the development of a distorted immune response to a single virus only. Thus, as many problems remain to be solved with influenza vaccines as with the development and use of specific anti-influenza virus inhibitors. The population groups in which more extended use of vaccine could be recommended are similar to those suggested for antivirals (Table 7.37).

An excellent summary of the *ca* virus studies carried out mainly in the USA and USSR has been published (Kendal et al., 1981) and we shall content ourselves here by re-examining some of this summarized data. Typical biological characteristics of these *ca* master or mistress viruses are outlined in Table 7.43, whilst a method of obtaining the *ca* Ann Arbor strain by passage in the laboratory at 35°C is illustrated in Table 7.44. These candidate vaccine strains are tested exhaustively in animal models, where they replicate well in the cooler parts of the upper respiratory tract of the ferret and mouse but not at all at 37°C in the lower respiratory tract. A protocol for phase I testing in volunteers is outlined in Table 7.45, and it is apparent that the candidate virus is tested for immunogenicity and side reactions in

TABLE 7.43.
Replication temperature markers of cold-adapted viruses used as parents in the preparation of recombinant vaccine candidates (after Kendal et al., 1981)

	Replication of cold-adapted parental virus						
	in eggs at (°C)				in CK cells at (°C)		
	25	28	33	39–40	25	33	39
A/Leningrad/9/46 (H1N1)	2.25	8.25	8.25	2.25	N.D.	N.D.	N.D.
A/Leningrad/134/57 (H2N2)	4.75	8.25	8.25	2.25	N.D.	N.D.	N.D.
A/Ann Arbor/6/60 (H2N2)	7.50	N.D.	8.50	1.30	8.30	8.70	<3.0

CK, chick kidney cells
N.D., not determined

TABLE 7.44.
Isolation and passage history of cold-adapted influenza A/Ann Arbor/6/60 mutant used for preparing recombinant vaccine candidates (after Kendal et al., 1981)

Passage level	Conditions
1	Throat swab, incubate at 36°C in CK cells
2	CK_1 isolate, incubate at 36°C in CK cells
3–9	Serial passages, incubate at 33°C in CK cells
10–16	Serial passages, incubate at 30°C in CK cells
17–23	Serial passages, incubate at 25°C in CK cells
24–29	Serial plaque purification, at 25°C in CK cells
30–32	Serial passage at 25°C in specific pathogen-free hens' eggs

TABLE 7.45.
Protocol for phase 1 (safety) testing of live vaccine in healthy young adults in the USA (after Kendal et al., 1981)

A. General procedure
   1. Pre-bleed potential vaccine participants.
   2. Test pre-study serum by HI, neutralization, or enzyme-linked immunosorbent (ELISA) assay, to identify volunteers who have maximum susceptibility to the vaccine virus.
   3. Quarantine all selected volunteers in restricted access area for 2–3 days to monitor for any developing illness.
   4. Under blind conditions, administer by drops vaccine or placebo (0.5 ml/nostril) to supine volunteers. Approximately 3/4 of participants receive vaccine, the remainder placebo.
   5. Monitor febrile, systemic and respiratory reactions and collect daily nasal wash specimens.
   6. After 2 weeks collect final serum specimen. Volunteers depart from restricted access area.

B. Laboratory tests on specimens collected
   1. Inoculation of tube cultures of monkey kidney (MK) or Madin–Darby canine kidney (MDCK) cells at the permissive temperature of 34°C with dilutions of nasal wash specimens to titrate post-inoculation replication of virus, and verify cessation of virus shedding. Additional virus isolation studies are undertaken to diagnose possible non-influenza infections in case of illness in participants.
   2. Inoculation of the tube cultures of MK or MDCK cells at the non-permissive temperature of 39°C with a low dilution of nasal wash specimens to detect possible revertant (non-*ts*) virus.
   3. Titration of virus isolates in primary chicken kidney cells at 25°C, 33°C and 39°C to examine stability of *ca* and *ts* markers.
   4. Titration of pre- and post-inoculation sera specimens by HI, and in some cases by neutralization and ELISA procedures, to detect and quantitate humoral antibody responses.
   5. In some cases titration of nasal wash specimens by neutralization and ELISA procedures to quantitate local antibody responses.

TABLE 7.46.
Infectiousness, immunogenicity and reactogenicity of cold-adapted H3N2 A/Ann Arbor recombinant vaccines in phase 1 studies with young adult volunteers (after Kendal et al., 1981)

H3N2 recombinants	Dose (log_{10} $TCID_{50}$	No. of volunteers	No. (%) infected[a]	No. (%) infected with	
				HI response	Febrile or systemic illness
CR 18 clone 7	7.5	10	8 (80)	6 (75)	1 (13)
	8.5	12	12 (100)	11 (92)	4 (33)
CR 19 clone 0	6.3	14	13 (93)	13 (93)	0 (0)
	7.5	13	12 (92)	10 (83)	1 (8)
CR 22 clone 1	6.8	9	9 (100)	9 (89)	1 (11)
CR 29 clone 2	7.5	24	18 (75)	16 (89)	0 (0)
CR 31 clone 3	7.7	12	12 (100)	7 (58)	0 (0)
CR 31 clone 10	7.7	17	13 (76)	10 (77)	1 (8)
H1N1 recombinant					
CR 35 clone 2	7.5	25	24 (96)	11 (46)	0 (0)

[a] Determined by virus isolation and antibody response.

a small group of volunteers in quarantined conditions. Samples are collected from the volunteers and examined for biological markers, including the important *ca* property. (A problem with *ts* viruses is that when tested in doubly sero-negative children with no antibody to either HA or NA, non *ts* 'revertant' viruses tend to appear. This has slowed the programme for development of *ts* influenza A viruses in the USA).

Typical results of immunogenicity from published trials with the Ann Arbor and Leningrad vaccine viruses are given in Table 7.46. Both viruses were shown to induce antibody in volunteers, whilst producing no serious side reactions. Most importantly subsequent challenge of these immunized volunteers showed them to be protected against clinical signs of influenza infection. Therefore, studies are progressing with *ca* mutants, but for reasons of safety have to progress slowly. One could ask, for example, whether recombination in nature with a *ca* and wild virus could lead to a more virulent recombinant? Would the *ca* viruses show clinical effects in chronic bronchitics or persons with other pre-existing respiratory diseases? Finally, as alluded to above and in Chapter 2, genetic engineering and biochemical methodology could lead to new vaccine candidates with lesions inserted at specific points in the genome.

7.15. Summary

An overwhelming amount of data has accumulated on the biochemistry, cell biology and epidemiology of influenza, but prospects of control of epidemics in the near future are dim. Meanwhile, a 'holding operation' can be achieved using inactivated vaccine and rimantadine (100 mg/daily) in special risk groups in the population until new more effective vaccines and broad spectrum antivirals (active against influenza A *and* B virus) are developed. Research work is centred now around biotechnology to produce immunogenic peptides and proteins and also more logical searches for antivirals using amino acid sequence data and also virus specific enzymes such as the virion transcriptase as targets.

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