CHEMOTHERAPY OF VIRAL DISEASES

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I. INTRODUCTION

It is a generally held belief that at the present time there are no known agents which are capable of exerting a real chemotherapeutic action in viral infections of man (189, 192). This, however, is not strictly true, as both Flumidin (N,N′-anhydrobis-β-hydroxymethyl-biguanide with methscopolamine and methatropine) (112) and Xenaldial (xenalamine, *p*-α-ethoxy-*p*-phenacylamine-benzoate) (30) have demonstrable activity in the prophylaxis and treatment of influenza, and isatin-β-thiosemicarbazone derivatives afford some protection against smallpox infection.

Earlier reviews (90, 109, 154) on this subject have tended to be catalogs of compounds which have been used in a variety of antiviral assays. It is the purpose of this review to summarize existing knowledge in this field under a few broad headings, including both physical and pharmacological data. Compounds which have shown the most extensive antiviral activity fall, with few exceptions, into four major groups, namely, urea derivatives, benzimidazoles, acridines and heterocyclic thiosemicarbazones; representatives of these groups are listed in Table 1. The importance of precise quantitative measurements where series of closely related compounds are being assayed is emphasized, and suitable methods are described. Structure-activity relationships of the more important active molecules are discussed in detail, as also are the experimental results and hypotheses concerning the mode of action. The last section deals with the special problems raised by the specificity of action of these agents.

TABLE 1

Compound	Stated Activity against Virus	Reference
Urea Derivatives		
Diphenylcarbamyl chloride	Influenza	191
4-Chloro-4′-fluorothiocarbanilide	Influenza	27
Guanidine salt of hydroxyaminomethylene malonitrile	Type 2 polio	100, 131
Guanidine HCl	Type 2 polio	100, 131
N′,N′-Anhydrobis-(β-hydroxymethyl)biguanidine hydrochloride	Influenza	112
Benzimidazoles		
5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole	Influenza virus	154, 167
Benzimidazole	Influenza B virus	155
4,5,6-Trichloro-β-D-ribofuranosylbenzimidazole	Influenza B virus	155, 162
5,6-Dichloro-D-arabinosylbenzimidazole	Poliomyelitis virus	165
2,5-Dimethylbenzimidazole	Influenza virus	157
2-α-Hydroxybenzylbenzimidazole	Poliovirus	60, 86, 124, 156
2-(*o*-Hydroxybenzyl)benzimidazole	Poliovirus type 2	120
Thiosemicarbazones		
Isatin-β-thiosemicarbazone	Neurovaccinia	14, 21
N-Alkylisatin-β-thiosemicarbazones	Vaccinia Variola	14, 15
1-Methyl-4′-4′-dibutylisatin-β-thiosemicarbazone	Poliovirus type 2	124
p-Amino-benzaldehyde-3-thiosemicarbazone	Vaccinia virus	77
Benzaldehyde thiosemicarbazone	Vaccinia virus	172
4-Methyl-5-thiazolecarboxyaldehyde thiosemicarbazone	Vaccinia	29
2,3-Butanedioneoxime thiosemicarbazone	Vaccinia	29
4′,4′-Dialkylthiosemicarbazones of isatin, methylisatin or N-ethylisatin	Ectromelia	183
Isatin-β-4′,4′-dimethylthiosemicarbazone		183
N-methylisatin-β-4′,4′-dibutylthiosemicarbazone	Type 2 polio	124, 139
Acridines		
Proflavine	Vaccinia	168
	Poliomyelitis	24
Mepacrine (quinacrine)	Vaccinia	168
	Equine encephalitis; Rift Valley fever; louping ill	72, 89
Chloro- and nitroacridines	Influenza A and B; mumps	56

II. FORMULATION OF EXPERIMENTAL DATA IN A QUANTITATIVE MANNER

A. Dose-response curves in vivo

Investigations involving large series of compounds possessing antiviral activity of varying degrees necessitate the development of some kind of assay which enables their relative activities to be expressed in fairly precise numerical terms. This is essential if meaningful conclusions are to be drawn from the biological findings and correlations made between structure and activity. Usually, of course, pharmacological assays are so designed that the plotting of dose-response curves is facilitated. However, in the case of most *in vivo* antiviral testing the response is quantal, *i.e.*, the animals either die or survive. It would be highly desirable to find also a response which is a continuous variable.

Johnson and Baker (94), using the Rous sarcoma virus, succeeded in obtaining a dose-response curve in birds with an antibiotic broth (M5-8450). Essentially, the assay consists in administering logarithmic dilutions of the virus, in tissue culture medium, by injection into the wing muscle. After 7 to 10 days, depending on the amount of virus inoculated, the gross and mean tumor weights per bird are recorded. The mean tumor weight per bird of a treated group when divided by the mean tumor weight of a control group gives the percentage growth of the treated tumors compared with the controls. The effect of an antibiotic broth or drug upon the virus infection is recorded as the percentage inhibition. Figure 1 shows a dose-response curve obtained from such data. This technique of comparing the mean tumor weights per bird appears to give a quantitative measure of the effect of drugs upon the oncogenic infection, and the investigators have suggested that this particular virus test system could be utilized as a general screen for antiviral activity (94).

An alternative method is that developed by Bauer (14). When the mean reciprocal survival time of groups of mice is plotted against the dose of compound on a logarithmic scale, a linear relationship is obtained. Dose-response curves obtained in this manner can be used in conventional 4-point assays of antiviral activity against a standard. Four groups of mice are infected with the same dose of virus; two groups are treated with the compound under test, the doses being in a fixed ratio, and the other two groups are treated with the standard compound at equivalent dose levels. The relative activity of the test compound may be calculated by standard methods from the displacement of its dose-response line to the left of that of the standard. This gives, with a fair degree of accuracy, numerical values relative to the standard for antiviral activities of various compounds.

Dose-response lines obtained in this fashion can also be used to measure the activity of the same compound against different viruses. The point at which the line intersects the ordinates corresponding to the mean reciprocal survival time of the animals of the untreated control group is a constant, and is independent of the dose of virus used for infection. Bauer (13) called the dose corresponding to the point of intersection the E_0, *i.e.*, the zero-effect dose (Fig. 2), and he demonstrated that when the dose of virus is varied over 1000-fold range the E_0

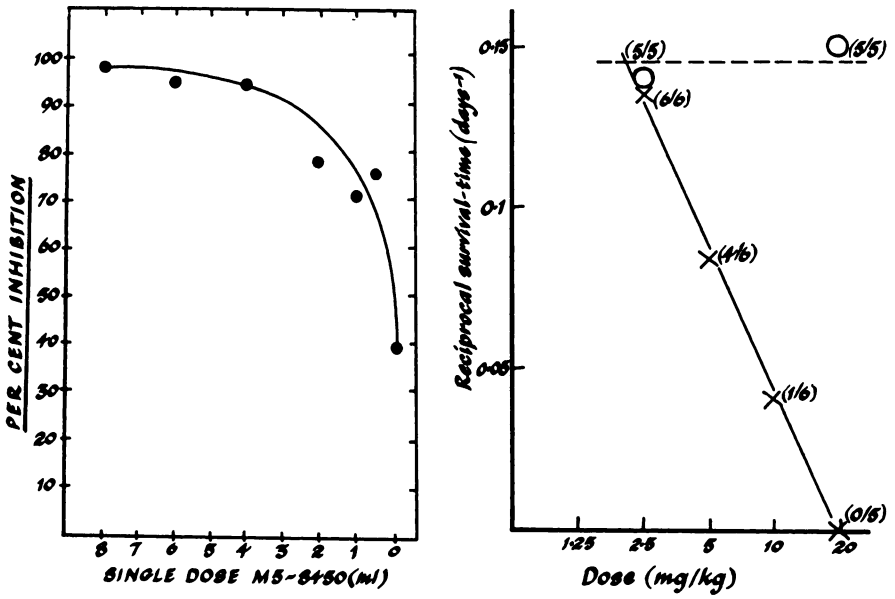


FIG. 1. (*left*) Dose-response curve with antibiotic broth M5-8450 based on % tumor inhibition [data from the publication by Johnson and Baker (94)].

FIG. 2. (*right*) Dose-response curve of antiviral chemotherapeutic effect of isatin β-4′,4′-diethylthiosemicarbazone in mice infected intracerebrally with 100 LD50 doses of ectromelia virus; ○, control; ×, treated.

Numerator, number of mice dying; denominator, number of mice in group. Data from the publication by Bauer and Sadler (16).

varies over a range of considerably less than 2-fold. The E_0 is therefore essentially an invariant, which can be used as an absolute numerical measure of the activity of a particular compound against a particular virus, and is quite independent of the use of a reference substance.

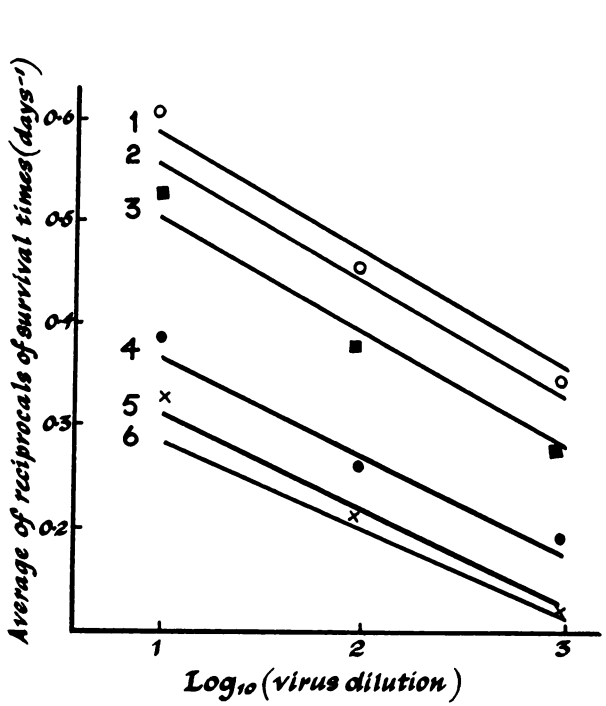
B. Dose-response curves of antiviral substances in tissue culture

Since the advent of the HeLa cell line (64), many lines of cells have been established in continuous culture (20, 35, 78, 96, 97, 194, 200), their varying susceptibilities to virus infection having greatly extended the resources of the virologist. When suspensions of such cells in tissue culture medium settle, they become attached to the glass wall of the vessel. Hence, they grow to form a confluent sheet of cells one cell thick, so enabling cytopathic changes following virus infection to be readily observed. Assays based on these cell lines are now widely used for primary screening, since they are less cumbersome and expensive than the corresponding *in vivo* methods, but they do not infallibly indicate whether an antiviral agent will be active in virus-infected animals.

Virus infection may be initiated in a number of ways. When all the cells are simultaneously infected by using a large infective dose, virus development is restricted to a single cycle, since no susceptible cells remain to support subsequent

cycles; on the other hand, if a small dose of virus is used there is a progressive spread of infection throughout the culture as developing virus is released into the medium. The rate of multiplication may then be followed by titration of virus yield or by observation of the cytopathic effects on the host cells. In most chemotherapeutic experiments, the virus is introduced in graded doses, usually on a logarithmic scale; the major problem in such experiments is the separation of virus cytopathogenicity from the toxicity of the test substance. One approach to this problem is to separate completely these two effects. O'Sullivan (124), using Type 2 poliovirus grown in ERK cells, has obtained dose-response curves for alkylated derivatives of isatin-β-thiosemicarbazone and hydroxybenzyl benzimidazole compounds. Different dilutions (10-fold) of the virus were added to monolayers of cells grown in ELP medium and the test substances were assayed at half their maximum tolerated doses. The tubes were examined twice daily for signs of cytopathic effects, and the end-point was taken when half the cells were affected. When the averages of reciprocals were plotted against the logarithms of virus dilution (Fig. 3), the relative activities of compounds were given by displacement (to the left) of the line from that of the standard (13). Because the toxicity of a test substance has to be established, a substantial amount of effort is required in these studies, as Rightsel *et al.* found (132) in their tissue culture antiviral tests in which two different cell lines, four viruses, and a number of dilutions of both the test substance and the virus were used.

For primary screening then, further simplification is desirable. Following the observation of Dulbecco and Vogt (53) that the spread of virus through the medium could be prevented by overlaying the monolayer with nutrient agar immediately after initial infection, a simple basic technique has been developed which has many special uses. Under these experimental conditions, only direct spread of virus is possible through the medium, and each focal lesion which develops represents one infective or plaque-forming unit of virus in the inoculum; this provides a ready method for accurate virus assay. Modifications of this technique permit even the study of individual infected cells (107), but the modification of the Dulbecco plaque technique (52) that has found the widest application is that in which the paper disc-agar technique, used in the testing and assay of antibacterial antibiotics (179), is combined with it (81). This method, which was originally reported as useful for the measurement of viral antibody (48), consists essentially in the use of a monolayer of cells infected with a quantity of virus which will produce numerous well isolated plaques. On the surface of the agar overlay are placed paper discs impregnated with the test material. After incubation and staining with a vital stain, cytotoxic effects due to the virus may be differentiated from the toxicity of the test compound, because the virus produces discrete plaques, while a toxic drug produces large zones of dead unstained cells. Considerable economy of effort is possible because only one disc need be used for each test substance, diffusion through the agar performing the function of an infinite number of dilutions. As the sizes of the zones of antiviral activity are related to the quantity of material in the discs (Fig. 4), it is possible to carry out simple assays by comparing the zones produced



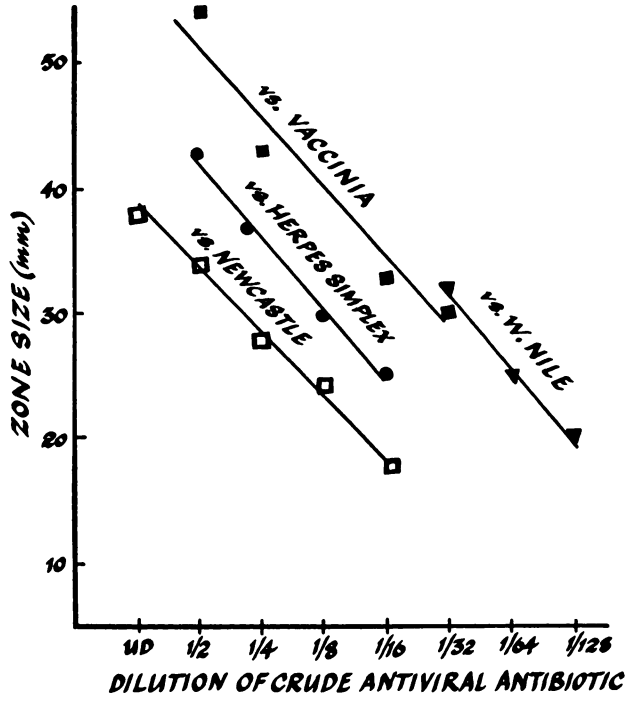


FIG. 3. (*left*) Poliovirus type 2 assays. Variation of average reciprocals of survival times (in days) with logarithms of virus dilutions (in presence of 50% of maximum tolerated dose of compounds). (1) Control line without antiviral compound. (2) 2-(*o*-Hydroxyphenyl)benzimidazole (10 μM). (3) 2-(*p*-Hydroxybenzyl)benzimidazole (100 μM). (4) 1-Methyl-4′,4′-dibutylisatin-β-thiosemicarbazone (30 μM). (5) 2-(*o*-Hydroxybenzyl)benzimidazole (50 μM). (6) 2-(α-Hydroxybenzyl)-benzimidazole (100 μM). For clarity, experimental points have been included only for line 3 (squares), line 4 (dots), line 5 (crosses), and for the control line (circles). Data from the publication by O'Sullivan and Sadler (124).

FIG. 4. (*right*) Representative assays of a crude microbial culture containing an inhibitor of plaque formation. The slope of the line is reasonably reproducible no matter which virus is used. The data further imply that there are differences in virus sensitivity that may be related largely to the multiplication rate of the virus involved. Data from the publication by Herrmann (80).

with a standard. Herrmann (80) stated that this method permits, with even a modest program, the testing of 200 to 300 substances per week against as many as four different plaque-forming viruses.

Another modification of the agar-disc technique utilizes Cooper's (40) agar-cell suspension method. In this case, a thin layer of semisolid agar containing virus mixed with host cells is overlaid on a base agar layer, and filter paper discs impregnated with potential antiviral agents are applied. The system is also of value in the identification of antiviral agents on paper chromatograms (82).

III. METHODS OF INVESTIGATION

A. Compounds with enzyme-inhibiting properties

Viruses are dependent for their growth on the enzymatic activity of the host cell, and interference with the basic metabolism of the cell can prevent virus multiplication. This has been shown in tissue cultures with a variety of agents which uncouple oxidative phosphorylation. This effect can also be demonstrated *in vivo*; even hypoxia resulting from reduced oxygen tension has been shown to reduce the titer of influenza virus in the lungs of mice (99).

Early work showed that fluoroacetate inhibited virus multiplication both in the mouse lung and in tissue culture. Fluoroacetate is essentially a poison for the Krebs tricarboxylic acid cycle, and the diminution in virus multiplication runs very closely parallel to the fall in aerobic respiration (2, 3). Similarly, 2,4-dinitrophenol (DNP) possesses a number of actions on cell metabolism, most of which are probably related to its capacity to activate adenosine triphosphate; they include a sharp increase in oxygen uptake and an increase in the release of organic phosphate.

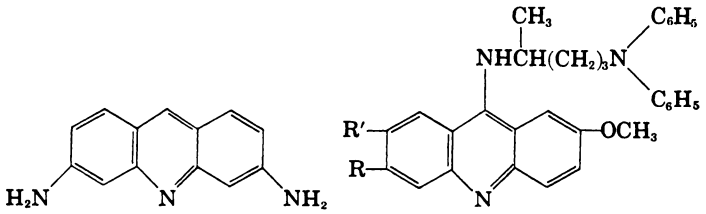
Considerable use has been made of the tissue culture technique in studying the inhibitory action of DNP on influenza virus. DNP in a concentration of 2×10^{-4} M doubles the O_2 uptake of tissue and almost completely prevents the multiplication of influenza virus in the tissue. If the DNP is removed virus multiplication progresses normally. The uncoupling of oxidative phosphorylation in the tissue is responsible for the inhibition of viral multiplication. Dinitrophenol has also been shown to inhibit the growth of vaccinia virus (168) and of the GD VII mouse encephalomyelitis virus in tissue culture (125).

Since the multiplication of several viruses may be inhibited by DNP, the conclusion appears justified that the Krebs cycle (and inferentially the mitochondria) plays a necessary role in supplying energy for virus multiplication, but poliovirus (Type 1, Mahoney strain) has been found to replicate in HeLa cells treated with critical concentrations of DNP which markedly stimulated oxygen uptake and glucose utilization under aerobic conditions. The DNP did not alter the latent period of virus synthesis. Apparently, the uncoupling effect on aerobic phosphorylating systems is offset by increased glycolysis, and the resulting fixation of phosphorus by systems not affected by DNP (65).

These results have stimulated interest in other compounds capable of "uncoupling" phosphorylation. Eaton and Perry (57) have demonstrated that butyl 3,5-diiodo-4-hydroxybenzoate had effects similar to those of DNP and inhibited

the growth of influenza virus in chorioallantoic membrane. Similarly, malonate retarded the production of influenza virus in the chorioallantoic membrane; here again the inhibition is probably related to blockage of the tricarboxylic acid cycle (3).

Thompson (169) has shown that sodium iodoacetate, and to a lesser extent sodium malonate, prevented multiplication of vaccinia virus in chick embryonic tissue; iodoacetate also inhibited the propagation of Theiler's GD VII strain of mouse encephalomyelitis virus in mouse brain tissue culture (126). This action was believed to result from the iodoacetates combining with, and so rendering unavailable for viral proliferation, vital thiol groups in the tissue enzyme systems. More recently the infectivity of several enteroviruses has been shown to be diminished by the sulfhydryl reagent *p*-chloromercuribenzoate, which under certain conditions prevents the adsorption of some enteroviruses to monolayer cultures of monkey kidney cells. Reduction of infectivity was reversed by the thiol compound, reduced glutathione. The results indicate that sulfhydryl groups on the enterovirus are involved in the establishment of infection, and that they play a role in the adsorption of virus to host cells (36).



I II

Following the original observation that the acridine enzyme inhibitors, proflavine (I) and mepacrine (II, R = Cl, R′ = H), are effective against vaccinia (168), and that proflavine is also active against poliomyelitis virus, extensive studies have been made on many related acridine derivatives (24). For example, near the maximum tolerated dose certain chloro- and nitro-acridines have retarded influenza A and B and mumps viruses in chick chorioallantoic and amniotic membrane cultures (56).

Proflavine inhibits the growth of fowl plague virus in tissue cultures of chick embryo cells. Production of virus particles is inhibited as an exponential function of the proflavine dose. S-antigen[1] production is not affected by low doses of proflavine, although such doses inhibit the production of infectious particles and hemagglutinin. This fits well with the observations that S-antigen multiplies in the cytoplasm, since proflavine inhibits cytoplasmic protein synthesis. Kinetic studies using proflavine block have shown that S-antigen or its precursors are formed before hemagglutinin or its precursors (62).

Proflavine also influences the synthesis of foot and mouth disease virus in pig kidney tissue culture cells. Incorporation of low concentrations of proflavine into

[1] S-antigen or soluble antigen is a nucleoprotein produced in infected cells before the appearance of mature virus, some of which may become incorporated in the virus particles.

the incubation medium decreases considerably the yield of infective virus, although the amounts of infective RNA and specific complement-fixing antigen are not proportionately reduced. In the presence of proflavine, the RNA in the cells remains sensitive to ribonuclease throughout the growth cycle, indicating that the compound prevents the incorporation of RNA into complete virus. The complement-fixing antigen produced in the proflavine-inhibited system is the 7 mμ, noninfective component of the virus (23).

Among several acridines examined by Hurst and his associates (72, 89), the antimalarial drug mepacrine was found to possess very marked protective activities against equine encephalitis, Rift Valley fever, and louping ill. A single oral dose of mepacrine given before or soon after virus infection protected a large number of mice, even when high infecting doses of virus were used. Growth of virus was wholly or partly suppressed according to the dose administered. This activity did not extend to many other viruses. The drug was active only in mice, and was inactive in guinea pigs, rabbits, chickens, and monkeys. Thus it would appear that either the course of the infection in the mouse is not comparable with that in other species, or the metabolism or distribution of mepacrine in the mouse is different, and that possibly a very active metabolite is formed. However, the latter does not seem to be the case, for 94% of the mepacrine extracted from the livers of treated mice was unchanged mepacrine, and the remainder, which was separated into three distinct fractions, had no significant biological activity against Eastern equine encephalomyelitis in the mouse (66).

Replacement of chlorine by a nitro group (II, R = NO_2, R′ = H) removed all activity against equine encephalitis, but this compound was active against psittacosis and lymphogranuloma. When the nitro group was shifted from the 6 to the 7 position in the acridine ring (II, R = H, R′ = NO_2) activity was regained against equine encephalitis and lost against psittacosis and lymphogranuloma. When the nitro group at position 7 was replaced by an amino group (II, R = H, R′ = NH_2), all therapeutic activity against both large and small viruses was lost (72). In fact, the simpler acridine derivative, proflavine, would appear to have a wider spectrum of activity. The action of proflavine is generally regarded as resulting from inhibition of cytoplasmic protein synthesis, with the result that infective RNA is still formed but the synthesis of infective virus is retarded. However, Dulbecco and Vogt (54) have shown that low concentrations of proflavine induce mutations in the virus of poliomyelitis, so the compound probably has some action on the nucleic acid of this virus.

An alternative to inhibiting the enzyme systems of the host cell is to inhibit an enzyme which is specific to the virus, such as the neuraminidase of influenza virus (68). Mucopolysaccharides and mucoproteins from many sources have been shown to inhibit the hemagglutination reaction with the virus (26, 70). This effect is due to their similarity to the receptor substance of the red blood cells and their affinity for the virus enzyme. The action is seen in substances which are slowly attacked by the enzyme, thus having an affinity for the enzyme while at the same time blocking the sites necessary for adsorption. It is seen even more markedly when the virus is slightly altered by heating so that the

enzymatic action, which leads to inactivation of the inhibitor, is more impaired than is the affinity. The functional unit in all these substances seems to be N-acetylneuraminic acid, since this substance is released by the virus and since, after its release, the inhibiting power on hemagglutination is lost (67).

It has been observed by Walop (180) and confirmed by Faillard (61) and Romanowska (133), that the neuraminidase from several strains of influenza virus can be inhibited by N-acetylneuraminic acid. Using Warren's thiobarbituric acid assay (182) for free N-acetylneuraminic acid, a method has been developed by Jacobs and Walop (93) for studying the interaction of neuraminidase and its substrates. With this method, the Michaelis constant of the system influenza virus neuraminidase and N-acetylneuraminyl-lactose was determined (181). The results indicated that the affinity of N-acetylneuraminic acid for the enzyme surface is increased 8-fold by a ketosidic linkage to the lactose moiety.

N-Acetylneuraminic acid is also released by the receptor-destroying enzyme of *Vibrio cholerae* (69). Intraperitoneal injection of this enzyme results in a chemoprophylactic protection against infection with Columbia SK virus by the same route (98). Therefore, the presence of N-acetylneuraminic acid at the sites of adsorption seems to be of importance for the mechanism of the first peripheral infection. As it is generally accepted that the red cell receptor concerned in hemagglutination by myxoviruses is an analog of the receptor substances of cells which are susceptible to infection, it follows that a hemagglutination inhibitor will inhibit virus multiplication only if it combines with the virus more firmly than does the cell receptor. An attempt to prepare influenza virus inhibitors with this requirement has been made by coupling diazotized aniline derivatives to a purified preparation of α-inhibitor (87). Marked reduction in the amount of virus multiplication was demonstrated in eggs with two strains of virus even when the inhibitor was injected 30 minutes after infection. Similar derivatives of neuraminic esters would be of considerable interest.

B. Metabolic analogs

The lack of knowledge of any specific reaction involved in the production of virus in the host cell has led to numerous attempts to inhibit the metabolic pathways leading to the synthesis of the constituents of the virus. Amino acid analogs were the first to be investigated; in this connection it is well to remember that only glucose, glutamine, and salts are needed to allow full poliovirus multiplication in HeLa cell cultures. This must mean that the supply of essential amino acids in the cells can be drawn on to provide all requirements for virus synthesis (55). The earliest work was on analogs of tryptophan (130) and methionine (1); these and the many related studies have been reviewed by Mathews and Smith (109).

This approach to the chemotherapy of virus disease still invites attention. Recent emphasis has been on the effects of *p*-fluorophenylalanine (FPA) on virus replication. The existence of several discrete steps in the reproduction of

fowl plague virus has been shown by exposure of cells to FPA at various periods before and after infection. The effect of FPA was reversible at various stages either by removing it from the medium or by adding phenylalanine. A correlation exists between the time of addition of FPA and the beginning of virus production after adding phenylalanine, which suggests that events which were preliminary to virus production (such as the synthesis of precursors) were preserved after the addition of FPA (199). In addition, Scholtissek and Rott (142) have investigated the effects of FPA on the production of viral RNA and protein during multiplication of fowl plague virus, and Brown *et al.* (22) have studied the effect of FPA on the multiplication of foot and mouth disease virus.

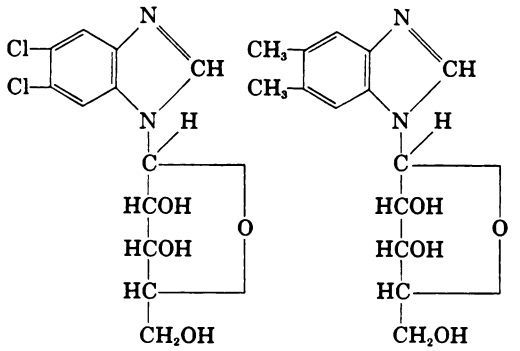
All these results were, of course, obtained in tissue culture systems, but in the case of β-phenylserine activity *in vivo* can also be demonstrated. Dickinson and Thompson (49) showed that β-phenylserine inhibited multiplication of influenza A virus in chorioallantoic membrane fragments, and Hosley (88) found that β-phenylserine inhibited the multiplication of rabies virus in cultures of minced mouse brain. The former, but not the latter, activity could be reversed by the addition of phenylalanine. Daily intraperitoneal administration of β-phenylserine protected rats against death from lethal amounts of rabies virus. The protective effect was most marked when the animals were treated for three days prior to infection. β-Phenylserine also inhibited the propagation of vaccinia virus in rabbit skin when the compound and virus were mixed prior to injection, but the compound had no apparent effect on the multiplication of the viruses of influenza A (PR_8), Eastern equine encephalitis, poliomyelitis (Lansing strain), or mouse encephalomyelitis (FA strain) in mice (128).

Interference with nucleic acid synthesis has several advantages over the inhibition of protein synthesis. For example, the nucleic acid molecule is considerably greater in size than the protein subunits, and the incorporation of unnatural bases or nucleosides into virus nucleic acid should necessarily impair replication. For the ribonucleic acid-containing viruses, it would seem important to inhibit specifically the synthesis of ribonucleic acid (RNA) without affecting the metabolism of deoxyribonucleic acid (DNA). It is generally believed that nuclear DNA acts as a kind of template for the synthesis of RNA, and that the RNA produced also forms a self-replicating system.

Many analogs of purines and pyrimidines inhibit both RNA and DNA synthesis in general. However, there is a possibility that the respective ribonucleoside or deoxyribonucleoside derivative could direct activity preferentially towards the inhibition of one nucleic acid or the other according to the degree of resemblance to the precursor. Some evidence for this structural specificity of the sugar component for the inhibition of virus synthesis can be seen, for instance, in the finding that ribonucleosides of some substituted benzimidazoles are more selective inhibitors of influenza virus multiplication than are the free benzimidazoles or their deoxyribonucleosides (154).

As the function of vitamin B_{12} seems to be closely connected with that of the folic acid group, it is not surprising that compounds similar in structure to the

benzimidazole portion of vitamin B_{12} have been used in attempts to interfere with viral proliferation.

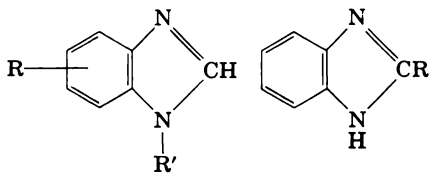


III IV

In particular, halogenated benzimidazoles, *e.g.*, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (III, DRB), designed as antagonists of 5,6-dimethylbenzimidazole ribonucleoside (IV), a component of vitamin B_{12}, were found to be more active inhibitors of the growth of influenza virus than was benzimidazole itself (154). Comparison has also been made between the concentrations which cause cytopathic changes and reduction in oxygen uptake of the chorioallantoic membrane of embryonated eggs *in vitro* with the concentrations necessary to inhibit multiplication of influenza B virus (155). The toxic concentration of benzimidazole was three times higher than the inhibitory concentration. β-D-Ribofuranosyl derivatives were not only active at much lower concentrations but also more "selective," *i.e.*, the ratio between toxic and inhibitory concentrations was twice as high with DRB and six times as high with 4,5,6-trichloro-β-D-ribofuranosylbenzimidazole as it was in the case of the parent compound. This selective action was found to be specific for the β-ribofuranoside; neither the β-D-ribopyranoside nor the arabinoside showed any higher selectivity than did the free base. As the β-D-ribofuranosyl form is the natural form for RNA nucleotides, it was believed that the "selective" action was due to a specific inhibition of RNA synthesis. Other evidence that the action of DRB is to interfere with the synthesis of RNA has been provided by Allfrey *et al.* (7), who found that DRB reduced the uptake of labeled orotic acid (uracil-4-carboxylic acid) into nuclear RNA (using thymus nuclei), and considered that its essential action was to block RNA synthesis in the nucleus. The finding that adenosine blocks the action of DRB on influenza virus would appear to corroborate this assumption (165).

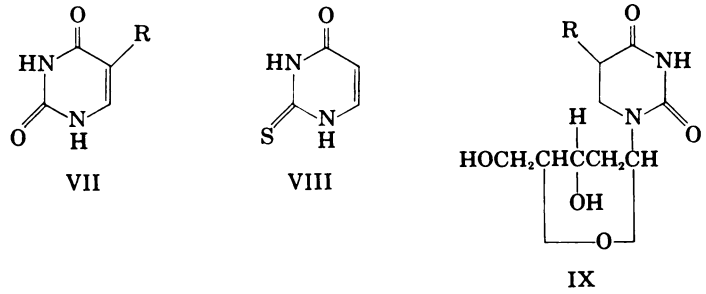
As would be expected, with vaccinia virus, which contains DNA rather than RNA, the ribonucleosides were found to be no more selective than were the free bases, when the virus was grown in chorioallantoic membranes (166); however, the selective effect of DRB does not extend to poliomyelitis virus, although this contains only RNA (165). In this case, the only compound which showed a more "selective" action than benzimidazole was 5,6-dichloro-D-arabinosylbenzimidazo'e. No clear relationship, therefore, seems to ex:st between the type of nucleic

acid of the virus and the selective action of β-D-ribofuranosides. These derivatives show only slight activity *in vivo*, for although DRB prolonged the survival time of mice infected with influenza virus it did not protect against death. It now seems that multi-substituted benzimidazoles of type V synthesized on this very rational basis are of less value than the simpler 2-substituted benzimidazoles (VI).



V VI

Another difference between RNA and DNA which might be exploited chemotherapeutically is the composition of the bases, since uracil is present in RNA and thymine in DNA. Pyrimidine analogs may therefore exert an action according to their structural similarity to either of these two bases. With halogenated pyrimidines, it has been found that the size of the halogen substituent determines the character of the compound; *e.g.*, the van der Waals radius of bromine closely resembles that of the methyl group, the size and shape of 5-bromouracil (VII, R = Br) being very similar to that of thymine (VII, R = CH_3).



VII VIII IX

5-Bromouracil was found to be an inhibitor of the synthesis of bacteriophages which contain DNA, and this effect is more marked with the deoxyribonucleoside derivative (39). Similarly, thiouracil (VIII), an antagonist of uracil (VII, R = H), inhibits replication of influenza virus. The increased synthesis of RNA in influenza infections, as demonstrated by incorporation experiments with C^{14}-guanine and P^{32}-phosphate, is reversed by 2-thiouracil, which may result in the production of noninfectious virus. Both 5-iodo- (IX, R = I) and 5-bromo-2′-deoxyuridine (IX, R = Br), when tested in the agar diffusion-plaque inhibition test, were found to inhibit plaque formation of DNA-containing vaccinia and herpes simplex virus but not RNA-containing West Nile and Newcastle disease viruses; this effect was readily reversed by thymidine (79). The 5-iodo compound (IX, R = I) has been shown to be remarkably effective in curing herpes simplex keratitis, in rabbits, when administered as eye drops (102a). This work has been confirmed clinically by Hertzberg (82a), who found that in a group of patients with an initial attack of herpes simplex keratitis, the drug was of definite value

especially in those cases in which there was no stromal involvement, and also by Davidson (46a), who found it to be effective in about 70 % of cases. In addition to its activity against herpes simplex keratitis, the compound, when given in large but nontoxic, parenteral doses, profoundly suppresses the development of the dermal lesions caused by vaccinia virus in rabbits (so that lesions are caused only by 10,000 to 100,000 times as much virus as in the control animals); in addition, vaccination "takes" were prevented in some patients under treatment for cancer with this drug (28a). Welch (191a) also reports that "profound suppression of the development of neoplasms, caused by human adenovirus type 12 (a DNA-virus) in newborn hamsters, can be caused by injections of nontoxic doses of 5-iodo-2′-deoxyuridine."

Such chemotherapeutic agents may, of course, act by being antimetabolites for the synthesis of precursors of the nucleic acids, in which case, incorporation might be of secondary importance. Sir Alexander Todd (174) suggested that instead of the simple purine and pyrimidine analogs which have so far been used, it might be desirable to try the effect of small synthetic oligonucleotides containing in them abnormal nucleotides—including those modified in the sugar or phosphate residues as well as those with unnatural base components. Evidence from the investigations of Ochoa and others (117) on polynucleotide phosphorylase seem to show that the purified enzyme needs an oligo- or polynucleotide primer. This suggests that oligonucleotides might well serve as much better starting points for RNA synthesis in the cell than a single base or nucleoside, and that such compounds might therefore be better as therapeutic agents.

An alternative approach, although not an antimetabolite one, is to utilise the differences in reactivity of the bases of RNA and DNA. In the case of DNA, the purine and pyrimidine bases are very unreactive, since they are involved in very strong hydrogen-bonding. In RNA, however, the amino groups are very reactive, which has some bearing on the effect of various antiviral substances, *e.g.*, certain dicarbonyl compounds, which inhibit the growth of influenza viruses in eggs, react specifically with the guanine groups of RNA, but not of DNA (147). As the virus protein and the virus nucleic acid are apparently formed at different intracellular sites, it is possible that such agents might react with the nucleic acid before it has combined with the protein.

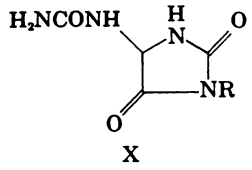
It has been suggested (174) that another possible chemical line of attack might be to use synthetic polymers with the object of blocking the virus polynucleotide chains by attachment either through covalent or hydrogen bonds, so as to prevent polynucleotide synthesis on the virus template. Little work has been done on this approach as yet, but the interesting experiments of Allfrey and Mirsky (6), in which they were able to show that replacement of a large proportion of the DNA in cell nuclei by a number of anionic polyelectrolytes allowed the nuclei to continue much of their normal biochemical activity, suggest that possible therapeutic agents of this type are worthy of serious consideration.

C. Active compounds derived from random screening

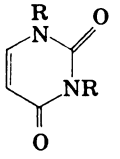
One approach to the chemotherapy of virus diseases entails empirically screening large series of compounds against a variety of infections either *in vivo*

or in tissue culture systems. In such studies useful compounds cannot be expected to appear more than one per several thousand compounds investigated, in which case the very simple tissue culture assay method developed by Herrmann (80) is to be preferred as a preliminary screen (Section IIB).

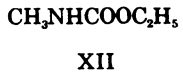
Pioneer work in animals was carried out by Coggeshall and Maier (38), who examined 67 substances, including many sulfonamides and sulfones, against mouse-adapted poliomyelitis given intracerebrally and influenza A given intranasally to mice. Andrewes *et al.* (10) screened 115 compounds against intranasally instilled influenza virus and 74 for *in vitro* activity against vaccinia. In experiments in which 190 substances were tested against mouse-adapted poliomyelitis (intracerebrally) and St. Louis encephalitis (intranasally) in mice, all the results were essentially negative (103). Cutting *et al.* (42) used 150 chemicals against herpes simplex (intraperitoneally), neurovaccinia (intracerebrally), and influenza (intranasally) in mice, and against egg-adapted vaccinia on the chorioallantoic membrane. Four compounds only, namely 3-methylallantoin (X, R = Me), 3-ethylallantoin (X, R = Et), 1,3-dimethyluracil (XI, R = Me), and ethyl-N-methylcarbamate (XII), evoked some interest by lengthening the average life of the chick embryos by a fraction of a day.



X

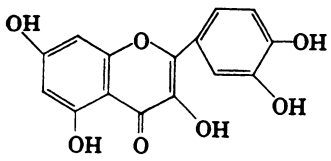


XI

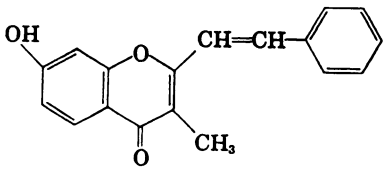


XII

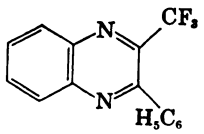
Cutting and Furst (45) reported further that, from a total of about 2,000 compounds which had been examined in their laboratory for antiviral effect, very few showed minimal but statistically significant effects: *e.g.*, benzoxazole against vaccinia in mice (43), quercetin (XIII) and 7-hydroxy-2-styryl-3-methylchromone (XIV) against ectromelia in mice (41, 44), 5-amino-6-methyluracil against neurovaccinia in mice, and the condensation product of 5-aminouracil and formic acid against neurovaccinia and rabies in mice (51). Using Columbia SK virus in mice, slight but genuine antiviral effects were found with 2-trifluoromethyl-3-phenylquinoxaline (XV), *m*-aminocyclohexanol, manganese gluconate, and phthalamide. On the basis of these findings, phthalamide derivatives were selected for further study (45).



XIII



XIV



XV

Obviously the labor involved in such studies is very considerable when judged by the number of potentially useful compounds emerging.

It would be an impracticable task to make mention of all the related investigations which have been carried out during the past twenty years, and indeed an unnecessary one, as several reviews of the subject have appeared during the latter part of this period (90, 109, 148); therefore attention is drawn only to studies which have given rise to promising leads. Antibiotics in general and the tetracyclines in particular have met with considerable success in treating diseases resulting from infections due to the larger viruses such as the psittacosis-lymphogranuloma group (71, 102), and this activity extends to the smaller "true" viruses. Tetracycline and oxytetracycline were found to prolong the latent period of multiplication of influenza virus type D in tissue culture, depressing its infectious and hemagglutinating properties, and inhibiting its cytopathogenic effect. They were effective when added to the culture medium 6 to 24 hours before infection, or during the first few hours after it. Chlortetracycline when added to the medium prior to infection decreased the titers of hemagglutination reactions. These antibiotics affect intracellular multiplication and are without effect *in vitro* (25) An antiviral agent called 1758 (a penicillin-like antibiotic) has been shown to inhibit the cytopathic action of Salisbury virus HGP, which is one of several strains reported to be of common cold origin (129). In addition, Japanese workers reported two new antibiotics with antiviral activity. Virocidin picrate, isolated from the broth filtrate of *Streptomyces flavoreticuli*, was found to have activity against rabies virus (63), and flavucidin (145) inhibited the growth of influenza virus at a dilution of 1 μg/ml. Little work as yet has been done on the possible mode of action of antibiotics which are specifically antiviral, but one such example is the action of mitomycin C on the rabbit kidney cell-pseudorabies virus system; the DNA formed does not seem to be functional, and the virus particles formed are noninfective (18).

Following earlier work on the bacteriostatic and bactericidal properties of urea derivatives (185, 186, 187, 188), Weinstein and his collaborators determined whether they were also effective against the viruses of influenza and encephalomyocarditis both *in vivo* and *in vitro* (191). Of 89 drugs studied, 17 were found to be highly active against influenza and 5 against encephalomyocarditis virus *in vitro*. Only diphenylcarbamyl chloride (XVI) produced a significant therapeutic effect in mice when given simultaneously with influenza virus. None of the drugs was active against encephalomyocarditis *in vivo*.

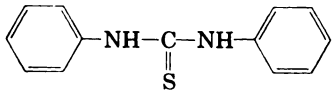
$$(C_6H_5)_2NCOCl$$

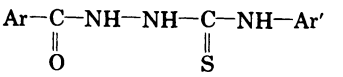
XVI

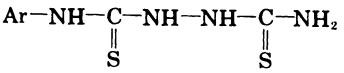
The difference in the activity *in vitro* of the urea compounds against two different viruses was very marked. The compounds that showed high activity against influenza virus were inactive against encephalomyocarditis, and *vice versa*. Weinstein considered that the results suggest that drugs with antiviral activity are highly specific in their effects, and that in this respect they are probably quite different from antimicrobial agents.

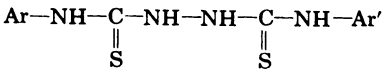
Thiourea derivatives have also been shown to have antiviral activity both

in vitro and *in vivo*, particularly against influenza virus (28). Since the original observation, a large number of sulfur-containing compounds bearing the thiourea group —NH—CS—NH— have been examined. These include substituted thiocarbanilides (XVII), 1-acyl-4-arylthiosemicarbazides (XVIII), 1-(arylaminothioformyl)thiosemicarbazides (XIX), and 4-aryl-1-(arylaminothioformyl)thiosemicarbazides (XX).

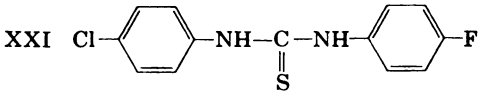
XVII 

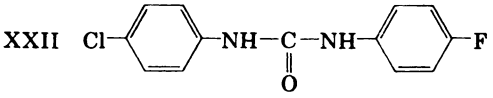
XVIII 

XIX 

XX 

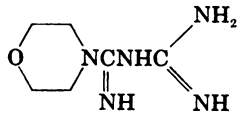
Several of these compounds were found to be chemotherapeutically active against influenza virus; 4-chloro-4′-fluorothiocarbanil de (XXI) was particularly active when administered by injection but less active by mouth (27). The importance of the $\rangle C{=}S$ structure is neatly demonstrated by the lack of activity of its oxygen analog, 4-chloro-4′-fluorocarbanilide (XXII).

XXI 

XXII 

4-Chloro-4′-fluorothiocarbanilide also shows additional but weak activity by retarding the development of experimental poliomyelitis in the mouse (27). The dependence of antivaccinial activity on the presence of a $\rangle C{=}S$ group has also been demonstrated by the relative activities of isatin-β-thiosemicarbazone and isatin-β-semicarbazone (14). Sulfur-containing compounds stil' maintain interest as potential antiviral agents both in the open-chain form, *e.g.*, 4 - (3 - hemisucc nyldeoxycholylamino) - 4′ - hemisuccinylaminodiphenylsulfone, which is an active inhibitor of the multiplication of the PR_8 strain of influenza virus A in the chick embryo (19), and in the cyclic form, as 2-amino-4-(*p*-tolyl)- and 2-amino-4-(*p* decylphenyl)thiazole show weak activity *in vivo* against the Nakamiya strain of Japanese B encephalitis virus (175).

The chemotherapeutic activity of compounds based on the urea structure seems almost limitless, for it has been found that soluble guanidine salts show an antiviral effect on poliovirus and on some other enterov.ruses (100, 131). The antiviral activity was observed initially with the guanidine salt of hydroxy-amino-methylene malononitrile on type 2 poliovirus, with human epithelium cells as the culture system. The drug was toxic to the cells at 630 μg/ml, but inhibited the cytopathic effect of poliovirus at one-tenth of this concentration. Subsequently, guanidine hydrochloride itself was found to reduce the infectivity titer of poliovirus by one log unit. Other guanidine salts showed activity that was approximately proportional to their solubility in the medium. Later, evidence was obtained that the compounds decreased the incidence of disease in polio-infected monkeys, although the activity was seen only at marginal toxic levels. These guanidine salts have a limited spectrum of antiviral activity for, among a number of viruses studied in tissue culture, they were effective only against members of the enterovirus groups; several influenza viruses were not inhibited (100, 131). However, when a series of biguanides was screened against mouse-adapted influenza, PR_8 and Lee I strains (112), it was found that one compound, N′,N′ - anhydro - *bis* - (β - hydroxymethyl)biguanide hydrochloride (XXIII), produced a marginal but reproducible effect.



XXIII

Compound XXIII, combined with the xerostomia-producing drugs methatropine nitrate and methscopolamine nitrate (Flumidin), has been found to minimize the degree of consolidation in mouse lungs investigated on the fourth or fifth day after inoculation. Both XXIII (which has a very low acute toxicity) and Flumidin have been tried clinically in various viroses, and they both show a statistically favourable effect when given early in the suppression of influenza. When administered prophylactically, Flumidin significantly protects against influenza (112).

D. Systematic structural modification of compounds with known antiviral activity

When it has been clearly demonstrated that a molecule has pronounced antiviral activity both *in vivo* and *in vitro*, a comprehensive research program is necessary to demonstrate whether or not a compound of clinical importance may emerge. The first and major portion of this program usually should be the synthesis of a very large number of derivatives and other compounds closely related in structure, to determine precisely which parts of the parent molecule are essential for the retention of antiviral activity. Compounds with enhanced activity against the test virus might be expected to result coincidentally from such an investigation, and subsequently derivatives of the most active structure should

be synthesized to increase the clinical efficacy. Concomitant biochemical and morphological studies should, of course, be carried out.

In view of the complexity of such investigations it is not surprising to find that they are extremely rare, and there would appear to be, in fact, only three examples, namely, acridine derivatives, substituted benzimidazoles, and derivatives of isatin-β-thiosemicarbazone; here, only the last-mentioned has been examined systematically.

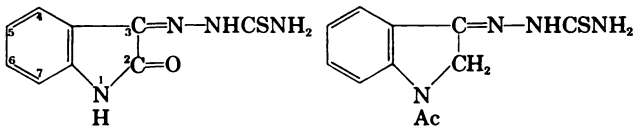
Marked dependence of inhibitory activity upon chemical structure was observed when an extensive series of compounds, based primarily on 3,4-xylidine, was examined for activity against influenza virus in tissue culture (37); but the limiting factor in all such studies must of course be the inherent activity of the parent molecule.

Earlier work on the action of mepacrine in a number of virus diseases (91) resulted in the synthesis of a number of analogs, the chemotherapeutic action of which has been reviewed by Hurst and Hull (90) (see also Section IIIA and IVB).

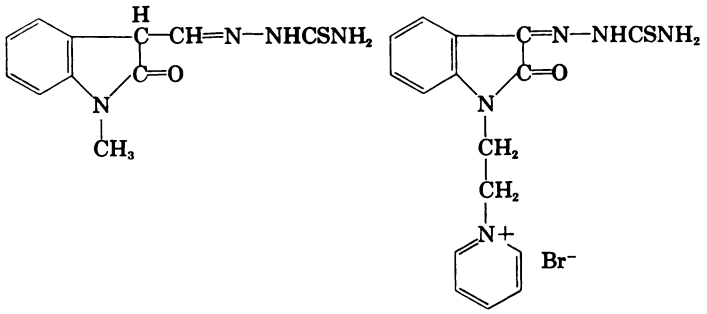
In the benzimidazole series, 2,5-dimethylbenzimidazole was selected by Tamm *et al.* (157) for further study as an inhibitor of influenza virus multiplication and further chemical modifications were made in the benzimidazole nucleus. These consisted mainly in introduction of methyl groups into other ring positions (158, 159, 160), but attempts to correlate the change in structure of the substituted benzimidazoles with corresponding enhancement or diminution in activity were not very successful. A closer correlation of the benzimidazole moiety with the naturally occurring benzimidazole "nucleoside" in vitamin B_{12} was attempted by studying the N-glycosides of the heterocyclic portion (161). Of the various pentosides and hexosides examined, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (167) exerted considerable activity against several strains of influenza virus, types A and B, in chorioallantoic membrane cultures (167). Extensive studies have been carried out to determine the mode of action of this compound (Section IIIB). Further substitution of chlorine into the benzenoid ring of this molecule increased the activity some 8-fold against influenza B virus in the chorioallantoic membrane (162). Tamm and Nemes (163) also showed that glycosides of chlorobenzimidazoles were active against poliovirus in tissue culture. However, more recent work has moved away from poly-substituted glycosides of benzimidazole and returned to the simpler derivatives such as 2-α-hydroxybenzylbenzimidazole (VI, R = PhCHOH) (60, 86, 124, 156).

Isatin-β-thiosemicarbazone (XXIV) was selected for further study since it was found to be the most active compound when a range of heterocyclic thioamides and thiouracils were screened for activity against neurovaccinia, using an *in vivo* assay method (14). As a first step, systematic structural modification was undertaken to determine which parts of this molecule are essential for retention of the antiviral activity (14). N-Acetylindoxyl-β-thiosemicarbazone (XXV), which lacks the carbonyl group in the 2 position, and N-methyl-β-formyloxindole-thiosemicarbazone (XXVI), a closely related molecule in which the side chain

has been lengthened by one carbon atom, are both inactive. Therefore, structural modifications in positions 2 and 3 are incompatible with the retention of high activity against neurovaccinia, which leaves positions 1, 4, 5, 6, and 7 available for further investigation.



XXIV XXV



XXVI XXVII

Substitution in the aromatic ring was explored systematically to give a range of steric, electronic, and mesomeric effects. The complete series of chloroisatin-β-thiosemicarbazones was synthesised and the effects of alkyl-, trifluoromethyl-, nitro-, carboxy-, and methoxy-substituents were investigated. To determine whether substituent effects were essentially chemical or physical, an examination was made of the infrared spectra. Comparison of shifts in the frequencies of the functional groups with changes in biological activity demonstrated that the substituent effects were almost entirely physical (135, 137). Large substituents in the 5 and 6 positions seriously decrease the activity (Table 2), and there is a gradation of antiviral activity with increasing Van der Waals radii of the substituents. The fluorine atom, which has the smallest radius apart from hydrogen, decreases the antiviral activity the least. Other large substituents such as the naphthisatins, in which another ring is fused to the 4,5, 5,6, or 6,7 positions, completely abolish activity. The 4 and 7 positions are less susceptible to substituent effects than the 5 and 6 positions but all structural modifications in these positions result in a diminution of antiviral activity. In general, these substituent effects are additive, so that any poly-substituted derivative containing large 5 or 6 substituents is inactive.

The situation with respect to position 1 is very different. Here there is almost endless scope for modifying the physical properties of the molecule, and a series of compounds with a very wide range of antiviral activity has been obtained. Table 3 shows the antiviral activities of a selection of the N-substituted isatin-β-thiosemicarbazones which have been prepared.

TABLE 2

Relationship of Van der Waals radii of ring-substituted isatin-β-thiosemicarbazones to antivaccinial activity

Substituent	Radius (A)	Relative Activity	
		5-Position	6-Position
None	1.2	100	100
Fluoro	1.35	35.5	43.1
Chloro	1.80	4.2	11.7
Bromo	1.95	3.1	10.5
Methyl	2.0	0	0.3
Iodo	2.15	0	3.9

TABLE 3

Antiviral activity of N-substituted isatin-β-thiosemicarbazones

Substituent	Relative Antiviral Activity
None	100
Methyl	202
Ethyl	286
Isopropyl	44
Propyl	28.5
Pentyl	3.4
Hydroxymethyl	42
2-Hydroxyethyl	204
Acetyl	87
Ethoxycarbonylmethyl	0
Diethoxycarbonylmethyl	0

The simple alkyl substituents produce a marked rise in activity against neurovaccinia with a maximum at ethyl, tailing off again to the pentyl derivative, which has only 3.4% of the activity of the parent compound. Similarly, in the series of hydroxylated compounds maximum activity is obtained by the hydroxyethyl compound. The N-acetyl derivative is quite active, but those with ester groupings are not. In general, most strongly polar groupings such as cyanoethyl or carboxymethyl abolish activity, and the useful range of compounds is restricted to the alkyl and hydroxyalkyl derivatives. This poses many problems from the point of view of chemotherapy, as all the derivatives which are readily water-soluble are inactive (138). For example, the sodium salt of 7-carboxyisatin-β-thiosemicarbazone and a quaternary derivative of the most active compound, 1-β-pyridinium ethylisatin-β-thiosemicarbazone (XXVII), show no activity; this is probably because they do not reach the neurotropic virus used in the *in vivo* assay method. There is, in fact, some correlation between lipid solubility and antivaccinial activity in the simple derivative (Table 4). When a wider range of compounds is considered and the logarithms of the solubilities in water

TABLE 4

Correlation between lipid solubility and activity in the ring-substituted isatin-β-thiosemicarbazone derivatives

Substituent	Solubility in Chloroform (mg/100 ml)	Relative Antiviral Activity
7-Carboxy	0	0
5-Methoxy	3	0.03
4-Methyl	8	3.4
4-Chloro	10	8.6
6-Fluoro	16	39.8
7-Chloro	29	85
None	32	100

or chloroform are plotted against antiviral activity, a correlation coefficient of 0.775 is obtained.

IV. METHODS FOR DETERMINING MODE OF ACTION OF ACTIVE MOLECULES

A. Results of biochemical investigations

As tissue culture techniques have improved, investigations of the chemical requirements for virus replication have become more numerous. Basically this type of work depends on accurate knowledge and control of nutrients required by metabolizing cells. It is possible, for instance, to culture cells in a completely synthetic medium, as has been done by Earle and his colleagues with their L strain of mouse fibroblasts (111). Unfortunately, the L strain will not support the multiplication of poliovirus with which much work on virus requirements has been carried out (193). Susceptible cell lines still require protein, usually in the form of serum, for multiplication, though not for maintenance, during the limited period required for virus replication. For instance, it has been found that a very simple medium consisting only of glutamine, glucose, and balanced salt solution will permit maximal yields of virus under particular test conditions (46, 55), and Westwood *et al.* (194) have found that under other conditions maximal yields of poliovirus may be obtained in the complete absence of sources of nutrients and energy other than those stored within the cell.

In studies on the effect of poliomyelitis virus on the metabolism of HeLa cells, from measurements of changes in cellular RNA, DNA, protein, and acid-soluble nucleotides, as well as the uptake of cytidine-2-C^{14}, an early effect of virus infection was found to be stimulation of cellular RNA activity rather than inhibition of cellular processes (140). Similar results were obtained by Maassab and Ackermann (108), who found that the cytoplasmic RNA content of HeLa cells 6 hours after infection with poliovirus was 2.5 times that of normal cells. The proportions of cytidylic, adenylic, guanylic, and uridylic acids in the infected cells did not differ significantly from those of normal cells. Synthesis of RNA closely paralleled that of protein for the first 4 hours, then increased, and ceased at 6 hours. The increase in cytoplasmic RNA appeared to be in excess of that

expected from the yield of virus. This was confirmed by studying the rate of incorporation of P^{32}-phosphate into RNA in experiments in which the amounts of protein, RNA, and virus were determined in various subcellular fractions of HeLa cells at various times during a single sequence of infection with poliovirus (5). On the other hand, in studies on the incorporation of orthophosphate labeled with P^{32} into the high molecular weight RNA fraction of HeLa cells infected with type 2 poliovirus, no increase in the amount of high molecular weight RNA was found in infected cells over that in normal cells. The specific activities of each of the nucleotides obtained from the infected cells were the same or lower than were those isolated from normal cells. These results suggested that the poliovirus RNA was synthesized *de novo* from acid-soluble precursors, or that the host RNA was used as a source of viral RNA (134).

Even in DNA-containing viruses, stimulation of RNA synthesis may occur early in infection. The uptake of C^{14}-labeled adenine in Earle's L cells during the first 12 hours after infection with vaccinia virus was confined to the microsomal fraction which contains no DNA; in fact, it was incorporated into polyribonucleotide. Joklik and Rodrick (95) interpreted their results as a reflection of the increased turnover of microsomal RNA as mechanisms for synthesizing protein and nucleic acid are stimulated. DNA production is increased at a later stage. In the case of herpes simplex virus growing in HeLa cells, infection initiates an increase in DNA, not in RNA. This increase is evident 6 to 9 hours after infection, and in 36 hours is double that in control cultures. The increase is confined to the nucleus (116). However, Moore and Randall (115) found both DNA and total protein to be significantly increased, whilst the RNA remained unchanged, in HeLa cells infected with equine abortion virus. Differences in the abilities of cell nuclei and psittacosis virus to utilise labeled precursors for the synthesis of DNA have also been noted (127).

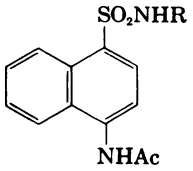
Wheelock and Tamm (196) explored the nature and extent of virus-induced metabolic alterations in a study of the biosynthetic capacities of HeLa cells infected with Newcastle disease virus (NDV). The incorporation of tritiated thymidine into DNA, of uridine and cytidine into RNA, and of amino acids into protein were determined at frequent intervals after infection of HeLa cells with NDV. Inhibition of DNA and protein synthesis became evident at about the same time as newly made virus antigen and infective virus particles first appeared. Progressive decrease in synthesis of DNA and protein coincided with rapid increase in virus antigen and infective particles, and synthesis of DNA and protein stopped as the amounts of virus materials produced were reaching maximum levels. Inhibition of DNA and protein synthesis preceded inhibition of mitosis by 2 to 3 hours, and the development of marked cellular damage by several hours. Incorporation of labeled uridine into RNA was reduced only slightly at the time of cessation of both virus production and synthesis of DNA and protein. These results, integrated with their earlier observations on biological aspects of infection, led them to the conclusion that the cessation of virus production is probably caused by inhibition of protein or RNA synthesis, and is not due to inhibition of DNA synthesis (195, 196).

Tissue culture also provides a ready method for investigating rates of virus adsorption to cells, both in suspension and in monolayers. Earlier measurements of adsorption of animal viruses by cell layers were based on infectivity determinations, and showed rather a wide scatter (101, 198), but the use of purified specimens of I^{131}-labeled vaccinia virus and P^{32}-labeled fowl plague virus have enabled more accurate measurements to be made (8, 178). Some of the host cells used in suspension were capable of supporting the multiplication of the viruses, others were not. In all cases, however, the rates of attachment of virus particles to cells were of the same order, which was several times less than that expected from Brownian theory. This stands in contrast to the rates of attachment of viruses to glass and other nonbiological surfaces, which under optimal conditions are very close to the calculated values. It is of interest that the rates of adsorption of vaccinia and fowl plague viruses were of the same order in cells in which the viruses multiply readily and in those in which they do not. Thus, the barrier to multiplication in these cases does not arise from a failure of the virus to be adsorbed to the cells. This finding contrasts with the behavior of poliovirus, which is reported to be adsorbed readily only to susceptible cells (110). Similar adsorption studies have been made using monolayers of chick embryo and HeLa cells, with essentially similar results. The adsorption of viruses by cells was found not to be depressed by protein but was dependent on the concentration of cations in the medium. It was suggested that the main interacting groups are the amino groups of the virus and phosphate groups of the host cell wall (9).

Holland and McLaren (85), working on the location and nature of enterovirus receptors in susceptible cells, found that the material in cell homogenates which binds enteroviruses is the same receptor which enables the intact cell to adsorb these viruses, since receptor activity of microsomes or other subcellular fractions was obtained only from susceptible cells which were capable of adsorption when intact. Furthermore, the ionic cofactor requirements for receptor activity in disrupted cells reflects the cofactor requirements for adsorption of virus to whole cells. Their results suggest that enterovirus receptor activity resides in the insoluble lipoproteins of the cell membranes, mainly in the microsome fraction. It seems probable that the weak virus-binding ability of nuclei and other subcellular fractions results from contamination of these fractions with microsomal lipoproteins.

Another surface property of particular interest to the chemotherapist results from the lipid content of certain viruses. Only the myxo- and arborviruses have lipid as a significant part of their structure, but although this lipid is essentially of cellular origin and is unmodified by the virus (184), its removal from either myxo- or arborviruses renders them noninfective. Studies of chemical inhibition of arborviruses in Japan led to the discovery of 4-acetylaminonaphthalene lauroylsulfonamide (XXVIII, R = lauroyl) (PANS), which is effective when administered prophylactically or therapeutically in experimentally produced or naturally occurring Japanese B encephalitis and Newcastle disease. This com-

pound (PANS) is virucidal on direct contact *in vitro*, for both crude and purified preparations of the Japanese B encephalitis virus (176, 177).



XXVIII

The possibility that PANS owes its antiviral properties to a surface effect has been investigated by Ito (92). This compound and other derivatives of XXVIII, in which R equals a long alkyl chain, showed extremely low surface tensions, which paralleled their antiviral activities. Penetration of these compounds through unimolecular layers of cholesterol and lecithin was also examined in the presence of serum albumin, and it was concluded that the strength of bonding of these compounds with serum albumin was one of the reasons for the retention of activity *in vivo*. Weinstein and Chang (190) have carried out further investigations with PANS. The drug was tested for activity against influenza, encephalomyocarditis, poliomyelitis, and herpes simplex viruses both *in vitro* and *in vivo*. Inhibition of multiplication of the PR_8 strain of influenza virus was observed in cultures of chicken chorioallantoic membrane, but PANS was not effective in altering the course of the various virus diseases in intact animals, with the exception of herpes simplex and influenza infection, in which a slight to moderate degree of prophylactic activity was demonstrable.

B. Physical methods for investigating the structural interactions of active molecules

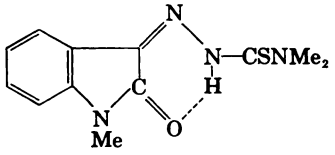
Physical methods have not received the attention they deserve as aids for interpretation of structure-activity relationships. Such tools as X-ray diffraction and the measurement of intrinsic viscosities and sedimentation rates may give considerable information concerning macromolecules, but such studies have rarely been undertaken in the realm of chemotherapy. One notable exception is the distinguished investigation by Lerman (105) on the interaction of DNA and acridines.

The mode of combination of acridine derivatives with DNA is of particular interest because of their general antiviral properties (Section IIIA); they have also been reported to be mutagenic (54). The planar configuration of the acridines and the probability of strong electronic interactions favor their flat, face-to-face binding to the bases of the nucleotides. Because these bases are in close Van der Waals contact, it is postulated that untwisting of the double helix provides sufficient space for the insertion of an acridine molecule, leaving undisturbed the hydrogen-bonded pairing of the nucleotides constituting each layer. Such an extended molecule containing intercalated acridine layers would differ from native DNA in several respects. The length of the molecule would be increased

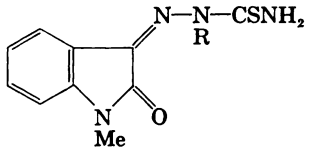
in proportion to the amount of acridine bound. Since the additional length due to each acridine is about the same as that of a nucleotide pair, while the mass increment is less than one-half of a nucleotide pair, the average mass per unit length would decrease. The sedimentation coefficient of such a molecule is expected to be nearly proportional to the mass per unit length (141). Where a molecule of molecular weight 210 (proflavine, free base) replaces, by intercalation, a nucleotide pair of mean molecular weight 670, while maintaining constant length, the sedimentation coefficient would be expected to decline about 0.69% for each substituted segment in a hundred. However, the length does not remain constant, but increases in direct proportion to the number of proflavine molecules bound, so the effect of the length-specific mass is in part compensated for by the increased size. Lerman (105) has clearly demonstrated that the depression in the sedimentation coefficient is in the region of that predicted for intercalation when moderate amounts of proflavine are bound. At higher concentrations the net effect suggests a balance between the consequences of intercalation and further external binding.

Taking into account information derived from viscosity measurements on DNA-acridine complexes and the X-ray diffraction pattern given by the complex of LiDNA with proflavine, Lerman (105) concluded that of all the possible ways of binding acridines to DNA, only intercalation into the helix by extension of the "backbone" is fully compatible with the available evidence.

Molecular spectroscopy may also be of use in structure-activity investigations, and this subject has been reviewed in general terms (135). Spectroscopic studies on suitable derivatives of isatin-β-thiosemicarbazone and related compounds have demonstrated that the parent compound possesses very strong *intra*molecular hydrogen bonds (137).



XXIX



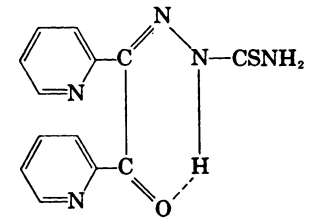
XXX

The 3-hydrazone, 3-phenylhydrazone, 3-thiosemicarbazone, and 3-4′-phenylthiosemicarbazone of 1-methylisatin all show bonded N—H frequencies at 3400 and 3200 cm^{-1} in potassium bromide discs. The broad band at 3400 to 3200 cm^{-1} shown by 1-methylisatin 3-4′,4′-dimethylthiosemicarbazone favors the intramolecular structure (XXIX), which is supported by the data for the 2′-methyl and the 2′-phenyl derivative (XXX; R = Me, Ph). Although these compounds in the solid state show bonded N—H frequencies, the carbonyl-stretching frequency of the 2′-phenyl derivative is raised to 1712 cm^{-1}, and a similar peak appears as a shoulder at 1710 cm^{-1} in the 2′-methyl derivative. Compounds of type XXX are unlikely to possess intramolecular hydrogen bonds and the intermolecular bonding appears to be rather weak. This is confirmed by results obtained with dilute solutions in carbon tetrachloride. The compounds are not

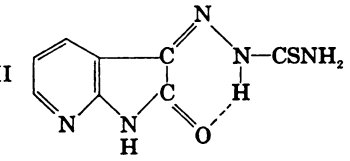
sufficiently soluble to provide good records in the 3 μ region, but 1-methylisatin-3-2′-phenyl- and 3-2′-methylthiosemicarbazone have peaks at 1722 and 1708 cm^{-1}, respectively. The demonstration that strong intramolecular hydrogen bonds are formed in compounds of type XXIX has been important in interpreting the antivaccinial and antivariola activities of N-alkylisatin-β-thiosemicarbazones (XLII) (14, 15).

The most notable structural difference between N-alkylisatin-β-thiosemicarbazones, which have pronounced antiviral activity, and the less active formylpyridine thiosemicarbazones is the absence of an α-carbonyl group from the latter. The α-carbonyl group of isatin-β-thiosemicarbazone has been shown to be essential for the retention of antivaccinial activity, and is also involved in the formation of an intramolecular hydrogen bond with the 2′-imino hydrogen atom. Two pyridine derivatives were prepared which could possess related intramolecularly bonded structures. Infrared spectra of α-pyridilmonothiosemicarbazone (XXXI) and 7-pyrisatin-β-thiosemicarbazone (XXXII) showed lowering and broadening of the 2′-imino N—H and carbonyl-stretching frequencies characteristic of hydrogen bonding, and dilution studies indicated that this was mainly *intra*molecular; however, neither XXXI nor XXXII possesses antivaccinial activity (138).

XXXI



XXXII



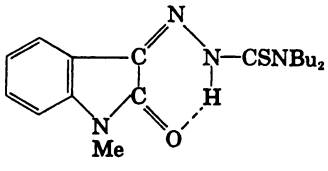
This finding is perhaps not surprising in the case of XXXI, since benzilmonothiosemicarbazone is also inactive, but the lack of activity of XXXII suggests that isatin and pyridine derivatives exert their antivaccinial effects by completely different means.

Correlation exists between σ values[2] of substituents (76), and both the α- and β-carbonyl-stretching frequencies of substituted isatins (122) and the latter frequencies are also directly related to the dehydrogenase activities of these compounds (123). However, solid state spectroscopic data for ring-substituted isatin-β-thiosemicarbazones have disclosed no sign of correlation between σ-values and either the α-carbonyl- or C=N-stretching frequency, in accord with

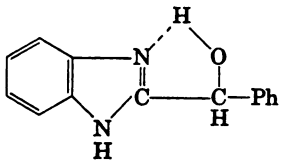
[2] These are numerical values for the effects of *meta* and *para* substituents in benzenoid systems containing a side-chain.

the lack of relation of antivaccinial activities to σ-values of substituted isatin-β-thiosemicarbazones. Therefore, substituent effects are entirely steric in nature in these derivatives (14).

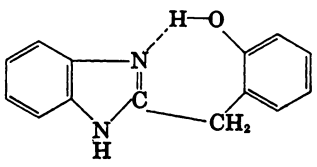
Since 1-methyl-4′-4′-dibutylisatin-β-thiosemicarbazone (XXXIII) at 30 μM concentration and 2-(α-hydroxybenzyl)benzimidazole (XXXIV) at 100 μM concentration have similar effects on poliovirus type 2 in tissue culture, the structures were examined for comparable features (124).



XXXIII



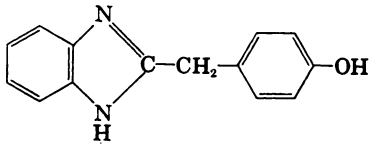
XXXIV



XXXV

The infrared spectrum (137) shows the presence of intramolecular hydrogen bonding in the isatin derivative, and intramolecular hydrogen bonds are also possible in XXXIV. In the latter case, however, this bonding will be weak in view of the strain present in the small ring and in view of the strong tendency of benzimidazole derivatives to form intermolecular hydrogen bonds (120). To shed further light on this, some related benzimidazoles were synthesized and tested. 2-(*o*-Hydroxybenzyl)benzimidazole (XXXV), which possesses the benzyl substituent and a hydroxyl group in the neighbourhood of the nitrogen atom, should form a slightly stronger intramolecular hydrogen bond similar to that in compound XXXIV. Tissue culture experiments show that the *o*-hydroxy compound (XXXV) has a selective action against poliovirus type 2 similar to that exerted by the α-hydroxy compound (XXXIV). The hydrogen bonding hypothesis was examined further by assaying the *p*-hydroxybenzylbenzimidazole (XXXVI), which is unable to form intramolecular hydrogen bonds but can associate by intermolecular hydrogen bonds. Since 2-benzylbenzimidazole itself shows slight activity, some activity might be expected in the *p*-hydroxy compound; but if the existence of an intramolecular hydrogen bond is of importance, then the activity should be low. The latter is, in fact, the case. Thus, the presence of intramolecular hydrogen bonding is a feature common to all antiviral agents of high activity in both the isatin-β-thiosemicarbazone and the benzimidazole series.

XXXVI

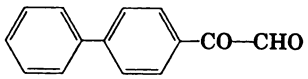


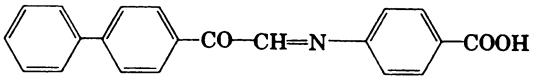
Many simpler benzimidazole (120) and benzotriazole (121) derivatives of lower activity (156) cannot form intramolecular hydrogen bonds, but have been shown by their infrared spectra to form intermolecular N—H----N links. Strong, but nonspecific, bonding to protein is possible with these compounds.

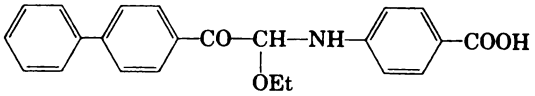
V. STRUCTURE-ACTIVITY RELATIONSHIPS

Information for use in elucidating structure-activity relationships in antiviral chemotherapy has in the past come mainly from biochemical studies on compounds which were designed as enzyme inhibitors or metabolic analogs, or which were subsequently found to possess these properties (Section IVA). More precise information is available from physical studies on active molecules; these studies may sometimes indicate a different mode of action from that suggested by biochemical investigation alone. This has been found in the case of acridine enzyme inhibitors which show antiviral activity, *e.g.*, the effect of proflavine on vaccinia and poliomyelitis viruses (168). More recent work has shown that this compound interacts directly with DNA (105).

A third approach is an entirely intuitive or deductive method. Cavallini and Massarani (32) have proposed the concept that substances possessing pharmacological activity have two features: 1) the "supporting moiety," which confers on the substance a selective affinity for biochemical substrates, and 2) the "radical moiety," which determines the type of pharmacological activity. Since it had been observed that some glyoxals display marked antiviral activity against the influenza A-PR_8 virus and the Newcastle (NDV) virus in the chick embryo (47, 114, 173, 197), and since previous investigations had shown that biphenyl was one of the most suitable "supporting moieties" (31, 34), a series of biphenylglyoxal derivatives was prepared (33) and found to have considerable activity *in vivo*. The compounds when administered to mice infected with influenza A-PR_8 virus, inoculated by the nasal route, and the hepatitic MHV 3 virus, inoculated by the subcutaneous route, displayed considerable activity against the two viruses.

XXXVII 

XXXVIII 

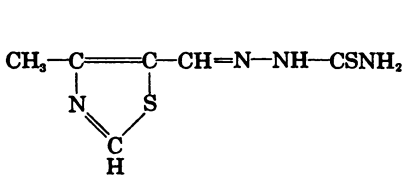
XXXIX 

The derivatives of biphenylglyoxal (XXXVII) which showed the lowest toxicity and the highest antiviral activity were *p*-(4-biphenylglyoxylidene)aminobenzoic acid (XXXVIII) and *p*-(α-ethoxy-*p*-phenacylamino)benzoic acid (XXXIX). The latter compound (xenalamine) has been the subject of several clinical trials and is commercially available; its antiviral properties have been reviewed (30).

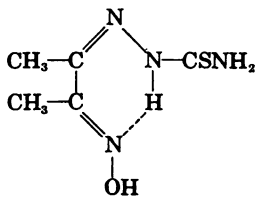
Apart from the acridines, only two series of compounds have been investigated in sufficient detail to provide a reasoned basis for speculation as to their mode of action; these are the substituted benzimidazoles and the isatin-β-thiosemicarbazone derivatives.

The chemotherapeutic activity of thiosemicarbazones against vaccinia was first demonstrated by Hamre *et al.* (77). *p*-Amino-benzaldehyde-3-thiosemicarbazone, when inoculated into the yolk sac, prolonged the survival time of infected chicken embryos, and when administered subcutaneously afforded some protection to mice which had been infected intranasally with vaccinia. The parent compound, benzaldehyde thiosemicarbazone, also protected a large proportion of mice infected intranasally with vaccinia when fed with the diet, and was also active when the virus was given by the cerebral route (172). Other derivatives, such as the *p*-nitro- and *p*-acetamido-benzaldehyde thiosemicarbazones, were reported to suppress the production of pulmonary lesions in cotton rats inoculated intranasally with the virus of primary atypical pneumonia (77). That a cyclic component, in addition to the thiosemicarbazone group, was essential for high activity was demonstrated by Thompson *et al.* (113, 171).

On re-examination, Bauer (11) found that very small doses of isatin-β-thiosemicarbazone would afford complete protection to mice against 100 to 1000 LD50 doses of the neurotropic International Health Division (IHD) strain of vaccinia. These results were confirmed by Bock (21), who reported subcutaneous injection to be a superior mode of administration compared with intraperitoneal injection. With the exception of benzaldehyde thiosemicarbazone and some of its derivatives, most of the thiosemicarbazones possessing the highest activity are those in which the thiosemicarbazone group, =N—NH—$CSNH_2$, is separated by two carbon atoms from a nitrogen or sulphur atom, such as thiosemicarbazones of certain α-oximinoketones and 2-thiophencarboxyaldehydes (170). In view of these results, it was of interest to prepare for antiviral screening thiosemicarbazones of thiazole and imidazole aldehydes, in which the position of the thiosemicarbazone group on the heterocyclic ring was such that the desired structure was obtained. In tests using the International Health Division (IHD) strain of vaccinia virus, it was found that both 4-methyl-5-thiazolecarboxyaldehyde thiosemicarbazone (XL) and 2,3-butanedioneoxime thiosemicarbazone (XLI) were equally effective against intranasally induced infection (29).



XL

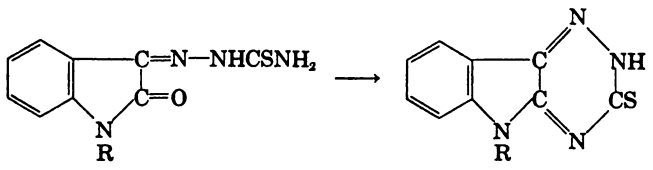


XLI

Isatin-β-thiosemicarbazone itself, of course, conforms to this specification, and the parts of the molecule which are essential for the retention of high antiviral activity have been clearly demonstrated (14).

In the course of extensive investigations, certain possibilities as to the mode of action of substituted isatin-β-thiosemicarbazones have been eliminated. The most active N-alkyl compounds (XLII) do not cyclize *in vivo* to form indolo-

thiotriazines (XLIII) which might be antimetabolites, and when these cyclic derivatives are prepared chemically they have no antiviral activity (136). This is in contrast with the findings of Lum and Smith (106) that the activity of some thiosemicarbazones against the PR_8 strain of influenza A virus in tissue cultures is prevented by the addition of small amounts of coenzyme A or pantothenic acid. The inhibition was believed to be competitive, since the necessary concentration of pantothenic acid was dependent on the concentration of thiosemicarbazone.



XLII XLIII

Furthermore, the compounds are not hydrolysed *in vivo* to release the parent isatin, which by virtue of its dehydrogenase activity could remove an essential metabolite; a series of isatin-β-thiosemicarbazones showed no correlation between antivaccinial activity and the dehydrogenase activities of the substituted isatins (123).

In the benzimidazole series also, the emphasis has moved away from the synthesis of compounds designed specifically as cellular antimetabolites, such as 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (III), and simpler derivatives related to 2-α-hydroxybenzylbenzimidazole (XXXIV) are now being extensively investigated (60, 124, 156). The structural feature which many active derivatives of both isatin and benzimidazole have in common is the presence of strong *intra*-molecular hydrogen bonds, *e.g.*, XXXIII and XXXV. A similar resonance-stabilized ring may also exist in the case of 2,3-butanedioneoxime thiosemicarbazone (XLI) and, of course, such structures facilitate the formation of stable metal chelates. The evidence relating the antiviral action to elements present in trace amounts is equivocal. During multiplication in mouse brain, some viruses (including neurovaccinia and ectromelia) have been shown to be sensitive, while 23 others are insensitive, to metal ions. Copper, rhodium, and platinum inhibit both viruses, whereas yttrium affects only the former and zinc only the latter (12). Ong (118) investigated the inhibitory action of 22 elements on type A influenza virus PR_8 and type B influenza virus. Among the elements tested, copper, gallium, lead, thallium, and selenium showed strong inhibitory action; it was further observed that lanthanum, tungsten, chromium, molybdenum, and vanadium all have strong inhibitory action and caused reduction in damage to mouse lung (119). Conversely, it has been clearly demonstrated that copper is a natural constituent of vaccinia virus (84). On balancing these findings, it would seem unlikely that removal of these elements by chelation, or even the transference of them to the virus particles would directly influence viral replication. What does appear to be far more probable is that these planar molecules, with

their hydrogen-bonding propensities, interact directly and specifically with viral nucleic acid intracellularly, without impairing normal cellular function.

VI. MORPHOLOGICAL CHANGES IN VIRUS PARTICLES IN TISSUE CULTURE SYSTEMS TREATED WITH ANTIVIRAL AGENTS

Investigation of the morphology of viruses by electron microscopy has now reached an advanced stage, and techniques are well developed for the study of many viruses in various types of cellular environment. Study of the development of viruses in the presence and absence of well-known antiviral agents is consequently of particular interest.

Certain straightforward lines of investigation may be suggested. The direct effects of antiviral agents of promise, such as the isatin-β-thiosemicarbazone derivatives, can be studied on tissues infected with various viruses. This type of investigation has been carried out using embryonic rat-kidney cells infected with neurovaccinia (104). In these experiments, cells were fixed for 100 minutes with osmium tetroxide, washed, and then placed for the specified times in 70 % alcohol (10 minutes), 90 % alcohol (10 minutes), and 100 % alcohol (20 minutes). At each stage, cells were separated by low speed centrifugation so that cellular contents were not disrupted. Cells were then embedded either with methacrylate monomer or with araldite resin monomer, which was then polymerised. Araldite, in general, proved to be superior to methacrylate.

The block containing the fixed cells was trimmed and cut with a Reichert ultramicrotome to 300 to 400 Å thickness, and sections were picked up with a copper grid coated with a carbon film. The grid was then examined with a Seimens electron microscope. These particular cells, when uninfected and untreated, appeared normal. Nuclei occupied about half the cell contents and had characteristic nucleoli; mitochondria with well-defined cristae were numerous, and both the endoplasmic reticulum and the Golgi apparatus were observed frequently. Some cells were seen which exhibited stages of degeneration, with vacuolated cytoplasm and swollen mitochondria. A few cells were seen in dividing stages. Cells treated with 20 μM isatin-β-thiosemicarbazone for periods up to 5 weeks looked healthy, with differences from the normal which were nearly imperceptible. These differences consisted in very slightly greater dispersion of the chromatic content of the nuclei in treated cells. Cells infected with vaccinia virus showed typical signs, with swollen mitochondria, cytoplasmic contents more sparsely arranged, and numerous vacuoles. During the infection of cells, half-moon-shaped virus membranes appeared with associated electron-dense material. These progressed to form the virus particles surrounded by the typical membrane. Dense clusters of virus particles were found.

Cells were then infected with neurovaccinia for 24 hours; half were processed for sectioning and half treated with isatin-β-thiosemicarbazone for 24 hours. Controls showed numerous virus particles, whilst these were almost absent from treated cells. The few particles seen were isolated, and no clusters were observed. A number of half-moon-shaped membrane fragments were present, and further experiments showed that these rarely developed into complete virus particles.

Most of the observed virus particles in treated cells appeared to possess abnormal, fragmented membranes. Although electron-microscopy studies in this field are still in their infancy, it is clear that isatin-β-thiosemicarbazone has a very marked inhibiting effect on the production of normal neurovaccinia virus particles in these cells.

This work has considerable potentialities, not only in the realm of the well-recognised virus diseases, but also in relation to cancer, as there is no doubt that virology offers great promise for future research on the cause and prevention of this disease. Following earlier work (73, 74, 75, 146), Stewart and her collaborators (149, 150, 151, 152, 153) have produced a parotid tumor filtrate by carrying it in tissue cultures. Newborn mice injected with this filtrate developed not only primary parotid tumors but also 22 other types of tumors as well, including tumors of the thymus, adrenal, and mammary glands. Consequently, this agent was named polyoma virus. Dmochowski (50) has obtained electron micrographs of virus-like particles in a variety of mouse and chicken tumor tissues, but the combined study on the morphology of oncogenic viruses in the presence of antiviral agents is still awaited.

VII. SPECIFICITY OF ACTION

The advent of a wide-spectrum antiviral chemotherapeutic agent still seems to be remote, and the possibility that each virus will require its own specialised treatment, which in turn will vary from host to host, seems far more likely; the balance of evidence is in favor of the development of chemotherapeutic agents with a narrow range of activity.

Selectivity of action may be highly desirable in certain circumstances, and earlier work indicated that a degree of selectivity against influenza B virus multiplication was conferred on benzimidazole derivatives by the introduction of a β-ribofuranoside group. As the β-D-ribofuranosyl form is the natural form for RNA nucleotides, it was thought that the selective action was due to a specific inhibition of RNA synthesis. This selective effect did not extend to poliomyelitis virus, although this contains only RNA, so there would appear to be no clear relationship between the nucleic acid of the virus and the selective action of benzimidazole-β-D-ribofuranosides (Section IIIB).

On the other hand, that the sensitivity to 2-α-hydroxybenzylbenzimidazole (HBB) reflects a fundamental property of certain enteroviruses has been clearly demonstrated (143). Although HBB shows pronounced activity against the major group of enteroviruses, *i.e.*, poliomyelitis, coxsackie, and ECHO, it is ineffective against herpes or pox viruses; while it has very high activity against poliomyelitis, it has no activity against influenza virus (156, 164). It has been suggested that the degree of sensitivity to HBB is a genetic characteristic, and that there is a correlation between the sensitivity of the virus to inhibition by HBB and its resistance to photodynamic inactivation when toluidine blue is used as a photosensitiser (83).

Eggers and Tamm (58, 59, 60) found that a number of different viruses are able to multiply in the presence of HBB in tissue culture, which demonstrates

that the essential biosynthetic processes of the cells are not impaired. This hypothesis is supported by the observations that HBB does not diminish the incorporation of labeled adenosine into RNA, or of labeled alanine into the host protein. Also, HBB has no effect on oxygen uptake, or the utilization of glucose, or the production of lactic acid; therefore, the cells are able to multiply in their natural rhythm in the presence of this substance.

These results indicate that HBB presents a new method for studying biochemical specificity in the majority of the enteroviruses. The evidence indicates that the presence of the hydroxybenzyl group in position 2 of the imidazole nucleus is of fundamental importance for the selective inhibitory action of HBB on the virus. It has been claimed that a more precise knowledge of the structural characteristics of HBB which are responsible for the "selective" inhibition will permit classification of the HBB-sensitive viruses in chemical terms (58, 59, 60).

In the case of isatin-β-thiosemicarbazone derivatives, the compounds which exhibit pronounced activity against neurovaccinia are also active against cowpox and rabbit-pox. This latter work was carried out with the Utrecht strain of rabbit-pox virus; the treated mice all survived, and the parent compound gave complete protection against infection with 100,000 LD50 doses of virus (17). The compounds also afford protection in alastrim infections (15), but they show no antiviral effect in mice infected with ectromelia. In view of the close antigenic relationship between these two viruses, this finding was very surprising, and cast the first doubt on the generally held view that an antiviral agent would have to act by affecting selectively some host-cell system which was required for the multiplication of the virus. In the specific case of vaccinia and ectromelia infections of mouse brain, the host cell is the same, with the same complement of enzymes and substrates, and only the virus is different. It is difficult to believe that such a closely related virus as ectromelia requires enzymes or substrates which are different from those required by vaccinia; hence, the observed fact that isatin-β-thiosemicarbazone is active against vaccinia, but not against ectromelia, suggests that it is acting against the virus rather than on the host cell. The force of this argument is not essentially reduced by the subsequent observation of Sheffield *et al.* (144) that isatin-β-thiosemicarbazone is active against ectromelia virus in tissue culture, since the activity was very low at the very limit of detectability. When 19 derivatives of isatin-β-thiosemicarbazone and related compounds were tested for activity against ectromelia in mice, none showed any evidence of activity (14). It is, therefore, of considerable interest that further work on the modification of the structure of isatin-β-thiosemicarbazone revealed a series of derivatives with high activity against ectromelia infection, and with little or no activity against vaccinia (16).

The compounds found so far to possess anti-ectromelia activity are all 4′,4′-dialkylthiosemicarbazones of isatin, N-methylisatin, or N-ethylisatin. Compounds with mixed alkyl groups and with cyclic groups, such as piperidine, pyrrolidine, and morpholine, were also prepared, and structural modifications were made in the 1 position and in the aromatic ring (183).

Earlier work showed that introduction of one or more alkyl substituents into

the side-chain completely abolishes antivaccinial activity (Section IIID), but compounds bearing identical alkyl substituents on the terminal nitrogen atom show a wide range of activity against ectromelia.

The most active compound is isatin-β-4′,4′-dimethylthiosemicarbazone, and activity is progressively reduced in the diethyl, dipropyl, and dibutyl analogs. The effect of alkylation in the 1 position is to reduce activity, which is in direct contrast with the situation in the antivaccinia compounds, with which substitution of alkyl groups in the 1 position enhances activity. The effect of substitution in the aromatic ring has disclosed another striking difference from the structure-function relationships of the antivaccinia compounds, for substitution in the 5 position is very damaging to antivaccinial activity; but with the anti-ectromelia compounds, substitution of large groups in the 5 position is tolerated with only moderate loss of activity. These results indicate that there is no general solution to the problem of antiviral chemotherapy, and that each virus must be treated individually. However, there is evidence which suggests that this is not necessarily the case, as N-methylisatin-β-4′,4′-dibutylthiosemicarbazone (XXXIII), which shows moderate activity against ectromelia, also possesses high activity against type 2 polio, in the quite unrelated group of enteroviruses (124, 139). This compound, which in tissue culture provides over 2 log units protection against infection with type 2 poliomyelitis virus, shows approximately the same activity as HBB. The question then, whether or not an antiviral agent with a wide spectrum of activity will be obtained, remains open; however, there are already clear indications that some of the present antiviral agents (such as certain isatin-β-thiosemicarbazone derivatives, certain hydroxybenzylbenzimidazole derivatives, and 5-iodo-2′-deoxyuridine) will find use in clinical practice.

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